ลิขสิทธิ์ของสมาคมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข
ห้ามมิให้ทำซ้ำ ดัดแปลง แก้ไข โดยไม่ได้รับอนุญาต

Thai Pharmacopoeia II 2011

Volume I Part 1

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ลิขสิทธิ์ กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข
ห้ามทำซ้ำ ดัดแปลง แก้ไข โดยไม่ได้รับอนุญาต

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PREFACE

The establishment of the Thai Pharmacopoeia (TP) and the Thai Herbal Pharmacopoeia (THP) has offered the national standards for modern and herbal drugs as well as biological products. After the publication of the first edition of the Thai Pharmacopoeia in 1987 and mandated in 1988, the succeeding publications extended the standards to cover the selected drugs and preparations. In parallel with TP, the establishment of THP was initiated in 1989 to provide the specifications for marketed herbal drugs. The work on both Pharmacopoeiae has gone through the remarkable development since the beginning era.

The revision of the first edition is aimed to provide the official quality standards that fit in with the actual situations in Thailand. The criteria of selecting drugs have been changed for the benefit of stakeholders and authorities involved. The monographs of raw materials and preparations are placed in the same volume to facilitate the users. Nevertheless, the pharmacological and toxicological information on the contra-indications, warnings, precautions, and other special information of drugs are maintained in the monographs to benefit the healthcare providers.

Monographs of biologicals are grouped under a separate chapter as in the previous edition. The standards set forth are intended to apply to the bulk materials to be later subdivided and the finished products themselves. The fundamental regulations for the control of biological products under manufacturing processes are compiled in the "Minimum Requirements for Biological Products" issued by the Ministry of Public Health.

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THE THAI PHARMACOPOEIA COMMITTEE AND SUBCOMMITTEES

The Thai Pharmacopoeia Committee was first appointed by the Ministerial Cabinet in 1979. The functions of the Thai Pharmacopoeia Commission are to

1. select therapeutically useful drugs for inclusion in the Pharmacopoeia;
2. establish compendial limits, tolerances and specifications for selected drugs;
3. specify appropriate tests and methods for quality control of the drugs;
4. compile complete text of the Thai Pharmacopoeia for publication;
5. issue supplements in compliance with the text;
6. revise the Thai Pharmacopoeia periodically;
7. appoint appropriate subcommittees to undertake the functions as entrusted;
8. perform any other tasks assigned by the Minister.

The Thai Pharmacopoeia Committee recruited assistance from the following subcommittees in carrying out its aforementioned functions:

1. The Subcommittee on Drug Selection

This subcommittee is responsible for selecting drugs to be included in or excluded from the Pharmacopoeia, taking into account their categories of actions and therapeutic uses by:

1.1 identifying the potential drugs for inclusion based on their therapeutic efficacy and safety;

1.2 determining the most effective formulations and compositions of the selected drugs;

1.3 specifying the official titles, synonym(s) for the monograph of each drug, and its preparations;

1.4 revising the text of the monograph that appeared in earlier editions.

2. The Subcommittee on Drug Safety

This subcommittee is responsible for detailing the safety, efficacy and potential side effects of the drugs by:

2.1 specifying information on category, dose, strengths available, contra-indication, warning, precaution, and additional information;

2.2 identifying for safety verification the different tests including the pyrogen test, the sterility test, etc.;

2.3 providing the appendices regarding testing methods stated in 2.2.

3. The Subcommittee on General Specifications and Reagents

This subcommittee is responsible for establishing additional drug specifications corollary to those already described in 4. by:

3.1 describing and identifying physical and chemical properties of the drugs, such as their solubility, melting and boiling temperatures, acid value, ester value, and pH value, and prescribing the purity tests such as those for heavy metals, related substances, etc.;

3.2 developing quality control methods for drug preparations, including leakage test, deliverable volume, minimum fill, completion of solutions after powder reconstitution, etc.;

3.3 preparing appendices regarding testing methods stating in 3.1 and 3.2;

3.4 surveying and preparing a list of reagents, volumetric solutions, standard solutions, pH indicators, buffer solutions, test solutions, reference substances, and any other test substances that are mentioned in the monographs or in the appendices of the Thai Pharmacopoeia, but their specifications have not been established;

3.5 assigning official names, synonyms, molecular formulae, and molecular weights to the above-mentioned reagents;

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3.6 drafting physico-chemical specifications, purity standards, methods of preparation as well as precautions and storage advices of the reagents mentioned in 3.4.

4. The Subcommittee on Standards and Analytical Methods

This subcommittee is responsible for specifying standards and analytical methods used in drug testing by:

4.1 selecting the most appropriate and economical method(s) from the different analytical methods described in established pharmacopoeia, published papers, or individual research works, with or without modifications, to be used in the analytical control of each drug and its preparations. The methods can be chemical, physico-chemical, microbiological, or biological in nature;

4.2 specifying the content limits of the drugs;

4.3 specifying the limits and identifying the methods for dissolution and content uniformity, where appropriate;

4.4 identifying the methods for identification, microbial limit, water content, or loss on drying for each monograph;

4.5 preparing appendices of the tests related to 4.1, 4.3 and 4.4;

4.6 specifying molecular weights of the drugs.

5. The Subcommittee on Drug Stability

This subcommittee is responsible for identifying the physical and chemical stability of the drugs by:

5.1 specifying the appropriate pharmaceutical containers and closures, and providing information on packaging, storage and labelling;

5.2 preparing appendices of the tests related to 5.1 and those beyond the responsibilities of the Subcommittee on General Specifications and Reagents and the Subcommittee on Standards and Analytical Methods;

5.3 defining and preparing general information on various dosage forms, e.g. tablets, parenteral preparations, suspensions.

6. The Subcommittee on the Establishment of the Thai Herbal Pharmacopoeia

This subcommittee is responsible for:

6.1 selecting the herbal drugs proven effective and safe for further consideration by the Thai Pharmacopoeia Committee;

6.2 establishing the specifications of herbal drugs which were preselected by the Thai Pharmacopoeia Committee and compiling to be monographs of the Thai Herbal Pharmacopoeia;

6.3 preparing the final English draft;

6.4 tending to pertinent matters related to the preparation of the Thai Herbal Pharmacopoeia.

7. The Subcommittee on Biological Products

This subcommittee is responsible for:

7.1 selecting the biological products that are therapeutically effective and safe for further consideration by the Subcommittee on Drug Selection;

7.2 prescribing the various specifications of the selected biologicals and their preparations;

7.3 providing any other information related to biological products that should be included in the Pharmacopoeia.

8. The Subcommittee on Editorial Style

This subcommittee is responsible for:

8.1 designing the format and style for printing;

8.2 editing the text;

8.3 keeping conformity of the molecular formula, chemical name, molecular weight, and expressions of the symbols of units throughout the text;

8.4 organizing the information compiled by the subcommittees into a pharmacopoeial form and completing the final draft of the Thai Pharmacopoeia;

8.5 tending to other matters related to editing the Pharmacopoeia.

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9. The Subcommittee on the Pharmacognostic and Botanic Specifications of the Thai Herbal Monographs

This subcommittee is responsible for:

9.1 establishing drafts of the pharmacognostic and botanic specifications of the Thai herbal monographs, i.e. nomenclature, definition, description of the plant, macroscopical and microscopical description, and other related information;

9.2 submitting the drafts to the Subcommittee on the Establishment of the Thai Herbal Pharmacopoeia for approval;

9.3 tending to pertinent matters related to the preparation of the pharmacognostic and botanic specifications.

10. The Subcommittee on the Physico-chemical Specifications and Safety of the Thai Herbal Monographs

This subcommittee is responsible for:

10.1 establishing drafts of the physico-chemical specifications of the Thai herbal monographs, i.e. constituents, packaging and storage, identification, assay, ashes, extractives, and other related information;

10.2 establishing information for safety of the Thai herbal monographs, i.e. category, contra-indication, warning, precaution, additional information, dose, and other related information;

10.3 submitting the drafts to the Subcommittee on the Establishment of the Thai Herbal Pharmacopoeia for approval;

10.4 tending to pertinent matters related to the preparation of the physico-chemical specifications.

11. The Ad-hoc Subcommittee on Statistical Analysis of the Results of Biological Assays and Tests

This ad-hoc subcommittee is responsible for:

11.1 identifying appropriate statistical methods for biological assays and tests;

11.2 preparing a preliminary description of statistical methods in 11.1 to be included in an appendix of the Pharmacopoeia;

11.3 completing the final draft of the appendix concerning the statistical analysis of the results of biological assays and tests.

12. The Ad-hoc Subcommittee on Standards of the Thai Herbal Drug Preparations

This ad-hoc subcommittee is responsible for:

12.1 establishing the specifications of Thai herbal preparation which were preselected by the Thai Pharmacopoeia Committee and compiling to be monographs of the Thai Herbal Pharmacopoeia;

12.2 preparing the final English draft;

12.3 tending to pertinent matters related to establishing the specifications of Thai herbal preparation in 10.1.

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Nidapan **Ruangrittinon**, B.Sc. in Pharm., M.Sc. in Pharm. (2003-2005), *Representative*

Pharmacist, expert, as assigned by Director General, Department of Medical Sciences (2005-)
Nantana **Sittichai**, B.Sc. in Pharm., M.S. (2005-2009)
Nidapan **Ruangrittinon**, B.Sc. in Pharm., M.Sc. in Pharm. (2009-)

Head, Thai Pharmacopoeia Section[1], Bureau of Drug and Narcotic, Department of Medical Sciences, Ministry of Public Health (2003-2008)
Nantana **Sittichai**, B.Sc. in Pharm., M.S., (2003- 2005)
Nidapan **Ruangrittinon**, B.Sc. in Pharm., M.Sc. in Pharm. (2005-2008)

Pharmacist as assigned by Director, Bureau of Drug and Narcotic, Department of Medical Sciences (2008-)
Nidapan **Ruangrittinon**, B.Sc. in Pharm., M.Sc. in Pharm. (2008-2009)
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[1] Effective from April 2005 (formerly Thai Pharmacopoeia and Reference Substances)

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Head, Department of Pharmacy, Faculty of Pharmacy, Mahidol University
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Director, Bureau of Drug and Narcotic, Department of Medical Sciences, Ministry of Public Health (2008-)
Nantana **Sittichai**, B.Sc. in Pharm., M.S. (2009-2010)
Yachit **Sriviroj**, B.Sc. in Pharm.
Nuansri **Niwattisaiwong**, B.Sc. in Pharm., Grad. Dip. FDA, M.Sc. (2005-)
Penpan **Aswakun**, B.Sc. in Pharm., M.Sc.
Puangkaew **Lukkanatinaporn**, B.Sc. in Pharm., M.Sc.
Walapa **Tatong**, B.Sc. in Pharm., M.S., Ph.D.
Arunee **Poompanich**, B.Sc. in Pharm. (2008-)
Rojana **Kovithvattanaphong**, B.S. (Pharm.), M.Sc. (2003-2008)
Yaowalak **Wattanapisit**, B.Sc. in Pharm., M.Sc. in Pharm. (2010-)
Anongnut **Noeimuangpak**, B.Sc. in Pharm.

Secretaries: Manas **Attawish**, B.S. (Pharm.)
Kornvika **Charupant**, B. Pharm., M.Sc. in Pharm., Ph.D. (2003)
Sasiwimon **Patasema**, B. Pharm., M.Sc. in Pharm.
Wimonwan **Warinpramote**, B. Pharm., M.Sc. St. (2003-2005)
Numtip **Pananookooln**, B. Pharm., M.Sc. (2005-2009)
Sasida **Yoosook**, B.Sc. in Pharm., M.Sc. in Pharm. (2009-)

4. THE SUBCOMMITTEE ON STANDARDS AND ANALYTICAL METHODS

Chairperson: Director, Bureau of Drug and Narcotic, Department of Medical Sciences, Ministry of Public Health (2003-2009)
Duangporn **Abhigantaphand**, B.Sc. in Pharm. (2003-2007)
Rojana **Kovithvattanaphong**, B.S. (Pharm.), M.Sc. (2007-2009)
Nantana **Sittichai**, B.Sc. in Pharm., M.S., *Representative* (2003-2009)
Yupadee **Payakkapan**, B.Sc. in Pharm., M.Sc. in Pharmaceutical Analysis (2009-2011)
Nantana **Sittichai**, B.Sc. in Pharm., M.S. (2011-)

Advisors: Sumana **Vardhanabhuti**, B.Sc. in Pharm., M.Sc. in Pharm., M.P.H., Cert. in Immunol. (WHO). (2009-)
Chongdee **Wongpinairat**, B.Sc. in Pharm., M.Sc., Ph.D.
Nuanta **Muangnoicharoen**, B.Sc. in Pharm., M.S., Dr. rer.nat.
Yupadee **Payakkapan**, B.Sc. in Pharm., M.Sc. in Pharmaceutical

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ห้ามทำซ้ำ จัดจำหน่าย ดัดแปลง ก่อนได้รับอนุญาต

Analysis (2011-)

Members: Director, Bureau of Drug and Narcotic, Department of Medical Sciences, Ministry of Public Health (2009-)
Nantana **Sittichai**, B.Sc. in Pharm., M.S. (2009-2010)
Director, Drug Control Division, The Food and Drug Administration, Ministry of Public Health
Suda **Dilokphatanamongkol**, B.Sc. in Pharm., M.Sc. in Pharm., *Representative*
Sukanya **Jiarapong**, B.Sc. in Pharm., M.Sc. in Pharm., *Representative*
Supattra **Phongsri**, B.Sc.(Pharm.), *Representative*
Sumana **Vardhanabhuti**, B.Sc. in Pharm., M.Sc. in Pharm., M.P.H., Cert. in Immunol. (WHO). (2003-2009)
Sumana **Khomvilai**, B.Sc. in Pharm., Hon. Ph.D. in Pharm.
Churairat **Rukwatin**, B.Sc. in Pharm.
Yupadee **Payakkapan**, B.Sc. in Pharm., M.Sc. in Pharmaceutical Analysis (2003-2009)
Puangkaew **Lukkanatinaporn**, B.Sc. in Pharm., M.Sc. (Pharmacy)
Somkiat **Rujirawat**, B.Sc. in Pharm., M.Sc. in Pharm. (2003-2009)
Metta **Treebamroong**, B.Sc.in Pharm., M.Sc. (2003-2005)
Arunee **Jutamanee**, B.S. Phar. (2003-2005)
Ahmpoon **Maha-utmr**, B.Sc. in Pharm. (deceased) (2007-2008)
Arpapan **Tongboonrawd**, B.Sc. in Pharm.
Kittisak **Sripha**, B.S. in Pharm., M.Sc. in Pharm., Dr.rer.nat.
Nidapan **Ruangrittinon**, B.Sc. in Pharm., M.Sc. in Pharm.
Jidapha **Kanogsunthornrat**, B.Sc. in Pharm., M.P.H. (2011-)
Poolsak **Phomsuwansiri**, B.Sc. in Pharm.
Sanhaluck **Buasuang**, B.S. (Pharm) (2003-2010)
Yaowalak **Wattanapisit**, B.Sc. in Pharm., M.Sc. in Pharm. (2009-)

Secretaries: Sirichai **Krabesri**, B.Sc. in Pharm., M. Pharm., LL. B., Barrister at Law
Yaowalak **Wattanapisit**, B.Sc. in Pharm., M.Sc. in Pharm. (2003-2009)
Sasiwimon **Patasema**, B. Pharm., M.Sc. in Pharm.
Kornvika **Charupant**, B. Pharm., M.Sc. in Pharm., Ph.D. (2009-2010)
Santi **Nimnoi**, B.S. in Pharm. (2010-)

5. THE SUBCOMMITTEE ON DRUG STABILITY

Chairperson: Sompol **Prakongpan**, B.Sc. in Pharm., M.Sc., M.S.P., Ph.D.

Members: Director, Drug Control Division, The Food and Drug Administration, Ministry of Public Health
Ahmpoon **Maha-utmr**, B.Sc. in Pharm., (deceased) *Representative*
Suda **Dilokphatanamongkol**, B.Sc. in Pharm., M.Sc. in Pharm., *Representative*
Nantarat **Ratchananon**, B. Pharm., *Representative*
Sukanya **Jiarapong**, B.Sc. in Pharm., M.Sc. in Pharm., *Representative*
Head, Department of Manufacturing Pharmacy, Faculty of Pharmaceutical Sciences, Chulalongkorn University
Garnpimol **C. Ritthidej**, B.Sc. in Pharm., M.S., Ph.D.
Wichien **Thanindratarn**, B.Sc. in Pharm., M. Pharm., *Representative*

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ห้ามทำซ้ำ ดัดแปลง เผยแพร่ โดยไม่ได้รับอนุญาต

Head, Department of Manufacturing Pharmacy, Faculty of Pharmacy, Mahidol University
Satit **Puttipipatkhachorn**, B. Pharm., M.Sc. (Pharm.), Ph.D., *Representative*
Monechoulie **Nitibhon**, B.Sc. in Pharm., Dr. en Pharm., *Representative*
Head, Department of Pharmacy, Faculty of Pharmaceutical Sciences, Chulalongkorn University
Porntip **Nimmannitya**, B.Sc. in Pharm., M.Sc. in Pharm.
Uthai **Suvanakoot**, B.Sc. in Pharm., M.Sc. in Pharm., Ph.D.
Suchada **Chutimaworaphan**, B.Pharm., M.Sc.(Pharm.), Ph.D., *Representative*
Panida **Vayumhasuwan**, B.Sc. in Pharm., M.Sc. in Pharm., Ph.D., *Representative*
Valaisiri **Muangsiri**, B.Sc. in Pharm., B.B.A, M.S., Ph.D., *Representative*
Pornpen **Weerawataganont**, B.Sc. in Pharm., M.Sc. in Pharm., Ph.D., Representative
Head, Department of Pharmacy, Faculty of Pharmacy, Mahidol University
Varaporn **Junyaprasert**, B.S. (Pharm.), Ph.D., *Representative*
Head, Stability Testing Section, Quality Assurance Department, The Government Pharmaceutical Organization
Pradittha **Santikul**, B. Pharm., *Representative*
Head, Physical and Chemical Testing Section, Bureau of Drug and Narcotic (2009-)
Yaowalak **Wattanapisit**, B.Sc. in Pharm., M.Sc. in Pharm.
Tasanee **Chokcharoenrat**, B. Pharm.
Supanee **Duangteerapreecha**, B.Sc.in Pharm., M.S., Ph.D., *Representative*
Representative, The Thai Pharmaceutical Manufacturers Association (2008-)
Wilai **Kaweewongworanan**, B.Sc (Pharm.), Representative
Churairat **Rakwatin**, B.Sc. in Pharm.
Sumana **Khomvilai**, B.Sc. in Pharm., Hon. Ph.D. in Pharm.
Nantana **Sittichai**, B.Sc. in Pharm., M.S.

Secretaries:
Sirichai **Krabesri**, B.Sc. in Pharm., M. Pharm., LL.B., Barrister at Law
Supattra **Phongsri**, B.Sc. (Pharm.) (2003-2009)
Sasiwan **Am-och**, B.Sc. in Pharm. (2003)
Sarinee **Lenapun**, B. Pharm., M.Sc. in Pharm.
Thanita **Patthamajinda**, B.S. in Pharm., M.A.(English for Specific Purposes) (2009-)

6. THE SUBCOMMITTEE ON THE ESTABLISHMENT OF THE THAI HERBAL PHARMACOPOEIA

Chairperson:
Vichiara **A. Jirawongse**, B.Sc. in Pharm., Ph.D., Hon.D.Sc. in Pharm. (CU), Hon. Ph.D. (KKU) (deceased) (2003-2006)
Rapepol **Bavovada**, B.Sc. in Pharm., M.Sc. in Pharm., Ph.D. (2006-)

Advisors:
Panida **Kanchanapee**, B.Sc. in Pharm. (2003-2008)
Kamol **Sawasdimongkol**, B.Sc. in Pharm., M.S. (2004-2008)
Thaweephol **Dechatiwongse Na Ayudhya**, B.Sc. in Pharm. (2008-)
Yupadee **Payakkapan**, B.Sc. in Pharm., M.Sc. in Pharmaceutical Analysis (2008-)
Kongkanda **Chayamarit**, B.Sc., M.Sc., D.Sc. (2008-)

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Chirayupin **Chandraprasong**, B.Sc., M.Sc., Hon. Ph.D. (2008-)

Members:

Kamol **Sawasdimongkol**, B.Sc. in Pharm., M.S. (2003)

Chamlong **Phengklai**, B.S. (Forestry), Hon.D.Sc. in Forestry (2003-2004)

Director, Medicinal Plant Research Institute, Department of Medical Sciences

Pranee **Chavalittumrong**, B.Sc. in Pharm., M.Sc. in Pharm. (2003-2007)

Nalinphat **Saktiyasunthorn**, B.Sc. in Pharm., M.Sc. in Pharm. (2008-)

Jaree **Bansiddhi**, B.Sc., M.Sc., *Representative* (2003-2010)

Pranom **Dechwisissakul**, B.Sc., M.Sc. in Pharm., *Representative* (2003, 2010-)

Kalaya **Anulukanapakorn**, B.Sc., M.Sc., Dr.rer.nat., *Representative* (2004-2009)

Director, Research and Development Institute, The Government Pharmaceutical Organization

Chada **Phisalaphong**, B.Sc. in Pharm., M.Sc., Ph.D., *Representative* (2003-2010)

Piyaporn **Prayakprom**, B.Sc. in Pharm., Ph.D., *Representative* (2010-)

Director, Raw Material Standard Division, The Government Pharmaceutical Organization

Weena **Sathianpokkasap**, B.Sc. in Pharm., Representative (2003-2004)

Jiraporn **Noppadech**, B.Sc. in Pharm., M. Pharm., *Representative* (2004-)

Chief, Phytochemistry Section, Medicinal Plant Research Institute, Department of Medical Sciences

Yenchit **Techadamrongsin**, B.Sc., B.S. Phar. (2003-2004)

Thidarat **Boonruad**, B.Sc., M.Sc. in Pharm. (2003-2004)

Prapai **Wongsinkongman**, B.Sc. in Pharm., M.Sc. in Pharm., Ph.D., *Representative* (2003-2004)

Chief, Herbal Quality Assurance Center, Medicinal Plant Research Institute, Department of Medical Sciences

Thidarat **Boonruad**, B.Sc., M.Sc. in Pharm. (2004-2008)

Prapai **Wongsinkongman**, B.Sc. in Pharm., M.Sc. in Pharm., Ph.D. (2008-)

Puritat **Ratanasiri**, B.Sc. in Pharm., *Representative* (2004-2009)

Jiranuch **Mingmuang**, B. Pharm., M.Sc. in Pharm., *Representative* (2004-)

Apirak **Sakpetch**, B. Pharm., *Representative* (2004-2009)

Duangpen **Pattamadilok**, B.Sc. in Pharm., M.Sc. in Pharm., Ph.D., *Representative* (2010-)

Chief, Pharmacognosy Section, Medicinal Plant Research Institute, Department of Medical Sciences

Pranom **Dechwisissakul**, B.Sc., M.Sc. in Pharm. (2003-2010)

Pairin **Thongkhoom**, B.Sc., M.Sc. in Pharm. (2010-)

Thaweephol **Dechatiwongse Na Ayudhya**, B.Sc. in Pharm. (2003-2008)

Supatra **Imerb**, B.Sc. in Pharm., M.Sc. (Pharm. Chem.) (2009-)

Yupadee **Payakkapan**, B.Sc. in Pharm., M.Sc. in Pharmaceutical Analysis (2003-2008)

Taweesak **Suntorntanasat**, B.Sc. in Pharm., M.Sc. in Pharm.

Rapepol **Bavovada**, B.Sc. in Pharm., M.Sc. in Pharm., Ph.D. (2003-2006)

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ห้ามทำซ้ำ ดัดแปลง ใช้ในเชิงพาณิชย์ โดยไม่ได้รับอนุญาต

Thatree **Phadungcharoen**, B.Sc. in Pharm., M.Sc. in Pharm.
Jaree **Bansiddhi**, B.Sc., M.Sc. (2010-)
Khanit **Suwanborirux**, B.Sc. in Pharm., M.Sc. in Pharm., Ph.D.
Chayan **Picheansoonthon**, B.S. in Pharm., Ph.D., FRI
Yenchit **Techadamrongsin**, B.Sc., B.S. Phar. (2008-)
Kanokwan **Watanayothin**, B.Sc. (Agriculture), M.S. (Horticulture), Ph.D. (2003-2008)
Sawanee **Sathornviriyapong**, B.S. (Agriculture), M.S. (Horticulture), Ph.D.
Nantana **Sittichai**, B.Sc. in Pharm., M.S. (2008-)
Chitra **Chaiyawat**, B. Pharm. (2010-)

Secretaries:
Nantana **Sittichai**, B.Sc. in Pharm., M.S. (2003-2008)
Kornvika **Charupant**, B. Pharm., M.Sc. in Pharm., Ph.D. (2003, 2008-)
Chitra **Chaiyawat**, B. Pharm. (2003-2009)
Supattra **Phongsri**, B.Sc. (Pharm.) (2003-2004)
Sarinee **Lenapun**, B. Pharm., M.Sc. in Pharm. (2003-2008)
Sasiwimon **Patasema**, B. Pharm., M.Sc. in Pharm. (2009)
Jiranuch **Jamtaweekul**, B.Sc. in Pharm., M.Sc. in Pharm. (2010-)
Thanita **Patthamajinda**, B.S. in Pharm., M.A. (English for Specific Purposes) (2009-)

7. THE SUBCOMMITTEE ON BIOLOGICAL PRODUCTS

Chairperson:
Sumana **Vardhanabhuti**, B.Sc. in Pharm. M.Sc. in Pharm., M.P.H., Cert. in Immunol. (WHO)

Advisors:
Boonchua **Dhorranintra**, M.D., Dr. med., FRCP(T)
Punnee **Pitisuttithum**, M.B., B.S., DTM&H, FRCP(T) (2008-)

Members:
Director, Biological Product Control Division, The Government Pharmaceutical Organization
Chalerm **Achamana**, B.Sc. in Pharm. (2003-2004)
Sumalee **Prangpratanporn**, B.Sc. (Biology), M.Sc. (Biology) (2004-)
Director, Biological Product Institute, Department of Medical Sciences
Teeranart **Jivapaisarnpong**, B.Sc.
Teerapon **Kachacheewa**, B.Sc. *Representative*
Wichuda **Jariyapan**, B.S., M.S., *Representative*
Puntawit **Natakul**, D.V.M., *Representative*
Pisan **Pangjunan**, D.V.M., *Representative*
Wereyarmarst **Jaroenkunathum**, B.Sc (Nursing and Midwife), M.Sc (Biochemistry), *Representative*
Nongyao **Somdach**, B.Sc. (Biology), M.Sc.(Tropical Medicine), *Representative*
Saiwarul **Jadoonkittinan**, B.Sc. (Chemistry), M.Sc. (Chemistry), *Representative*
Apichai **Supasansatorn**, B.Sc. (Biology), M.Sc. (Tropical Medicine), *Representative*
Head, Biological Testing Section, Bureau of Drug and Narcotic
Daweesapya **Chaisomboonpan**, B.Sc. in Pharm.
Chantapong **Wasi**, M.D. (2006-)
Sumana **Khomvilai**, B.Sc. in Pharm.
Punnee **Pitisuttithum**, M.B., B.S., DTM&H, FRCP(T) (2005-2008)

ลิขสิทธิ์ กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข
ห้ามทำซ้ำ ดัดแปลง และใช้ในรูปแบบอื่นใดโดยไม่ได้รับอนุญาต

Yupadee **Payakkapan**, B.Sc. in Pharm., M.Sc. in Pharmaceutical Analysis
Aroonrat **Chantanakajornfung**, B.Sc. in Pharm., Hon. Ph.D. in Pharm. Sci. (biopharmacy)
Vimolmas **Lipipun**, B.Sc. in Pharm., M.Sc. in Pharm., Ph.D.
Rochapon **Wacharotayankun**, B.Sc. in Pharm., Ph.D. (2003-2007)
Dumrong **Thitikornkovit**, B.Sc. in Pharm.
Sirichai **Krabesri**, B.Sc. in Pharm., M. Pharm., LL.B., Barrister at Law (2008-)

Secretaries: Sirichai **Krabesri**, B.Sc. in Pharm., M. Pharm., LL.B., Barrister at Law (2003-2008)
Sasiwimon **Patasema**, B. Pharm., M.Sc. in Pharm.
Supattra **Phongsri**, B.Sc. (Pharm.) (2003)
Areeya **Disratthakit**, B.Sc. in Pharm (2003-2004)
Sarinee **Lenapun**, B. Pharm., M.Sc. in Pharm. (2004-2008)
Walailuk **Matapatara**, B.Sc. in Pharm., M.Sc. in Pharm., Ph.D. (2008-)
Sumanee **Lertkanokkun**, B.Sc. (Pharm.) (2008)
Thanita **Patthamajinda**, B.S. in Pharm., M.A. (English for Specific Purposes) (2009-)

8. THE SUBCOMMITTEE ON EDITORIAL STYLE

Chairperson: Sumana **Vardhanavhuti**, B.Sc. in Pharm. M.Sc. in Pharm., M.P.H., Cert. in Immunol. (WHO) (2003-2010)
Boonchua **Dhorranintra**, M.D., Dr. med., FRCP(T) (2010-)

Advisors: Nadhirat **Sangkawibha**, M.D., M.P.H. (deceased) (2004-2009)
Sumana **Vardhanavhuti**, B.Sc. in Pharm. M.Sc. in Pharm., M.P.H., Cert. in Immunol. (WHO) (2010-)
Manat **Pohmakotr**, B.Sc., M.Sc., Dr. rer. nat. (2005-)
Kamphol **Raksrivong**, B.Sc. in Pharm.
Rachanee **Pinthaworn**, B.Sc. in Pharm. (2003-2005, 2009-2010)

Members: Vichiara A. **Jirawongse**, B.Sc. in Pharm., Ph.D., Hon. D.Sc. in Pharm. (CU), Hon. Ph.D. (KKU) (deceased) (2003-2006)
Wantana **Ngamwat**, B.Sc., B.Sc. in Pharm., M.Sc.
Boonchua **Dhorranintra**, M.D., Dr. med., FRCP(T) (2003-2010)
Manat **Pohmakotr**, B.Sc., M.Sc., Dr. rer. nat. (2003-2005)
Rachanee **Pinthaworn**, B.Sc. in Pharm. (2005-2009)
M.L. Othong **Sawasdimongkol**, B.Sc. in Pharm.
Arunee **Poompanich**, B.Sc. in Pharm.
Rapepol **Bavovada**, B.Sc. in Pharm., M.Sc. in Pharm., Ph.D. (2006-2008)
Churairat **Rukwatin**, B.Sc. in Pharm. (2006-)
Suntana **Sutadarat**, B.Ed., M.A., Ph.D.
Nantana **Sittichai**, B.Sc. in Pharm., M.S. (2005-)
Nongluck **Sookvanichsilp**, B.Sc. in Pharm., M.Sc. in Pharm., Dr.Phm.Sc., LL.B., B.B.A. (2005-)
Supong **Akesiripong**, B. Pharm., Ph.D.
Parkpoom **Tengamnuay**, B. Pharm., Ph.D.
Nidapan **Ruangrittinon**, B.Sc. in Pharm., M.Sc. in Pharm. (2005-)
Kornvika **Charupant**, B.S. in Pharm., M.Sc. in Pharm., Ph.D. (2008-)
Sirichai **Krabesri**, B.Sc. in Pharm., M. Pharm., LL. B., Barrister at Law (2009-)

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ห้ามทำซ้ำ ดัดแปลง นำไปใช้ประโยชน์ทางการค้า โดยไม่ได้รับอนุญาต

Secretaries: Nantana **Sittichai**, B.Sc. in Pharm., M.S. (2003-2005)
Manas **Attawish**, B.S. (Pharm.)
Kornvika **Charupant**, B. Pharm., M.Sc. in Pharm., Ph.D. (2003)
Sasiwimon **Patasema**, B. Pharm., M.Sc. in Pharm.
Sarinee **Lenapun**, B. Pharm., M.Sc. in Pharm. (2005-)

9. THE SUBCOMMITTEE ON THE PHARMACOGNOSTIC AND BOTANIC SPECIFICATIONS OF THE THAI HERBAL MONOGRAPHS (2010-)

Chairperson: Chayan **Picheansoonthon**, B.S. in Pharm., Ph.D., FRI

Vice-chairperson: Thatree **Phadungcharoen**, B.Sc. in Pharm., M.Sc. in Pharm.

Advisors: Chirayupin **Chandraprasong**, B.Sc., M.Sc., Hon. Ph.D.
Kongkanda **Chayamarit**, B.Sc., M.Sc., D.Sc.

Members: Kalaya **Pharadai**, B.Sc. in Pharm., M.Eng.
Jaree **Bansiddhi**, B.Sc., M.Sc.
Sawanee **Sathornviriyapong**, B.S. (Agriculture), M.S. (Horticulture), Ph.D.
Pranom **Dechwisissakul**, B.Sc., M.Sc. in Pharm.
Jiranuch **Jamtaweekul**, B.Sc. in Pharm., M.Sc. in Pharm.

Secretaries: Kornvika **Charupant**, B. Pharm., M.Sc. in Pharm., Ph.D.
Thanita **Patthamajinda**, B.S. in Pharm., M.A. (English for Specific Purposes)

10. THE SUBCOMMITTEE ON THE PHYSICO-CHEMICAL SPECIFICATIONS AND SAFETY OF THE THAI HERBAL MONOGRAPHS (2010-)

Chairperson: Khanit **Suwanborirux**, B.Sc. in Pharm., M.Sc. in Pharm., Ph.D.

Vice-chairperson: Nantana **Sittichai**, B.Sc. in Pharm., M.S.

Advisor: Yupadee **Payakkapan**, B.Sc. in Pharm., M.Sc. in Pharmaceutical Analysis

Members: Taweesak **Suntorntanasat**, B.Sc. in Pharm., M.Sc. in Pharm.
Chada **Phisalapong**, B.Sc. in Pharm., M.Sc., Ph.D.
Chitra **Chaiyawat**, B. Pharm.

Secretaries: Kornvika **Charupant**, B. Pharm., M.Sc. in Pharm., Ph.D.
Santi **Nimnoi**, B.S. in Pharm.

11. THE AD-HOC SUBCOMMITTEE ON STATISCAL ANALYSIS OF THE RESULTS OF BIOLOGICAL ASSAYS AND TESTS (2007-)

Chairperson: Dechavudh **Nityasuddhi**, B.S.(Mathematics), M.S.(Biostatistics), M.Sc.(Medical Statistics), M.Sc.(Computer Sciences), Ph.D.(Statistics)

Advisor: Sumana **Vardhanavhuti**, B.Sc. in Pharm. M.Sc. in Pharm., M.P.H., Cert. in Immunol. (WHO)

Members: Yupadee **Payakkapan**, B.Sc. in Pharm., M.Sc. in Pharmaceutical Analysis
Wongdyan **Pandii**, B.Sc. (Public Health Nursing), M.Sc. (Biostatistics), M.Sc.(Public Health), Dr.P.H. (Epidemiology)

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ห้ามทำซ้ำ ดัดแปลง ใช้ในเชิงพาณิชย์ โดยไม่ได้รับอนุญาต

Arpapan **Tongboonrawd**, B.Sc. in Pharm.
Nantana **Sittichai**, B.Sc. in Pharm., M.S. (2009-)
Walapa **Tatong**, B.Sc. in Pharm., M.S., Ph.D. (2007-2010)
Sunee **Sangkiew**, B. Pharm. (2007-2009)
Poolsak **Phomsuwansiri**, B.Sc. in Pharm.

Secretaries: Kornvika **Charupant**, B. Pharm., M.Sc. in Pharm., Ph.D.
Amorn **Sahamethapat**, B. Pharm., M.Sc. in Pharm. (2007-2010)
Sasiwimon **Patasema**, B. Pharm., M.Sc. in Pharm.
Santi **Nimnoi**, B.S. in Pharm. (2010-)

12. THE AD-HOC SUBCOMMITTEE ON STANDARDS OF THE THAI HERBAL DRUG PREPARATIONS (2010-)

Chairperson: Yupadee **Payakkapan**, B.Sc. in Pharm., M.Sc. in Pharmaceutical Analysis

Members: Churairat **Rukwatin**, B.Sc. in Pharm.
Nantana **Sittichai**, B.Sc. in Pharm., M.S.
Nidapan **Ruangrittinon**, B.Sc. in Pharm., M.Sc. in Pharm.
Prapai **Wongsinkongman**, B.Sc. in Pharm., M.Sc. in Pharm., Ph.D.

Secretaries: Sirichai **Krabesri**, B.Sc. in Pharm., M. Pharm., LL. B., Barrister at Law
Sarinee **Lenapun**, B.Pharm., M.Sc. in Pharm.

Contributors: The following persons were engaged in the production of the Thai Pharmacopoeia and the Thai Herbal Pharmacopoeia:

Thipchuta **Bharnthong**, B.Sc., M.Sc. (Medical Technology)
Juthamas **Bhura**, B.Sc. in Pharm., M.Sc. in Pharm.
Chalermchoke **Boonchit**, B.F.A.
Nuchattra **Chansuwanit**, B.Sc., M.S.
Montri **Chiobamroongkiat**, B.Sc., D.V.M.
Supanee **Duangteraprecha**, B.Sc. in Pharm., M.Sc. in Pharm., Ph.D.
Pajaree **Inthachub**, B.Sc. (Biology), M.Sc. (Botany)
Jiranuch **Jamtaweekul**, B.Sc. in Pharm., M.Sc. in Pharm.
Sasima **Juntryam**, B.Sc. (Biology)
Tanwarat **Kajsongkram**, B.S., M.S. (Chemistry)
Supachai **Koonterm**, B.Sc. (Environmental Sciences)
Sirichai **Krabesri**, B.Sc. in Pharm., M. Pharm., LL.B., Barrister at Law
Sumanee **Lertakanokkun**, B.Sc. (Pharm.)
Pichitra **Maekprayoon**, B.Sc.
Tathaporn **Maison**, B.Sc.
Jiranuch **Mingmuang**, B. Pharm., M.Sc. in Pharm.
Piya **Mokkamul**, B.S. (Forestry)
Narumole **Mongkolchaipak**, B. Pharm., M.Sc. (Biochem.)
Duangpen **Pattamadilok**, B.Sc. in Pharm., M.Sc. in Pharm., Ph.D.
Wittaya **Pongamornkul**, B.Sc. (Agriculture), M.S. (Biology)
Ladda **Poolsawat**, B. Pharm.
Pattamawadee **Setakanna** (Biology), M.Sc. (Microbiology)
Sununta **Srisopon**, B. Pharm., M. Pharm. (Community Pharmacy)
Ananya **Supanvanich**, B.Sc.
Areerat **Surathanjanya**, B.S. (Agriculture Sciences), M.S. (Agriculture)

ลิขสิทธิ์ กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข
ห้ามทำซ้ำ ดัดแปลง หรือจำหน่ายโดยไม่ได้รับอนุญาต

Akekmol **Suttisun**, Cert. in Medical Science Technology
Parkpoom **Tengamnuay**, B. Pharm., Ph.D.
Thanawat **Thongchin**, B.S. (Chemistry)
Pairin **Thongkhoom**, B.Sc., M.Sc. in Pharm.
Rawat **Tiasakul**, B.Sc. (Pharm.)
Niran **Vipunngeun**, B.Sc. (Fishery Biology), M.Sc. (Biology)
Wimonwan **Warinpramote**, B. Pharm., M.Sc. St.
Sopidawan **Wichienkul**, B.S.

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ห้ามทำซ้ำ ดัดแปลง แก้ไข โดยไม่ได้รับอนุญาต

INTRODUCTION

Upon the first approval of the Ministerial Cabinet in 1979, the appointed Thai Pharmacopoeia Committee has since carried out its tasks to establish the Thai Pharmacopoeia with the following major objectives:

a. to establish official standards for pharmaceutical products imported, manufactured and sold in the country;

b. to develop more economical, yet reliable, methods of analysis independent of sophisticated and more expensive instruments;

c. to serve as an official compendium to be recognized and used;

d. for use as a reference handbook for physicians, pharmacists and those in the pharmaceutical profession.

Some changes are to be noted. The Thai Pharmacopoeia, second edition, or TP II, combines monographs of raw materials and their pharmaceutical preparations in a single publication for convenience.

To fulfill the aforementioned objectives, the criteria for drug selecting and preparations have been revised to the following:

1. As some identical pharmaceutical substances and preparations listed in the Pharmacopoeiae promulgated by Thai Minister of Public Health have different methods of analysis or standards, appropriate methods or their modifications are adopted to suit the Thai pharmaceutical industries.

2. Modern drugs which are not listed in the Pharmacopoeiae promulgated by Thai Minister of Public Health will be considered based on one or more of the following criteria:

2.1 Medicines must be registered in Thailand in at least two categories, original and generic. Capability of local manufacturers to produce the selected preparations is also considered.

2.2 Medicines listed in the Thai National List of Essential Medicines, WHO Model List of Essential Medicines as well as medicines used in hospitals with high consumption are of priority.

2.3 Certain essential medicines which are not listed in the Thai National List of Essential Medicines, such as life-saving drugs, orphan drugs and antineoplastic drugs are also taken into account.

2.4 For medicines in the same category, only those with high consumption or high import value will be selected.

2.5 For medicines in the same category and with similar mode of action, only the items with top-ranking consumption will be selected. At least one preparation from each class will be considered.

2.6 For medicines with various dosage forms, only suitable dosage forms will be selected.

3. Herbal drugs, both raw materials and their preparations following the policy of the Ministry of Public Health, are considered.

4. The selected medicines must have sufficient scientific evidence and supportive information to establish the monographs on such medicines.

5. Other pharmaceutical substances and preparations suggested by the Subcommittee on Drug Selection.

The appendices appeared in the Thai Pharmacopoeia II (2011) are only those referred to in the monographs; some appendices from TP I have been removed.

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DEDICATION

The Thai Pharmacopoeia Committee wishes to express its appreciation of the services of all those who have contributed to this important task and is indebted to the past activities of the followings:

1. Prof. Chamlong Suvagondha (Deceased), Director General, Department of Medical Sciences, Ministry of Public Health (1957-1969)
2. Prof. Dr. Komol Pengsritong (Deceased), Deputy Director General, Department of Medical Sciences (1958-1960); Permanent Secretary, Ministry of Public Health (1970-1974)
3. Prof. Dr. Prakorb Tuchinda (Deceased), Director General, Department of Medical Sciences (1969-1974); Permanent Secretary, Ministry of Public Health (1975-1982)
4. Prof. Dr. Vichiara A. Jirawongse (Deceased), Dean, Faculty of Pharmaceutical Sciences, Chulalongkorn University (1972-1973); Fellow (Pharmaceutical Sciences), Royal Institute (1981-2006)
5. Than Phuying Preeya Kashemsant Na Ayudhya, Director General, Department of Medical Sciences, Ministry of Public Health (1988-April 1991, October 1991-September 1992); Secretary General, The Food and Drug Administration, Ministry of Public Health (April 1991-September 1991)
6. Dr. Nadhirat Sangkawibha (Deceased), Director General, Department of Medical Sciences, Ministry of Public Health (1982-1985).

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ลิขสิทธิ์ กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข
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GENERAL NOTICES

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ลิขสิทธิ์ กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข
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The main objective of the Thai Pharmacopoeia is to establish the Thai National Standards of selected pharmaceutical substances and preparations. Apart from their standards and requirements set forth in the monographs, the non-mandatory information given includes their main classes of actions and applications (categories) and their safety (contra-indications, warnings, etc.). Subjected to other pertinent laws and ministerial regulations and notifications promulgated by Thai Minister of Public Health, the various standards of articles set up in this book, together with their pharmacopoeial requirements, are legally recognized by the Royal Thai Government.

The General Notices and general requirements appearing in appendices provide the basic guidelines for the interpretation and application of the standards, tests, assays, and other specifications of the Pharmacopoeia. Where exceptions to the General Notices or Appendices are made, the expressions such as "unless otherwise specified", "unless otherwise directed", or "unless otherwise indicated" are employed.

Title

The full title of this book, including its supplements, is the Pharmacopoeia of the Kingdom of Thailand, second edition. This title may be abbreviated to Thai Pharmacopoeia, second edition, or to TP II. Where the term TP is used, without further qualification, during the period in which this Pharmacopoeia is officially effective, it refers to TP II.

"Official", "Official Name" and "Official Standards"

The word "official", as used in this Pharmacopoeia or with reference hereto, is synonymous with "Pharmacopoeial", with "TP", and with "compendial".

The "official name" implies the "main title" of each Pharmacopoeial substance or preparation which is given in English and printed with capital letters. Any "other name" or "subsidiary title", where included, has the same status as the main title. It is mostly derived from the main title of that substance or preparation as it was used in the National List of Essential Medicine and the other recognized pharmacopoeiae such as the International Pharmacopoeia, the British Pharmacopoeia, and the United States Pharmacopeia. The spelling "sulph-" may be substituted for "sulf-" in the main title. Similarly, the spelling "aluminum" may also be substituted for "aluminium".

In a monograph of pharmaceutical preparations, "other name" or "subsidiary title" is nevertheless excluded.

The terminology "article" is an item for which a monograph is provided, whether a substance or a preparation.

The requirements stated in the monographs of the Pharmacopoeia apply to articles that are intended only for medicinal use. An article intended or labelled for medicinal use that is described by means of an official title must comply with the requirements of the relevant monograph. A formulated preparation must comply throughout its assigned shelf-life (period of validity). The subject of any other monograph must comply throughout its period of use.

Unless otherwise indicated in the General Notices or in the monographs, statements in monographs constitute mandatory requirements. The chemical formula and solubility statements are presented for informative purposes only and are not to be considered in the same category as the standards or tests for purity. The tests or methods in general appendices become mandatory when referred to in a monograph, unless such reference is made in a way to indicate that it is not the intention to make the text referred to mandatory but rather to cite it for information.

An article is not of pharmacopoeial quality unless it complies with all of the requirements stated. The general notice on Assays and Tests indicates that analytical methods other than those described in the Pharmacopoeia may be employed for routine purposes.

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Where a monograph on a biological substance or preparation refers to a strain, a test, a method, a substance, etc., using the qualifications "suitable" or "appropriate" without further definition in the text, the choice of such strain, test, method, substance, etc., is made in accordance with any international agreements or national regulations affecting the subject concerned.

The designation "TP" in conjunction with the official title or elsewhere on the label of article indicates that a monograph is included in the Thai Pharmacopoeia and that the article must comply with all applicable TP standards.

Printing Types

In the text, words which refer to reagents, which conform to the requirements specified in the appendices of other parts of the book, and the systematic names of plants, animals and micro-organisms are usually printed in italics or other types to distinguish them from the other words in that portion of the text.

Graphic Formulae, Molecular Formulae, Molecular Weights, CAS Registry Numbers, and Chemical Names

When the chemical composition of a pharmaceutical substance is known or generally accepted, the graphic and molecular formulae, the molecular weight, the Chemical Abstracts Service (CAS) registry number and the chemical name are normally given at the beginning of the monograph for the purpose of information. This information refers to chemically pure substances and is not to be regarded as an indication of the purity of the official drugs. Elsewhere, in statements of standards of purity and strength and in descriptions of processes of assay, it is evident from the context that the formulae denote chemically pure substances.

Consonant with the employment of Chemical Abstracts (CA) nomenclature, and also in the interest of uniformity of style, the orientation of ring systems and the depiction of stereoisomeric features in graphic formulae are generally consistent with CAS practices.

The molecular formulae and the molecular weights also generally conform to CAS practices.

The chemical names given in the monographs are those Index names used by the CAS of the American Chemical Society. It is the inverted form of the systemic chemical name developed by CAS, in general accordance with the rules established over the years by the International Union of Pure and Applied Chemistry (IUPAC) and the International Union of Biochemistry (IUB), and employed in the current issues of CA.

Monographs carrying chemical names generally carry also CAS registry numbers. These italicized numbers function, independently of nomenclature, as invariant numerical designators of unique unambiguous chemical substances in the CAS registry and thus find wide, convenient use.

Atomic Weights

The atomic weights used in computing molecular weights and factors in the assays and elsewhere are those recommended in 2005 by the International Commission on Atomic Weights.

Molecular Weights

The molecular weights of compounds (or where appropriate, atomic weights) are given at the beginning of the monographs. These molecular weights are calculated by adding together the atomic weights, or multiples thereof, using all the figures of the International Table of Atomic Weights; the total is then rounded off to two decimal places as follows:

a. If this first digit to be dropped is less than 5, the last digit retained is left unchanged.

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b. If the first digit to be dropped is greater than 5 or is 5 followed by digits more than zero, the last digit retained is increased by one.

c. If the first digit to be dropped is 5 followed by zeros, the last digit retained is left unchanged if even and is increased by one if odd.

d. In applying the foregoing rounding operations, all digits to be dropped are dropped in one operation.

The molecular weights shown in the monographs are theoretical values and neither these nor the molecular and graphic formulae constitute analytical standards for the substances described.

Significant Figures and Tolerances

Where limits are expressed numerically herein, the upper and lower limits of a range include the two values themselves and all intermediate values, but no values outside the limits. The limits expressed in monograph definitions and tests, regardless of whether the values are expressed as percentages or as absolute numbers, are considered significant to the last digit shown.

EQUIVALENCE STATEMENTS IN TITRIMETRIC PROCEDURES In the equivalence statement of titrimetric procedures, the number of significant figures in the concentration of the titrant corresponds to the number of significant figures in the weight of the analyte.

TOLERANCES The limits specified in the monographs for Pharmacopoeial articles are established with a view to the use of these articles as drugs, except where it is indicated otherwise. The use of the molecular formula for the active ingredient(s) named in defining the required strength of a Pharmacopoeial article is intended to designate the chemical entity or entities, as given in the complete chemical name of the article, having absolute (100 per cent) purity.

A dosage form shall be formulated with the intent to provide 100 per cent of the quantity of each ingredient declared on the label. The tolerances and limits stated in the Definitions in the monographs for Pharmacopoeial articles allow for analytical error, for unavoidable variations in manufacturing and compounding, and for deterioration to an extent considered acceptable under practical conditions. No further tolerances should be applied to the values obtained in an assay to determine whether the product being examined complies with the requirements of monograph.

The specified tolerances are based upon such attributes of quality as might be expected to characterize an article produced from suitable raw materials under recognized principles of good manufacturing practice.

INTERPRETATION OF REQUIREMENTS Analytical results in the laboratory (observed or calculated from experimental measurements) are compared with stated limits to determine whether there is conformance with compendial assay or test requirements. The observed or calculated values usually will contain more significant figures than there are in the stated limit, and a reportable result is to be rounded off to the number of places that is in agreement with the limit expression by the following procedure. Intermediate calculations (e.g., slope for linearity in validation of compendial method) may be rounded for reporting purposes, but the original value (not rounded) should be used for any additional required calculations. Rounding off should not be done until the final calculations for the reportable value have been completed. (**Note** Limits, which are fixed numbers, are not rounded off.) When rounding off is required, consider only one digit in the decimal place to the right of the last place in the limit expression. If this digit is smaller than 5, it is eliminated and the preceding digit is unchanged. If this digit is equal to 5 or more than 5, it is eliminated and the preceding digit is increased by one.

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Illustration of Rounding Numerical Values for Comparison with Requirements

Compendial Requirement	Unrounded Value	Rounded Result	Conforms
Assay limit ≥99.0 per cent	98.97 per cent	99.0 per cent	Yes
	98.93 per cent	98.9 per cent	No
	98.95 per cent	99.0 per cent	Yes
Assay limit ≤102.5 per cent	102.55 per cent	102.6 per cent	No
	102.54 per cent	102.5 per cent	Yes
	102.56 per cent	102.6 per cent	No
Limit test ≤0.03 per cent	0.035 per cent	0.04 per cent	No
	0.034 per cent	0.03 per cent	Yes
	0.037 per cent	0.04 per cent	No
Limit test ≤2 ppm	2.4 ppm	2 ppm	Yes
	2.5 ppm	3 ppm	No
	2.6 ppm	3 ppm	No

Limit of Content

When limits of content are prescribed in a monograph, they are determined by the method described under "Assay".

When the result of a test or assay is referred to the "dried", "anhydrous" or "ignited" substance, the determination of loss on drying, water content or loss on ignition is carried out by the method prescribed under the heading "Loss on drying", "Water" or "Loss on ignition", respectively, in the monograph concerned.

Units of Potency of Antibiotics

The "Unit" referred to in an individual monograph is the specific antimicrobial activity contained in such an amount of the respective antibiotic Reference Substance as the appropriate international or national organization may from time to time indicate. (The necessary information is usually provided with the samples of the Reference Substance).

Unless otherwise specified in the individual monographs, the activity assigned to each "Unit" or "μg" is equivalent to an "International Unit (IU)", "Unit" or "μg" used in other recognized pharmacopoeia.

Freshly and Recently Prepared

The direction that a preparation must be freshly prepared indicates that it must be made not more than 24 hours before it is issued for use. The direction that a preparation should be recently prepared indicates that deterioration is likely if the preparation is stored for longer than about 4 weeks at 15° to 25°.

Added Substances

The word "added substances" as used in this Pharmacopoeia or with reference hereto is synonymous with "auxiliary substances".

An official substance, as distinguished from an official preparation, contains no added substances except where specifically permitted in the individual monograph. Where such addition is permitted, the label indicates the name(s) and amount(s) of any added substance(s).

Unless otherwise specified in the individual monograph, or elsewhere in the General Notices, suitable substances such as antimicrobial agents, bases, carriers, coatings, colours, flavours, preservatives, stabilizers, and vehicles may be added to an official preparation to enhance its stability, usefulness, or elegance or to facilitate its preparation. Such substances are regarded as unsuitable and are prohibited unless (a) they are harmless in the amounts used, (b) they do not exceed the minimum quantity required to provide their intended effect, (c) their presence does not impair the bioavailability or the therapeutic

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efficacy or safety of the official preparation, and (d) they do not interfere with the assays and tests prescribed for determining compliance with the Pharmacopoeial standards.

Colouring Agents

Added substances employed solely to impart colour may be incorporated into official preparations, except those intended for parenteral or ophthalmic use, in accordance with the regulations pertaining to the use of colouring agents issued by the Thai Food and Drug Administration provided such added substances are otherwise appropriate in all respects.

Description

Information on the "description" pertaining to an article, which is relatively general in nature, is provided for those who use, prepare, and dispense drugs, solely to indicate the properties of an article complying with monograph standards. The properties are not in themselves standards or tests for purity even though they may indirectly assist in the preliminary evaluation of the integrity of an article.

In the case of crude drugs, the macroscopical description of a drug includes those features which can be seen by the unaided eye or by the use of a hand lens. The diagnostic characters given under a powdered crude drug are to be read in conjunction with the microscopical description given under the whole drug.

Statements of the distinctive microscopic structural elements in powdered crude drugs may be included in the individual monograph as a means of determining identity, quality, or purity.

Solubility

Statements given in the monographs under "Solubility" are intended as information on the approximate solubility only and are not regarded as part of the standard for a substance. Where no temperature is given, statements of solubility apply at ordinary room temperature.

Statements given under "Solubility test" express exact requirements and constitute part of the standards for the substances under which they occur.

When the term "parts" is used in expressing the solubility of a substance, it means parts by weight (grams) of a solid in parts by volume (millilitres) of the solvent, or parts by volume (millilitres) of a liquid in parts by volume (millilitres) of the solvent. The following table indicates the meanings of such terms:

Descriptive Term	Parts of Solvent Required for 1 Part of Solute
Very soluble	less than 1
Freely soluble	from 1 to 10
Soluble	from 10 to 30
Sparingly soluble	from 30 to 100
Slightly soluble	from 100 to 1000
Very slightly soluble	from 1000 to 10,000
Practically insoluble or insoluble	more than 10,000

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Safety of Drugs

Important information on drug hazards is only partially and concisely included in the safety sections of this Pharmacopoeia. This part of a monograph does not constitute standards. Selection of information is based on what is considered practical and clinically significant in the effective and safe use of a drug. It is intended to aid the professional in minimizing the risks and enhancing the benefits of its use. It should also be noted that there are still many other potential adverse reactions which have been omitted. Additional relevant information should therefore be obtained from other sources.

The risks of certain drug used during pregnancy are well recognized and the general principle is to give a drug only when the benefit to the individual mother outweighs the risk to the fetus. Caution should always be exercised in administering drugs during the first trimester of pregnancy. Unless the life of the patient is actually threatened by her morbidity, the desirability of postponing treatment until after delivery should be considered.

Where there is a clear risk, this is noted under the heading "Warning" or "Precaution", but safety should not be inferred from the absence of this statement for any drugs.

Although the results of certain studies, both *in vitro* and in certain experimental animals, have demonstrated that a drug does not produce any teratogenic, carcinogenic, nor mutagenic effect, it does not always imply that it could also be safely used without producing such adverse effects in patients. Cautions and care should be observed in this respect, especially during pregnancy, and should also be extended to the case of administering a drug to nursing mothers, where the potential risk of the presence of the drug in the mother's milk could arise.

An increasing number of drugs that may produce hypersensitivity in some patients have been recognized, and it is well established that a person with known hypersensitivity to one or more particular drugs should inform the physician and avoid using them. It is also well established that the hypersensitivity to the drug limits its use; it is, therefore, not stated under the heading "Contra-indication". When there is no other alternative to using a drug, the use of such a drug should be attempted with due careful consideration of its potential risk against its actual benefit. The possible risk of hypersensitivity is emphasized under the heading "Warning" or "Precaution" in certain drug monographs, but, in its absence, it should not be assumed that a drug does not carry such a potential hazard.

Category

A statement of Category usually is provided for each pharmacopoeial substance. This indicates the class of its main pharmacological action and its application. Where there are several main actions or applications, usually not more than the two most important are selected, with due consideration to the prevailing medical situation. No attempt has been made to describe in detail the actions and uses of a drug nor to specify its indications. The statement of Category is thus not intended to limit in any way the choice or use of the drug nor to indicate that it has no other activity or utility.

Strength(s) Available

Statements under the side-headings Strength or Strengths Available are included as a guide and are not necessarily comprehensive. For solid dosage forms such as Capsules and Tablets, the strength is given as the amount of active ingredient in each unit. For liquid dosage forms such as Injections and semi-solid dosage forms such as Creams, the strength is given as a concentration. For Powders for Injections, the strength is given as the amount of active ingredient in each sealed container. Unless otherwise stated, the strength is given in terms of the weight or concentration of the official medicinal substance used in making the formulation.

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Dose

The statements given under "Dose" in the monographs of the Pharmacopoeia are primarily intended to serve only as a guide to physicians or pharmacists who may vary it in the best interests of patients and in accordance with the variables that affect the action of the drug.

The specific terms used to classify the age of the patients are as follows:

- Adults: over 12 years of age
- Adolescents: 12 to 15 years of age
- Children: 1 to 12 years of age
- Infants: 5 weeks to 1 year of age
- Neonates: from newborn to 1 month of age

Unless otherwise specified, the route of administration is oral.

The statements of dosage in the case of Capsules and Tablets are expressed in terms of the content of active ingredient and seldom represent the total weight of the capsule contents or of the tablets.

In some instances, the dosage may be stated in terms of the pharmacologically active portion (moiety) of the molecule in order to permit the prescriber or dispenser to correlate the weight equivalent for salts, esters, or other chemical forms of the same drug moiety. However, it is not to be inferred that all chemical forms in which the active moiety may be presented are therapeutically equivalent.

Where the body surface area is stated as a basis for the specified dose, the "Table of Body Surface Area from Height and Weight (m^2)" in Appendix 1.17 may be employed to simplify the calculation of square metres of body surface.

The dose given in each monograph is that which may ordinarily be expected to produce in the patients with normal renal/hepatic function, following administration in the manner indicated, at such time intervals as may be stated, the diagnostic, therapeutic or prophylactic effect for which the monograph is recognized.

Contra-indication

This section specifies those conditions in which the drug should NOT be used.

Warning and Precaution

Warnings of the possible risk of certain hazards from the use of a drug are to be observed and taken care of before prescribing or administering it to a patient. Caution and careful consideration on the risk-benefit ratio of the drug should therefore be contemplated on an individual basis prior to the decision to use it.

On the other hand, important notes to be observed and carefully followed during and after the administration of a drug are described under the heading "Precaution".

Only the more important warnings and precautions are selected and included under the headings "Warning" and "Precaution" on the basis of their common or usual clinical significance to the population as a whole and it should not be assumed that the omission of a warning or a precaution in any particular monograph means that warning or precaution may not be of clinical significance for a specific patient.

Additional Information

The specific route of administration to be used for a particular drug, any pertinent personal observation or care on the part of the patient, and other special relevant information concerning an individual drug are to be grouped under the heading "Additional information".

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Packaging and Storage

The substances and preparations described in the Pharmacopoeia are stored in such a way as to prevent contamination and, as far as possible, deterioration. Precautions that should be taken in relation to the effects of the atmosphere, moisture, heat, and light are indicated, where appropriate, in the monographs.

CONTAINERS

The container is the device that holds the substance, either in the form of the raw material or of the finished dosage form. The closure of the container, including the stopper, the cap, the attached dropper, etc., is considered as a part of the container.

The *immediate container* is the one which is in direct contact with the substance.

The container should be cleaned before use, and no extraneous matter should be introduced into it or into the substance placed in it. It must, likewise, not interact physically or chemically with the substance which it holds so as to alter the latter's quality, purity, or therapeutic potency to a level below its Pharmacopoeial requirements.

1. Well-closed container

A well-closed container must protect the contents from extraneous matter or from loss of the substance under ordinary or customary conditions of handling, shipment, storage, or sale.

2. Tightly closed container

A tightly closed container must protect the contents from contamination by extraneous matter or moisture, from loss of the substance, and from efflorescence, deliquescence, or evaporation under the ordinary or customary conditions of handling, shipment, storage, or sale, and shall be capable of tight reclosure. Where a tightly closed container is specified, it may be replaced by a hermetically closed container for a single-dose of the substance.

3. Hermetically closed container

A hermetically closed container must be impervious to air or any other gas under the ordinary or customary conditions of handling, shipment, storage, or sale.

4. Light-resistant container

A light-resistant container is the one which prevents transmission of light, such as an opaque container or a bottle of black, dark red or dark brown glass.

5. Single-unit container

A single-unit container is one that is designed to hold a quantity of drug product intended for administration as a single dose or a single finished device intended for use promptly after the container is opened. Preferably, the immediate container and/or the outer container or protective packaging shall be so designed as to show evidence of any tampering with the contents. Each single-unit container shall be labelled to indicate the identity, quantity and/or strength, name of the manufacturer, lot number, and expiration date of the article.

6. Single-dose container

A single-dose container is a single-unit container for articles intended for parenteral administration only a single-dose container is labelled as such. Examples of single-dose containers include pre-filled syringes, cartridges, fusion-sealed containers, and closure-sealed containers when so labelled.

7. Unit-dose container

A unit-dose container is a single-unit container for articles intended for administration by other than the parenteral route as a single dose, direct from the container.

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8. Multiple-unit container

A multiple-unit container is a container that permits withdrawal of successive portions of the contents without changing the strength, quality, or purity of the remaining portion.

9. Multiple-dose container

A multiple-dose container is a multiple-unit container for articles intended for parenteral administration only.

10. Tamper-evident container

A tamper-evident container is a closed container fitted with a device that reveals irreversibly whether the container has been opened.

STORAGE

The following expressions are used in monographs under Packaging and storage with the meaning shown.

Protected from light means that the product is to be stored either in a light-resistant container or in a container enclosed in an outer cover that provides such protection or stored in a place from which all such light is excluded.

Protected from moisture means that the product is to be stored in a tightly closed container. Care is to be taken when the container is opened in a damp atmosphere. A low moisture content may be maintained, if necessary, by the use of a desiccant in the container provided that direct contact with the product is avoided.

Protected from freezing Where, in addition to the risk of breakage of the container, freezing subjects an article to loss of strength or potency, or to destructive alteration of its characteristics, the container label bears an appropriate instruction to protect the article from freezing.

STORAGE TEMPERATURES

When special conditions of storage are necessary, including limits of temperature, they are prescribed in the monograph. Where, in a monograph, the storage conditions are mentioned using the general expressions "at room temperature", "in a cold place", and the like, these terms are generally defined as follows.

Freezing temperature Any temperature not higher than –10°. An article for which storage at freezing temperature is directed may, alternatively, be stored in a freezer, unless otherwise specified in the individual monograph.

Very cold temperature Any temperature above –10° but not higher than 8°. A *refrigerator* is a very cold place in which the temperature is maintained thermostatically between 2° and 8°.

Cold temperature Any temperature above 8° but not higher than 16°.

Cool temperature Any temperature above 16° but not higher than 23°.

Room temperature Any temperature above 23° but not higher than 35°.

Controlled room temperature A temperature maintained thermostatically (30°±2°) that encompasses the usual and customary working environment of 23° to 35°; that results in a mean kinetic temperature calculated to be 30°.

Hot temperature Any temperature above 35° but not higher than 40°.

Very hot temperature or Excessive heat Any temperature higher than 40°.

STORAGE UNDER NONSPECIFIC CONDITIONS

For articles, regardless of quantity, where no specific storage directions or limitations are provided in the individual monograph, it is to be understood that conditions of storage and distribution include protection from moisture, freezing and excessive heat.

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Labelling

Every article in this Pharmacopoeia shall bear one or more labels indicating its identity and certain pertinent characteristics. This label(s) shall be affixed or firmly fastened to, or indelibly written on, the article's immediate container and, if any, its package. The label shall comply, in addition to the requirements prescribed in the Pharmacopoeia, with those labelling requirements currently promulgated by the Thai Ministry of Public Health. In cases where contradiction might arise between these requirements the latter bears more legal authority.

A *shipping* container, or a shipping package, unless such container or package is also essentially the immediate container or the outside of the consumer package, is exempt from the labelling requirements of this Pharmacopoeia.

Labelling requirements, also, do not necessarily apply when the article is supplied in compliance with a medical prescription.

The label on the container and the label on the package of every official article state:

(1) the name (commercial and/or generic) of the article, together with its dosage form;

(2) the name and address of the manufacturer;

(3) amount(s) of active ingredient(s) per dosage unit (capsule, tablet, or other unit dosage form), per gram (or 100 grams), per millilitre (or 100 millilitres), or per other measurement, as the case may be;

(4) total amount of the article in the immediate container;

(5) batch or lot, or quality control, number or code of the product;

(6) the expiration date;

(7) where applicable, the name(s) of any antimicrobial preservative(s);

(8) other statements specifically required under "Labelling" in that article's monograph.

Where an expiration date is required to be stated on the label, this date identifies the time during which the article may be expected to meet the requirements of the Pharmacopoeial monograph, provided it is kept under the prescribed storage conditions. The expiration date limits the time during which the product may be dispensed or used. Where an expiration date is stated only in terms of the month and the year, it is a representation that the intended expiration date is the last day of the stated month. For articles requiring constitution prior to use, a suitable beyond-use date for the constituted product shall be identified on the label. The beyond-use date placed on the label shall not be later than the expiration date on the manufacturer's container.

Identification

Identification tests are provided for the verification of the identity of the substance described in the monograph. A judgment by the analyst is needed as to the extent of testing required, taking into account the available instrumentation.

It is generally recognized that the infrared spectrum provides the best method of identification because of the uniqueness of a well-developed "finger print" region of the spectrum for a given drug substance.

Wherever possible, infrared spectrum characteristics are used as the primary test of identification. Usually this can stand by itself without any additional test. However, where the article is a salt, it is necessary to include a "specific ion test". Further identification tests provided in an individual monograph, taken together, are intended to provide verification of identity, should the use of an infrared spectrophotometer be precluded. Such identification tests, however specific, are not necessarily sufficient to establish proof of identity; but failure of an article taken from a labelled container to meet the requirements of a prescribed identification test indicates that the article may be mislabelled. Other tests and specifications in the monograph often contribute to establishing or confirming the identity of the article under examination.

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It should further be noted that whenever a melting temperature is provided under the heading "Identification", an approximate value is usually given, since no exact reproduction of the quoted temperature is necessary.

Water and Loss on Drying

Where the water of hydration or adsorbed water of a Pharmacopoeial article is determined by the titrimetric method, the test is generally given under the heading "Water". Where the determination is made by drying under specified conditions, the test is generally given under the heading "Loss on drying". However, "Loss on drying" is most often given as the heading where the loss in weight is known to represent residual volatile constituents including organic solvents as well as water.

Impurities

Inorganic, organic, biochemical, isomeric, or polymeric components can all be considered impurities.

Related substances Related substances are known impurities which may be identified or unidentified. These substances are structurally related to a drug substance. They include intermediates and by-products from a synthetically produced organic substance, co-extracted substances from a natural product and degradation products of the substance. The residual organic solvents, water, inorganic impurities, residues from cells and micro-organisms or culture media used in a fermentation process are excluded from this definition.

Ordinary impurities Ordinary impurities are innocuous because they have no significant, undesirable biological activity in the amounts present. They may arise from the synthesis, preparation, or degradation of the substance.

Tests for *related substances* or *chromatographic purity* may control the presence of ordinary impurities.

Tests for the presence of impurities are provided to limit such impurities to amounts that are unobjectionable under conditions in which the medicinal agent is customarily employed. The quantity is expressed in parts per million (ppm) weight in weight where the quantity is not greater than 100 ppm, and as a percentage in the case of quantities greater than 100 ppm. These quantities are approximations for comparative tests. Acceptance or rejection is determined on the basis of compliance or non-compliance with the stated test.

Assays and Tests

The Assays and Tests described are the official methods upon which the standards of the Pharmacopoeia depend. Assay and test procedures are provided for determining compliance with the Pharmacopoeial standards of identity, strength, quality and purity. The analysts are not precluded from employing alternative methods, including methods of micro-analysis, in any Assay or Test if they are satisfied that the method which they use will give the results of equivalent accuracy. In the event of doubt or dispute, the methods of analysis described in the Pharmacopoeia alone are authoritative.

When the solvent used for a solution is not named, the solvent is Purified Water.

The use of a proprietary designation to identify a material used in an Assay or Test does not imply that another equally suitable material may not be used.

APPARATUS

A specification for a definite size or type of container or apparatus in a test or assay is given solely as a recommendation. Where volumetric flasks or other exact measuring, weighing, or sorting devices are specified, this or other equipment of at least equivalent accuracy shall be employed. Where low-actinic or light-resistant containers are specified, clear containers that have been rendered opaque by application of a suitable coating or wrapping may be used.

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Where an instrument for physical measurement, such as a spectrophotometer, is specified in a test or assay by its distinctive name, another instrument of equivalent or greater sensitivity and accuracy may be used. In order to obtain solutions having concentrations that are adaptable to the working range of the instrument being used, solutions of proportionately higher or lower concentrations may be prepared according to the solvents and proportions thereof that are specified for the procedure. Where a particular brand or source of a material, instrument, or piece of equipment, or the name and address of a manufacturer or distributor, is mentioned, this identification is furnished solely for informational purposes as a matter of convenience, without implication of approval, endorsement, or certification. Items capable of equal or better performance may be used if these characteristics have been validated.

Where the use of a centrifuge is indicated, unless otherwise specified, the directions are predicated upon the use of apparatus having an effective radius of about 20 cm (8 inches) and driven at a speed sufficient to clarify the supernatant layer within 15 minutes.

Unless otherwise specified, for chromatographic tubes and columns the diameter specified refers to internal diameter; for other types of tubes and tubing the diameter specified refers to outside diameter.

While one of the primary objectives of the Pharmacopoeia is to assure the user of official articles of their identity, strength, quality, and purity, it is manifestly impossible to include in each monograph a test for every impurity, contaminant, or adulterant that might be present, including microbial contamination. These may arise from a change in the source of material or from a change in the processing, or may be introduced from extraneous sources. Tests suitable for detecting such occurrences, the presence of which is inconsistent with applicable good manufacturing practice or good pharmaceutical practice, should be employed in addition to the tests provided in the individual monograph.

PROCEDURE

In performing the assay or test procedures in this Pharmacopoeia, it is expected that safe laboratory practices will be followed. This includes the use of precautionary measures, protective equipment, and work practices consistent with the chemicals and procedures used. Prior to undertaking any assay or procedure described in this Pharmacopoeia, the individual should be aware of the hazards associated with the chemicals and the procedures and means of protecting against them. This Pharmacopoeia is not designed to describe such hazards or protective measures.

Automated procedures employing the same basic chemistry as those assay and test procedures given in the monograph are recognized as being equivalent in their suitability for determining compliance. Conversely, where an automated procedure is given in the monograph, manual procedures employing the same basic chemistry are recognized as being equivalent in their suitability for determining compliance.

In the performance of assay or test procedures, not less than the specified number of dosage units should be taken for analysis. Proportionately larger or smaller quantities than the specified weights and volumes of assay or test substances and Reference Standards may be taken, provided the measurement is made with at least equivalent accuracy and provided that any subsequent steps, such as dilutions, are adjusted accordingly to yield concentrations equivalent to those specified and are made in such manner as to provide at least equivalent accuracy. To minimize environmental impact or contact with hazardous materials, apparatus and chemicals specified in Pharmacopoeial procedures also may be proportionally changed.

Where it is directed in an assay or a test that a certain quantity of substance or a counted number of dosage units is to be examined, the specified quantity or number is a minimal figure (the singlet determination) chosen only for convenience of analytical manipulation; it is not intended to restrict the total

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quantity of substance or number of units that may be subjected to the assay or test or that should be tested in accordance with good manufacturing practices.

Where it is directed in the assay of Tablets to "weigh and finely powder not less than" a given number, usually 20, of the Tablets, it is intended that a counted number of Tablets shall be weighed and reduced to a powder. The portion of the powdered tablets taken for assay is representative of the whole Tablets and is, in turn, weighed accurately. The result of the assay is then related to the amount of active ingredient per Tablet by multiplying this result by the average Tablet weight and dividing by the weight of the portion taken for the assay.

Similarly, where it is directed in the assay of Capsules to remove, as completely as possible, the contents of not less than a given number, usually 20, of the Capsules, it is intended that a counted number of Capsules should be carefully opened and the contents quantitatively removed, combined, mixed, and weighed accurately. The portion of mixed Capsules contents taken for the assay is representative of the contents of the Capsules and is, in turn, weighed accurately. The result of the assay is then related to the amount of active ingredient per Capsule by multiplying this result by the average weight of Capsule content and dividing by the weight of the portion taken for the assay.

Where the definition in a monograph states the tolerances as being "calculated on the dried (or anhydrous or ignited) basis," the directions for drying or igniting the sample prior to assaying are generally omitted from the *Assay* procedure. Assay and test procedures may be performed on the undried or unignited substance and the results calculated on the dried, anhydrous, or ignited basis, provided a test for Loss on drying, or Water, or Loss on ignition, respectively, is given in the monograph. Results are calculated on an "as-is" basis unless otherwise specified in the monograph. Where the presence of moisture or other volatile material may interfere with the procedure, previous drying of the substance is specified in the individual monograph and is obligatory.

Throughout a monograph that includes a test for Loss on drying or Water, the expression "previously dried" without qualification signifies that the substance is to be dried as directed under Loss on drying or Water (gravimetric determination).

In stating the appropriate quantities to be taken for assays and tests, the use of the word "about" indicates a quantity within 10 per cent of the specified weight or volume. However, the weight or volume taken is accurately determined, and the calculated result is based upon the exact amount taken. The same tolerance applies to specified dimensions.

Where the use of a pipette is directed for measuring a specimen or an aliquot in conducting a test or an assay, the pipette conforms to the standards set forth under "Volumetric Apparatus" (Appendix 1.9), and is to be used in such manner that the error does not exceed the limit stated for a pipette of its size. Where a pipette is specified, a suitable burette, conforming to the standards set forth under "Volumetric Apparatus" (Appendix 1.9), may be substituted. Where a "to contain" pipette is specified, a suitable volumetric flask may be substituted.

Expressions such as "25.0 ml" and "25.0 mg," used with respect to volumetric or gravimetric measurements, indicate that the quantity is to be "accurately measured" or "accurately weighed" within the limits stated under "Volumetric Apparatus" (Appendix 1.9) or under "Weights and Balances" (Appendix 1.10).

The term "transfer" is used generally to specify a quantitative manipulation.

The term "concomitantly," used in such expressions as "concomitantly determine" or "concomitantly measured," in directions for assays and tests, is intended to denote that the determinations or measurements are to be performed in immediate succession.

Blank Determination Where it is directed that "any necessary correction" be made by a blank determination, the determination is to be conducted using

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the same quantities of the same reagents treated in the same manner as the solution or mixture containing the portion of the substance under assay or test, but with the substance itself omitted.

Desiccator The expression "in a desiccator" specifies the use of a tightly closed container of suitable size and design that maintains an atmosphere of low moisture content by means of self-indicating silica gel or other suitable desiccant.

Drying to Constant Weight The specification "dried to constant weight" means that the drying shall be continued until two consecutive weighings do not differ by more than 0.5 mg per g of substance taken, (2.5 mg per g in case of crude drug), the second weighing following an additional hour of drying at the prescribed conditions.

Ethanol The term "ethanol" used without other indication means ethanol 95 per cent v/v. Where other strengths are intended, the term "ethanol" is used followed by the statement of the strength.

Filtration Where it is directed to "filter", without further qualification, the intent is that the liquid be filtered through suitable filter paper or equivalent device until the filtrate is clear.

Ignition to Constant Weight The specification "ignite to constant weight" means that the ignition shall be continued until two consecutive weighings do not differ by more than 0.5 mg per g of substance taken, the second weighing following an additional 15-minute ignition period.

Indicators Unless otherwise directed, where starch is specified in a test or an assay as an indicator, 3 ml of *starch TS* shall be used; in cases of the other test solutions, approximately 0.2 ml, or 4 drops, of the specified solution shall be added.

Logarithms Logarithms used in this Pharmacopoeia are to the base 10 and are denoted by the abbreviation "log". Natural logarithms, if used, are denoted by the abbreviation "ln".

Negligible This term indicates a quantity not exceeding 0.5 mg.

Odour A suitable quantity is left open to the air in an evaporating dish for 15 minutes, after which any odour is defined. An odour designation is descriptive only and is not to be regarded as a standard of purity for a particular lot of an article, except in those cases where a particular odour is specifically prohibited in the individual monograph.

Percentage Expressions Percentage concentrations are expressed as follows:

Per cent weight in weight (w/w) expresses the number of g of a constituent in 100 g of solution or mixture.

Per cent weight in volume (w/v) expresses the number of g of a constituent in 100 ml of solution, and is used regardless of whether water or another liquid is the solvent.

Per cent volume in volume (v/v) expresses the number of ml of a constituent in 100 ml of solution.

The term "per cent" used without qualification means, for mixtures of solids and semisolids, per cent weight in weight; for solutions or suspensions of solids in liquids, per cent weight in volume; for solutions of liquids in liquids, per cent volume in volume; and for solutions of gases in liquids, per cent weight in volume. For example, a 1 per cent solution is prepared by dissolving 1 g of a solid or semisolid, or 1 ml of a liquid, in sufficient solvent to make 100 ml of the solution.

In the dispensing of prescription medications, slight changes in volume owing to variations in room temperatures may be disregarded.

Pressure Pressure is expressed in kPa.

- 1 kPa is 7.5006 Torr.
- 1 Torr is the pressure exerted by a column 1 mm of mercury.

Reagents The proper conduct of the analytical procedures described in the Pharmacopoeia and the reliability of the results depend, in part, upon the quality of the reagents used. The reagents, including the indicators and solu-

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tions required for the Assay and Tests of the Pharmacopoeia, are defined in the Appendices.

Reference Standards Reference Standards are authentic specimens that have been verified for suitability for use as comparison standards in Pharmacopoeial tests and assays. (See "Reference Substances", Appendix 1.8).

Where a Reference Substance is referred to in an assay or a test in this pharmacopoeia, the words "Reference Substance" are abbreviated to "RS".

Where a test or an assay calls for the use of a Pharmacopoeial article rather than a Reference Substance, as a material standard of reference, a substance meeting all of the requirements of the monograph for that article is to be used.

Any information necessary for proper use of the reference substance or reference preparation is given on the label or in the accompanying leaflet or brochure. Where no drying conditions are stated in the leaflet or on the label, the substance is to be used as received. The stability of the contents of opened containers cannot be guaranteed.

Solutions Unless otherwise specified in the individual monograph, all solutions called for in tests and assays are prepared with *water*.

An expression such as "(1 in 10)" means that 1 part *by volume* of a liquid is to be diluted with, or 1 part *by weight* of a solid is to be dissolved in, sufficient amount of the diluent or solvent to make the volume of the finished solution 10 parts *by volume*.

An expression such as "(20:5:2)" means that the respective numbers of parts, by volume, of the designated liquids are to be mixed, unless otherwise indicated.

Steam-Bath Where the use of a steam-bath is directed, exposure to actively flowing steam or to another form of regulated heat, corresponding in temperature to that of flowing steam, may be used.

Temperatures Unless otherwise specified, all temperatures in this Pharmacopoeia are expressed in Celsius degrees, and all measurements are made at 25°. Where "room temperature" is stated, a temperature from 20° to 30° is intended. Absolute temperatures are expressed in Kelvin degrees; the absolute temperature is related to a Celsius temperature by the equation °K = °C + 273.16.

Vacuum The term "in vacuum" denotes exposure to a pressure not exceeding 0.7 kPa (about 5 Torr), unless otherwise indicated.

Water Where the use of *water* is specified or implied in identifications, tests and assays, for the preparation of reagents, or as a diluent in any of these circumstances, water complying with the requirements of the monograph on Purified Water is used. The term "distilled water" indicates purified water prepared by distillation.

Water-Bath Where the use of a water-bath is directed without qualification with respect to temperature, a bath of vigorously boiling water is intended.

Weights and Measures The metric system of weights and measures is employed in the Pharmacopoeia; the International System of Units (SI) has been adopted wherever practicable. Unless otherwise stated, all measurements involved in the analytical operations of the Pharmacopoeia are intended to be made at 25°.

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MONOGRAPHS

PHARMACEUTICAL SUBSTANCES AND PREPARATIONS

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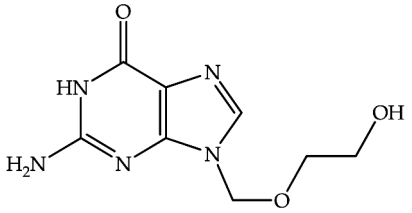


$C_8H_{11}N_5O_3$ 225.20 *59277-89-3*

6*H*-Purin-6-one, 2-amino-1,9-dihydro-9-[(2-hydroxyethoxy)methyl]-.

Category Antiviral.

Acyclovir contains not less than 98.0 per cent and not more than 101.0 per cent of $C_8H_{11}N_5O_3$, calculated on the anhydrous basis.

Description White to off-white, crystalline powder.

Solubility Slightly soluble in *water*; soluble in *dilute hydrochloric acid*; insoluble in *ethanol*.

Warning

1. Dose reduction may be required in acute or chronic renal impairment.
2. It may cause renal impairment, increased serum bilirubin and liver enzymes, gastro-intestinal disturbance, neuropsychiatric toxicity and hair loss.
3. Concurrent use with nephrotoxic medications may increase the potential for nephrotoxicity, especially in the presence of renal function impairment.
4. Risk-benefit should be considered if it is to be used in pregnant or nursing women.

Precaution Periodic determinations of blood urea nitrogen and serum creatinine are recommended.

Additional information

1. Acyclovir tablets may be taken with meals since absorption has not been shown to be significantly affected by food; however, they may be taken on an empty stomach.
2. Intermittent short-term treatment of recurrent herpes genitalis infections may be effective for some patients, especially when treatment is patient-initiated during the prodrome or first sign of lesion formation.
3. Patients allergic to ganciclovir may also be allergic to acyclovir because of the chemical similarity of the two medications.
4. Use of acyclovir has not been shown to prevent the transmission of herpes simplex virus to sexual partners.
5. Herpes genitalis may be sexually transmitted even if the partner is asymptomatic.

Packaging and storage Acyclovir shall be kept in tightly closed containers.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Acyclovir RS (Appendix 2.1) or with the reference spectrum of Acyclovir.

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

Water Not more than 6.0 per cent w/w (Karl Fischer Method, Appendix 4.12).

Limit for guanine Not more than 0.7 per cent w/w, using the chromatogram from the *Assay*. Calculate the quantity, in µg, of guanine in the portion of Acyclovir taken from the peak corresponding to the peak of Guanine standard preparation.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Prepare a 0.1 per cent v/v solution of *glacial acetic acid*. Make adjustments if necessary.

System suitability solution 1 Dissolve accurately weighed quantities of Acyclovir RS and *guanine* in 0.1 M *sodium hydroxide*, and dilute quantitatively, and stepwise if necessary, with *water* to obtain a solution having known concentrations of about 100 µg of each per ml.

System suitability solution 2 Dissolve an accurately weighed quantity of *guanine* in 0.1 M *sodium hydroxide*, and dilute quantitatively, and stepwise if necessary, with *water* to obtain a solution having a known concentration of about 0.7 µg per ml.

Guanine standard preparation Transfer about 8.75 mg of *guanine*, accurately weighed, to a 500-ml volumetric flask. Dissolve in 50 ml of 0.1 M *sodium hydroxide*, dilute with *water* to volume, and mix. Transfer 2.0 ml of this solution to a 50-ml volumetric flask, dilute with 0.01 M *sodium hydroxide* to volume, and mix to obtain a solution having a known concentration of about 0.7 µg per ml.

Standard preparation Dissolve about 25 mg of Acyclovir RS, accurately weighed, in 5 ml of 0.1 M *sodium hydroxide* in a 50-ml volumetric flask, dilute with *water* to volume, and mix. Transfer 10.0 ml of this solution to a 50-ml volumetric flask, dilute with 0.01 M *sodium hydroxide* to volume, and mix to obtain a solution having a known concentration of about 100 µg of Acyclovir RS per ml.

Assay preparation Dissolve about 100 mg of Acyclovir, accurately weighed, in 20 ml of 0.1 M *sodium*

hydroxide in a 200-ml volumetric flask, dilute with *water* to volume, and mix. Transfer 10.0 ml of this solution to a 50-ml volumetric flask, dilute with 0.01 M *sodium hydroxide* to volume, and mix.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (25 cm × 4.6 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles, (b) *Mobile phase* at a flow rate of about 3 ml per minute, and (c) an ultraviolet photometer set at 254 nm.

To determine the suitability of the chromatographic system, chromatograph *System suitability solution 1*, *System suitability solution 2*, and record the peak responses as directed under *Procedure*: the resolution factor between acyclovir and guanine is not less than 2.0, the symmetry factor for the analyte peak is not more than 2, and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Procedure Separately inject equal volumes (about 20 µl) of *Standard preparation*, *Guanine standard preparation* and *Assay preparation* into the chromatograph, record the chromatograms, and measure the responses for all the peaks.

Calculation Calculate the content of $C_8H_{11}N_5O_3$ in the Acyclovir taken, using the declared content of $C_8H_{11}N_5O_3$ in Acyclovir RS.

ACYCLOVIR TABLETS

Category Antiviral.

Acyclovir Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the labelled amount of $C_8H_{11}N_5O_3$.

Strengths available 200, 400 and 800 mg.

Dose Adults—

Herpes simplex, treatment: 200 mg (400 mg in the immunocompromised or if absorption impaired) five times daily, usually for 5 days.

Genital herpes infection, initial episode: 200 mg every 4 hours while awake, five times a day, for 10 days.

Recurrent infections, intermittent therapy (<6 episodes per year): 200 mg every 4 hours while awake, five times a day, for 5 days.

Recurrent infections, chronic suppressive therapy (≥6 episodes per year): 400 mg twice a day, or 200 mg three to five times a day, for up to 12 months.

Herpes zoster (shingles): 800 mg every 4 hours while awake, five times a day, for 7 to 10 days.

Varicella (chickenpox): 800 mg four times a day for 5 days. Treatment should be initiated at the earliest sign or symptom of chickenpox.

(**Note** Adults with acute or chronic renal impairment may require a reduction in dose as indicated in the

Normal Dosing Regimen	Creatinine (ml/min)	Clearance (ml/sec)	Adjusted Dosing Regimen
200 mg every 4 hours, five times daily while awake	>10	0.17	200 mg every 4 hours, five times daily while awake
	0 to 10	0 to 0.17	200 mg every 12 hours
400 mg every 12 hours	>10	0.17	400 mg every 12 hours
	0 to 10	0 to 0.17	200 mg every 12 hours
800 mg every 4 hours, five times daily while awake	>25	0.42	800 mg every 4 hours, five times daily while awake
	10 to 25	0.17 to 0.42	800 mg every 8 hours
	0 to 10	0 to 0.17	800 mg every 12 hours

ลิขสิทธิ์ กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข
ห้ามทำซ้ำ ดัดแปลง แก้ไข โดยมิได้รับอนุญาต

table.) Children 2 years of age and over—Varicella (chickenpox): 20 mg per kg of body weight, up to 800 mg per dose, four times a day for 5 days. Treatment should be initiated at the earliest sign or symptom of chickenpox.

Warning; Precaution; Additional information See under *Acyclovir*, p. 21.

Packaging and storage Acyclovir Tablets shall be kept in tightly closed containers, protected from light, and stored at a temperature not exceeding 25°.

Identification The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

Related substances Not more than 2.0 per cent w/w of guanine and not more than 0.5 per cent w/w of any other impurities, using the chromatograms from the *Assay*. Calculate the quantity, in µg, of guanine in the portion of Acyclovir Tablets taken from the peak corresponding to the peak of Guanine standard preparation and calculate the percentage of other impurities by the normalization procedure.

Dissolution Carry out the test as described in the "Dissolution Test" (Appendix 4.24).

Dissolution medium: 0.1 M *hydrochloric acid*; 900 ml.

Apparatus 2: 50 rpm.

Time: 45 minutes.

Procedure Determine the amount of $C_8H_{11}N_5O_3$ dissolved from absorbances at the maximum at about 254 nm of filtered portions of the test solution, suitably diluted with *Dissolution medium*, if necessary, in comparison with a standard solution having a known concentration of Acyclovir RS in the same medium (Appendix 2.2).

Tolerances Not less than 80 per cent (*Q*) of the labelled amount of $C_8H_{11}N_5O_3$ is dissolved in 45 minutes.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase, System suitability solution 1 and **Standard preparation** Prepare as directed in the Assay under *Acyclovir*, p. 21.

Guanine standard preparation Dissolve an accurately weighed quantity of guanine in 0.1 M *sodium hydroxide,* and dilute quantitatively, and stepwise if necessary, with *water* to obtain a solution having a known concentration of about 2.0 µg per ml.

System suitability solution 2 Use *Guanine standard preparation*.

Assay preparation Weigh and finely powder not less than 10 Acyclovir Tablets. Transfer an accurately weighed quantity of the powder, containing about 10 mg of acyclovir, to a 100-ml volumetric flask, dissolve in 10 ml of 0.1 M *sodium hydroxide*, dilute with *water* to volume, mix, and filter.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (25 cm × 4.6 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles maintained at a temperature of 40°, (b) *Mobile phase* at a flow rate of about 1.5 ml per minute, and (c) an ultraviolet photometer set at 254 nm.

To determine the suitability of the chromatographic system, chromatograph *System suitability solution 1*, *System suitability solution 2*, and record the peak responses as directed under *Procedure*: the relative retention times are about 1.0 for guanine and 1.6 for acyclovir, the resolution factor between acyclovir and guanine is not less than 2.0, and the relative standard deviation for replicate injections is not more than 2.0 per cent.

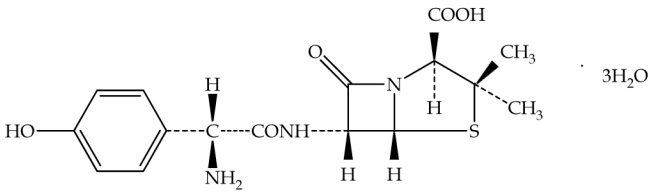
Procedure Separately inject equal volumes (about 20 µl) of *Standard preparation*, *Guanine standard preparation* and *Assay preparation* into the chromatograph, record the chromatograms, and measure the responses for all the peaks.

Calculation Calculate the content of $C_8H_{11}N_5O_3$ in the portion of the Tablets taken, using the declared content of $C_8H_{11}N_5O_3$ in Acyclovir RS.

Other requirements Comply with the requirements described under "Tablets" (Appendix 1.16).

AMOXICILLIN

Amoxycillin Trihydrate



$C_{16}H_{19}N_3O_5S.3H_2O$ 419.45 *61336-70-7*
4-Thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid, 6-[[amino(4-hydroxyphenyl)acetyl]amino]-3,3-dimethyl-7-oxo-, [2*S*-[2α,5α,6β(*S**)]]-, trihydrate.
Anhydrous 365.40 *26787-78-0*

Category Antibacterial.

Amoxicillin contains not less than 90.0 per cent and not more than 105.0 per cent of $C_{16}H_{19}N_3O_5S$, calculated on the anhydrous basis.

Description White or almost white, crystalline powder.

Solubility Slightly soluble in *water*, in *ethanol*, and in *methanol*; practically insoluble in *chloroform*, in *ether*, and in fixed oils.

Contra-indication; Warning; Precaution; Additional Information See under *Penicillin V Potassium*, p. 140.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Amoxicillin RS (Appendix 2.1) or with the reference spectrum of Amoxicillin.

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

C. Suspend 10 mg in 1 ml of *water* and add 2 ml of a mixture of 2 ml of *potassium cupric-tartrate TS* and 6 ml of *water*: a magenta colour is immediately produced.

D. Dissolve 2 drops of *aniline* in a mixture of 1 ml of *hydrochloric acid* and 3 ml of *water*. Cool the solution in ice and add 1 ml of a freshly prepared 20 per cent w/v solution of *sodium nitrite*. Add the resulting mixture dropwise to a cold solution of 100 mg of the test substance in 2 ml of 5 M *sodium hydroxide*: the solution becomes deep cherry-red and a copious dark brown precipitate is produced.

Pakaging and storage Amoxicillin shall be kept in tightly closed containers and stored at a temperature not exceeding 25°.

Labelling The label on the container states storage condition.

Clarity of solution Dissolve 1.0 g in 10 ml of 0.5 M *hydrochloric acid*. Dissolve separately 1.0 g in 10 ml of diluted ammonia prepared by diluting 14 g of *strong ammonia solution* to 100 ml with *water*. (**Note** The solution contains not less than 3.3 per cent and not more than 3.5 per cent of NH_3 (about 2 M).) Immediately after dissolution, the solutions are not more opalescent than *reference suspension II* (Appendix 4.1).

Crystallinity It is crystalline (Method I, Appendix 4.14).

pH 3.5 to 6.0, in a 0.2 per cent w/v solution (Appendix 4.11).

Specific rotation +290° to +315°, calculated on the anhydrous basis, determined in a 0.2 per cent w/v solution (Appendix 4.8).

Water Not less than 11.5 per cent w/w and not more than 14.5 per cent w/w (Karl Fischer Method, Appendix 4.12).

Dimethylaniline Not more than 20 ppm. Carry out the determination as described in the "Gas Chromatography" (Appendix 3.4).

Internal standard solution Dissolve 50 mg of *naphthalene* in *cyclohexane* and dilute to 50 ml with the same solvent. Dilute 5 ml of this solution to 100 ml with *cyclohexane*.

Standard solution To 50.0 mg of N,N-*dimethylaniline* add 2 ml of *hydrochloric acid* and 20 ml of *water*, shake to dissolve and dilute to 50.0 ml with *water*. Dilute 5.0 ml of this solution to 250.0 ml with *water*. To 1.0 ml of the latter solution in a ground-glass-stoppered tube add 5 ml of 1 M *sodium hydroxide* and 1.0 ml of *Internal standard solution*. Stopper the tube and shake vigorously for 1 minute. Centrifuge if necessary and use the upper layer.

Test solution To 1.00 g of the substance to be examined in a ground-glass-stoppered tube add 5 ml of 1 M *sodium hydroxide* and 1.0 ml of *Internal standard solution*. Stopper the tube and shake vigorously for 1 minute. Centrifuge if necessary and use the upper layer.

Chromatographic system The chromatographic procedure may be carried out using (a) a glass column 2 m long and 2 mm in internal diameter packed with *silanized diatomaceous support* impregnated with 3 per cent w/w of *polymethylphenylsiloxane*, (b) *nitrogen* as the carrier gas at a flow rate of about 30 ml per minute, and (c) a flame-ionization detector, maintaining the tempera-

ture of the column at 120° and that of the injection port and of the detector at 150°.

Procedure Inject 1 µl of *Test solution* and 1 µl of *Standard solution*.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Diluent Dissolve 13.6 g of *potassium dihydrogen phosphate* in *water* to make 2000 ml of solution, and adjust with a 45 per cent w/w solution of *potassium hydroxide* to a pH of 5.0±0.1.

Mobile phase Prepare a mixture of 4 volumes of *acetonitrile* and 96 volumes of *Diluent*. Make adjustments if necessary. Decrease the acetonitrile concentration to increase the retention time of amoxicillin.

Standard preparation Dissolve an accurately weighed quantity of Amoxicillin RS, quantitatively in *Diluent* to obtain a solution having a known concentration of about 1.2 mg per ml. Use this solution within 8 hours.

Assay preparation Transfer about 240 mg of Amoxicillin, accurately weighed, to a 200-ml volumetric flask, dissolve in and dilute with *Diluent* to volume, and mix. Use this solution within 6 hours.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (25 cm × 4 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticle (3 to 10 µm), (b) *Mobile phase* at a flow rate of about 1.5 ml per minute, and (c) an ultraviolet photometer set at 230 nm.

To determine the suitability of the chromatographic system, chromatograph *Standard preparation*, and record the peak responses as directed under *Procedure*: the relative standard deviation for replicate injections is not more than 2.0 per cent. The capacity factor, *K'*, is between 1.1 and 2.8, and the symmetry factor for the amoxicillin peak is not more than 2.5.

Procedure Separately inject equal volumes (about 10 µl) of *Standard preparation* and *Assay preparation* into the chromatograph, record the chromatograms, and measure the responses for the major peaks.

Calculation Calculate the content of $C_{16}H_{19}N_3O_5S$ in the Amoxicillin taken, using the declared content of $C_{16}H_{19}N_3O_5S$ in Amoxicillin RS.

AMOXICILLIN CAPSULES

Category Antibacterial.

Amoxicillin Capsules contain the equivalent of not less than 90.0 per cent and not more than 120.0 per cent of the labelled amount of $C_{16}H_{19}N_3O_5S$.

Strengths available 250 and 500 mg (anhydrous).

Dose Adults: 250 to 500 mg every 8 hours, or 500 to 1000 mg every 12 hours.

Contra-indication; Warning; Precaution; Additional information See under *Penicillin V Potassium*, p. 140.

Pakaging and storage Amoxicillin Capsules shall be kept in tightly closed containers.

Labelling The label on the container states the quantity equivalent to the amount of anhydrous amoxicillin.

Identification

A. The infrared absorption spectrum of the capsule contents is concordant with the spectrum obtained from Amoxicillin RS (Appendix 2.1) or with the reference spectrum of Amoxicillin.

B. Comply with the tests for Identification B, C and D described under *Amoxicillin*, p. 24.

Water Not more than 14.5 per cent w/w (Karl Fischer Method, Appendix 4.12).

Dissolution Carry out the test as described in the "Dissolution Test" (Appendix 4.24).

Dissolution medium: *water*; 900 ml.

Apparatus 1: 100 rpm.

Time: 60 minutes.

Procedure Determine the amount of $C_{16}H_{19}N_3O_5S$ dissolved from absorbances at the maximum at about 272 nm of filtered portions of the test solution, suitably diluted with *water*, if necessary, in comparison with a standard solution having a known concentration of Amoxicillin RS in the same medium (Appendix 2.2).

Tolerances Not less than 80 per cent (*Q*) of the labelled amount of $C_{16}H_{19}N_3O_5S$ is dissolved in 60 minutes.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Diluent, Mobile phase, Standard preparation, Chromatographic system, and **Procedure** Proceed as directed in the Assay under *Amoxicillin*, p. 24.

Assay preparation Remove as completely as possible, the contents of not less than 20 Amoxicillin Capsules and weigh accurately. Mix the combined contents, and transfer an accurately weighed quantity,

containing about 200 mg of anhydrous amoxicillin, to a 200-ml volumetric flask, add *Diluent* to volume and mix. Sonicate if necessary to ensure complete dissolution. Filter a portion of this solution through a suitable filter of 1-µm or finer porosity, and use the filtrate as *Assay preparation*. Use this solution within 6 hours.

Calculation Calculate the content of $C_{16}H_{19}N_3O_5S$ in the Capsules taken, using the declared content of $C_{16}H_{19}N_3O_5S$ in Amoxicillin RS.

Other requirements Comply with the requirements described under "Capsules" (Appendix 1.16).

AMOXICILLIN FOR ORAL SUSPENSION

Category Antibacterial.

Amoxicillin for Oral Suspension contains the equivalent of not less than 90.0 per cent and not more than 120.0 per cent of the labelled amount of $C_{16}H_{19}N_3O_5S$ when constituted as directed. It contains one or more suitable buffers, colours, flavours, preservatives, stabilizers, sweeteners, and suspending agents.

Strengths available 125 and 250 mg (anhydrous) per 5 ml.

Dose Adults and children 20 kg of body weight and over: 250 to 500 mg every 8 hours.

Infants and children 8 to 20 kg of body weight: 6.7 to 13.3 mg per kg of body weight every 8 hours.

Infants 6 to 8 kg of body weight: 50 to 100 mg every 8 hours.

Infants up to 6 kg of body weight: 25 to 50 mg every 8 hours.

Contra-indication; Warning; Precaution; Additional information See under *Penicillin V Potassium*, p. 140.

Packaging and storage Amoxicillin for Oral Suspension shall be kept in tightly closed containers and stored at a temperature not exceeding 30°. After constitution, it should be used within the period and stored as stated on the label.

Labelling The label on the container states the quantity equivalent to the amount of anhydrous amoxicillin.

Identification The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the Chromatogram of the Standard preparation, as obtained in the *Assay*.

pH 4.0 to 7.5 (Appendix 4.11).

Water Not more than 3.0 per cent w/w (Karl Fischer Method, Appendix 4.12).

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Diluent, Mobile phase, Standard preparation, Chromatographic system, and **Procedure** Proceed as directed in the Assay under *Amoxicillin*, p. 24.

Assay preparation Dilute an accurately measured volume of Amoxicillin for Oral Suspension, constituted as directed in the labelling, freshly mixed and free from air bubbles, quantitatively and stepwise in *Diluent* to obtain a solution containing about 1 mg of anhydrous amoxicillin per ml. Filter a portion of this solution through a suitable filter of 1-µm or finer porosity, and use the filtrate as *Assay preparation*. Use this solution within 6 hours.

Calculation Calculate the content of $C_{16}H_{19}N_3O_5S$ in each ml of the constituted Oral Suspension taken, using the declared content of $C_{16}H_{19}N_3O_5S$ in Amoxicillin RS.

Other requirements Complies with the requirements described under "Oral Liquids" (Appendix 1.16).

AMOXICILLIN AND CLAVULANATE POTASSIUM FOR ORAL SUSPENSION

Category Antibacterial.

Amoxicillin and Clavulanate Potassium for Oral Suspension contains the equivalent of not less than 90.0 per cent and not more than 120.0 per cent of the labelled amount of $C_{16}H_{19}N_3O_5S$ and the equivalent of not less than 90.0 per cent and not more than 125.0 per cent of the labelled amount of $C_8H_9NO_5$. It contains one or more suitable buffers, colours, flavours, preservatives, stabilizers, sweeteners, and suspending agents.

Strengths available 125 mg and 31.25 mg; 200 mg and 28.5 mg; 250 mg and 62.5 mg; and 400 mg and 57 mg of amoxicillin (anhydrous) and clavulanic acid, respectively, per 5 ml.

Dose Adults: 250 mg of amoxicillin and 62.5 mg of clavulanic acid every 8 hours for 7 to 10 days.

Infants and children up to 40 kg of body weight: 6.7 mg of amoxicillin and 1.7 mg of clavulanic acid per kg of body weight every 8 hours for 7 to 10 days.

Contra-indication; Warning; Precaution; Additional information See under *Penicillin V Potassium*, p. 140.

Packaging and storage Amoxicillin and Clavulanate Potassium for Oral Suspension shall be kept in tightly closed containers and stored at a temperature not exceeding 25°. After constitution, it should be used within the period stated on the label.

Labelling The label on the container states (1) the quantity equivalent to the amount of amoxicillin; (2) the quantity equivalent to the amount of clavulanic acid.

Identification The retention times of the major peaks in the chromatogram of the Assay preparation correspond to those in the chromatogram of the Standard preparation, as obtained in the *Assay*.

pH 4.2 to 6.6, in the suspension constituted as directed in the labelling, the test being performed immediately after reconstitution (Appendix 4.11).

Water Not more than 7.5 per cent w/w, where the label indicates that after constitution as directed, the suspension contains an amount of amoxicillin that is less than 40 mg per ml; not more than 8.5 per cent where the label indicates that after constitution as directed the suspension contains an amount of amoxicillin that is equal to or more than 40 mg per ml and is less than or equal to 50 mg per ml; not more than 11.0 per cent w/w where the label indicates that after constitution as directed the suspension contains an amount of amoxicillin that is more than 50 mg per ml and is less than or equal to 80 mg per ml; and not more than 12.0 per cent w/w where the label indicates that after constitution as directed the suspension contains an amount of amoxicillin that is more than 80 mg per ml (Karl Fischer Method, Appendix 4.12).

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

pH 4.4 Sodium phosphate buffer Dissolve 7.8 g of *sodium dihydrogenphosphate* in 900 ml of *water*, adjust with *phosphoric acid* or 10 M *sodium hydroxide* to a pH of 4.4±0.1, dilute with *water* to make 1000 ml, and mix.

Mobile phase Prepare a suitable mixture of 95 volumes of *pH 4.4 sodium phosphate buffer* and 5 volumes of *methanol*. Make adjustments if necessary.

Standard preparation Dissolve accurately weighed quantities of Amoxicillin RS and Clavulanate Lithium RS in *water* to obtain a solution having known concentrations of about 500 µg per ml and 200 µg per ml, respectively.

Assay preparation Dilute an accurately measured volume of Amoxicillin and Clavulanate Potassium for Oral Suspension, constituted as directed in the labelling, quantitatively with *water* to obtain a solution containing about 500 µg of amoxicillin per ml. Stir by mechanical means for 10 minutes, and filter. Use the filtrate as *Assay preparation* within 1 hour of the dilution of the suspension.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (30 cm × 4 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles (3 to 10 µm), (b) *Mobile phase* at a flow rate of about 2 ml per minute, and (c) an ultraviolet photometer set at 220 nm.

To determine the suitability of the chromatographic system, chromatograph *Standard preparation*, and record the peak responses as directed under *Procedure*: the relative standard deviation for replicate injections is not more than 2.0 per cent, the resolution factor between the amoxicillin and clavulanic acid peaks is not less than 3.5, and the symmetry factor for the amoxicillin peak is not more than 1.5.

Procedure Separately inject equal volumes (about 20 µl) of *Standard preparation* and *Assay preparation* into the chromatograph, record the chromatograms, and measure the responses for the major peaks. The relative retention times are about 0.5 for clavulanic acid and 1.0 for amoxicillin.

Calculation Calculate the contents of amoxicillin ($C_{16}H_{19}N_3O_5S$) and of clavulanic acid ($C_8H_9NO_5$) in each ml of the constituted Oral Suspension using the declared content of $C_{16}H_{19}N_3O_5S$ in Amoxicillin RS and the declared content of $C_8H_9NO_5$ in Clavulanate Lithium RS.

Other requirements Complies with the requirements described under "Oral Liquids" (Appendix 1.16).

AMOXICILLIN AND CLAVULANATE POTASSIUM TABLETS

Category Antibacterial.

Amoxicillin and Clavulanate Potassium Tablets contain the equivalent of not less than 90.0 per cent and not more than 120.0 per cent of the labelled amounts of $C_{16}H_{19}N_3O_5S$ and $C_8H_9NO_5$.

Strengths available 250, 500 and 875 mg of amoxicillin (anhydrous) with 125 mg of clavulanic acid.

Dose Adults: 250 mg of amoxicillin and 125 mg of clavulanic acid every 8 hours for 7 to 10 days.

Contra-indication; Warning; Precaution; Additional information See under *Penicillin V Potassium*, p. 140.

Packaging and storage Amoxicillin and Clavulanate Potassium Tablets shall be kept in tightly closed containers, protected from light, and stored at a temperature not exceeding 25°.

Labelling The label on the container states (1) the quantity equivalent to the amount of amoxicillin; (2) the quantity equivalent to the amount of clavulanic acid.

Identification The retention times of the major peaks in the chromatogram of the Assay preparation correspond to those in the chromatogram of the Standard preparation, as obtained in the *Assay*.

Water Not more than 7.5 per cent w/w where the labelled amount of amoxicillin in each tablet is 250 mg or less; not more than 10.0 per cent w/w where the labelled amount of amoxicillin in each tablet is more than 250 mg but less than or equal to 500 mg; not more than 11.0 per cent w/w where the labelled amount of amoxicillin in each tablet is more than 500 mg (Karl Fischer Method, Appendix 4.12).

Dissolution Carry out the test as described in the "Dissolution Test" (Appendix 4.24). (**Note** Tablets labelled for veterinary use only are exempt from this requirement.)

Dissolution Medium: *water*; 900 ml.

Apparatus 2: 75 rpm.

Time: 30 minutes.

Procedure Determine the amount of $C_{16}H_{19}N_3O_5S$ and $C_8H_9NO_5$ dissolved, employing the procedure set forth in the *Assay* making any necessary volumetric adjustments.

Tolerances Not less than 85 per cent (*Q*) of the labelled amount of $C_{16}H_{19}N_3O_5S$ and not less than 80 per cent (*Q*) of the labelled amount of $C_8H_9NO_5$ are dissolved in 30 minutes.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

pH 4.4 Sodium phosphate buffer, Mobile phase, Standard preparation and **Chromatographic system** Proceed as directed in the Assay under *Amoxicillin and Clavulanate Potassium for Oral Suspension*, p. 26.

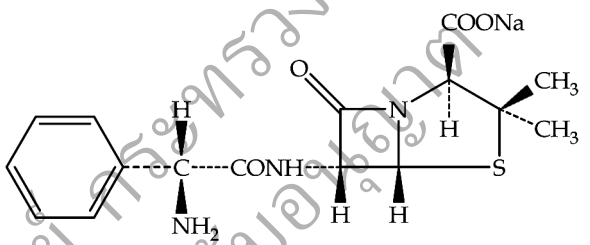
Assay preparation Dissolve not less than 10 Amoxicillin and Clavulanate Potassium Tablets, accurately counted, in *water* with the aid of mechanical stirring, transfer to a suitable volumetric flask, dilute with *water* to volume, and mix. Filter a portion of this solution, discarding the first 10 ml of the filtrate. Dilute an accurately measured volume of the filtrate quantitatively and stepwise with *water* to obtain a solution containing about 0.5 mg of amoxicillin per ml. Use this *Assay preparation* within 1 hour.

Procedure Proceed as directed under Procedure in the Assay under *Amoxicillin and Clavulanate Potassium for Oral Suspension*, p. 26.

Calculation Calculate the contents of amoxicillin ($C_{16}H_{19}N_3O_5S$) and of clavulanic acid ($C_8H_9NO_5$) in the Tablets taken, using the declared content of $C_{16}H_{19}N_3O_5S$ in Amoxicillin RS and the declared content of $C_8H_9NO_5$ in Clavulanate Lithium RS.

Other requirements Comply with the requirements described under "Tablets" (Appendix 1.16).

AMPICILLIN SODIUM



$C_{16}H_{18}N_3O_4S.Na$ 371.39 *69-52-3*

4-Thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid, [6-(aminophenylacetyl)amino]-3,3-dimethyl-7-oxo-, [2*S*-[2α,5α,6β(*S**)]]-, monosodium salt.

Category Antibacterial.

Ampicillin Sodium contains not less than 87.0 per cent and not more than 100.5 per cent of $C_{16}H_{19}N_3O_4S$, calculated on the anhydrous basis.

Description White to off-white, crystalline powder.

Solubility Soluble in 2 parts of *water* and in 50 parts of *acetone*; slightly soluble in *chloroform*; insoluble in *ether*, in *liquid paraffin* and in fixed oils.

Stability It is hygroscopic.

Contra-indication; Warning; Precaution; Additional information See under *Penicillin V Potassium*, p. 140.

Warning It should be given with caution to patients with infectious mononucleosis since they are susceptible to ampicillin-induced skin rashes.

See also under *Penicillin V Potassium*, p. 140.

Packaging and storage Ampicillin Sodium shall be kept in tightly closed containers and stored at a temperature not exceeding 25°. If it is intended for parenteral administration, it shall also be kept under sterile condition.

Labelling The label on the container states (1) storage condition; (2) parenteral grade.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Ampicillin Sodium RS (Appendix 2.1) or with the reference spectrum of Ampicillin Sodium.

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

C. It yields the *reactions* characteristic of sodium salts (Appendix 5.1).

Crystallinity It is crystalline (Method I, Appendix 4.14).

pH 8.0 to 10.0, in a 1.0 per cent w/v solution (Appendix 4.11).

Specific rotation +258° to +287°, calculated on the anhydrous basis. Dissolve 62.5 mg in a 0.4 per cent w/v solution of *potassium hydrogenphthalate* and dilute to 25.0 ml with the same solvent (Appendix 4.8).

Water Not more than 2.0 per cent w/w (Karl Fischer Method, Appendix 4.12).

Heavy metals Not more than 20 ppm (Method II, Appendix 5.2). Use 2.0 g; for the standard preparation, use *lead standard solution* (*2 ppm Pb*).

***N*,*N*-Dimethylaniline** Not more than 20 ppm (Appendix 5.16).

Limit of dichloromethane Not more than 0.2 per cent w/w. Carry out the determination as described in the "Gas Chromatography" (Appendix 3.4).

Internal standard solution Prepare a solution of *dioxane* in *dimethyl sulfoxide* containing about 2.1 mg per ml.

Standard solution Dissolve an accurately weighed quantity of *dichloromethane* in *Internal standard solution* to obtain a solution having a known concentration of about 330 µg per ml.

Test solution Dissolve about 500 mg of Ampicillin Sodium, accurately weighed, in 3.0 ml of *Internal standard solution*.

Chromatographic system The chromatographic procedure may be carried out using (a) a glass column (1.8 m × 4 mm) packed with a 10 per cent w/w polyethylene glycol (average molecular weight about 1500) on *silanized diatomaceous support*, maintained at 65°, (b) the injection port and the detector block maintained at 100° and 260°, respectively, (c) *nitrogen* as the carrier gas at a flow rate of about 60 ml per minute, and (d) a flame ionization detector. Chromatograph *Standard solution*, and record the peak responses as directed under *Procedure:* the relative retention times are about 0.5 for dichloromethane and 1.0 for dioxane, the resolution factor between the dichloromethane peak and the dioxane peak is not less than 4, and the relative standard deviation for replicate injections is not more than 5 per cent.

Procedure Separately inject equal volumes (about 1 µl) of *Standard solution* and *Test solution* into the chromatograph, record the chromatograms, and measure the responses for the dichloromethane and dioxane peaks. Calculate the percentage of dichloromethane in Ampicillin Sodium.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Diluent Mix 10 ml of 1 M *potassium dihydrogenphosphate* and 1 ml of 1 M *acetic acid*, dilute with *water* to 1000 ml, and mix.

Resolution solution Dissolve *caffeine* in *Standard preparation* to obtain a solution containing about 120 µg per ml.

Mobile phase Prepare a suitable mixture of 909 volumes of *water*, 80 volumes of *acetonitile* 10 volumes of 1 M *potassium dihydrogenphosphate* and 1 volume of 1 M *acetic acid*. Make adjustments if necessary.

Standard preparation Dissolve a suitable quantity of Ampicillin RS, accurately weighed, in *Diluent* to obtain a solution having a known concentration of about 1 mg per ml, using shaking and sonication, if necessary, to achieve complete dissolution. Use this solution promptly after preparation.

Assay preparation (**Note** Ampicillin Sodium is hygroscopic. Minimize exposure to the atmosphere, and weigh promptly.) Transfer an accurately weighed quantity of Ampicillin Sodium, containing about 100 mg of anhydrous ampicillin, to a 100-ml volumetric flask, dissolve in and dilute with *Diluent* to volume, and mix. Use this solution promptly after preparation.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (30 cm × 4 mm) and a pre-column (5 cm × 4 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles (5 to 10 µm), (b) *Mobile phase* at a flow rate of about 2 ml per minute, and (c) an ultraviolet photometer set at 254 nm.

To determine the suitability of the chromatographic system, chromatograph *Resolution solution* and record the peak responses as directed under *Procedure*: the resolution factor between the caffeine and the ampicillin peaks is not less than 2.0 and the relative retention times are about 0.5 for ampicilin and 1.0 for caffeine. Chromatograph *Standard preparation*, and record the peak

responses as directed under *Procedure*: the relative standard deviation for replicate injections is not more than 2.0 per cent and the symmetry factor for the amoxicillin peak is not more than 1.4.

Procedure Separately inject equal volumes (about 20 µl) of *Standard preparation* and *Assay preparation* into the chromatograph, record the chromatograms, and measure the responses for the major peaks.

Calculation Calculate the content of $C_{16}H_{19}N_3O_4S$ using the declared content of $C_{16}H_{19}N_3O_4S$ in Ampicillin RS.

Other requirements Ampicillin Sodium intended for parenteral administration complies with the following additional requirements.

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains not more than 0.15 Endotoxin Unit per mg of ampicillin.

Sterility Complies with the "Sterility Test" (Method I, Appendix 10.1).

AMPICILLIN SODIUM FOR INJECTION

Category Antibacterial.

Ampicillin Sodium for Injection contains an amount of Ampicillin Sodium equivalent to not less than 90.0 per cent and not more than 115.0 per cent of the labelled amount of $C_{16}H_{19}N_3O_4S$.

Strengths available 125, 250 and 500 mg; 1 and 2 g (base); and 10 g (base, pharmacy bulk package.)

Dose Adults and children 20 kg of body weight and over: *Intramuscular* or *intravenous*, 250 to 500 mg every 6 hours. The total dosage should not exceed 14 g daily.

Infants and children up to 20 kg of body weight: *Intramuscular* or *intravenous*, 12.5 mg per kg of body weight every 6 hours.

Contra-indication See under *Penicillin V Potassium*, p. 140.

Warning

1. Phlebitis or thrombophlebitis may occur after repeated intravenous injections, particularly with use of concentrated solutions or continuous infusion.

2. It should be given with caution to patients with infectious mononucleosis since they are susceptible to ampicillin-induced skin rashes.

3. Intrathecally injection or too rapid intravenous administration may result in convulsive seizures.

See also under *Penicillin V Potassium*, p. 140.

Precaution; Additional information See under *Penicillin V Potassium*, p. 140.

Packaging and storage Ampicillin Sodium for Injection shall be kept in Containers for Sterile Solids as described under "Parenteral Preparations" (Appendix 1.16), and stored at a temperature not exceeding 30°. The constituted solution should be used immediately after preparation and protected from freezing.

Labelling The label on the container states the quantity equivalent to the amount of ampicillin.

Identification

A. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

B. It yields the reactions characteristic of sodium salts (Appendix 5.1).

pH; Water Complies with the tests described under *Ampicillin Sodium*, p. 29.

Crystallinity It is crystalline (Method I, Appendix 4.14). (**Note** Ampicillin for Injection in the freeze-dried form is exempt from this requirement.)

Constituted solution At the time of use, it complies with the requirements described under "Constituted Solutions" (Appendix 4.20).

Particulate matter Complies with the requirements described under "Particulate Matter in Injections" (Small-volume Injections, Appendix 4.27).

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains not more than 0.15 Endotoxin Unit per mg of ampicillin.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase, Diluent, Standard preparation, Resolution Solution, and **Chromatographic system** Proceed as directed in the Assay under *Ampicillin Sodium*, p. 29.

Assay preparation 1 (Where it is represented as being in a single dose container,) Constitute Ampicillin Sodium for Injection in an accurately measured volume of *Diluent*, corresponding to the volume of solvent specified in the labeling. Withdraw all of the withdrawable contents, using a suitable hypodermic needle and syringe, and dilute quantitatively with *Diluent* to obtain a solution containing about 1 mg of ampicillin per ml. Use this solution promptly after preparation.

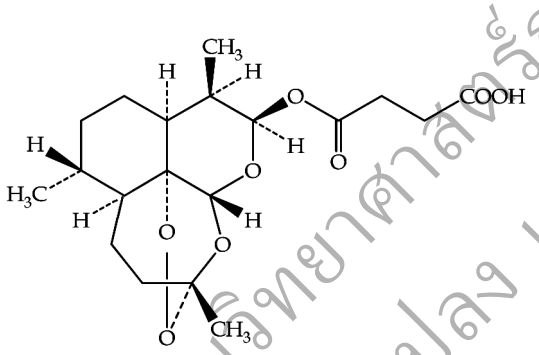
Assay preparation 2 (Where the label states the quantity of ampicillin in a given volume of constituted solution) Constitute 1 container of Ampicillin Sodium for Injection in a volume of *Diluent*, accurately measured, corresponding to the volume of solvent specified in the labelling. Dilute an accurately measured portion of the constituted solution quantitatively with *Diluent* to obtain a solution having a concentration of about 1 mg of ampicillin per ml. Use this solution promptly after preparation.

Procedure Proceed as directed under Procedure in the Assay under *Ampicillin sodium*, p. 29.

Calculation Calculate the quantity, in mg, of $C_{16}H_{19}N_3O_4S$ in the container and in the volume of constituted solution taken using the declared content of $C_{16}H_{19}N_3O_4S$ in Ampicillin RS. Where the test for *Uniformity of dosage units* has been performed using the procedure for content uniformity, use the average of these determinations as the Assay value.

Other requirements Complies with the requirements described under "Parenteral Preparations" (Appendix 1.16).

ARTESUNATE



$C_{19}H_{28}O_8$ 384.42 *88495-63-0*

(3*R*,5a*S*,6*R*,8a*S*,9*R*,10*R*,12*R*,12a*R*)-Decahydro-3,6,9-trimethyl-3,12-epoxy-12*H*-pyrano[4,3-*j*]-1,2-benzodioxepin-10-ol, hydrogen succinate.

Category Antiprotozoal (antimalarial).

Artesunate contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{19}H_{28}O_8$, calculated on the anhydrous basis.

Description Fine, white crystalline powder.

Solubility Very slightly soluble in *water*; very soluble in *dichloromethane*; freely soluble in *acetone* and in *ethanol*.

Warning

1. It should not be used at all for prophylaxis of malaria.

2. It should not be used during the first trimester of pregnancy. Risk-benefit should be considered if it is to be used in pregnant women.

3. It may cause mild gastro-intestinal disturbances, dizziness, tinnitus, neutropenia, elevated liver enzyme values, electrocardiogram abnormalities including prolongation of the QT interval, acute cerebellar dysfunction, bone marrow depression or reticulocytopenia.

4. It should be used with caution in patients with cardiac, hepatic, renal and gastro-intestinal diseases.

Packaging and storage Artesunate shall be kept in well-closed containers, protected from light, and stored at a temperature not exceeding 25°.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Artesunate RS (Appendix 2.1) or with the reference spectrum of Artesunate.

B. Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel GF254* as the coating substance and a mixture of 5 volumes of *ethyl acetate* and 95 volumes of *toluene* as the mobile phase. Apply separately to the plate, 2 µl of the following two solutions in *toluene* containing (A) 100 µg of the test substance per ml, and (B) 100 µg of Artesunate RS per ml. After removal of the plate, allow it to dry in air, spray with *anisaldehyde TS*, heat the plate to 120° for 5 minutes and examine under ultraviolet light (254 nm): the principal spot obtained from solution (A) corresponds in position, appearance and intensity to that obtained from solution (B).

C. Dissolve 100 mg of the test substance in 40 ml of *absolute ethanol*, shake and filter. To half of the filtrate (keep the remaining filtrate for test D) add about 0.5 ml of *hydroxylamine* in *ethanol TS* and 5 drops of *sodium hydroxide TS*. Heat the mixture in a water-bath to boiling, cool, and add 2 to 3 drops of *dilute hydrochloric acid* and 1 drop of *iron(III) chloride TS*: a light red-violet colour is produced.

D. Evaporate the remaining filtrate from Test C on a water-bath to a volume of about 5 ml. Place a few drops of the mixture on a white porcelain dish, add one drop of *vanillin-sulfuric acid TS1*, and allow to stand for 30 minutes: a red colour is produced.

Melting range 132° to 135° (Appendix 4.3).

pH 3.5 to 4.5, in a 1 per cent w/w suspension (Appendix 4.11).

Specific rotation +2.5° to +3.5°, determined in a 1.0 per cent w/v solution in *dichloromethane* (Appendix 4.8).

Water Not more than 0.5 per cent w/w (Karl Fischer Method, Appendix 4.12).

Heavy metals Not more than 20 ppm (Method II, Appendix 5.2). Use 1.0 g; for the Standard Preparation, use *lead standard solution (1 ppm Pb)*.

Sulfated ash Not more than 0.1 per cent w/w (Appendix 5.3).

Related substances Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel G* as the coating substance.

Reference solution 1 Prepare a 0.005 per cent w/v solution of the test substance in *dichloromethane*.

Reference solution 2 Prepare a 0.0025 per cent w/v solution of the test substance in *dichloromethane*.

Test solution Prepare a 0.5 per cent w/v solution of the test substance in *dichloromethane*.

Mobile phase Prepare a mixture of 48 volumes of *petroleum ether (boiling range, 30° to 40°)*, 36 volumes of *ethyl acetate* and 1 volume of *glacial acetic acid*.

Procedure Apply separately to the plate, 10 µl of each of the solutions. After removal of the plate, allow it to dry in air, spray with *vanillin-sulfuric acid TS1*, and examine the chromatogram in daylight. Any spot obtained from *Test solution*, other than the principal spot, is not more intense than that obtained from *Reference solution 1* (1.0 per cent). Furthermore, not more than one such spot is more intense than that obtained from *Reference solution 2* (0.5 per cent).

Assay Dissolve about 250 mg of Artesunate, accurately weighed, in 25 ml of *neutralized ethanol* and titrate with 0.05 M *sodium hydroxide VS*, using *dilute phenolphthalein TS* as indicator. Perform a blank determination, and make any necessary correction. Each ml of 0.05 M *sodium hydroxide* is equivalent to 19.22 mg of $C_{19}H_{28}O_8$.

ARTESUNATE TABLETS

Category Antiprotozoal (antimalarial).

Artesunate Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the labelled amount of $C_{19}H_{28}O_8$.

Strength available 50 mg.

Dose Initial, 5 mg per kg of body weight on the first day. Maintenance, 2.5 mg per kg of body weight for a minimum of 4 days.

To effect radical cure, a 3-day course of artesunate should be used in combination with a single oral dose of mefloquine 15 mg per kg of body weight (or occasionally 25 mg per kg of body weight if necessary) on the second day.

Warning See under *Artesunate*, p. 31.

Packaging and storage Artesunate Tablets shall be stored at a temperature not exceeding 25°.

Identification

A. To a quantity of the powdered tablets equivalent to 50 mg of artesunate add 25 ml of *acetone*, shake to dissolve, and filter. Evaporate the filtrate at low temperature and dry overnight over *self-indicating silica gel*. The infrared absorption spectrum is concordant with the spectrum obtained from Artesunate RS (Appendix 2.1) or with the reference spectrum of Artesunate.

B. Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel GF254* as the coating substance and a mixture of 5 volumes of *ethyl acetate* and 95 volumes of *toluene* as the mobile phase. Apply separately to the plate, 2 µl of the following two solutions in *toluene*. For solution (A) shake a quantity of the powdered tablets equivalent to 0.1 mg of artesunate in *absolute ethanol*, filter and evaporate. Dissolve the residue in 1.0 ml of *toluene*. For solution (B) use 0.10 mg of Artesunate RS per ml. After removal of the plate, allow it to dry in air, spray with *anisaldehyde TS*, heat the plate to 120° for 5 minutes and examine under ultraviolet light (254 nm): the principal spot obtained from solution (A) corresponds in position, appearance and intensity to that obtained from solution (B).

C. To a quantity of the powdered tablets equivalent to 100 mg of artesunate add 40 ml of *absolute ethanol*, shake to dissolve, and filter. To half of the filtrate (keep the remaining filtrate for test D) add about 0.5 ml of *hydroxylamine in ethanol (60 per cent) TS* and 0.25 ml of *sodium hydroxide TS*. Heat the mixture in a water-bath to boiling, cool, and add 2 to 3 drops of *dilute hydrochloric*

ลิขสิทธิ์ กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข
ห้ามทำซ้ำ ดัดแปลง แก้ไข ขายหรือใช้ประโยชน์ทางการค้า

acid and 1 drop of *iron(III) chloride TS*: a light red-violet colour is produced.

D. Evaporate the remaining filtrate from Test C on a water-bath to a volume of about 5 ml. Place a few drops of the mixture on a white porcelain dish, add one drop of *vanillin-sulfuric acid TS1*, and allow to stand for 30 minutes: a red colour is produced.

Related substances Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel G* as the coating substance.

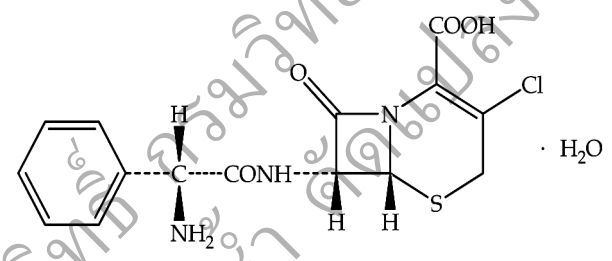
Reference solution 1, Reference solution 2, Mobile phase, and **Procedure** Proceed as directed in the Related substances under *Artesunate*, p. 32.

Test solution Shake a quantity of the powdered tablets equivalent to about 10 mg of artesunate with 2 ml of *dichloromethane*, filter, and use the filtrate.

Assay Weigh and powder not less than 20 Artesunate Tablets. To a quantity of the powder equivalent to about 500 mg of artesunate, accurately weighed, add 50.0 ml of *neutralized ethanol*, shake thoroughly, filter, and discard about 10 ml of the initial filtrate. Titrate 25.0 ml of the filtrate with 0.05 M *sodium hydroxide VS*, using *dilute phenolphthalein TS* as indicator. Perform a blank determination, and make any necessary correction. Each ml of 0.05 M *sodium hydroxide* is equivalent to 19.22 mg of $C_{19}H_{28}O_8$.

Other requirements Comply with the requirements described under "Tablets" (Appendix 1.16).

CEFACLOR



$C_{15}H_{14}ClN_3O_4S.H_2O$ 385.82 *70356-03-5*
5-Thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid, 7-[(aminophenylacetyl)amino]-3-chloro-8-oxo-, [6*R*-[6α,7β(*R**)]]-, monohydrate.
Anhydrous 367.81 *53994-73-3*

Category Antibacterial (second-generation cephalosporin).

Cefaclor contains not less than 95.0 per cent and not more than 102.0 per cent of $C_{15}H_{14}ClN_3O_4S$, calculated on the anhydrous basis.

Description White to off-white crystalline powder.

Solubility Slightly soluble in *water*; practically insoluble in *methanol* and in *dichloromethane*.

Stability In aqueous solutions, it rapidly loses activity.

Contra-indication It is contra-indicated in patients who have shown hypersensitivity in any member of the cephalosporins.

Warning Serum sickness-like reactions consisting of erythema multiforme or maculopapular pruritic rash or urticaria have been reported most frequently in pediatric patients younger than 6 years of age.

See also under *Cephalexin*, p. 58.

Additional information

1. Group A beta-hemolytic streptococcal infections should be treated for at least 10 days to prevent the development of acute rheumatic fever or acute glomerulonephritis.

2. Continue administration for a minimum of 48 to 72 hours after fever abates or after evidence of bacterial eradication has been obtained.

See also under *Cephalexin*, p. 58.

Packaging and storage Cefaclor shall be kept in tightly closed containers.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Cefaclor RS (Appendix 2.1) or with the reference spectrum of Cefaclor.

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

Crystallinity It is crystalline (Method I, Appendix 4.14).

pH 3.0 to 4.5, in a 2.5 per cent w/v suspension (Appendix 4.11).

Water Not less than 3.0 per cent w/w and not more than 6.5 per cent w/w (Karl Fischer Method, Appendix 4.12). Use 200 mg.

Chromatographic purity Not more than 0.5 per cent of any individual cefaclor-related substance is found, and the sum of all cefaclor-related substances found is not more than 2.0 per cent. In an acceptable determination, the difference between duplicate determinations of total cefaclor-related compounds is not more than 0.2 per cent absolute, or the variation from the mean of the two values is not more than 10 per cent, whichever is greater. Carry out the test as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Solvent Dissolve 2.7 g of *sodium dihydrogenphosphate* in 1000 ml of *water*, and adjust with *phosphoric acid* to a pH of 2.5.

Solution A Dissolve 7.8 g of *sodium dihydrogenphosphate* in 1000 ml of *water*, and adjust with *phosphoric acid* to a pH of 4.0. Make adjustments if necessary.

Solution B Prepare a mixture of 11 volumes of *Solution A* and 9 volume of *acetonitrile*, degassing for not more than 2 minutes. Make adjustments if necessary. Reducing the acetonitrile content increases the retention time of cefaclor and increases the resolution between the delta-3-cefaclor and cefaclor.

Mobile phase Use variable mixtures of *Solution A* and *Solution B* as directed for *Chromatographic system*.

Standard solutions Dissolve an accurately weighed quantity of Cefaclor RS in *Solvent* to obtain a solution having a known concentration of about 50 µg per ml. Sonicate briefly, if necessary, to achieve dissolution, and avoid heating (Standard solution 1). Dilute accurately 2 volumes of *Standard solution 1* with *1* volume of *Solvent* to obtain a solution having a known concentration of about 33 µg per ml (Standard solution 2). Dilute accurately 1 volume of *Standard solution 1* with 3 volumes of *Solvent* to obtain a solution having a known concentration of about 12.5 µg per ml (Standard solution 3). Use these *Standard solutions* on the day prepared.

Resolution solution Dissolve a quantity of Delta-3-cefaclor RS in *Standard solution 2* to obtain a solution containing 50 µg per ml.

Test solutions Transfer about 50 mg of the test substance, accurately weighed, to each of two 10-ml volumetric flasks, dilute with *Solvent* to volume, and mix. Sonicate briefly, if necessary, to achieve dissolution, and avoid heating. (**Note** Use these *Test solutions* within 2 hours when stored at room temperature or within 20 hours when stored under refrigeration.)

Blank Use the *Solvent*.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (25 cm × 4.6 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles (3 to 10 µm), (b) *Mobile phase* at a flow rate of about 1 ml per minute, (c) an ultraviolet photometer set at 220 nm. The system is equilibrated with a mixture of 95 per cent *Solution* A and 5 per cent *Solution B*, and immediately following the injection of *Standard solution*, *Resolution solution*, *Test solution*, or *Blank*, the proportion of *Solution B* is increased linearly from 5 to 25 per cent over a period of 30 minutes. The proportion of *Solution B* is then increased linearly to 100 per cent over a period of 15 minutes, and held for 10 minutes. (**Note** After this the proportion of *Solution B* is returned to 5 per cent, and the system is allowed to equilibrate for 10 minutes at the initial composition before the next injection.) To determine the suitability of chromatographic system, chromatograph *Resolution solution* as directed under *Procedure:* the retention time for the cefaclor peak is between 23 and 29 minutes. Chromatograph *Standard solution*, and record the responses as directed under *Procedure*: the symmetry factor is not more than 1.5, and the resolution factor between the delta-3-cefaclor peak and the cefaclor peak is not less than 2.0. Chromatograph *Blank* as directed under *Procedure*. Examine the chromatogram for any extraneous peaks, and disregard any corresponding peaks observed in the chromatogram of *Test solutions*. (**Note** Take care to ensure that any extraneous peaks observed do not represent carryover from previous injections.) Chromatograph *Standard solutions* as directed under *Procedure*. Plot the responses for the cefaclor peaks in the three chromatograms versus their concentrations, in µg of cefaclor ($C_{15}H_{14}ClN_3O_4S$) per ml, and draw a straight line through the three points and zero (*Standard response line*). The relative standard deviation of replicate injections is not more than 3 per cent.

Procedure (**Note** Use peak areas where peak responses are indicated.) Separately inject equal volumes (about 20 µl) of the two *Test solutions* into the chromatograph, record the chromatograms, and measure the responses for all of the peaks except that for cefaclor. From *Standard response line*, determine the concentration, in mg per ml, of cefaclor-related compound represented by each peak other than the cefaclor peak. Calculate the percentage of cefaclor-related compound represented by each such peak by the expression:

$$IP/W,$$

in which I is the concentration, in mg per ml, of cefaclor-related substance in the respective *Test solution* as determined from *Standard response line*, P is the designated potency, in µg per mg, of Cefaclor RS, and W is the weight, in mg, of the portion of the test substance taken to prepare the respective *Test solution*. Determine the mean values for each cefaclor-related substance.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Dissolve 1 g of *sodium 1-pentanesulfonate* in a mixture of 780 ml of *water* and 10 ml of *triethylamine*. Adjust with *phosphoric acid* to a pH of 2.5±0.1,

add 220 ml of *methanol*, and mix. Make adjustments if necessary.

Resolution solution Prepare a solution in *Mobile phase* containing about 300 µg of cefaclor and 300 µg of Delta-3-cefaclor RS per ml.

Standard preparation Transfer about 15 mg of Cefaclor RS, accurately weighed, to a 50-ml volumetric flask, dilute with *Mobile phase* to volume, and mix. Sonicate briefly, if necessary, to achieve dissolution, and avoid heating the solution. (**Note** Use this *Standard preparation* within 8 hours if stored at room temperature, or within 20 hours if stored under refrigeration.)

Assay preparation Transfer about 15 mg of Cefaclor, accurately weighed, to a 50-ml volumetric flask, dilute with *Mobile phase* to volume, and mix. Sonicate briefly, if necessary, to achieve dissolution, and avoid heating the solution. (**Note** Use this *Assay preparation* within 8 hours if stored at room temperature, or within 20 hours if stored under refrigeration.)

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (25 cm × 4.6 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles (5 µm), (b) *Mobile phase* at a flow rate of about 1.5 ml per minute, and (c) an ultraviolet photometer set at 265 nm.

To determine the suitability of the chromatographic system, chromatograph *Resolution solution*, and record the peak responses as directed under *Procedure*: the resolution factor between cefaclor and delta-3-cefaclor peaks is not less than 2.5, the relative retention times are about 0.8 for cefaclor and 1.0 for delta-3-cefaclor, the relative standard deviation for replicate injections is not more than 2.0 per cent and the symmetry factor for the cefaclor peak is not more than 1.5.

Procedure Separately inject equal volumes (about 20 µl) of *Standard preparation* and *Assay preparation* into the chromatograph, record the chromatograms, and measure the responses for the major peaks.

Calculation Calculate the content of $C_{15}H_{14}ClN_3O_4S$ in the Cefaclor taken, using the declared content of $C_{15}H_{14}ClN_3O_4S$ in Cefaclor RS.

CEFACLOR CAPSULES

Category Antibacterial (second-generation cephalosporin).

Cefaclor Capsules contain the equivalent of not less than 90.0 per cent and not more than 120.0 per cent of the labelled amount of $C_{15}H_{14}ClN_3O_4S$.

Strengths available 250 and 500 mg (base).

Dose Adults: 250 to 500 mg every 8 hours. The maximum total dose should not exceed 4 g a day.

Children: 20 mg per kg of body weight daily in equally divided doses every 8 hours, for severe infections, this dosage may be doubled. The maximum total dose should not exceed 1 g a day.

Contra-indication; Warning; Additional information See under *Cefaclor*, p. 33.

Packaging and storage Cefaclor Capsules shall be kept in tightly closed containers.

Identification

A. Shake a quantity of the contents of the capsules containing the equivalent of 300 mg of cefaclor with 100 ml of *water*, filter and dilute 1 ml of the filtrate to 100 ml of *water*. The ultraviolet absorption spectrum of the resulting solution, when observed between 190 nm to 310 nm, exhibits a maximum only at 264 nm (Appendix 2.2).

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

Dissolution Carry out the test as described in the "Dissolution Test" (Appendix 4.24).

Dissolution medium: *water*, 900 ml.

Apparatus 2: 50 rpm.

Time: 30 minutes.

Procedure Determine the amount of $C_{15}H_{14}ClN_3O_4S$ dissolved from absorbances at the maximum at about 264 nm of filtered portions of the test solution, suitably diluted with *water*, in comparison with a standard solution having a known concentration of Cefaclor RS in the same medium (Appendix 2.2).

Tolerance Not less than 80 per cent (*Q*) of the labelled amount of $C_{15}H_{14}ClN_3O_4S$ is dissolved in 30 minutes.

Water Not more than 8.0 per cent w/w (Karl Fischer Method, Appendix 4.12).

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase, Resolution solution, Standard preparation, Chromatographic system and **Procedure** Proceed as directed in the Assay described under *Cefaclor*, p. 34.

Assay preparation Remove, as completely as possible, the contents of not less than 20 Cefaclor Capsules, and weigh accurately. Mix the combined contents, and transfer an accurately weighed portion of the powder, containing about 75 mg of cefaclor, to a 250-ml volumetric flask, dilute with *Mobile phase* to volume, mix and filter.

Calculation Calculate the content of $C_{15}H_{14}ClN_3O_4S$, in the portion of the Capsules taken, using the declared content of $C_{15}H_{14}ClN_3O_4S$ in Cefaclor RS.

Other requirements Comply with the requirements described under "Capsules" (Appendix 1.16).

CEFACLOR FOR ORAL SUSPENSION

Category Antibacterial (second-generation cephalosporin).

Cefaclor for Oral Suspension is a dry mixture of cefaclor and one or more suitable buffers, colours, diluents, and flavours. It contains the equivalent of not less than 90.0 per cent and not more than 120.0 per cent of the labelled amount of $C_{15}H_{14}ClN_3O_4$.

Strengths available 125, 187, 250, and 375 mg (base) per 5 ml.

Dose Adults: 250 to 500 mg every 8 hours. The maximum total dose should not exceed 4 g a day.

Children: 20 mg per kg of body weight daily in equally divided doses every 8 hours, for severe infections, this dosage may be doubled. The maximum total dose should not exceed 1 g a day.

Contra-indication; Warning; Additional information See under *Cefaclor*, p. 33.

Packaging and storage Cefaclor for Oral Suspension shall be kept in tightly closed containers and stored at a temperature not exceeding 30°. After constitution, it should be used within the period stated on the label.

Identification

A. Shake a quantity of the oral suspension containing the equivalent of 300 mg of cefaclor with 500 ml of *water*, filter and use the filtrate. The ultraviolet absorption spectrum of the resulting solution, when observed between 190 nm to 310 nm, exhibits a maximum only at 264 nm (Appendix 2.2).

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

pH 2.5 to 5.0, in the suspension constituted as directed in the labelling (Appendix 4.11).

Water Not more than 2.0 per cent w/w (Karl Fischer Method, Appendix 4.12).

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

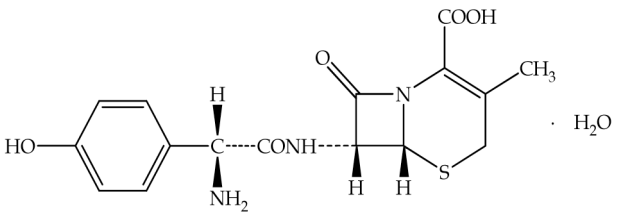
Mobile phase, Resolution solution, Standard preparation, Chromatographic system and **Procedure** Proceed as directed in the Assay described under *Cefaclor*, p. 34.

Assay preparation Constitute a container of Cefaclor for Oral Suspension as directed in the labelling. Transfer an accurately measured volume of the resulting suspension, freshly mixed and free from air bubbles, dilute quantitatively with *Mobile phase* to obtain a final solution containing about 300 µg of cefaclor per ml. Sonicate if necessary to ensure complete dissolution of the cefaclor. Filter to obtain clear *Assay preparation*.

Calculation Calculate the content of $C_{15}H_{14}ClN_3O_4S$, in the portion of the Oral Suspension taken, using the declared content of $C_{15}H_{14}ClN_3O_4S$ in Cefaclor RS.

Other requirements Complies with the requirements described under "Oral Liquids" (Appendix 1.16).

CEFADROXIL



$C_{16}H_{17}N_3O_5S.H_2O$	381.40	*66592-87-8*

5-Thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid, 7-[[amino(4-hydroxyphenyl)acetyl]amino]-3-methyl-8-oxo-, [6*R*-[6α,7β(*R**)]]-, monohydrate.

Hemihydrate	372.39	*119922-85-9*
Anhydrous	363.40	*50370-12-2*

Category Antibacterial (first-generation cephalosporin).

Cefadroxil contains not less than 95.0 per cent and not more than 102.0 per cent of $C_{16}H_{17}N_3O_5S$, calculated on the anhydrous basis.

Description White or almost white powder.

Solubility Slightly soluble in *water*; very slightly soluble in *ethanol*.

Contra-indication; Warning; Precaution See under *Cephalexin*, p. 58.

Additional information Cefadroxil is used as an alternative to amoxicillin or ampicillin for prophylaxis against alpha-hemolytic (viridans group) streptococcal endocarditis in penicillin-allergic individuals considered to be at risk for bacterial endocarditis following certain dental or upper respiratory tract procedures.

Packaging and storage Cefadroxil shall be kept in tightly closed containers, protected from light, and stored at a temperature not exceeding 25°.

Labelling The label on the container states (1) storage condition; (2) whether it is in the monohydrate or hemihydrate form.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Cefadroxil RS (Appendix 2.1) or with the reference spectrum of Cefadroxil.

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

C. Place 2 mg in a test-tube. Moisten with one drop of *water*, add 2 ml of *sulfuric-formaldehyde TS* and mix: the solution is yellow. Place the test-tube in a water-bath for 1 minute: an orange colour develops.

pH 4.0 to 6.0, in a 5.0 per cent w/v suspension (Appendix 4.11).

Specific rotation +165° to +178°, calculated on the anhydrous basis, determined in a 1.0 per cent w/v solution (Appendix 4.8).

Water Not less than 4.2 per cent w/w and not more than 6.0 per cent w/w except that where it is labelled as being in the hemihydrate form it is not less than 2.4 per cent w/w and not more than 4.5 per cent w/w (Karl Fischer Method, Appendix 4.12).

Absorbance Dissolve 20.0 mg in *phosphate buffer solution pH* 6.0 and dilute to 100.0 ml with the same solvent. The absorbance of the solution measured at 330 nm is not greater than 0.05. Dilute 10.0 ml of the solution to 100.0 ml with *phosphate buffer solution pH 6.0*. The light absorption spectrum of the diluted solution, when observed between 235 nm and 340 nm, exhibits a maximum at about 264 nm; the specific absorbance at this maximum is between 225 and 250, calculated on the anhydrous basis (Appendix 2.2).

***N,N*-Dimethylaniline** Not more than 20 ppm (Appendix 5.16).

Related substances Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase

Mobile phase A Dissolve 2.72 g of *potassium dihydrogenphosphate* in 800 ml of *water*, adjust the pH to 5.0 with 1 M *potassium hydroxide* and dilute to 1000 ml with *water*.

Mobile phase B Use *methanol*.

Phosphate buffer solution pH 7.0 Dissolve 28.4 g of *anhydrous disodium hydrogenphosphate* in 800 ml of *water*. Adjust the pH using a 30 per cent w/w solution of *phosphoric acid* and dilute to 1000 ml with *water*.

Standard solution (a) Dissolve 10.0 mg of *D*-α-(4-Hydroxyphenyl) glycine RS (impurity A) in *Mobile phase A* and dilute to 10.0 ml with *Mobile phase A*.

Standard solution (b) Dissolve 10.0 mg of 7-Aminodes-acetoxycephalosporanic Acid RS (impurity B) in *Phosphate buffer solution pH* 7.0, and dilute to 10.0 ml with the same buffer solution.

Standard solution (c) Dilute 1.0 ml of *Standard solution (a)* and 1.0 ml of *Standard solution (b)* to 100.0 ml with *Mobile phase A*.

Standard solution (d) Dissolve 10.0 mg of *dimethylformamide* and 10.0 mg of *dimethylacetamide* in *Mobile phase A* and dilute to 10.0 ml with *Mobile phase A*. Dilute 1.0 ml to 100.0 ml with *Mobile phase A*.

Standard solution (e) Dilute 1.0 ml of *Standard solution (c)* to 25.0 ml with *Mobile phase A*.

Test solution Dissolve 50.0 mg of the test substance in *Mobile phase A* and dilute to 50.0 ml with *Mobile phase A*.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (10 cm × 4.6 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles (5 μm), (b) *Mobile phases* at a flow rate of about 1.5 ml per minute, and (c) an ultraviolet photometer set at 220 nm. The step gradient of mobile phases is as follows:

Time (minutes)	*Mobile phase A* (per cent v/v)	*Mobile phase B* (per cent v/v)
0 - 1	98	2
1 - 20	98 → 70	2 → 30
20 - 23	70 → 98	30 → 2
23 - 30	98	2

To determine the suitability of the chromatographic system, separately chromatograph *Standard solutions* (c) and (*e*), and record the peak responses as directed under

Procedure: the resolution factor between the peaks due to impurity A and to impurity B is not less than 5.0 in the chromatogram obtained from *Standard solution (c)* and the signal-to-noise ratio is not less than 10 for the second peak in the chromatogram obtained from *Standard solution (e)*.

In the chromatogram obtained from *Test solution*, determine the percentage content of related substances by using the areas of the first peak (impurity A) and the second peak (impurity B) in the chromatogram obtained from *Standard solution (c)* as a comparison area (1.0 per cent).

Limits

Disregard limit Disregard the peaks due to dimethylformamide and dimethylacetamide; not more than 0.05 times the area of the second peak in the chromatogram obtained from Standard solution (c) (0.05 per cent).

Impurity A Not more than the area of the first peak in the chromatogram obtained from Standard solution (c) (1.0 per cent).

Any other impurity Not more than the area of the second peak in the chromatogram obtained from Standard solution (c) (1.0 per cent).

Total Not more than 3 times the area of the second peak in the chromatogram obtained from Standard solution (c) (3.0 per cent).

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Prepare a mixture of 4 volumes of *acetonitrile* and 96 volumes of a 0.272 per cent w/v solution of *potassium dihydrogenphosphate*. Make adjustments if necessary.

Resolution solution Dissolve about 5 mg of Cefadroxil RS and about 50 mg of Amoxicillin Trihydrate RS in *Mobile phase* and dilute to 100.0 ml with the same solvent.

Standard preparation Dissolve about 50 mg of Cefadroxil RS, accurately weighed, in *Mobile phase* and dilute to 100.0 ml with the same solvent.

Assay preparation Dissolve about 50 mg of Cefadroxil, accurately weighed, in *Mobile phase* and dilute to 100.0 ml with the same solvent.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (25 cm × 4.6 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles (5 µm), (b) *Mobile phase* at a flow rate of about 1.0 ml per minute, and (c) an ultraviolet photometer set at 254 nm.

To determine the suitability of the chromatographic system, chromatograph *Resolution solution*, and record the peak responses as directed under *Procedure*: the resolution factor between cefadroxil and amoxicillin peaks is not less than 5.0. Chromatograph *Standard preparation*, and record the peak responses as directed under *Procedure:* the relative standard deviation for replicate injections is not more than 2.0 per cent and the symmetry factor for the cefadroxil peak is not more than 2.2.

Procedure Separately inject equal volumes (about 20 µl) of *Standard preparation* and *Assay preparation* into the chromatograph, record the chromatograms, and measure the responses for the major peaks.

Calculation Calculate the content of $C_{16}H_{17}N_3O_5S$ in the Cefadroxil taken, using the declared content of $C_{16}H_{17}N_3O_5S$ in Cefadroxil RS.

CEFADROXIL CAPSULES

Category Antibacterial (first-generation cephalosporin).

Cefadroxil Capsules contain the equivalent of not less than 90.0 per cent and not more than 120.0 per cent of the labelled amount of $C_{16}H_{17}N_3O_5S$.

Strength available 500 mg (anhydrous).

Dose Adults: 1 to 2 g daily as a single dose or in two divided doses.

Children: 30 mg per kg of body weight as a single dose or in two divided doses.

Contra-indication; Warning; Precaution See under *Cephalexin*, p. 58.

Additional information See under *Cefadroxil*, p. 37.

Packaging and storage Cefadroxil Capsules shall be kept in tightly closed containers.

Labelling The label on the container states (1) the quantity equivalent to the amount of anhydrous cefadroxil; (2) whether it is in the monohydrate or hemihydrate form.

Identification

A. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

B. Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel H* as the coating substance and a mixture of 3 volumes of a 6.7 per cent w/v solution of *ninhydrin* in *acetone*,

80 volumes of 0.1 M *disodium hydrogenphosphate* and 120 volumes of 0.1 M *citric acid* as the mobile phase. Impregnate the plate by development with a 5 per cent v/v solution of n-*tetradecane* in *hexane*. Allow the solvent to evaporate and carry out the chromatography in the same direction as the impregnation. Apply separately to the plate, 20 µl of each of the following solutions. For solution (A) stir a quantity of the contents of the capsules containing the equivalent of 200 mg of anhydrous cefadroxil with 100 ml of *water*, filter and use the filtrate. Solution (B) contains 0.2 per cent w/v of Cefadroxil RS in *water*. After removal of the plate, allow it to dry in air, spray with a 0.2 per cent w/v solution of *ninhydrin* in *absolute ethanol*, heat the plate at 110° for 10 minutes and allow it to cool. The principal spot in the chromatogram obtained from solution (A) is similar in position and size to that in the chromatogram obtained from solution (B).

Dissolution Carrry out the test as described in the "Dissolution Test" (Appendix 4.24).

Dissolution medium: *water*; 900 ml.

Apparatus 1: 100 rpm.

Time: 30 minutes.

Procedure Determine the amount of $C_{16}H_{17}N_3O_5S$ dissolved from absorbances at the maximum at about 263 nm of filtered portion of the test solution, suitably diluted with *Dissolution medium*, if necessary, in comparison with a standard solution having a known concentration of Cefadroxil RS in the same medium (Appendix 2.2).

Tolerances Not less than 80 per cent (*Q*) of the labelled amount of $C_{16}H_{17}N_3O_5S$ is dissolved in 30 minutes.

Water Not more than 7.0 per cent w/w (Karl Fischer Method, Appendix 4.12). Use 500 mg.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase, Resolution solution, Chromatographic system and **Procedure** Proceed as directed in the Assay described under *Cefadroxil*, p 37.

Standard preparation Dissolve an accurately weighed quantity of Cefadroxil RS, in *Buffer phosphate solution* and dilute quantitatively to obtain a solution having a known concentration of about 1 mg per ml.

Assay preparation Remove as completely as possible, the contents of not less than 20 Cefadroxil Capsules and weigh accurately. Mix the combined contents, and transfer an accurately weighed quantity, containing about 200 mg of cefadroxil, to a 200-ml volumetric flask. Add 150 ml of *Buffer phosphate solution*, and shake for 5 minutes. Dilute with the same solvent to volume and filter.

Calculation Calculate the content of $C_{16}H_{17}N_3O_5S$ in the portion of the Capsules taken, using the declared content of $C_{16}H_{17}N_3O_5S$ in Cefadroxil RS.

Other requirements Comply with the requirements described under "Capsules" (Appendix 1.16).

CEFADROXIL FOR ORAL SUSPENSION

Category Antibacterial (first-generation cephalosporin).

Cefadroxil for Oral Suspension is a dry mixture of cefadroxil and one or more suitable buffers, colours, diluents, and flavours. It contains the equivalent of not less than 90.0 per cent and not more than 120.0 per cent of the labelled amount of $C_{16}H_{17}N_3O_5S$.

Strengths available 125, 250 and 500 mg (anhydrous) per 5 ml.

Dose Adults: 1 to 2 g daily as a single dose or in two divided doses.

Children: 30 mg per kg of body weight as a single dose or in two divided doses.

Contra-indication; Warning; Precaution See under *Cephalexin*, p. 58.

Additional information See under *Cefadroxil*, p. 37.

Packaging and storage Cefadroxil for Oral Suspension shall be kept in tightly closed containers. After constitution, it should be used within the period stated on the label.

Labelling The label on the container states (1) the quantity equivalent to the amount of anhydrous cefadroxil; (2) whether it is in the monohydrate or hemihydrate form.

Identification

A. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

B. Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel H* as the coating substance and a mixture of 3 volumes of a 6.7 per cent w/v solution of *ninhydrin* in *acetone*, 80 volumes of 0.1 M *disodium hydrogenphosphate* and 120 volumes of 0.1 M *citric acid* as the mobile phase. Impregnate the plate by development with a 5 per cent v/v solution of n-*tetradecane* in *hexane*. Allow the

solvent to evaporate and carry out the chromatography in the same direction as the impregnation. Apply separately to the plate, 20 µl of each of the following solutions. For solution (A) dilute a volume of the oral suspension containing 200 mg of cefadroxil to 100 ml with *water*, filter and use the filtrate. Solution (B) contains 0.2 per cent w/v of Cefadroxil RS in *water*. After removal of the plate, allow it to dry in air, spray with a 0.2 per cent w/v solution of *ninhydrin* in *absolute ethanol*, heat the plate at 110° for 10 minutes and allow it to dry in air. The principal spot in the chromatogram obtained from solution (A) is similar in position and size to that in the chromatogram obtained from solution (B).

pH 4.5 to 6.0, in the suspension constituted as directed in the labelling (Appendix 4.11).

Water Not more than 2.0 per cent w/w (Karl Fischer Method, Appendix 4.12).

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase, Resolution solution, Chromatographic system and **Procedure** Proceed as directed in the Assay described under *Cefadroxil*, p 38.

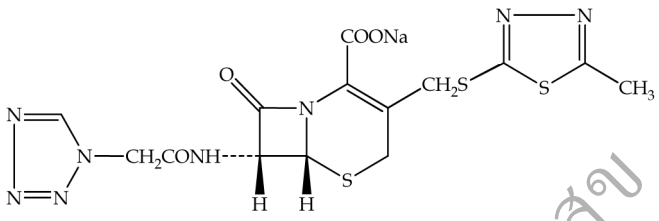
Buffer phosphate solution and **Standard preparation** Proceed as directed in the Assay described under *Cefadroxil Capsules*, p 20.

Assay preparation Constitute a container of Cefadroxil for Oral Suspension as directed in the labelling. Transfer an accurately measured volume of the resulting suspension, containing about 250 mg of cefadroxil, to a 250-ml volumetric flask, dilute with *Buffer phosphate solution*, stir by mechanical means for 5 minutes. Dilute with the same solvent to volume and filter. (**Note** this solution on the day prepared.)

Calculation Calculate the content of $C_{16}H_{17}N_3O_5S$, in the portion of the constituted Oral Suspension taken, using the declared content of $C_{16}H_{17}N_3O_5S$ in Cefadroxil RS.

Other requirements Complies with the requirements described under "Oral Liquids" (Appendix 1.16).

CEFAZOLIN SODIUM



$C_{14}H_{13}N_8O_4S_3.Na$ 476.48 *27164-46-1*

5-Thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid, 3-[[(5-methyl-1,3,4-thiadiazol-2-yl)thio]methyl]-8-oxo-7-[[1*H* tetrazol-1-yl)acetyl]amino]-, (6*R-trans*)-, monosodium salt.

Category Antibacterial (first-generation cephalosporin).

Cefazolin Sodium contains not less than 89.1 per cent and not more than 110.1 per cent of $C_{14}H_{13}N_8O_4S_3.Na$, calculated on the anhydrous basis.

Description White or almost white powder.

Solubility Freely soluble in *water*; very slightly soluble in *ethanol*; practically insoluble in *chloroform* and in *ether*.

Contra-indication It is contra-indicated in patients who have shown hypersensitivity to any member of the cephalosporins.

Warning

1. Cephalosporins should be avoided in patients who have had an immediate-type (anaphylactic) hypersensitivity reaction to penicillins and should be administered with caution in patients who have had a delayed-type (e.g., rash, fever, eosinophilia) reaction to penicillins or other drugs.

2. It may cause local reaction at the injection site, phlebitis and thrombophlebitis upon intraveneous administration, CNS side effects, hematological abnormalities especially hypoprothrombinemia, gastrointestinal disturbances, pseudomembranous colitis, and hypersensitivity reactions.

3. It should be administered with caution in patients with markedly impaired renal function or with a history of gastro-intestinal diseases, particularly colitis.

4. Risk-benefit should be considered if it is to be used in pregnant or nursing women.

Additional information

1. Cefazolin is used as an alternative to amoxicillin or ampicillin for prophylaxis against beta-hemolytic (viridans group) streptococcal endocarditis in penicillin-allergic individuals considered to be at risk for bacterial endocarditis following certain dental or upper respiratory tract procedures.

2. Cefazolin sodium can be administered intramuscularly by injection into a large muscle mass or intravenously by direct injection or by intermittent or continuous infusion. Total daily dosages are the same for intraveneous and intramuscular administration.

3. Cefazolin causes less pain on intramuscular injection than other first-generation cephalosporins. In addition, cefazolin is preferred to other first-generation cephalosporins because it has superior pharmacokinetics properties that result in higher and more sustained serum concentrations.

Packaging and storage Cefazolin Sodium shall be kept in tightly closed containers, protected from light and stored at a temperature not exceeding 25°. If it is intended for parenteral administration, it shall also be kept under sterile condition.

Labelling The label on the container states (1) storage condition; (2) parenteral grade.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Cefazolin Sodium RS (Appendix 2.1) or with the reference spectrum of Cefazolin Sodium.

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

C. It yields the *reactions* characteristic of sodium salts (Appendix 5.1).

Appearance of solution Dissolve 2.50 g in *carbon dioxide-free water* and dilute to 25.0 ml with the same solvent. The resulting solution is clear (Appendix 4.1) and its absorbance at 430 nm is not more than 0.15 (Appendix 2.2).

pH 4.0 to 6.0, in a 10.0 per cent w/v solution (Appendix 4.11).

Specific rotation –10.0° to –24.0°, calculated on the anhydrous basis, determined in a 5.5 per cent w/v solution in 0.1 M *sodium hydrogencarbonate* (Appendix 4.8).

Absorbance Dissolve 100 mg in *water* and dilute to 100.0 ml with the same solvent. Dilute 2.0 ml of the solution to 100.0 ml with a 4.2 per cent w/v solution of *sodium hydrogencarbonate*. The light absorption spectrum of the diluted solution, when observed between 220 nm and 350 nm, exhibits a maximum at about 272 nm; the specific absorbance at this maximum is between 260 and 300, calculated on the anhydrous basis (Appendix 2.2).

Water Not more than 6.0 per cent w/w (Karl Fischer Method, Appendix 4.12). Use 300 mg.

***N,N*-Dimethylaniline** Not more than 20 ppm (Appendix 5.16).

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Phosphate buffer pH 3.6 Dissolve 900 mg of *anhydrous disodium hydrogenphosphate* and 1.298 g of *citric acid monohydrate* in *water* to make 1000 ml.

Phosphate buffer pH 7.0 Dissolve 5.68 g of *anhydrous disodium hydrogenphosphate* and 3.63 g of *potassium dihydrogenphosphate* in *water* to make 1000 ml.

Mobile phase Prepare a mixture of 1 volume of *acetonitrile* and 9 volumes of *Phosphate buffer pH 3.6*. Make adjustments if necessary.

Internal standard solution Transfer 750 mg of *salicylic acid* to a 100-ml volumetric flask, dissolve in 10 ml of *methanol*, dilute with *Phosphate buffer pH 7.0* to volume, and mix.

Standard preparation Transfer about 25 mg of Cefazolin RS, accurately weighed, to a 25-ml volumetric flask, dissolve in and dilute with *Phosphate buffer pH 7.0* to volume and mix. Transfer 5.0 ml of this solution to a 100-ml volumetric flask, add 5.0 ml of *Internal standard solution*, dilute with *Phosphate buffer pH 7.0* to volume and mix.

Assay preparation Transfer about 50 mg of Cefazolin Sodium, accurately weighed, to a 50-ml volumetric flask, dissolve in and dilute with *Phosphate buffer pH 7.0* to volume, and mix. Transfer 5.0 ml of this solution to a 100-ml volumetric flask, add 5.0 ml of *Internal standard solution*, dilute with *Phosphate buffer pH 7.0* to volume, and mix.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (30 cm × 4.0 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles (10 μm), (b) *Mobile phase* at a flow rate of about 2 ml per minute, and (c) an ultraviolet photometer set at 254 nm.

To determine the suitability of the chromatographic system, chromatograph *Standard preparation*, and record the peak responses as directed under *Procedure*: the resolution factor between cefazolin and salicylic acid peaks is not less than 4.0, the symmetry factor is not more than 1.5 and the relative standard deviation for

replicate injections is not more than 2.0 per cent. The relative retention times are about 0.7 for salicylic acid and 1.0 for cefazolin.

Procedure Separately inject equal volumes (about 10 µl) of *Standard preparation* and *Assay preparation* into the chromatograph, record the chromatograms, and measure the responses for the major peaks.

Calculation Calculate the content of $C_{14}H_{13}N_8O_4S_3$.Na in the Cefazolin Sodium taken, using the declared content of $C_{14}H_{14}N_8O_4S_3$ in Cefazolin RS. Each mg of $C_{14}H_{14}N_8O_4S_3$ is equivatent to 1.0484 mg of $C_{14}H_{13}N_8O_4S_3$.Na.

CEFAZOLIN SODIUM FOR INJECTION

Category Antibacterial (first-generation cephalosporin).

Cefazolin Sodium for Injection is a sterile material consisting of cefazolin sodium with or without excipients equivalent to not less than 90.0 per cent and not more than 115.0 per cent of the labelled amount of $C_{14}H_{14}N_8O_4S_3$.

Strengths available 250 and 500 mg, 1 g (base); 5, 10 and 20 g (base, pharmacy bulk package).

Dose Adults: Deep *intramuscular* or *intravenous*, 1 to 6 g daily in divided doses every 6 to 8 hours.

Children and infants: Deep *intramuscular* or *intravenous*, 25 to 100 mg per kg of body weight daily in divided doses every 6 to 8 hours.

Contra-indication; Warning; Additional information See under *Cefazolin sodium*, p. 40.

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains not more than 0.15 Endotoxin Unit per mg of cefazolin.

Packaging and storage Cefazolin Sodium for Injection shall be kept in Containers for Sterile Solids as described under "Parenteral Preparations" (Appendix 1.16), protected from light and stored at a temperature not exceeding 30°.

Labelling The label on the container states the quantity equivalent to the amount of cefazolin.

Identification

A. The ultraviolet absorption spectrum of a 0.002 per cent w/v solution of the injection in 0.1 M *sodium hydrogencarbonate* exhibits maxima at the same wavelengths as that of a similar solution of Cefazolin Sodium RS, concomitantly measured (Appendix 2.2).

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

C. It yields the *reactions* characteristic of sodium salts (Appendix 5.1).

pH 4.0 to 6.0, in a 10.0 per cent w/v solution (Appendix 4.11).

Water Not more than 6.0 per cent w/w (Karl Fischer Method, Appendix 4.12). Use 300 mg.

Particulate matter Complies with the requirements described under "Particulate Matter in Injections" (Small-volume Injections, Appendix 4.27).

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Phosphate buffer pH 3.6, Phosphate buffer pH 7.0, Mobile phase, Internal standard solution, Standard preparation, Chromatographic system and **Procedure** Proceed as directed in the Assay described under *Cefazolin sodium*, p 40.

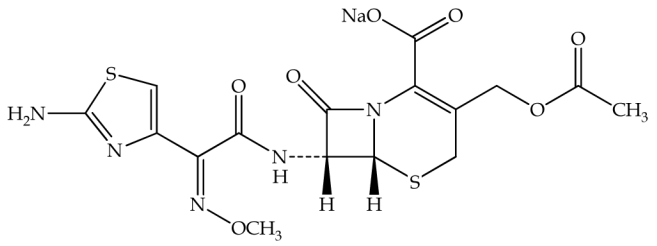
Assay preparation 1 (where it is packaged for dispensing and is represented as being in a single-dose container) Constitute Cefazolin Sodium for Injection in a volume of *water*, accurately measured, corresponding to the volume of solvent specified in the labelling. Withdraw all of the contents, using a suitable hypodermic needle and syringe, and dilute quantitatively with *Phosphate buffer pH 7.0* to obtain a stock solution containing about 1 mg of cefazolin per ml. Transfer 5.0 ml of this solution to a 100-ml of volumetric flask, add 5.0 ml of *Internal standard solution*, dilute with *Phosphate buffer pH 7.0* to volume, and mix.

Assay preparation 2 (where the label states the quantity of cefazolin in a given volume of constituted solution) Constitute Cefazolin Sodium for Injection in an accurately measured volume of *water* corresponding to the volume of solvent specified in the labelling. Dilute an accurately measured volume of the constituted solution quantitatively with *Phosphate buffer pH 7.0* to obtain a solution containing about 1 mg of cefazolin per ml. Transfer 5.0 ml of this solution to a 100-ml of volumetric flask, add 5.0 ml of *Internal standard solution*, dilute with *Phosphate buffer pH 7.0* to volume, and mix.

Calculation Calculate the content of $C_{14}H_{14}N_8O_4S_3$ in each ml of the constituted Injection taken, using the declared content of $C_{14}H_{14}N_8O_4S_3$ in Cefazolin RS.

Other requirements Complies with the requirements described under "Parenteral Preparations" (Appendix 1.16).

CEFOTAXIME SODIUM



$C_{16}H_{16}N_5O_7S_2.Na$ 477.44 *64485-93-4*

5-Thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid, 3-(acetyloxy)methyl-7-[[(2-amino-4-thiazolyl)(methoxyimino)acetyl]amino]-8-oxo-, [6*R*-[6α,7β(*Z*)]]-, monosodium salt.

Category Antibacterial (third-generation cephalosporin).

Cefotaxime Sodium contains not less than 96.0 per cent and not more than 101.0 per cent of $C_{16}H_{16}N_5O_7S_2.Na$, calculated on the dried basis.

Description White or slightly yellow powder. It is hygroscopic.

Solubility Freely soluble in *water*; sparingly soluble in *methanol*; practically insoluble in *ether*.

Contra-indication It is contra-indicated in patients who have shown hypersensitivity to any member of the cephalosporins.

Warning

1. It should be avoided in patients who have had an immediate-type (anaphylactic) hypersensitivity reaction to penicillins and should be administered with caution in patients who have had a delayed-type (e.g., rash, fever, eosinophilia) reaction to penicillins or other drugs.

2. It should be used with caution in patients with a history of gastro-intestinal diseases, particularly colitis.

3. It should be administered with caution and in reduced dosage in the presence of markedly impaired renal function.

4. It may cause local reaction at the injection site, gastro-intestinal disturbances, headache, hypersensitivity reactions, pseudomembranous colitis, and hematological abnormalities.

5. Its overdosage can cause CNS irritation leading to seizures.

6. Prolonged use may result in the overgrowth of nonsusceptible organisms, especially Pseudomonas and Candida.

7. Risk-benefit should be considered if it is to be used in pregnant or nursing women.

Precaution Cefotaxime therapy should be discontinued if resistant strains of some organisms, especially Enterobacter, *Pseudomonas aeruginosa*, and Serratia have developed during therapy.

Additional information

1. Group A beta-hemolytic streptococcal infections should be treated for at least 10 days to prevent the development of acute rheumatic fever or acute glomerulonephritis.

2. Continue administration for a minimum of 48 to 72 hours after fever abates or after evidence of bacterial eradication has been obtained.

Packaging and storage Cefotaxime Sodium shall be kept in tightly closed containers, protected from light.

Labelling The label on the container states (1) storage condition; (2) parenteral grade.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Cefotaxime Sodium RS (Appendix 2.1) or with the reference spectrum of Cefotaxime Sodium.

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation 1, as obtained in the *Assay*.

C. Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel HF254* as the coating substance and a mixture of 15 volumes of *acetone* and 85 volumes of a 15.4 per cent w/v solution of *ammonium acetate*, previously adjusted to pH 6.2 with *glacial acetic acid* as the mobile phase. Apply separately to the plate, 1 µl of each of the following solutions. For solution (A) dissolve 20 mg of the test substance in 5.0 ml of a mixture of equal volumes of *methanol* and *0.067 M mixed phosphate buffer pH 7.0*. For solution (B) dissolve 20 mg of Cefotaxime Sodium RS in 5.0 ml of a mixture of equal volumes of *methanol* and *0.067 M mixed phosphate buffer pH 7.0*. For solution (C) dissolve 20 mg of Cefotaxime Sodium RS and 20 mg of Cefoxitin Sodium RS in 5.0 ml of a mixture of equal volumes of *methanol* and *0.067 M mixed phosphate buffer pH 7.0*. After removal of the plate, allow it to dry and examine under ultraviolet light (254 nm). The principal spot in the chromatogram obtained from solution (A) is similar in position and size to that obtained from solution (B). The test is not valid unless the chromatogram obtained from solution (C) shows two clearly separated spots.

D. It yields the *reactions* characteristic of sodium salts (Appendix 5.1).

Clarity and colour of solution Transfer 2.5 g of the test substance to a 25-ml volumetric flask. Dissolve in and dilute with *carbon dioxide-free water* to volume, mix, and examine immediately: the solution is clear. Measure the absorbance of this solution at 430 nm in a 1-cm cell, using *carbon dioxide-free water* as the blank: its absorbance is not more than 0.20. Transfer 10 ml of the solution to a glass test-tube, add 1 ml of *glacial acetic acid*, mix, and examine immediately: the solution is clear.

pH 4.5 to 6.5, in a 10.0 per cent w/v solution (Appendix 4.11).

Specific rotation +58° to +64°, calculated on the dried basis, determined in a 1.0 per cent w/v solution (Appendix 4.8).

Loss on drying Not more than 3.0 per cent w/w after drying at 105° for 3 hours (Appendix 4.15).

Absorbance Dissolve 20.0 mg in *water* and dilute to 100.0 ml with the same solvent. Dilute 10.0 ml of the solution to 100.0 ml with *water*. The specific absorbance at the maximum at 235 nm is between 360 to 390, calculated on the dried basis (Appendix 2.2).

Related substances Carry out the test as described under *Assay*, using Assay preparation and Standard preparation 2. Continue the chromatography for at least eight times the retention time of the peak.

In the chromatogram obtained from the Assay preparation, determine the percentage content of related substances by using the area of the principal peak in the chromatogram obtained from the Standard preparation 2 (1.0 per cent) as a comparison area.

Limits

Any impurity Not more than the comparison area (1.0 per cent).

Total Not more than 3 times the comparison area (3.0 per cent).

***N,N*-Dimethylaniline** Not more than 20 ppm (Appendix 5.16.).

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Dissolve 3.5 g of *potassium dihydrogenphosphate* and 11.6 g of *disodium hydrogenphosphate* in 1000 ml of *water* at pH 7.0 and add 180 ml of *methanol*. Make adjustments if necessary.

Standard preparation 1 Dissolve 25 mg of Cefotaxime Sodium RS in *Mobile phase* and dilute to 25.0 ml with the same solvent.

Standard preparation 2 Dilute 1.0 ml of *Standard preparation 1* to 100.0 ml with *Mobile phase*.

Assay preparation Dissolve about 25 mg of Cefotaxime Sodium, accurately weighed, in *Mobile phase* and dilute to 25.0 ml with the same solvent.

Resolution solution Add 1.0 ml of *dilute hydrochloric acid* to 4.0 ml of *Assay preparation*. Heat the solution at 40° for 2 hours. Add 5.0 ml of *phosphate buffer solution pH 6.6* and 1.0 ml of *sodium hydroxide TS*.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (25 cm × 4.6 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles (5 μm), (b) *Mobile phase* at a flow rate of about 1 ml per minute, and (c) an ultraviolet photometer set at 235 nm.

To determine the suitability of the chromatographic system, chromatograph *Resolution solution*, and record the peak responses as directed under *Procedure*: the resolution factor between two principal peaks is not less than 3.5 and the symmetry factor of cefotaxime peak is less than 2.0. Chromatograph *Standard preparation 1*, and record the peak responses as directed under *Procedure*: the relative standard deviation for six replicate injections is not more than 1.0 per cent.

Procedure Separately inject equal volumes (about 10 μl) of *Standard preparation 1* and *Assay preparation* into the chromatograph, record the chromatograms, and measure the responses for the major peaks.

Calculation Calculate the content of $C_{16}H_{16}N_5O_7S_2$.Na in the Cefotaxime Sodium taken, using the declared content of $C_{16}H_{16}N_5O_7S_2$.Na in Cefotaxime Sodium RS.

Other requirements Cefotaxime Sodium intended for parenteral administration complies with the following additional requirements.

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains not more than 0.20 Endotoxin Unit per mg of cefotaxime.

Sterility Complies with the "Sterility Test" (Method I, Appendix 10.1).

CEFOTAXIME SODIUM FOR INJECTION

Category Antibacterial (third-generation cephalosporin).

Cefotaxime Sodium for Injection is a sterile material consisting of Cefotaxime Sodium with or without excipients. It contains an amount of cefotaxime sodium equivalent to not less than 90.0 per cent and not more than 115.0 per cent of the labelled amount of $C_{16}H_{17}N_5O_7S_2$.

Strengths available 0.5, 1 and 10 g (base).

Dose Adults: Deep *intramuscular* or *intravenous*, 2 to 6 g daily in 2 to 4 divided doses or up to 12 g daily.

Children: Deep *intramuscular* or *intravenous*, 100 to 150 mg per kg of body weight daily in 2 to 4 divided doses.

Contra-indication; Warning; Precaution; Additional information See under *Cefotaxime Sodium*, p. 43.

Packaging and storage Cefotaxime Sodium for Injection shall be kept in Containers for Sterile Solids as described under "Parenteral Preparations" (Appendix 1.16), protected from light, and stored at a temperature not exceeding 30°.

Labelling The label on the container states the quantity equivalent to the amount of cefotaxime.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Cefotaxime Sodium RS (Appendix 2.1) or with the reference spectrum of Cefotaxime Sodium.

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

C. Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel HF254* as the coating substance and a mixture of 15 volumes of *acetone* and 85 volumes of a 15.4 per cent w/v solution of *ammonium acetate*, previously adjusted to pH 6.2 with *glacial acetic acid* as the mobile phase. Apply separately to the plate, 1 µl of each of the following solutions. For solution (A) dissolve a quantity of the contents of a sealed container in sufficient of a mixture of equal volumes of *methanol* and *0.067 M mixed phosphate buffer pH 7.0* to produce a solution containing the equivalent of 4 mg per ml of cefotaxime. Solution (B) contains 4 mg per ml of Cefotaxime Sodium RS in a mixture of equal volumes of *methanol* and *0.067 M mixed phosphate buffer pH 7*. Solution (C) contains 4 mg per ml each of Cefotaxime Sodium RS and Cefoxitin Sodium RS in a mixture of equal volumes of *methanol* and *0.067 M mixed phosphate buffer pH 7*. After removal of the plate, allow it to dry in air and examine under ultraviolet light (254 nm). The principal spot in the chromatogram obtained from solution (A) corresponds to that obtained from solution (B). The test is not valid unless the chromatogram obtained with solution (C) shows two clearly separated spots.

D. It yields the *reactions* characteristic of sodium salts (Appendix 5.1).

Clarity of solution A solution containing the equivalent of 10.0 per cent w/v of cefotaxime in *carbon dioxide-free water* is clear (Appendix 4.1). The absorbance of the solution at 430 nm is not greater than 0.60 (Appendix 2.2).

pH 4.5 to 6.5, in a 10.0 per cent w/v solution (Appendix 4.11).

Loss on drying Not more than 3.0 per cent w/w after drying at 100° to 105° for 3 hours, (Appendix 4.15).

Particulate matter Complies with the requirement described under "Particulate Matter in Injections" (Small-volume Injections, Appendix 4.27).

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains not more than 0.20 Endotoxin Unit per mg of cefotaxime.

Related substances Carry out the test as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Use a mixture prepared by dissolving 3.5 g of *potassium dihydrogenphosphate* and 11.6 g of *disodium hydrogenphosphate* in 1000 ml of *water* at pH 7.0 and adding 375 ml of *methanol*.

Standard preparation Use a 0.0011 per cent w/v solution of Cefotaxime Sodium RS in *Mobile phase*.

Test solution Dissolve a quantity of the contents of the sealed container in sufficient of *Mobile phase* to produce a solution containing the equivalent of 0.1 per cent w/v of cefotaxime.

The chromatographic conditions described under *Assay* may be used.

Procedure Separately inject 10 µl each solution. Allow the chromatography to proceed for at least 8 times the retention time (about 6 minutes) of cefotaxime. In the chromatogram obtained from *Test solution* the area of any secondary peak is not more than the area of the principal peak in the chromatogram obtained from *Standard solution* (1 per cent) and the sum

of the areas of all the secondary peaks is not more than 4 times the area of the principal peak in the chromatogram obtained from *Standard solution* (4 per cent).

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Dissolve 3.5 g of *potassium dihydrogenphosphate* and 11.6 g of *disodium hydrogenphosphate* in 1000 ml of *water* at pH 7.0 and add 375 ml of *methanol*. Make adjustments if necessary.

Standard preparation Dissolve an accurately weighed quantity of Cefotaxime Sodium RS in *Mobile phase* and dilute quantitatively to obtain a solution having a known concentration of about 100 µg per ml.

Assay preparation Dissolve an accurately weighed quantity of the mixed contents of the 10 containers of Cefotaxime Sodium for Injection in *Mobile phase* and dilute quantitatively to produce a solution containing about 100 µg of cefotaxime per ml.

Resolution solution Add 1.0 ml of 2 M *hydrochloric acid* to 4.0 ml of *Assay preparation*. Heat the solution at 40° for 2 hours. Add 5.0 ml of *Phosphate buffer solution pH 6.6* and 1.0 ml of 2 M *sodium hydroxide*.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (25 cm × 4.6 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles (5 µm), (b) *Mobile phase* at a flow rate of about 1 ml per minute, and (c) an ultraviolet photometer set at 235 nm.

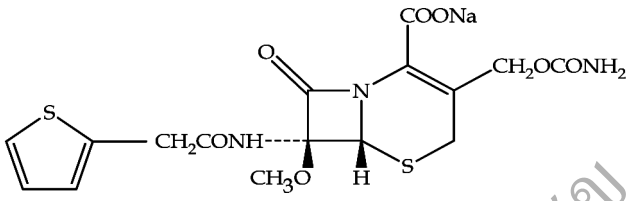
To determine the suitability of the chromatographic system, chromatograph *Resolution solution*, and record the peak responses as directed under *Procedure*: the resolution factor between two principal peaks is not less than 3.5 and the symmetry factor of cefotaxime peak is less than 2.0. Chromatograph *Standard preparation*, and record the peak responses as directed under *Procedure*: the relative standard deviation for replicate injections is not more than 2.0 per cent.

Procedure Separately inject equal volumes (about 10 µl) of *Standard preparation* and *Assay preparation* into the chromatograph, record the chromatograms, and measure the responses for the major peaks.

Calculation Calculate the content of $C_{16}H_{17}N_5O_7S_2$ in the portion of the Injection taken, using the declared content of $C_{16}H_{16}N_5O_7S_2$.Na in Cefotaxime Sodium RS. Each mg of $C_{16}H_{16}N_5O_7S_2$.Na is equivalent to 0.9540 mg of $C_{16}H_{17}N_5O_7S_2$.

Other requirements Complies with the requirements described under "Parenteral Preparations" (Appendix 1.16)

CEFOXITIN SODIUM



$C_{16}H_{16}N_3O_7S_2$.Na 449.43 *33564-30-6*

5-Thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid, 3-[[(aminocarbonyl)oxy]methyl]-7-methoxy-8-oxo-7-[(2-thienylacetyl)amino]-, (6*R-cis*)-, sodium salt.

Category Antibacterial (second-generation cephalosporin).

Cefoxitin Sodium contains the equivalent of not less than 95.0 per cent and not more than 102.0 per cent of $C_{16}H_{17}N_3O_7S_2$, calculated on the anhydrous basis.

Description White to off-white, granules or powder; odour, slight and characteristic.

Solubility Very soluble in *water*; soluble in *methanol*; sparingly soluble in *dimethylformamide*; slightly soluble in *acetone*; insoluble in *chloroform* and in *ether*.

Contra-indication; Additional information See under *Cephalexin*, p. 58.

Warning

1. Pain, tenderness and induration have been reported with intramuscular administration and thrombophlebitis may occur with intravenous administration.

2. Concurrent use of nephrotoxic agents may increase the risk of nephrotoxicity.

3. It may cause transient increase in serum aspartate transminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH) and alkaline phosphatase concentrations and jaundice.

See also under *Cephalexin*, p. 58.

Packaging and storage Cefoxitin Sodium shall be kept in tightly closed containers and stored at a temperature between 2° and 8°.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Cefoxitin RS (Appendix 2.1) or with the reference spectrum of Cefoxitin.

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

C. The ultraviolet absorption spectrum, when observed between 220 and 400 nm, of a 0.002 per cent w/v solution in phosphate buffer prepared by dissolv-

ing 1.0 g of *potassium dihydrogenphosphate* and 1.8 g of *anhydrous sodium hydrogenphosphate* in *water* to make 1000 ml, exhibits maxima at the same wavelengths as those of a similar solution of Cefoxitin Sodium RS, concomitantly measured (Appendix 2.2).

D. It yields the *reactions* characteristic of sodium salts (Appendix 5.1).

Crystallinity It is crystalline (Method I, Appendix 4.14).

pH 4.2 to 7.0, in a 10.0 per cent w/v solution (Appendix 4.11).

Specific rotation +206° to +214°, calculated on the anhydrous basis, determined in a solution prepared by dissolving 250 mg in *methanol* and diluting to 25.0 ml with the same solvent (Appendix 4.8).

Water Not more than 1.0 per cent w/w (Karl Fischer Method, Appendix 4.12). Use 500 mg.

Absorbance Dissolve 100.0 mg in *water* and dilute to 100.0 ml with the same solvent. Dilute 2.0 ml of the solution to 100.0 ml with a 4.2 per cent w/v solution of *sodium hydrogencarbonate*. The light absorption spectrum of the diluted solution, when observed between 220 nm and 350 nm, exhibits a maximum at about 236 nm and a broad absorption maximum at about 262 nm; the specific absorbance at this broad maximum is 190 to 210, calculated on the anhydrous basis (Appendix 2.2).

Related substances Carry out the test as described in the "High-pressure Liquid Chromatography" (Appendix 3.5). Prepare the solutions immediately before use.

Mobile phase

Mobile phase A Use *water* adjusted to pH 2.7 with *anhydrous formic acid*.

Mobile phase B Use *acetonitrile*.

Solution A Dilute 20 ml of a 34.8 g/l solution of *dipotassium hydrogenphosphate* adjusted to pH 6.8 with *phosphoric acid* to 1000 ml with *water*.

Test solution Dissolve 50.0 mg of the test substance in *Solution A* and dilute to 10.0 ml with the same solution.

Reference solution (a) Dilute 1.0 ml of *Test solution* to 100.0 ml with *Solution A*.

Reference solution (b) To 1.0 ml of *Test solution*, add 7.0 ml of *water* and 2.0 ml of *methanol*. Add 25 mg of *sodium carbonate*, stir for 10 minutes at room temperature, and then heat in a water-bath at 70° for 30 minutes. Allow to cool. Add 3 drops of *glacial acetic acid* and 1 ml of *Test solution* and mix.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (25 cm × 4.6 mm) packed with phenylsilyl silica gel for chromatography or ceramic microparticles (5 µm) with a specific surface area of 300 m^2/g and a pore size of 7 nm, (b) *Mobile phase* at a flow rate of about 1 ml per minute, and (c) an ultraviolet photometer set at 235 nm. The step gradient of mobile phases is as follows:

Time (minutes)	*Mobile phase A* (per cent v/v)	*Mobile phase B* (per cent v/v)
0 - 12	90	10
12 - 37	90 → 80	10 → 20
37 - 50	80 → 60	20 → 40
50 - 55	60 → 20	40 → 80
55 - 60	20	80
60 - 62	20 → 90	80 → 10
62 - 70	90	10

To determine the suitability of the chromatographic system, chromatograph *Reference solution (b)*, and record the peak responses as directed under *Procedure*: theresolution factor between the two principal peaks is not less than 5.0.

Procedure Separately inject equal volumes (about 50 µl) of *Reference solutions (a)* and *(b)*, and *Test solution* into the chromatograph, record the chromatograms, and measure the responses for the major peaks. The relative retention times are about 0.82 for impurity A, about 1.16 for impurity B, about 1.27 for impurity C, about 1.31 for impurity D and 1.0 for cefoxitin (retention time about 34 minutes).

In the chromatogram obtained from *Test solution*, determine the percentage content of related substances by using the area of the principal peak in the chromatogram obtained from *Reference solution (a)* (1.0 per cent) as a comparison area.

Limits

Disregard limit Not more than 0.05 times the comparison area (0.05 per cent).

Any impurity Not more than 0.5 times the comparison area (0.5 per cent).

Total Not more than 4 times the comparison area (4.0 per cent).

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Prepare a mixture of 84 volumes of *water*, 16 volumes of *acetonitrile* and 1 volume of *glacial acetic acid*. Make adjustments if necessary.

Phosphate buffer Dissolve 1.0 g of *potassium dihydrogenphosphate* and 1.8 g of *sodium*

hydrogenphosphate in 900 ml of *water*, adjust with *phosphoric acid* or 10 M *sodium hydroxide* to a pH of 7.1±0.1, dilute with *water* to make 1000 ml, and mix.

Standard preparation Dissolve an accurately weighed quantity of Cefoxitin RS in *Phosphate buffer pH 7.1* to obtain a solution having a known concentration of about 300 μg per ml. (Sonicate, if necessary, to dissolve the sample. Use this solution within 5 hours.)

Assay preparation Transfer about 150 mg of Cefoxitin Sodium, accurately weighed, to a 500-ml volumetric flask, dissolve in and dilute with *Phosphate buffer pH 7.1* to volume, and mix. (Sonicate, if necessary, to dissolve the specimen. Use this solution within 5 hours.)

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (30 cm × 3.9 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles (5 to 10 μm), (b) *Mobile phase* at a flow rate of about 1 ml per minute, and (c) an ultraviolet photometer set at 254 nm.

To determine the suitability of the chromatographic system, chromatograph *Standard preparation*, and record the peak responses as directed under *Procedure*: the relative standard deviation for replicate injections is not more than 1.0 per cent and the symmetry factor for the cefoxitin peak is not more than 1.5.

Procedure Separately inject equal volumes (about 10 μl) of *Standard preparation* and *Assay preparation* into the chromatograph, record the chromatograms, and measure the responses for the major peaks.

Calculation Calculate the content of $C_{16}H_{17}N_3O_7S_2$ in the Cefoxitin Sodium taken, using the declared content of $C_{16}H_{17}N_3O_7S_2$ in Cefoxitin RS.

Other requirements Cefoxitin Sodium intended for parenteral administration complies with the following additional requirements.

Bacterial endotoxins When test as described the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains not more than 0.13 Endotoxin Unit per mg of cefoxitin.

Sterility Complies with the "Sterility Test" (Method I, Appendix 10.1).

CEFOXITIN SODIUM FOR INJECTION

Category Antibacterial (second-generation cephalosporin).

Cefoxitin Sodium for Injection is a sterile material consisting of Cefoxitin Sodium with or without excipients equivalent to not less than 90.0 per cent and not more than 120.0 per cent of the labelled amount of $C_{16}H_{17}N_3O_7S_2$.

Strengths available 1, 2 and 10 g (base).

Dose Adults: Deep *Intramuscular* or *intravenous*, 3 to 12 g daily in divided doses every 4 to 8 hours.

Children and infants over 3 months: Deep *Intramuscular* or *intravenous*, 80 to 160 mg per kg of body weight daily divided into 4 to 6 equal doses. The maximum total dose should not exceed 12 g a day.

Contra-indication See under *Cefoxitin Sodium*, p. 46.

Warning

1. Pain, tenderness and induration have been reported with intramuscular administration and thrombophlebitis may occur with intravenous administration.

2. Concurrent use of nephrotoxic agents may increase the risk of nephrotoxicity.

3. It may cause transient increase in serum aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH) and alkaline phosphatase concentrations and jaundice.

See also under *Cephalexin*, p. 58.

Additional information See under *Cephelexin*, p. 58.

Packaging and storage Cefoxitin Sodium for Injection shall be kept in Containers for Sterile Solids as described under "Parenteral Preparations" (Appendix 1.16).

Labelling The label on the container states the quantity equivalent to the amount of cefoxitin.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Cefoxitin RS (Appendix 2.1) or with the reference spectrum of Cefoxitin.

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

C. The ultraviolet absorption spectrum, when observed between 220 and 400 nm, of a 0.002 per cent w/v solution of the injection in phosphate buffer prepared by dissolving 1.0 g of *potassium dihydrogenphosphate* and 1.8 g of *anhydrous disodium hydrogenphosphate* in *water* to make 1000 ml, exhibits

maxima at the same wavelengths as those of a similar solution of Cefoxitin Sodium RS, concomitantly measured (Appendix 2.2).

D. It yields the *reactions* characteristic of sodium salts (Appendix 5.1).

pH 4.2 to 7.0, in a 1.0 per cent w/v solution (Appendix 4.11).

Water Not more than 1.0 per cent w/w (Karl Fischer Method, Appendix 4.12). Use 500 mg.

Constituted solution At the time of use, complies with the requirements described under "Constituted Solutions" (Appendix 4.20).

Particulate matter Complies with the requirements described under "Particulate Matter in Injections (Small-volume Injections, Appendix 4.27).

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains not more than 0.13 Endotoxin Unit per mg of cefoxitin.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase, Phosphate buffer, **Standard preparation**, **Chromatographic system** and **Procedure** Proceed as directed in the Assay described under *Cefoxitin Sodium*, p 47.

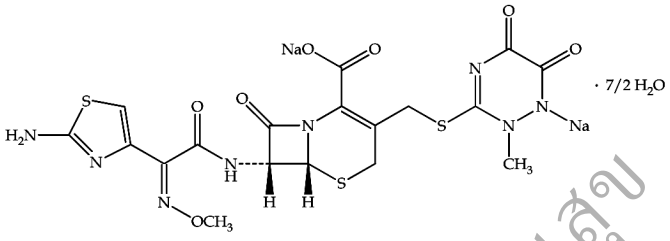
Assay preparation 1 (where it is represented as being in a single-dose container) Constitute Cefoxitin Sodium for Injection in a volume of *water*, accurately measured, corresponding to the volume of solvent specified in the labelling. Withdraw all of the contents, using a suitable hypodermic needle and syringe, and dilute quantitatively with *water* to obtain a solution containing about 300 µg of cefoxitin per ml. (**Note** Use this solution within 5 hours.)

Assay preparation 2 (where the label states the quantity of cefoxitin in a given volume of constituted solution) Constitute Cefoxitin Sodium for Injection in a volume of *water*, accurately measured, corresponding to the volume of solvent specified in the labelling. Dilute an accurately measured volume quantitatively with *water* to obtain a solution containing about 300 µg of cefoxitin per ml. (**Note** Use this solution within 5 hours.)

Calculation Calculate the content of $C_{16}H_{17}N_3O_7S_2$ in the portion of the constituted Injection taken, using the declared content of $C_{16}H_{17}N_3O_7S_2$, in Cefoxitin RS.

Other requirements Complies with the requirements described under "Parenteral Preparations" (Appendix 1.16).

CEFTRIAXONE SODIUM



$C_{18}H_{16}N_8O_7S_3.2Na.3½H_2O$ 661.59 *104376-79-6*

5-Thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid, 7-[[(2-amino-4-thiazolyl)(methoxyimino)acetyl]amino]-8-oxo-3-[[(1,2,5,6-tetrahydro-2-methyl-5, 6-dioxo-1,2,4-triazin-3-yl)thio]methyl]-, [6*R*-[6α,7β(*Z*)]]-, disodium salt, hydrate, (2:7).

Anhydrous 598.53

Category Antibacterial (third-generation cephalosporin).

Ceftriaxone Sodium contains not less than 96.0 per cent and not more than 102.0 per cent of $C_{18}H_{16}N_8O_7S_3.2Na$, calculated on the anhydrous basis.

Description Almost white or yellowish, crystalline powder; hygroscopic.

Solubility Freely soluble in *water*; sparingly soluble in *methanol*; very slightly soluble in *ethanol*.

Stability It is slightly hygroscopic.

Contra-indication It is contra-indicated in patients who have shown hypersensitivity to any member of the cephalosporins.

Warning

1. It should be avoided in patients who have had an immediate-type (anaphylactic) hypersensitivity reaction to penicillins and should be administered with caution in patients who have had a delayed-type (e.g., rash, fever, eosinophilia) reaction to penicillins or other drugs.

2. It should be used with caution in patients with a history of gastro-intestinal diseases, particularly colitis and in hyperbilirubinemic neonates, especially premature neonates.

3. It should be administered with caution and in reduced dosage in patients with markedly impaired renal function or in those with impaired hepatic and renal functions.

4. It may cause gastro-intestinal disturbances, hypersensitivity reactions, pseudomembranous colitis, pseudolithiasis (or biliary "sludge"), and hematological abnormalities.

5. Prolonged use may result in the overgrowth of nonsusceptible organisms, especially Enterobacter, Pseudomonas, Enterococci, or Candida.

6. Risk-benefit should be considered if it is to be used in pregnant or nursing women.

Precaution Group A beta-hemolytic streptococcal infections should be treated for at least 10 days to prevent the development of acute rheumatic fever or acute glomerulonephritis.

Additional information The admixture of beta-lactam antibacterials (penicillins and cephalosporins) and aminoglycosides may result in substantial mutual inactivation. If they are administered concurrently, they should be administered in separate sites and do not mix them in the same intravenous bag or bottle.

Packaging and storage Ceftriaxone Sodium shall be kept in tightly closed containers, protected from light.

Labelling The label on the container states parenteral grade.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Ceftriaxone Sodium RS (Appendix 2.1) or with the reference spectrum of Ceftriaxone Sodium.

B. The retention time of the major peak in the chromatogram of the Assay preparation 1 corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

C. It yields the *reactions* characteristic of sodium salts (Appendix 5.1).

Clarity of solution A solution containing the equivalent of 12.0 per cent w/v of ceftriaxone in *carbon dioxide-free water* is clear (Appendix 4.1).

Crystallinity It is crystalline (Appendix 4.14).

pH 6.0 to 8.0, in a 1.20 per cent w/v solution (Appendix 4.11).

Specific rotation –155° to –170°, calculated on the anhydrous basis, determined in a 1.0 per cent w/v solution (Appendix 4.8).

Related substances Carry out the test as described under *Assay* using Assay preparation 1, Resolution solution, and Assay preparation 2. Allow the chromatography to proceed for at least twice the retention time of the principal peak.

In the chromatogram obtained from the Assay preparation 1, determine the percentage content of related substances by using the area of the principal peak in the chromatogram obtained from the Assay preparation 2 (1.0 per cent) as a comparison area.

Limits

Disregard limit Not more than 0.1 times the comparison area (0.1 per cent).

Any impurity Not more than the comparison area (1.0 per cent).

Total Not more than 4 times the comparison area (4.0 per cent).

***N,N*-Dimethylaniline** Not more than 20 ppm (Appendix 5.16).

Water 8.0 to 11.0 per cent w/w (Karl Fischer Method, Appendix 4.12). Use 100 mg.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Dissolve 2.0 g of *tetradecylammonium bromide* and 2.0 g of *tetraheptylammonium bromide* in a mixture of 440 ml of *water*, 55 ml of 0.067 M *phosphate buffer solution pH 7.0*, 5 ml of citrate buffer solution pH 5.0 prepared by dissolving 20.17 g of *citric acid* in 800 ml of *water*, adjusting to pH 5.0 with 10 M *sodium hydroxide* and diluting to 1000.0 ml with *water*, and 500 ml of *acetronitrile*. Make adjustments if necessary.

Standard preparation Dissolve 30.0 mg of Ceftriaxone Sodium RS, in *Mobile phase* and dilute to 100.0 ml with the same solvent.

Assay preparation 1 Dissolve 30 mg of Ceftriaxone Sodium, accurately weighed, in *Mobile phase* and dilute to 100.0 ml with the same solvent.

Assay preparation 2 Dilute 1.0 ml of *Assay preparation 1* to 100.0 ml with the *Mobile phase*.

Resolution solution Dissolve an accurately weighed quantity of each of Ceftriaxone Sodium RS and Ceftriaxone Sodium *E*-isomer RS in *Mobile phase* to obtain a solution having each known concentration of 50 µg per ml.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (25 cm × 4.6 mm) packed with octadecylsilane chemically bonded to porous silica microparticles (5 µm), (b) *Mobile phase* at a flow rate of about 1.5 ml per minute, and (c) an ultraviolet photometer set at 254 nm.

To determine the suitability of the chromatographic system, chromatograph *Resolution solution*, and record the peak responses as directed under *Procedure*: the resolution factor between the two principal peaks is not

less than 3.0. Chromatograph *Standard preparation*, and record the peak responses as directed under *Procedure*: the relative standard deviation for replicate injections is not more than 2.0 per cent.

Procedure Separately inject equal volumes (about 20 µl) of *Standard preparation* and *Assay preparation 1* into the chromatograph, record the chromatograms, and measure the responses for the major peaks.

Calculation Calculate the content of $C_{18}H_{16}N_8O_7S_3.2Na$ in the Ceftriaxone Sodium taken, using the declared content of $C_{18}H_{16}N_8O_7S_3.2Na.3½H_2O$ in Ceftriaxone Sodium RS. Each mg of $C_{18}H_{16}N_8O_7S_3.2Na.3½H_2O$ is equivalent to 0.9047 mg of $C_{18}H_{16}N_8O_7S_3.2Na$.

Other requirements Ceftriaxone Sodium intended for parenteral administration complies with the following additional requirements.

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains not more than 0.20 Endotoxin Unit per mg of ceftriaxone.

Sterility Complies with the "Sterility Test" (Method I, Appendix 10.1).

CEFTRIAXONE SODIUM FOR INJECTION

Category Antibacterial (third-generation cephalosporin).

Ceftriaxone Sodium for Injection is a sterile material prepared from Ceftriaxone Sodium with or without excipients. It contains an amount of ceftriaxone sodium equivalent to not less than 90.0 per cent and not more than 115.0 per cent of the labelled amount of $C_{18}H_{18}N_8O_7S_3$.

Strengths available 0.25, 0.5, 1, 2, and 10 g (base).

Dose Adults: Deep *intramuscular* or *intravenous*, 1 to 2 g daily as a single dose or in 2 divided doses.

Children and infants: Deep *intramuscular* or *intravenous*, 20 to 50 mg per kg of body weight once daily.

Contra-indication; Precaution; Additional information See under *Ceftriaxone Sodium*, p. 49.

Warning The preparation reconstituted with bacteriostatic water containing benzyl alcohol should not be used for intramuscular injection in neonates.

See also under *Ceftriaxone Sodium*, p. 49.

Packaging and storage Ceftriaxone Sodium for Injection shall be kept in Containers for Sterile Solids as described under "Parenteral Preparations" (Appendix 1.16), protected from light, and stored at a temperature not exceeding 30°.

Labelling The label on the container states the quantity equivalent to the amount of ceftriaxone.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Ceftriaxone Sodium RS (Appendix 2.1) or with the reference spectrum of Ceftriaxone Sodium.

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

C. It yields the reactions characteristic of sodium salts (Appendix 5.1).

Clarity of solution A solution containing the equivalent of 1.20 per cent w/v of ceftriaxone in *carbon dioxide-free water* is clear (Appendix 4.1).

Crystallinity It is crystalline (Appendix 4.14).

pH 6.0 to 8.0, in a 12.0 per cent w/v solution (Appendix 4.11).

Water Not more than 11.0 per cent w/w (Karl Fischer Method, Appendix 4.12). Use 100 mg.

Particulate matter Complies with the requirement described under "Particulate Matter in Injections" (Small-volume Injections, Appendix 4.27).

Related substances Carry out the test as described under *Assay* using Assay preparation 1, and Assay preparation 2. Allow the chromatography to proceed for at least twice the retention time of the principal peak.

In the chromatogram obtained from the Assay preparation 1, determine the percentage content of related substances by using the area of the principal peak in the chromatogram obtained from the Assay preparation 2 (1.0 per cent) as a comparison area.

Limits

Disregard limit Not more than 0.1 times the comparison area (0.1 per cent).

Any impurity Not more than the comparison area (1.0 per cent).

Total Not more than 5 times the comparison area (5.0 per cent).

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains not more than 0.20 Endotoxin Unit per mg of ceftriaxone.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Dissolve 2 g of *tetradecylammonium bromide* and 2 g of *tetraheptylammonium bromide* in a mixture of 440 ml of *water*, 55 ml of 0.067 M *phosphate buffer solution pH 7.0*, 5 ml of citrate buffer solution pH 5.0 prepared by dissolving 20.17 g of *citric acid* in 800 ml of *water*, adjusting to pH 5.0 with 10 M *sodium hydroxide* and diluting to 1000.0 ml with *water,* and 500 ml of *acetronitrile*. Make adjustments if necessary.

Standard preparation Dissolve about 30 mg of Ceftriaxone Sodium RS, accurately weighed, in *Mobile phase* and dilute to 100.0 ml with the same solvent.

Resolution solution Dissolve an accurately weighed quantity of each of Ceftriaxone Sodium RS and Ceftriaxone Sodium E-isomer RS in *Mobile phase* to obtain a solution having each known concentration of 50 µg per ml.

Assay preparation 1 Dissolve an accurately weighed quantity of the mixed contents of the 10 containers of Ceftriaxone Sodium for Injection with *Mobile phase* to produce a solution containing the equivalent of about 300 µg of ceftriaxone per ml.

Assay preparation 2 Dilute 1.0 volume of *Assay preparation 1* to 100.0 volumes with the *Mobile phase*.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (25 cm × 4.6 mm) packed with octadecylsilane silica microparticles (5 µm), (b) *Mobile phase* at a flow rate of about 1.5 ml per minute, and (c) an ultraviolet photometer set at 254 nm.

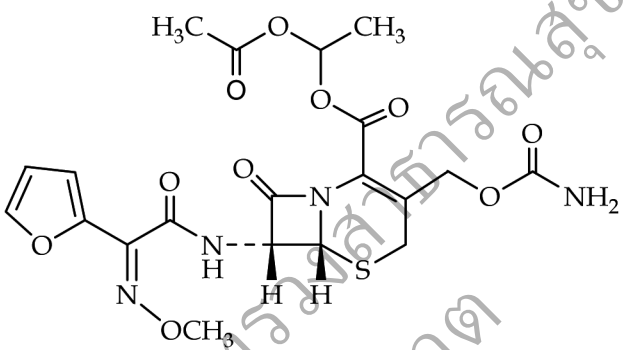
To determine the suitability of the chromatographic system, chromatograph *Resolution solution*, and record the peak responses as directed under *Procedure*: the resolution factor between ceftriaxone and ceftriaxone sodium E isomer peaks is not less than 3.0. Chromatograph *Standard preparation*, and record the peak responses as directed under *Procedure:* the relative standard deviation for replicate injections is not more than 2.0 per cent.

Procedure Separately inject equal volumes (about 20 µl) of *Standard preparation* and *Assay preparation 1* into the chromatograph, record the chromatograms, and measure the responses for the major peaks.

Calculation Calculate the content of $C_{18}H_{18}N_8O_7S_3$ in each ml of the Ceftriaxone Sodium for Injection taken, using the declared content of $C_{18}H_{16}N_8O_7S_3.2Na.3½H_2O$ in Ceftriaxone Sodium RS. Each mg of $C_{18}H_{16}N_8O_7S_3.2Na.3½H_2O$ is equivalent to 0.8382 mg of $C_{18}H_{18}N_8O_7S_3$.

Other requirements Complies with the requirements described under "Parenteral Preparations" (Appendix 1.16).

CEFUROXIME AXETIL



$C_{20}H_{22}N_4O_{10}S$ 510.48 *64544-07-6*

5-Thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid, 3-[[(aminocarbonyl)oxy]methyl]-7-[[2-furanyl-(methoxyimino)acetyl]amino]-8-oxo-, 1-(acetyloxy)ethyl ester, [6*R*- [6α,7β(*Z*)]]-.

Category Antibacterial (second-generation cephalosporin).

Cefuroxime Axetil contains the equivalent of not less than 96.0 per cent and not more than 102.0 per cent of a mixture of the two diastereoisomers of $C_{20}H_{22}N_4O_{10}S$, calculated on the anhydrous and acetone-free basis.

Description White or almost white powder.

Solubility Slightly soluble in *water* and in *ethanol*; soluble in *acetone*, *ethyl acetate* and in *methanol*.

Contra-indication; Additional information See under *Cephalexin*, p. 58.

Warning It may cause transient increase in serum aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH) and alkaline phosphatase concentrations and jaundice.

See also under *Cephalexin*, p. 58.

Packaging and storage Cefuroxime Axetil shall be kept in tightly closed containers, protected from light and stored at a temperature not exceeding 25°.

Labelling The label on the container states whether it is amorphous or crystalline.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Cefuroxime Axetil RS

(Appendix 2.1) or with the reference spectrum of Cefuroxime Axetil.

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

Diastereoisomer ratio Carry out the test as described under *Assay*.

In the chromatogram obtained from *Assay preparation 1*, the ratio of the peak due to cefuroxime axetil diastereoisomer A to the sum of the peaks due to cefuroxime axetil diastereoisomers A and B is between 0.48 and 0.55 by normalization.

Acetone Not more than 1.1 per cent. Carry out the test as described in the "Gas Chromatography" (Appendix 3.4).

Standard preparation Transfer 5.0 ml of *acetone* to a 1000-ml volumetric flask, dilute with *water* to volume, and mix (Solution A). Transfer 50.0 ml of *Solution A* to a 500-ml volumetric flask, dilute with *water* to volume, and mix to obtain a solution having concentration of *acetone* of 0.050 per cent v/v.

Test preparation Transfer 5.0 g of Cefuroxime Axetil to a 50-ml volumetric flask, dissolve in and dilute with *water* to volume, and mix. Transfer 3.0 ml of the resulting solution to a 15-ml centrifuge tube, cool in an ice-water-bath for 2 minutes, and add 3.0 ml of 0.24 M *hydrochloric acid* while swirling vigorously. Centrifuge to obtain a clear solution.

Chromatographic system The chromatographic procedure may be carried out using (a) a glass column (1.8 m × 6.3 mm) containing styrene-divinylbenzene copolymer packed with 60- to 80-mesh silane-treated glass beads with a nominal surface area of less than 50 m^2 per g and an average pore diameter of 0.3 to 0.4 μm and maintained at 110°, (b) the injection port and the detector block are maintained at 100° and 200°, respectively, (c) *nitrogen* as the carrier gas at a flow rate of about 50 ml per minute, and (d) a flame ionization detector.

Chromatograph *Standard preparation*, and record the peak responses as directed under *Procedure*.

Procedure Separately inject equal volumes of about 2 μl of *Standard preparation* and *Test preparation* into the chromatograph, record the chromatograms, and measure the area for the acetone peak. The test is not valid unless the acetone peak is not less than 160 theoretical plates, the symmetry factor for the acetone peak is not more than 1.3, and the relative standard deviation for replicate injections is not more than 5 per cent.

Calculation Calculate the percentages of acetone from the expression:

$$15.8P(r_u/r_s),$$

in which P is the percentage (v/v) of acetone in *Standard preparation*, and r_u and r_s are the acetone peak responses of *Test preparation* and *Standard preparation*, respectively.

Water Not more than 1.5 per cent w/w (Karl Fischer Method, Appendix 4.12). Use 400 mg.

Related substances Carry out the test as described under *Assay* using Standard preparation, Assay preparation 1, Assay preparation 2, Assay preparation 3, and Assay preparation 4. Prepare the solutions immediately before use.

In the chromatogram obtained from the Assay preparation 1, determine the percentage content of related substances by using the sum of areas of the two principal peaks (Cefuroxime axetil diastereoisomer B and Cefuroxime axetil diastereoisomer A), in the chromatogram obtained from the Assay preparation 2 (1.0 per cent) as a comparison area.

Limits

Disregard limit Not more than 0.05 times the comparison area (0.05 per cent).

***E*-isomers** The areas of the pair of peaks corresponding to the *E*-isomers in the Assay preparation 4 is not more than the comparison area (1.0 per cent).

Δ^3-isomers The areas of the pair of peaks corresponding to the Δ^3-isomers in the Assay preparation 3 is not more than 1.5 times the comparison area (1.5 per cent).

Any impurity Not more than 0.5 times the comparison area (0.5 per cent).

Total Not more than 3 times the comparison area (3.0 per cent).

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Prepare a mixture of 38 volumes of *methanol* and 62 volumes of a 2.3 per cent w/v solution of *ammonium dihydrogenphosphate*. Make adjustments if necessary.

Standard preparation Dissolve about 10 mg of Cefuroxime Axetil RS, accurately weighed, in *Mobile phase* and dilute to 50.0 ml with *Mobile phase*. Prepare the solution immediately before use.

Assay preparation 1 Dissolve about 10 mg of Cefuroxime Axetil, accurately weighed, in *Mobile phase* and dilute to 50.0 ml with *Mobile phase*. Prepare the solution immediately before use.

Assay preparation 2 Dilute 1.0 ml of *Assay preparation 1* to 100.0 ml with *Mobile phase*.

Assay preparation 3 Heat 5 ml of *Assay preparation 1* at 60° for 1 hour to generate Δ^3-isomers.

Assay preparation 4 Expose 5 ml of *Assay preparation 1* to ultraviolet light at 254 nm for 24 hours to generate *E*-isomers.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (25 cm × 4.6 mm) packed with trimethylsilyl group chemically bonded to porous silica microparticles (5 μm), (b) *Mobile phase* at a flow rate of about 1.0 ml per minute, and (c) an ultraviolet photometer set at 278 nm.

To determine the suitability of the chromatographic system, chromatograph *Assay preparations 2, 3, 4* and *Standard preparation*, and record the peak responses as directed under *Procedure*: the relative retention times are about 0.9 for cefuroxime axetil diastereoisomer B, 1.2 for cefuroxime axetil-Δ^3-isomers, 1.7 and 2.1 for *E*-isomer and 1.0 for Cefuroxime axetil diastereoisomer A. Chromatograph *Assay preparation 2* and record the peak responses as directed under *Procedure*: the resolution factor between cefuroxime axetil diastereoisomer A and cefuroxime axetil-Δ^3-isomer peaks is not less than 1.5. Chromatograph *Standard preparation*, and record the peak responses as directed under *Procedure*: the resolution factor between cefuroxime axetil diastereoisomer A and B peaks is not less than 1.5 and the relative standard deviation for six replicate injections is not more than 2.0 per cent.

Procedure Separately inject equal volumes (about 20 μl) of *Standard preparation* and *Assay preparation 1* into the chromatograph, record the chromatograms, and measure the responses for the major peaks.

Calculation Calculate the content of $C_{20}H_{22}N_4O_{10}S$ in the Cefuroxime Axetil taken from the sum of areas of the two diastereoisomer peaks, using the declared content of $C_{20}H_{22}N_4O_{10}S$ in Cefuroxime Axetil RS.

CEFUROXIME AXETIL TABLETS

Category Antibacterial (second-generation cephalosporin).

Cefuroxime Axetil Tablets contain the equivalent of not less than 90.0 per cent and not more than 110.0 per cent of the labelled amount of $C_{16}H_{16}N_4O_8S$.

Strengths available 125, 250 and 500 mg (base).

Dose Adults: 250 or 500 mg twice daily.
Children: 125 or 250 twice daily.

Contra-indication See under *Cefuroxime Axetil*, p 52.

Warning It may cause transient increase in serum aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH) and alkaline phosphatase concentrations and jaundice.

See also under *Cephalexin*, p. 58.

Additional information See under *Cephalexin*, p. 59.

Packaging and storage Cefuroxime Axetil Tablets shall be kept in tightly closed containers.

Labelling The label on the container states (1) the quantity equivalent to the amount of cefuroxime; (2) whether it is amorphous or crystalline.

Identification

A. Extract a quantity of the powdered tablets containing the equivalent of 100 mg of cefuroxime with 5 ml of *dichloromethane*, filter and evaporate the filtrate to dryness: the infrared absorbtion spectrum of the residue is concordant with the spectrum obtained from Cefuroxime Axetil RS (Appendix 2.1) or with the reference spectrum Cefuroxime Axetil.

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

Related substances Carry out the test as described under *Assay* using Standard preparation, Assay preparation 1, Assay preparation 2, and Assay preparation 3. Prepare the solutions immediately before use.

Calculate the percentage content of related substances from the areas in chromatogram obtained from the Assay preparation 1 by normalization.

Limits

***E*-isomers** The sum of the areas of the pair of peaks corresponding to the *E*-isomers in the Assay preparation 3 is not more than 1.5 per cent.

Δ^3-isomers The sum of the areas of the pair of peaks corresponding to the Δ^3-isomers in the Assay preparation 2 is not more than 2.0 per cent.

Any impurity The area of any other secondary peak is not more than 1.0 per cent.

Dissolution Carry out the test as described in the "Dissolution Test" (Appendix 4.24).

Dissolution medium: 0.07 M *hydrochloric acid*; 900 ml.

Apparatus 2: 55 rpm.

Time: 15 and 45 minutes.

Procedure Determine the amount of $C_{16}H_{16}N_4O_8S$ dissolved from absorbances at the maximum at about 278 nm of a filtered portion of the test solution, suitably diluted with *Dissolution medium*, if necessary, in com-

parison with a standard solution having a known concentration of Cefuroxime Axetil RS equivalent to about 10 to 20 µg of cefuroxime per ml in the same medium (Appendix 2.2).

Tolerances Not less than 60 per cent (*Q*) of the labelled amount of $C_{16}H_{16}N_4O_8S$ is dissolved in 15 minutes, and not less than 75 per cent (*Q*) is dissolved in 45 minutes, except that where Tablets are labelled to contain the equivalent of 500 mg of cefuroxime, not less than 50 per cent (*Q*) of the labelled amount of $C_{16}H_{16}N_4O_8S$ is dissolved in 15 minutes and not less than 70 per cent (*Q*) is dissolved in 45 minutes.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5). (**Note** All solutions containing cefuroxime axetil, if not to be used for immediate analysis, should be stored in the dark at a temperature between 2° and 8° before analysis.)

Mobile phase Prepare a mixture of 38 volumes of *methanol* and 62 volumes of a 0.2 M *ammonium dihydrogenphosphate*. Make adjustments if necessary.

Standard preparation Dissolve an accurately weighed quantity of Cefuroxime Axetil RS in *Mobile phase* and dilute quantitatively to obtain a solution having a known concentration of about 300 µg per ml.

Assay preparation 1 Disperse 10 Cefuroxime Axetil Tablets in 0.2 M *ammonium dihydrogenphosphate*, previously adjusted to pH 2.4 with *phosphoric acid*, using about 10 ml per g of the stated content of cefuroxime, accurately measured. Immediately add sufficient *methanol* to produce a solution containing the equivalent of about 0.5 per cent w/v of cefuroxime and shake vigorously. Filter and dilute a quantity of the filtrate with sufficient of *Mobile phase* to produce a solution containing the equivalent of about 0.025 per cent w/v of cefuroxime.

Assay preparation 2 Heat a quantity of *Assay preparation* at 60° for 1 hour or until sufficient impurities (Δ^3-isomers) have been generated.

Assay preparation 3 Expose a quantity of *Assay preparation* to ultraviolet light at 254 nm for 24 hours or until sufficient impurities (*E*-isomers) have been generated.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (25 cm × 4.6 mm) packed with trimethyl group chemically bonded to porous silica microparticles (5 µm), (b) *Mobile phase* at a flow rate of about 1.2 ml per minute, and (c) an ultraviolet photometer set at 278 nm.

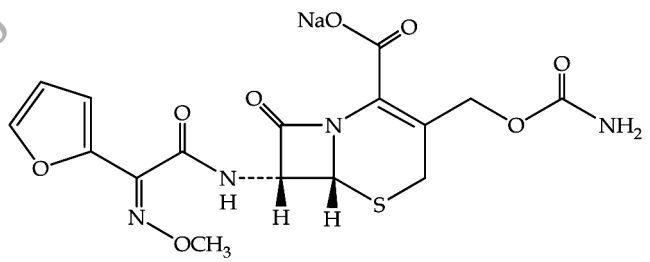
To determine the suitability of the chromatographic system, chromatograph *Assay preparation 2*, and record the peak responses as directed under *Procedure*: the resolution factor between cefuroxime axetil diastereoisomer A and cefuroxime axetil Δ^3-isomer peaks is not less than 1.5. Chromatograph *Standard preparation*, and record the peak responses as directed under *Procedure*: the relative standard deviation for replicate injections is not more than 2.0 per cent and the resolution factor between cefuroxime axetil diastereoisomer A and B peaks is not less than 1.5. The relative retention times are about 0.9 for cefuroxime axetil diastereoisomer B, 1.2 for cefuroxime axetil Δ^3-isomer, 1.7 and 2.1 for *E*-isomers, and 1.0 for cefuroxime axetil diastereoisomer A.

Procedure Separately inject equal volumes (about 20 µl) of *Standard preparation* and *Assay preparation 1* into the chromatograph, record the chromatograms, and measure the responses for the major peaks.

Calculation Calculate the content of $C_{16}H_{16}N_4O_8S$ in the portion of the Tablets taken, using the declared content of $C_{20}H_{22}N_4O_{10}S$ in Cefuroxime Axetil RS. Each mg of $C_{20}H_{22}N_4O_{10}S$ is equivalent to 0.8313 mg of $C_{16}H_{16}N_4O_8S$.

Other requirements Comply with the requirements described under "Tablets" (Appendix 1.16).

CEFUROXIME SODIUM



$C_{16}H_{15}N_4O_8S.Na$ 446.37 *56238-63-2*

5-Thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid, 3-[[(aminocarbonyl)oxy]methyl]-7-[[2-furanyl(methoxyimino)acetyl]amino]-8-oxo-, [6*R*-[6α,7β(*Z*)]]-, monosodium salt.

Category Antibacterial (second-generation cephalosporin).

Cefuroxime Sodium contains not less than 96.0 per cent and not more than 102.0 per cent of $C_{16}H_{15}N_4O_8S.Na$, calculated on the anhydrous basis.

Description White or almost white powder.

Solubility Freely soluble in *water*; very slightly soluble in *ethanol*, in *chloroform* and in *ethyl acetate*.

Contra-indication; Additional information See under *Cephalexin*, p. 58.

Warning It may cause transient increase in serum aspartate transaminase (AST), alanine transaminase (AST), lactate dehydrogenase (LDH) and alkaline phosphatase concentrations and jaundice.

See also under *Cephalexin*, p. 58.

Packaging and storage Cefuroxime Sodium shall be kept in tightly closed containers.

Labelling The label on the container states (1) storage condition; (2) parenteral grade.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Cefuroxime Sodium RS (Appendix 2.1) or with the reference spectrum of Cefuroxime Sodium.

B. It yields the *reactions* characteristic of sodium salts (Appendix 5.1).

Appearance of solution Dissolve 2.0 g in *carbon dioxide-free water* and dilute to 20.0 ml with the same solvent. The resulting solution is not more opalescent than *reference suspension II* (Appendix 4.1) and its absorbance at 450 nm is not more than 0.25 (Appendix 2.2).

pH 5.5 to 8.5, in a 1.0 per cent w/v solution (Appendix 4.11).

Specific rotation +59° to +66°, calculated on the anhydrous basis, determined in a 2.0 per cent w/v solution of *acetate buffer solution pH 4.6* (Appendix 4.8).

Water Not more than 3.5 per cent w/w (Karl Fischer Method, Appendix 4.12). Use 400 mg.

***N,N*-Dimethylaniline** Not more than 20 ppm (Appendix 5.16).

Related substances Carry out the test as described under *Assay* using Assay preparation 1, Assay preparation 2 and Resolution solution.

In the chromatogram obtained from the Assay preparation 1, determine the percentage content of related substances by using the area of the principal peak in the chromatogram obtained from the Assay preparation 2 (1.0 per cent) as a comparison area.

Limits

Disregard limit Not more than 0.05 times the comparison area (0.05 per cent).

Impurity A (descarbamoylcefuroxime) Not more than the comparison area (1.0 per cent).

Any other impurity Not more than the comparison area (1.0 per cent).

Total Not more than 3 times the comparison area (3.0 per cent).

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5)

Mobile phase Prepare a mixture of one volume of *acetonitrile* and 99 volumes of an acetate buffer solution pH 3.4 prepared by dissolving 6.01 g of *glacial acetic acid* and 0.68 g of *sodium acetate* in *water* and diluting to 1000.0 ml with *water*. Make adjustments if necessary.

Standard preparation Dissolve about 25 mg of Cefuroxime Sodium RS, accurately weighed, in *water* and dilute to 25.0 ml with the same solvent.

Assay preparation 1 Dissolve about 25 mg of Cefuroxime Sodium, accurately weighed, in *water* and dilute to 25.0 ml with the same solvent.

Assay preparation 2 Dilute 1.0 ml of *Assay preparation 1* to 100.0 ml with *water*.

Resolution solution Heat 20 ml of *Standard preparation* in a water-bath at 60° for 10 minutes. Cool and inject immediately.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (12.5 cm × 4.6 mm) packed with hexylsilyl group chemically bonded to porous silica microparticles (5 µm), (b) *Mobile phase* at a flow rate of about 1.5 ml per minute, and (c) an ultraviolet photometer set at 273 nm.

To determine the suitability of the chromatographic system, chromatograph *Resolution solution*, and record the peak responses as directed under *Procedure*: the resolution factor between cefuroxime and descarbamoylcefuroxime is not less than 2.0. Chromatograph *Standard preparation*, and record the peak responses as directed under *Procedure*: the relative standard deviation for replicate injections is not more than 2.0 per cent.

Procedure Separately inject equal volumes (about 20 µl) of *Standard preparation* and *Assay preparation 1* into the chromatograph, record the chromatograms, and measure the responses for the major peaks.

Calculation Calculate the content of $C_{16}H_{15}N_4O_8S.Na$ in the Cefuroxime Sodium taken, using the declared content of $C_{16}H_{15}N_4O_8S.Na$ in Cefuroxime Sodium RS.

Other requirements Cefuroxime Sodium intended for parenteral administration complies with the following additional requirements.

Bacterial endotoxins When tested as described the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains not more than 0.10 Endotoxin Unit per mg of cefuroxime.

Sterility Complies with the "Sterility Test" (Method I, Appendix 10.1).

CEFUROXIME SODIUM FOR INJECTION

Category Antibacterial (second-generation cephalosporin).

Cefuroxime Sodium for Injection is a sterile material consisting of Cefuroxime Sodium with or without excipients. It contains the equivalent of not less than 90.0 per cent and not more than 120.0 per cent of the labelled amount of $C_{16}H_{16}N_4O_8S$.

Strengths available 0.75, 1.5 and 7.5 g (base).

Dose Adults: Deep *Intramuscular* or *intravenous*, 750 mg to 3 g every 8 hours.

Children and infants over 3 months: Deep *Intramuscular* or *intravenous*, 50 to 100 mg per kg of body weight daily in equally divided doses every 6 to 8 hours.

Contra-indication; Additional information See under *Cephalexin*, p. 58.

Warning

1. Pain at the injection site has been reported with intramuscular administration, and thrombophlebitis may occur with intravenous administration.

2. It may cause transient increase in serum aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH) and alkaline phosphatase concentrations and jaundice.

See also under *Cephalexin*, p. 58.

Packaging and storage Cefuroxime Sodium for Injection shall be kept in Containers for Sterile Solids as described under "Parenteral Preparations" (Appendix 1.16), protected from light, and stored at a temperature not exceeding 30°.

Labelling The label on the container states the quantity equivalent to the amount of cefuroxime.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Cefuroxime Sodium RS (Appendix 2.1) or with the reference spectrum of Cefuroxime Sodium.

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

C. Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel HF254* as the coating substance and a mixture of 10 volumes of *tetrahydrofuran* and 90 volumes of a 15.0 per cent w/v solution of *ammonium acetate*, previously adjusted to pH 6.2 with *glacial acetic acid* as the mobile phase. Apply separately to the plate, 1 µl of each of the following solutions. For solution (A) dissolve a quantity of the contents of a sealed container in sufficient of a mixture of equal volumes of *methanol* and 0.067 M *mixed phosphate buffer pH 7.0* to produce a solution containing the equivalent of 400 µg ml of cefuroxime. Solution (B) contains 4 mg per ml of Cefuroxime Sodium RS in a mixture of equal volumes of *methanol* and 0.067 M *mixed phosphate buffer pH 7.0*. Solution (C) contains 400 µg per ml each of Cefuroxime Sodium RS and Cefoxitin Sodium RS in a mixture of equal volumes of *methanol* and 0.067 M *mixed phosphate buffer pH 7.0*. After removal of the plate, allow it to dry in a current of warm air, and examine under ultraviolet (254 nm): the principal spot in the chromatogram obtained from solution (A) corresponds to that obtained from solution (B). The test is not valid unless the chromatogram obtained from solution (C) shows two clearly separated principal spots.

D. It yields the *reactions* characteristic of sodium salts (Appendix 5.1).

Clarity of solution A solution containing the equivalent of 10.0 per cent w/v of cefuroxime in *carbon dioxide-free water* is not more opalescent than *reference suspension II* (Appendix 4.1).

Constituted solution At the time of use, complies with the requirements described under "Constituted Solutions" (Appendix 4.20).

pH 5.5 to 8.5, in a 10.0 per cent w/v solution (Appendix 4.11).

Water Not more than 3.5 per cent w/w (Karl Fischer Method, Appendix 4.12). Use 400 mg.

Particulate matter Complies with the requirements described under "Particulate Matter in Injections" (Small-volume Injections, Appendix 4.27).

Related substances Carry out the test as described under *Assay* using Assay preparation, Resolution solution, and Standard preparation 2.

In the chromatogram obtained from the Assay preparation, determine the percentage content of related substances by using the area of the principal peak in the chromatogram obtained from the Standard preparation 2 (1.0 per cent) as a comparison area.

Limits

Disregard limit Not more than 0.1 times the comparison area (0.1 per cent).

Impurity A (descarbamoylcefuroxime) Not more than the comparison area (1.0 per cent).

Any other impurity Not more than the comparison area (1.0 per cent).

Total Not more than 3 times the comparison area (3.0 per cent).

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains not more than 0.10 Endotoxin Unit per mg of cefuroxime.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Prepare a mixture of 1 volume of *acetonitrile* and 99 volumes of an acetate buffer solution pH 3.4 prepared by dissolving 6.01 g of *glacial acetic acid* and 0.68 g of *sodium acetate* in *water* and diluting to 1000.0 ml with the *water*. Make adjustments if necessary.

Standard preparation 1 Dissolve an accurately weighed quantity of Cefuroxime Sodium RS in *water* to obtain a solution having a known concentration of about 1 mg per ml.

Standard preparation 2 Dilute 1 volume of *Standard preparation 1* to 100 volumes.

Assay preparation Dissolve an accurately weighed quantity of mixed contents of the 10 containers of Cefuroxime Sodium for Injection in *water* to produce a solution containing the equivalent of about 1 mg per ml.

Resolution solution Heat 20 ml of *Standard preparation 1* in a water-bath at 60° for 10 minutes, cool and inject immediately.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (12.5 cm × 4.6 mm) packed with trimethyl group chemically bonded to porous silica or ceramic microparticles (5 µm), (b) *Mobile phase* at a flow rate of about 1.5 ml per minute, and (c) an ultraviolet photometer set at 273 nm.

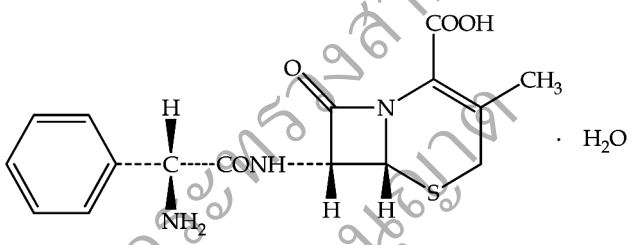
To determine the suitability of the chromatographic system, chromatograph *Resolution solution*, and record the peak responses as directed under *Procedure*: the resolution factor between cefuroxime and descabamoylcefuroxime peaks is not less than 2.0 and the symmetry factor of cefuroxime peak is not more than 1.5.

Procedure Separately inject equal volumes (about 20 µl) of *Standard preparation 1* and *Assay preparation* into the chromatograph, record the chromatograms, and measure the responses for the major peaks.

Calculation Calculate the content of $C_{16}H_{16}N_4O_8S$, in each ml of the Injection taken, using the declared content of $C_{16}H_{15}N_4O_8S.Na$ in Cefuroxime Sodium RS. Each mg of $C_{16}H_{15}N_4O_8S.Na$ is equivalent of 0.9508 mg of $C_{16}H_{16}N_4O_8S$.

Other requirements Complies with the requirements described under "Parenteral Preparations" (Appendix 1.16).

CEPHALEXIN



$C_{16}H_{17}N_3O_4S.H_2O$ 365.40 *23325-78-2*
5-Thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid, 7-[(aminophenylacetyl)amino]-3-methyl-8-oxo-, [6*R*-[6α, 7β(*R**)]]-, monohydrate.
Anhydrous 347.39 *15686-71-2*

Cephalexin contains not less than 95.0 per cent and not more than 101.0 per cent of $C_{16}H_{17}N_3O_4S$, calculated on the anhydrous basis.

Category Antibacterial (first-generation cephalosporin)

Description White or off-white, crystalline powder.

Solubility Slightly soluble in *water*; practically insoluble in *chloroform*, in *ethanol* and in *ether*.

Stability It is slightly hygroscopic. In neutral or alkaline solutions, cephalexin degrades rapidly. In acid solution, it is relatively more stable especially when kept at pH 4.5 under refrigeration.

Contra-indication It is contra-indicated in patients who have shown hypersensitivity to any member of the cephalosporins.

Warning

1. It should be avoided in patients who have had an immediate-type (anaphylactic) hypersensitivity reaction to penicillins and should be administered with caution in patients who have had a delayed-type (e.g., rash, fever, eosinophilia) reaction to penicillins or other drugs.

2. It should be used with caution in patients with a history of gastro-intestinal diseases, particularly colitis.

3. It should be administered with caution and in reduced dosage in the presence of markedly impaired renal function.

4. It may cause gastro-intestinal disturbances, headache, hypersensitivity reactions, pseudomembranous colitis, and hematological abnormalities.

5. Its overdosage can cause CNS irritation leading to seizures.

6. Prolonged use may result in the overgrowth of nonsusceptible organisms, especially Enterobacter, Pseudomonas, Enterococci, or Candida.

7. Risk-benefit should be considered if it is to be used in pregnancy or nursing women.

Additional information

1. Group A beta-hemolytic streptococcal infections should be treated for at least 10 days to prevent the development of acute rheumatic fever or acute glomerulonephritis.

2. Continue administration for a minimum of 48 to 72 hours after fever abates or after evidence of bacterial eradication has been obtained.

Packaging and storage Cephalexin shall be kept in tightly closed containers, protected from light, and stored at a temperature not exceeding 25°.

Labelling The label on the container states storage condition.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Cephalexin RS (Appendix 2.1) or with the reference spectrum of Cephalexin.

B. Place 2 mg in a test-tube about 150 mm long and 15 mm in diameter. Moisten with one drop of *water* and add 2 ml of *sulfuric-formaldehyde TS*. Mix the contents of the tube by swirling; the solution is pale yellow. Place the test-tube in a water-bath for 1 minute: a dark yellow colour develops.

C. Mix 20 mg with 5 drops of a 1 per cent v/v solution of *glacial acetic acid* and add 2 drops of a 1 per cent w/v solution of *copper(II) sulfate* and 1 drop of 2 M *sodium hydroxide*: an olive-green colour is produced.

Crystallinity It is crystalline (Method I, Appendix 4.14).

pH 3.0 to 5.5, in a 0.5 per cent w/v solution (Appendix 4.11).

Water Not less than 4.0 per cent w/w and not more than 8.0 per cent w/w (Karl Fischer Method, Appendix 4.12). Use 300 mg.

Sulfated ash Not more than 0.2 per cent w/w (Appendix 5.3).

Specific rotation +149° to +158°, calculated on the an hydrous basis, determined in a 0.5 per cent w/v in phthalate buffer solution pH 4.4 prepared by dissolving 2.042 g of *potassium hydrogenphthalate* in 50 ml of *water*, adding 7.5 ml of 0.20 M *sodium hydroxide* and diluting to 200 ml with *water* (Appendix 4.8).

Absorbance Dissolve 50 mg in *water* and dilute to 100.0 ml with the same solvent. The absorbance of the solution determined at 330 nm is not greater than 0.05. Dilute 2.0 ml of the solution to 50.0 ml with *water*. Examined between 220 nm and 300 nm, the diluted solution shows an absorption maximum at about 262 nm. The specific absorbance at this maximum is 220 to 245, calculated on the anhydrous basis (Appendix 2.2).

Related substances Not more than 1.0 per cent w/w of any individual cephalexin-related substance and not more than 5.0 per cent w/w of the sum of all cephalexin-related substances. Carry out the test as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Solution A Dissolve 1 g of *sodium 1-pentanesulfonate* in a mixture of 1000 ml of *water* and 15 ml of *triethylamine*. Adjust with *phosphoric acid* to a pH of 2.5±0.1. Make adjustments if necessary.

Solution B Dissolve 1 g of *sodium 1-pentanesulfonate* in a mixture of 300 ml of *water* and 15 ml of *triethylamine*. Adjust with *phosphoric acid* to a pH of 2.5±0.1, add 350 ml of *acetonitrile* and 350 ml of *methanol*, and mix. Make adjustments if necessary.

Mobile phase Use variable mixtures of *Solution A* and *Solution B* as directed for *Chromatographic system*.

Solvent Dissolve 18 g of *potassium dihydrogenphosphate* in 1000 ml of *water*.

Standard solution Dissolve accurately weighed quantities of Cephalexin RS quantitatively in *Solvent* to obtain solutions having known concentrations of about 80 and 160 µg of cephalexin per ml, respectively, taking into account the stated potency of the Cephalexin RS.

Test solution Transfer about 25 mg of the substance being examined in, accurately weighed, to a 5-ml volumetric flask, dissolve in and dilute with *Solvent* to volume, and mix.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (4.6 mm × 25 cm) packed with low acidity octadecylsilane chemically bonded to porous silica or ceramic microparticles (3 to 10 µm), (b) *Mobile phase* at a flow rate of about 1 ml per minute, and (c) an ultraviolet photometer set at 254 nm. The chromatograph is programmed as follows:

Time (minutes)	*Solution A* (per cent)	*Solution B* (per cent)	Elution
0	100	0	equilibration
0-1	100	0	isocratic
1-33.3	100→0	0→100	linear gradient
33.3-34.3	0	100	isocratic

Procedure Separately inject equal volumes (about 20 µl) of *Standard solutions* and *Test solution* into the chromatograph, record the chromatograms, and measure the responses for the cephalexin peaks in the chromatograms obtained from *Standard solutions* and for all of the peaks, other than that from cephalexin, in the chromatogram obtained from *Test solution*. Plot the responses of the cephalexin peaks in the chromatograms obtained from *Standard solutions* versus their concentrations, calculated on the anhydrous basis, in mg per ml, and draw a straight line through the two points and zero. From the line so obtained and the peak responses obtained from *Test solution*, determine the concentration, *I*, in mg per ml, of each cephalexin-related substance obtained from *Test solution* other than the cephalexin peak. Calculate the percentage of each cephalexin-related substance by the expression:

$$500I/W,$$

in which *W* is the quantity, calculated on the anhydrous basis, in mg, of Cephalexin taken to prepare the Test solution.

***N,N*-Dimethylaniline** Not more than 20 ppm (Appendix 5.16).

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Prepare a mixture of 5 volumes of *acetonitrile*, 2 volumes of *methanol*, 10 volumes of a 1.36 per cent w/v solution of *potassium dihydrogenphosphate* and 83 volumes of *water*. Make adjustments if necessary.

Resolution solution Dissolve about 10 mg of Cephalexin RS and 10 mg of Cephradine RS, accurately weighed, in *water* and dilute to 100.0 ml with the same solvent.

Standard preparation Dissolve about 50 mg of Cephalexin RS, accurately weighed, in *water* and dilute to 100.0 ml with the same solvent.

Assay preparation Dissolve about 50 mg of Cephalexin, accurately weighed, in *water* and dilute to 100.0 ml with the same solvent.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (25 cm × 4.6 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles (5 µm or 10 µm), (b) *Mobile phase* at a flow rate of about 1.5 ml per minute, and (c) an ultraviolet photometer set at 254 nm.

To determine the suitability of the chromatographic system, chromatograph *Resolution solution*, and record the peak responses as directed under *Procedure*: the resolution factor between cephalexin and cephradrine peaks is not less than 4.0. Chromatograph *Standard preparation*, and record the peak responses as directed under *Procedure*: the relative standard deviation for six replicate injections is not more than 1.0 per cent.

Procedure Separately inject equal volumes (about 20 µl) of *Standard preparation* and *Assay preparation* into the chromatograph, record the chromatograms, and measure the responses for the major peaks.

Calculation Calculate the content of $C_{16}H_{17}N_3O_4S$ of the Cephalexin taken, using the declared content of $C_{16}H_{17}N_3O_4S$ in Cephalexin RS.

CEPHALEXIN CAPSULES

Category Antibacterial (first-generation cephalosporin).

Cephalexin Capsules contain the equivalent of not less than 90.0 per cent and not more than 120.0 per cent of the labelled amount of $C_{16}H_{17}N_3O_4S$.

Strengths available 250 and 500 mg (anhydrous).

Dose Adults: 1 to 4 g daily in divided doses every 6 hours. Children: 25 to 50 mg per kg of body weight daily in divided doses every 6 hours. For severe infections, these dosages may be doubled.

Contra-indication; Warning; Precaution; Additional information See under *Cephalexin*, p. 58.

Packaging and storage Cephalexin Capsules shall be kept in tightly closed containers, protected from light.

Labelling The label on the container states the quantity equivalent to the amount of anhydrous cephalexin.

Identification

A. Shake a portion of the capsule contents, equivalent to 500 mg of cephalexin, with 1 ml of *water* and 1.4 ml of 1 M *hydrochloric acid*, filter and wash the filter with 1 ml of *water*. Add slowly to the filtrate a saturated solution of *sodium acetate* until precipitation occurs. Add 5 ml of *methanol*, filter and wash the precipitate

with two 1-ml portions of *methanol*. The residue, after drying at a pressure not exceeding 0.7 kPa (about 5 Torr), complies with the tests for *Identification* described under *Cephalexin*, p 58.

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of Standard preparation, as obtained in the *Assay*.

Water Not more than 10.0 per cent w/w (Karl Fischer Method, Appendix 4.12).

Dissolution Carry out the test as described in the "Dissolution Tests" (Appendix 4.24).

Dissolution medium: *water*; 900 ml.

Apparatus 1: 100 rpm.

Time: 30 minutes.

Procedure Determine the amount of $C_{16}H_{17}N_3O_4S$ dissolved from absorbances at the maximum at about 262 nm of filtered portions of the test solution, suitably diluted with *Dissolution medium*, if necessary, to a concentration of about 20 µg per ml, in comparison with a standard solution having a known concentration of Cephalexin RS in the same medium (Appendix 2.2).

Tolerances Not less than 80 per cent (*Q*) of the labelled amount of $C_{16}H_{17}N_3O_4S$ is dissolved in 30 minutes.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase, Standard preparation, Chromatographic system and **Procedure** Proceed as directed in the *Assay* under *Cephalexin*, p 60.

Assay preparation Remove as completely as possible, the contents of not less than 20 Cephalexin Capsules and weigh accurately. Mix the combined contents, and transfer an accurately weighed quantity, containing about 250 mg of cephalexin, to a 250-ml volumetric flask. Add 100 ml of *water* and shake for 30 minutes. Adjust to volume with *water* and filter. Dilute 25.0 ml of the filtrate to 50.0 ml with *water*.

Calculation Calculate the quantity of $C_{16}H_{17}N_3O_4S$, in mg, in a portion of the Capsules taken, using the declared content of $C_{16}H_{17}N_3O_4S$ in Cephalexin RS.

Other requirements Comply with the requirements described under "Capsules" (Appendix 1.16).

CEPHALEXIN FOR ORAL SUSPENSION

Category Antibacterial (first-generation cephalosporin).

Cephalexin for Oral Suspension is a dry mixture of Cephalexin and one or more suitable buffers, colours, diluents, and flavours. It contains the equivalent of not less than 90.0 per cent and not more than 120.0 per cent of the labelled amount of $C_{16}H_{17}N_3O_4S$ when constituted as directed in the labelling.

Strengths available 125 and 250 mg (anhydrous) per 5 ml.

Dose Adults: 1 to 4 g daily in divided doses every 6 hours.

Children: 25 to 50 mg per kg of body weight daily in divided doses every 6 hours. For severe infections, these dosages may be doubled.

Contra-indication; Warning; Precaution; Additional information See under *Cephalexin*, p. 58.

Packaging and storage Cephalexin for Oral Suspension shall be kept in tightly closed containers and stored at a temperature not exceeding 30°, protected from light. After constitution, it should be used within the period stated on the label.

Labelling The label on the container states the quantity equivalent to the amount of anhydrous cephalexin.

Identification

A. Shake a portion of the oral suspension, equivalent to 100 mg of cephalexin, with 70 ml of *methanol*, filter and evaporate the filtrate to dryness using a rotary evaporator. Dissolve the residue in the minimum volume of a 1 per cent v/v solution of *glacial acetic acid*, decolourize if necessary by the addition of sufficient *decolourizing charcoal*, shake and filter. To 5 drops of the resulting solution, add 2 drops of a 1 per cent w/v solution of *copper(II) sulfate* and 1 drop of 2 M *sodium hydroxide*: an olive-green colour is produced.

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

pH 3.0 to 6.0, in the suspension constituted as directed in the labelling (Appendix 4.11).

Water Not more than 2.0 per cent w/w (Karl Fischer Method, Appendix 4.12).

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase, Standard preparation, Chromatographic system and **Procedure** Proceed as directed in the *Assay* under *Cephalexin*, p. 60.

Assay preparation Transfer an accurately measured volume of Cephalexin for Oral Suspension, constituted as directed in the labelling, containing about 250 mg of cephalexin, to a 250-ml volumetric flask. Add 100 ml of *water* and shake for 30 minutes. Adjust to volume with *water* and filter. Dilute 25.0 ml of the filtrate to 50.0 ml with *water*.

Calculation Calculate the content of $C_{16}H_{17}N_3O_4S$, in mg, in each ml of the constituted Oral Suspension taken, using the declared content of $C_{16}H_{17}N_3O_4S$ in Cephalexin RS.

Other requirements Complies with the requirements described under "Oral Liquids" (Appendix 1.16).

CEPHALEXIN TABLETS

Category Antibacterial (first-generation cephalosporin).

Cephalexin Tablets contain the equivalent of not less than 90.0 per cent and not more than 120.0 per cent of the labelled amount of $C_{16}H_{17}N_3O_4S$.

Strengths available 250, 500 mg and 1 g (anhydrous).

Dose Adults: 1 to 4 g daily in divided doses every 6 hours.

Children: 25 to 50 mg per kg of body weight daily in divided doses every 6 hours. For severe infections, these dosages may be doubled.

Contra-indication; Warning; Precaution; Additional information See under *Cephalexin*, p. 58.

Packaging and storage Cephalexin Tablets shall be kept in tightly closed containers, protected from light.

Labelling The label on the container states the quantity equivalent to the amount of anhydrous cephalexin.

Identification

A. Remove any coating. Shake a portion of the powdered tablet cores, equivalent to about 500 mg of cephalexin, with 1 ml of *water* and 1.4 ml of 1 M *hydrochloric acid*, add 100 mg of *decolourizing charcoal*. Shake, filter and wash the filter with 1 ml of *water*. Add slowly to the filtrate a saturated solution of *sodium acetate* until precipitate occurs. Add 5 ml of *methanol*, filter and wash the precipitate with two 1-ml portions of *methanol*. The residue, after drying at a pressure not exceeding 0.7 kPa (about 5 Torr), complies with the tests for *Identification* described under *Cephalexin*, p 58.

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

Water Not more than 9.0 per cent w/w where Tablets contain Cephalexin (Karl Fischer Method, Appendix 4.12).

Dissolution Carry out the test as described in the "Dissolution Tests" (Appendix 4.24).

Dissolution medium: *water*; 900 ml.

Apparatus 1: 100 rpm.

Time: 30 minutes.

Procedure Determine the amount of $C_{16}H_{17}N_3O_4S$ dissolved from absorbances at the maximum at about 262 nm of filtered portions of the test solution, suitably diluted with *Dissolution medium*, if necessary, to a concentration of about 20 µg per ml, in comparison with a standard solution having a known concentration of Cephalexin RS in the same medium (Appendix 2.2).

Tolerances Not less than 80 per cent (*Q*) of the labelled amount of $C_{16}H_{17}N_3O_4S$ is dissolved in 30 minutes.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

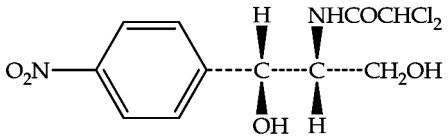
Mobile phase, Standard preparation, Chromatographic system and **Procedure** Proceed as directed in the Assay under *Cephalexin*, p 60.

Assay preparation Weigh and finely powder not less than 20 Cephalexin Tablets. Transfer an accurately weighed portion of the powder, containing about 250 mg of cephalexin, to a 250-ml volumetric flask. Add 100 ml of *water* and shake for 30 minutes. Adjust to volume with *water* and filter. Dilute 25.0 ml of the filtrate to 50.0 ml with *water*.

Calculation Calculate the content of $C_{16}H_{17}N_3O_4S$, in a portion of the Tablets taken, using the declared content of $C_{16}H_{17}N_3O_4S$ in Cephalexin RS.

Other requirements Comply with the requirements described under "Tablets" (Appendix 1.16).

CHLORAMPHENICOL



$C_{11}H_{12}Cl_2N_2O_5$ 323.13 *56-75-7*

Acetamide, 2,2-dichloro-*N*-[2-hydroxy-1-(hydroxymethyl)-2-(4-nitrophenyl)ethyl]-, [*R*-(*R**,*R**)].

Category Antibacterial; antirickettsial.

Chloramphenicol contains not less than 97.0 per cent and not more than 103.0 per cent of $C_{11}H_{12}Cl_2N_2O_5$, calculated on the dried basis.

Description Fine, white to greyish white or yellowish white, crystalline powder or crystals, needles or elongated plates.

Solubility Slightly soluble in *water*; freely soluble in *ethanol* and in *propylene glycol*; slightly soluble in *ether*.

Stability It is very stable unless being exposed to excessive light or moisture. In aqueous solutions, chloramphenicol degrades mainly by hydrolysis with the release of chloride ions. Hydrolysis is lowest at pH 6. It may also degrade by a photolytic reaction.

Warning

1. Jarisch-Herxheimer-like reactions may occur in patients receiving chloramphenicol for the treatment of typhoid fever.

2. It should be reserved for serious infections in which less toxic antibacterials are ineffective or contraindicated.

3. It may depress bone marrow or may cause fatal idiosyncratic aplastic anemia. It may also interfere with the development of immunity and, thus, should not be given during active immunization.

4. It should be used with extreme caution in premature or very young infants to avoid "grey syndrome" toxicity; in patients with impaired renal or hepatic function; or in those who are receiving oral anticoagulants, oral hypoglycemics, phenytoin, or bone marrow depressants (e.g., antineoplastics, phenylbutazone, etc.).

5. It is not recommended in pregnancy at term, during labour or in nursing mothers.

Precaution

1. Complete blood count should be performed prior to and approximately every 2 days during chloramphenicol therapy. Careful observation for sudden sore throat or development of additional infection are also recommended.

2. Long-term therapy may cause headache, mental confusion, delirium, optic and/or peripheral neuritis, superinfection, or abnormal bleeding.

3. If superinfection occurs, the drug should be discontinued and appropriate therapy instituted.

Packaging and storage Chloramphenicol shall be kept in tightly closed containers, protected from light.

Labelling The label on the container states (1) storage condition; (2) sterile or non-sterile grade.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Chloramphenicol RS (Appendix 2.1) or with the reference spectrum of Chloramphenicol.

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

Crystallinity It is crystalline (Appendix 4.14).

Melting range 149° to 153° (Appendix 4.3).

pH 4.5 to 7.5, in a 2.5 per cent w/v suspension (Appendix 4.11).

Specific rotation +17° to +20°, determined in a 5.0 per cent w/v solution in *absolute ethanol* (Appendix 4.8).

Chromatographic purity Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel HF254* as the coating substance and a mixture of 79 volumes of *chloroform*, 14 volumes of *methanol* and 7 volumes of *glacial acetic acid* as the mobile phase. Apply separately to the plate, 20 µl of each of the following solutions. For solution (A) dissolve 10 mg of the test substance in 1 ml of *methanol*. For solution (B) dissolve 10 mg of Chloramphenicol RS in 1 ml of *methanol*. For solution (C) dilute portions of solution (B) quantitatively with *methanol* to obtain 100 µg per ml. For solution (D) dilute portions of solution (B) quantitatively with *methanol* to obtain 50 µg per ml. After removal of the plate, allow it to dry and examine under ultraviolet light (254 nm). Any spot other than the principal spot obtained from solution (A) does not exceed in size or intensity the principal spot obtained from solution (C) (1 per cent), and the sum of the impurities represented by all of the spots other than the principal spot, based on a comparison of the intensities of such spots with the intensities of the principal spots obtained from solutions (C) and (D), does not exceed 2 per cent.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Prepare a suitable filtered mixture of 55 volumes of *water*, 45 volumes of *methanol* and 0.1 volume of *glacial acetic acid*. Make adjustments if necessary.

Standard preparation Dissolve an accurately weighed quantity of Chloramphenicol RS in *Mobile phase* and dilute quantitatively, and stepwise if necessary, with *Mobile phase* to obtain a solution having a known concentration of about 80 µg per ml.

Assay preparation Transfer about 200 mg of Chloramphenicol, accurately weighed, to a 100-ml volumetric flask, add *Mobile phase* to volume, and mix. Transfer 4.0 ml of the resulting solution to a 100-ml volumetric flask, dilute with *Mobile phase* to volume, and mix.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (10 cm × 4.6 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles (5 µm), (b) *Mobile phase* at a flow rate of about 1 ml per minute, and (c) an ultraviolet photometer set at 280 nm.

To determine the suitability of the chromatographic system, chromatograph *Standard preparation* and record the peak responses as directed under *Procedure*: the symmetry factor is not more than 2.0, and the relative standard deviation for replicate injections is not more than 1.0 per cent.

Procedure Separately inject equal volumes (about 10 µl) of *Standard preparation* and *Assay preparation* into the chromatograph, record the chromatograms, and measure the responses for the major peaks.

Calculation Calculate the content of $C_{11}H_{12}Cl_2N_2O_5$ in the Chloramphenicol taken, using the declared content of $C_{11}H_{12}Cl_2N_2O_5$ in Chloramphenicol RS.

CHLORAMPHENICOL CAPSULES

Category Antibacterial; antirickettsial.

Chloramphenicol Capsules contain not less than 90.0 per cent and not more than 120.0 per cent of the labelled amount of $C_{11}H_{12}Cl_2N_2O_5$.

Strength available 250 mg.

Dose Adults: 12.5 mg per kg of body weight every 6 hours. The maximum total dose should not exceed 4 g daily.

Where there is no alternative to the use of chloramphenicol.

Children, infants and neonates 2 weeks of age and over: 12.5 mg per kg of body weight every 6 hours; or 25 mg per kg of body weight every 12 hours.

Neonates up to 2 weeks of age and premature: 6.25 mg per kg of body weight every 6 hours.

Serum determinations are recommended in patients with immature or impaired hepatic and/or renal function.

Warning; Precaution See under *Chloramphenicol*, p. 63.

Packaging and storage Chloramphenicol Capsules shall be kept in tightly closed containers.

Identification Suspend a portion of the capsule contents containing 100 mg of chloramphenicol in 60 ml of *water* and extract with two 20-ml portions of *petroleum ether (boiling range, 120° to 160°)*. Wash the combined extracts with two 15-ml portions of *water*, add the washings to the aqueous layer, extract with four 50-ml portions of *ether*, and evaporate the combined ether extracts. The residue complies with test A.

A. Dissolve 10 mg of the residue in 2 ml of *ethanol (50 per cent)*, add 4.5 ml of 1 M *sulfuric acid* and 50 mg of *zinc powder* and allow to stand for 10 minutes. Decant the supernatant liquid or filter if necessary. Cool the resulting solution in ice and add 0.5 ml of *sodium nitrite TS*. Allow to stand for 2 minutes and add 1 g of *urea*, followed by 1 ml of *2-naphthol TS* and 2 ml of 10 M *sodium hydroxide*: a red colour is produced. Repeat the test omitting the zinc powder: no red colour is produced.

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

Dissolution Carry out the test as described in the "Dissolution Test" (Appendix 4.24).

Dissolution medium: 0.01 M *hydrochloric acid*; 900 ml.

Apparatus 1: 100 rpm.

Time: 30 minutes.

Procedure Determine the amount of $C_{11}H_{12}Cl_2N_2O_5$ dissolved from absorbances at the maximum at about 278 nm of filtered portions of the test solution, suitably diluted with *Dissolution medium*, if necessary, in comparison with a standard solution having a known concentration of Chloramphenicol RS in the same medium (Appendix 2.2).

Tolerances Not less than 85 per cent (*Q*) of the labelled amount of $C_{11}H_{12}Cl_2N_2O_5$ is dissolved in 30 minutes.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase, Chromatographic system and **Procedure** Proceed as directed in the Assay under *Chloramphenicol*, p. 63.

Standard preparation Transfer about 25 mg of Chloramphenicol RS, accurately weighed, to a 200-ml volumetric flask, add 10 ml of *water*, and heat on a water-bath until completely dissolved. Cool to room temperature, dilute with *Mobile phase* to volume and mix.

Assay preparation Dissolve a quantity of the mixed contents of 20 Chloramphenicol Capsules, accurately weighed, containing 2.5 g of chloramphenicol in 800 ml of *water*, warming if necessary to effect solution, and add sufficient *water* to produce 1000.0 ml. Dilute 5.0 ml to 100.0 ml with *Mobile phase*.

Calculation Calculate the content of $C_{11}H_{12}Cl_2N_2O_5$ in the portion of the Capsules taken, using the declared content of $C_{11}H_{12}Cl_2N_2O_5$ in Chloramphenicol RS.

Other requirements Comply with the requirements described under "Capsules" (Appendix 1.16).

CHLORAMPHENICOL EAR DROPS

Category Antibacterial (otic).

Chloramphenicol Ear Drops are a sterile solution of Chloramphenicol in a suitable solvent. They contain not less than 90.0 per cent and not more than 130.0 per cent of the labelled amount of $C_{11}H_{12}Cl_2N_2O_5$.

Strength available 0.5 per cent w/v.

Dose *Topical*, to the ear canal, 2 or 3 drops every 6 to 8 hours.

Contra-indication It is contra-indicated in patients with a history of toxic reaction to chloramphenicol.

Warning It should not be used in the presence of a perforated tympanic membrane.

Precaution

1. Long-term use of chloramphenicol otic preparation may cause bone marrow hypoplasia.

2. If superinfection occurs, the drug should be discontinued and appropriate therapy instituted.

Packaging and storage Chloramphenicol Ear Drops shall be kept in tightly closed containers, protected from light and not be frozen.

Identification

A. Dilute a volume of the ear drops containing 50 mg of chloramphenicol to 10 ml with *ethanol (50 per cent)*: 2 ml of the resulting solution complies with the test for Identification A described under *Chloramphenicol Capsules*, p. 64.

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

pH 4.0 to 8.0, when diluted with an equal volume of *water* (Appendix 4.15).

Water Not more than 2.0 per cent w/w (Karl Fischer Method, Appendix 4.12).

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase, Chromatographic system and **Procedure** Proceed as directed in the Assay under *Chloramphenicol*, p. 64.

Standard preparation Dissolve an accurately weighed quantity of Chloramphenicol RS in *Mobile phase*, and dilute quantitatively, and stepwise if necessary, with *Mobile phase* to obtain a solution having a known concentration of about 100 µg per ml.

Assay preparation Transfer an accurately measured volume of Chloramphenicol Ear Drops, containing about 50 mg of chloramphenicol, to a 100-ml volumetric flask, dilute with *Mobile phase* to volume, and mix. Transfer 5.0 ml of the resulting solution to a 25-ml volumetric flask, dilute with *Mobile phase* to volume, and mix.

Calculation Calculate the content of $C_{11}H_{12}Cl_2N_2O_5$ in the portion of the Ear Drops taken, using the declared content of $C_{11}H_{12}Cl_2N_2O_5$ in Chloramphenicol RS.

Other requirements Comply with the requirements described under "Ear Preparations" (Appendix 1.16).

CHLORAMPHENICOL EYE DROPS

Category Antibacterial (ophthalmic).

Chloramphenicol Eye Drops are a sterile, buffered solution of Chloramphenicol. They contain not less than 90.0 per cent and not more than 130.0 per cent of the labelled amount of $C_{11}H_{12}Cl_2N_2O_5$.

Strength available 0.5 per cent w/v.

Dose *Topical*, to the conjunctiva, 1 drop every 1 to 4 hours.

Contra-indication It is contra-indicated in patients with a history of toxic reaction to chloramphenicol.

Precaution

1. Long-term use of chloramphenicol ophthalmic preparation may cause bone marrow hypoplasia.
2. If superinfection occurs during chloramphenicol therapy, the drug should be discontinued and appropriate therapy instituted.

Additional information Therapy should be continued for at least 48 hours after the eye appears normal.

Packaging and storage Chloramphenicol Eye Drops shall be kept in tightly closed containers, protected from light, stored at a temperature between 2° and 8° and not be frozen.

Identification

A. To a volume containing 50 mg of chloramphenicol, add 15 ml of *water* and extract with four 25-ml portions of *ether*. Combine the extracts and evaporate to dryness. The dried residue complies with the test for Identification A described under *Chloramphenicol Capsules*, p. 64.

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

pH 7.0 to 7.5, except in case of Eye Drops that is unbuffered the pH is 3.0 to 6.0 (Appendix 4.15).

2-Amino-1-(4-nitrophenyl)propane-1,3-diol Carry out the test as described in the "High-Pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Mix 85 volumes of a 0.21 per cent w/v solution of *sodium pentanesulfonate*, 15 volumes of *acetonitrile* and 1 volume of *glacial acetic acid*.

Standard solution Prepare a solution containing 0.0040 per cent w/v of 2-Amino-1-(4-nitrophenyl) propane-1,3-diol RS in *Mobile phase*.

Test solution Dilute the eye drops with *Mobile phase* to contain 0.050 per cent w/v of chloramphenicol.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (10 cm × 4.6 mm) packed with octadecylsilane chemically bonded to silica gel (5 µm) (Nucleosil C 18 or equivalent is suitable), (b) *Mobile phase* at a flow rate of 2 ml per minute, and (c) an ultraviolet photometer set at 272 nm.

Procedure Separately inject equal volumes of *Standard solution* and *Test solution* into the chromatograph, record the chromatograms, and measure the responses for the major peaks.

In the chromatogram obtained from the test solution the area of any peak corresponding to 2-amino-1-(4 nitrophenyl)propane-1,3-diol is not more than the area of the peak in the chromatogram obtained from the standard solution.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase; Chromatographic system and **Procedure** Proceed as directed in the Assay under *Chloramphenicol*, p. 64.

Standard preparation Dissolve an accurately weighed quantity of Chloramphenicol RS in *Mobile phase*, and dilute quantitatively, and stepwise if necessary, with *Mobile phase* to obtain a solution having a known concentration of about 100 µg per ml.

Assay preparation Transfer an accurately measured volume of Chloramphenicol Eye Drops, containing about 50 mg of chloramphenicol, to a 100-ml volumetric flask, dilute with *Mobile phase* to volume, and mix. Transfer 5.0 ml of the resulting solution to a 25-ml volumetric flask, dilute with *Mobile phase* to volume, and mix.

Calculation Calculate the content of $C_{11}H_{12}Cl_2N_2O_5$ in the portion of the Eye Drops taken, using the declared content of $C_{11}H_{12}Cl_2N_2O_5$ in Chloramphenicol RS.

Other requirements Comply with the requirements described under "Eye Preparations" (Appendix 1.16).

CHLORAMPHENICOL EYE OINTMENT

Category Antibacterial (ophthalmic).

Chloramphenicol Eye Ointment is a sterile preparation containing Chloramphenicol in a suitable base. It contains not less than 90.0 per cent and not more than 130.0 per cent of the labelled amount of $C_{11}H_{12}Cl_2N_2O_5$.

Strength available 1 per cent w/w.

Dose *Topical*, to the conjunctiva, a thin strip (approximately 1 cm) of ointment every 3 hours or more frequently.

Contra-indication; Precaution; Additional information See under *Chloramphenicol Eye Drops*, p. 66.

Packaging and storage Chloramphenicol Eye Ointment shall be stored at a temperature not exceeding 30°.

Identification

A. Mix a portion of the eye ointment containing 50 mg of chloramphenicol with 10 ml of *petroleum ether (boiling range, 40° to 60°)*, centrifuge and discard the supernatant liquid. Repeat this procedure using three 10-ml portions of the same solvent. The dried residue complies with the test for Identification A described under *Chloramphenicol Capsules*, p. 64.

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase; Chromatographic system and **Procedure** Proceed as directed in the Assay under *Chloramphenicol*, p. 63.

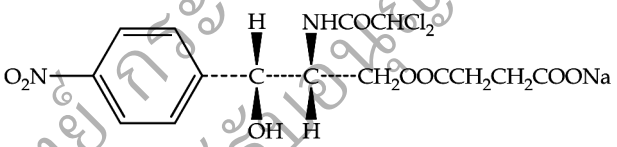
Standard preparation Transfer about 25 mg of Chloramphenicol RS, accurately weighed, to a 100-ml volumetric flask, dissolve in *methanol*, dilute with *methanol* to volume, and mix. Transfer 10.0 ml of the resulting solution to a 25-ml volumetric flask, dilute with *Mobile phase* to volume, and mix.

Assay preparation Transfer an accurately weighed quantity of Chloramphenicol Eye Ointment, containing about 25 mg of chloramphenicol, to a suitable conical flask, add 20 ml of *cyclohexane*, mix and sonicate for about 2 minutes. Add 60 ml of *methanol*, and mix. Filter this mixture, collecting the filtrate in a 100-ml volumetric flask. Wash the filter with *methanol*, collecting the washings in the volumetric flask. Dilute with *methanol* to volume, and mix. Transfer 50.0 ml of the resulting solution to a suitable round-bottomed flask, and evaporate to dryness by rotating the flask under vacuum in a water-bath at 35°. Dissolve the residue in 50.0 ml of *methanol*. Transfer 10.0 ml of the resulting solution to a 25-ml volumetric flask, dilute with *Mobile phase* to volume, and mix.

Calculation Calculate the content of $C_{11}H_{12}Cl_2N_2O_5$ in the portion of the Eye Ointment taken, using the declared content of $C_{11}H_{12}Cl_2N_2O_5$ in Chloramphenicol RS.

Other requirements Complies with the requirements described under "Eye Preparations" (Appendix 1.16).

CHLORAMPHENICOL SODIUM SUCCINATE



$C_{15}H_{15}Cl_2N_2O_8.Na$ 445.19 *982-57-0*

Butanedioic acid, mono[2-[(2,2-dichloroacetyl)amino]-3-hydroxy-3-(4-nitrophenyl)propyl]ester, [*R*-(*R**,*R**)]-, monosodium salt.

Category Antibacterial; antirickettsial.

Chloramphenicol Sodium Succinate contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{15}H_{15}Cl_2N_2O_8.Na$, calculated on the anhydrous basis.

Description White or yellowish white powder; hygroscopic.

Solubility Very soluble in *water*; freely soluble in *ethanol*; practically insoluble in *ether*.

Stability It is very hygroscopic. In aqueous solutions, it loses the potency relatively rapidly especially at higher temperatures.

Warning; Precaution See under *Chloramphenicol*, p. 63.

Packaging and storage Chloramphenicol Sodium Succinate shall be kept in tightly closed containers under sterile condition, protected from light.

Labelling The label on the container states (1) storage condition; (2) parenteral grade.

Identification

A. The ultraviolet absorption spectrum of a 0.002 per cent w/v solution, when observed between

230 and 350 nm, exhibits a maximum only at 276 nm; the absorbance of a 1-cm layer at this wavelength is about 0.43 (Appendix 2.2).

B. Dissolve 10 mg in 2 ml of *ethanol (50 per cent)*, and add 4.5 ml of 1 M *sulfuric acid* and 50 mg of *zinc powder*. Allow to stand for 10 minutes, and decant the supernatant liquid or filter if necessary. Cool the resulting solution in ice and add 0.5 ml of *sodium nitrite TS*. After 2 minutes, add 1 g of *urea* followed by 1 ml of *2-naphthol TS* and 2 ml of 10 M *sodium hydroxide*: a red colour develops. Repeat the test omitting the zinc powder: no red colour is produced.

C. To 5 ml of a 0.1 per cent w/v solution, add a few drops of 0.1 M *silver nitrate*: no precipitate is produced. Then heat 50 mg with 2 ml of *ethanolic potassium hydroxide TS* on a water-bath for 15 minutes. Add 15 mg of *decolourizing charcoal*, shake, and filter. The filtrate, when treated with 0.1 M *silver nitrate*, yields a curdy precipitate which is insoluble in *nitric acid*, but soluble, after being well washed with *water*, in 5 M *ammonia* from which it is reprecipitated by the addition of *nitric acid*.

D. To 100 mg, add 200 mg of *resorcinol* and 4 drops of *sulfuric acid*. Heat gently until a deep red solution is produced, and then pour the solution carefully into a large volume of *water*: an orange-yellow solution with an intense green fluorescence is produced.

E. It yields the *reactions* characteristic of sodium salts (Appendix 5.1).

pH 6.4 to 7.0, in a 25 per cent w/v solution (Appendix 4.11).

Specific rotation +5.0° to +8.0°, calculated on the anhydrous basis, determined in a 5.0 per cent w/v solution (Appendix 4.8).

Water Not more than 5.0 per cent w/w (Karl Fischer Method, Appendix 4.12).

Chloramphenicol and **chloramphenicol disodium disuccinate** Not more than 2.0 per cent of each. Carry out the test as described in the "High-Pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Mix 5 volumes of a 2 per cent w/v solution of *phosphoric acid*, 40 volumes of *methanol* and 55 volumes of *water*.

Reference solution (a) Dissolve 10.0 mg of Chloramphenicol RS in *Mobile phase* and dilute to 100.0 ml with *Mobile phase* (stock solution A). Dilute 5.0 ml of this solution to 100.0 ml with *Mobile phase*.

Reference solution (b) Dissolve 10.0 mg of Chloramphenicol Disodium Disuccinate RS in *Mobile phase* and dilute to 100.0 ml with *Mobile phase* (stock solution B). Dilute 5.0 ml of this solution to 100.0 ml with *Mobile phase*.

Reference solution (c) Dissolve 25 mg of the test substance in *Mobile phase*, add 5 ml of *stock solution A* and 5 ml of *stock solution B* and dilute to 100 ml with *Mobile phase*.

Test solution Dissolve 25.0 mg of the test substance in *Mobile phase* and dilute to 100.0 ml with *Mobile phase*.

Chromatographic system The chromatographic procedure may be carried out using (a) stainless steel column (25 cm × 4.6 mm) packed with octadecylsilyl silica gel for chromatography (5 µm), (b) *Mobile phase* at a flow rate of about 1 ml per minute, (c) an ultraviolet photometer set at 275 nm, and (d) a 20 µl fixed-loop injector.

Procedure Separately inject the test solution and each of the reference solutions. The test is not valid unless, in the chromatogram obtained from reference solution (c), the two peaks corresponding to those in the chromatograms obtained from reference solutions (a) and (b) are clearly separated from the peaks corresponding to the two principal peaks in the chromatogram obtained from the test solution. If necessary, adjust the methanol content of the mobile phase.

In the chromatogram obtained from the test solution the areas of any peaks corresponding to chloramphenicol and chloramphenicol disodium disuccinate are not greater than those of the principal peaks in the chromatograms obtained from Reference solutions (a) and (b) respectively.

Assay

Standard preparation Dissolve an accurately weighed quantity of Chloramphenicol RS in *water* and dilute quantitatively with *water* to obtain a solution having a known concentration of about 20 µg per ml.

Assay preparation Dissolve about 200 mg of Chloramphenicol Sodium Succinate, accurately weighed, in *water*, add sufficient *water* to produce 500.0 ml. Dilute 5.0 ml of the solution to 100.0 ml with *water*.

Procedure Concomitantly measure the absorbances of *Standard preparation* at the maximum at about 278 nm and *Assay preparation* at the maximum at about 276 nm, using *water* as the blank (Appendix 2.2).

Calculation Calculate the content of $C_{15}H_{15}Cl_2N_2O_8.Na$ in the Chloramphenicol Sodium Succinate taken, using the declared content of $C_{11}H_{12}Cl_2N_2O_5$ in Chloramphenicol RS. Each mg of $C_{11}H_{12}Cl_2N_2O_5$ is equivalent to 1.3778 mg of $C_{15}H_{15}Cl_2N_2O_8.Na$.

Other requirements Chloramphenicol Sodium Succinate intended for parenteral administration complies with the following additional requirements.

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains not more than 0.20 Endotoxin Unit per mg of chloramphenicol.

Sterility Complies with the "Sterility Test" (Method I, Appendix 10.1).

CHLORAMPHENICOL SODIUM SUCCINATE FOR INJECTION

Category Antibacterial; antirickettsial.

Chloramphenicol Sodium Succinate for Injection contains an amount of Chloramphenicol Sodium Succinate equivalent to not less than 90.0 per cent and not more than 115.0 per cent of the labelled amount of $C_{11}H_{12}Cl_2N_2O_5$.

Strength available 1 g.

Dose Adults: *Intravenous*, 12.5 mg per kg of body weight every 6 hours. The maximum total dose should not exceed 4 g daily.

Children, infants and neonates 2 weeks of age and over: Where there is no alternative to the use of chloramphenicol, *intravenous*, 12.5 mg per kg of body weight every 6 hours; or 25 mg per kg of body weight every 12 hours.

Neonates up to 2 weeks of age and premature: Where there is no alternative to the use of chloramphenicol, *intravenous*, 6.25 mg per kg of body weight every 6 hours.

In severe infections, such as bacteremia or meningitis, doses up to 75 to 100 mg per kg of body weight daily may be used.

Serum determinations are recommended in patients with impaired hepatic and/or renal function, and in neonates with immature metabolic functions may require a reduction in dose.

Warning; Precaution See under *Chloramphenicol*, p. 63.

Packaging and storage Chloramphenicol Sodium Succinate for Injection shall be kept in Containers for Sterile Solids as described under "Parenteral Preparations" (Appendix 1.16), protected from light.

Labelling The label on the container states the quantity equivalent to the amount of chloramphenicol.

Identification

A. Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel GF254* as the coating substance and a mixture of 1 volume of 2 M *acetic acid*, 14 volumes of *methanol* and 85 volumes of *chloroform* as the mobile phase. Apply separately to the plate, 2 µl of each of the following solutions. For solution (A) dissolve a suitable quantity of the injection in *acetone* to produce a solution containing the equivalent of 1 per cent w/v of chloramphenicol. Solution (B) contains 1 per cent w/v of Chloramphenicol Sodium Succinate RS and solution (C) contains 1 per cent w/v of Chloramphenicol RS. After removal of the plate, allow it to dry in air, and examine under ultraviolet light (254 nm): the two principal spots in the chromatogram obtained from solution (A) are similar in position and size to those in the chromatogram obtained from solution (B) and their positions are different from that of the principal spot in the chromatogram obtained from solution (C).

B. Dissolve 10 mg in 1 ml of *ethanol (50 per cent)*, add 3 ml of a 1 per cent w/v solution of *calcium chloride* and 50 mg of *zinc powder* and heat on a water-bath for 10 minutes. Filter the hot solution, allow to cool, add 0.1 ml of *benzoyl chloride* and shake for 1 minute. Add 0.5 ml of *iron(III) chloride TS* and 2 ml of *chloroform* and shake. The aqueous layer is light violet-red to purple.

C. It yields the *reactions* characteristic of sodium salts (Appendix 5.1).

pH 6.4 to 7.0, in a 25 per cent w/v solution (Appendix 4.11).

Particulate matter Complies with the requirement described under "Particulate Matter in Injections" (Small-volume Injections, Appendix 4.27).

Specific rotation +5.0° to +8.0°, calculated on the anhydrous basis, determined in a 5.0 per cent w/v solution (Appendix 4.8).

Water Not more than 5.0 per cent w/w (Karl Fischer Method, Appendix 4.12).

Chloramphenicol and chloramphenicol disodium disuccinate Not more than 2.0 per cent of each. Carry out the test as described in the "High-Pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase; Reference solution (a); Reference solution (b); Chromatographic system; and **Procedure** Proceed as directed in the Chloramphenicol and chloramphenicol disodium disuccinate under *Chloramphenicol Sodium Succinate*, p. 68.

Reference solution (c) Dissolve a sufficient amount of the contents of the sealed container to obtain a solution containing the equivalent of 0.18 per cent w/v of chloramphenicol in *Mobile phase* (stock solution C). Mix 10.0 ml of *stock solution C*, 5.0 ml of *stock solution A* and

5.0 ml of *stock solution B* and dilute to 100.0 ml with *Mobile phase*.

Test solution Dilute 10.0 ml of *stock solution C* to 100.0 ml with *Mobile phase*.

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains not more than 0.2 Endotoxin Unit per mg of chloramphenicol.

Assay

Standard preparation Dissolve an accurately weighed quantity of Chloramphenicol RS in *water*, and dilute quantitatively with *water* to obtain a solution having a known concentration of about 20 μg per ml.

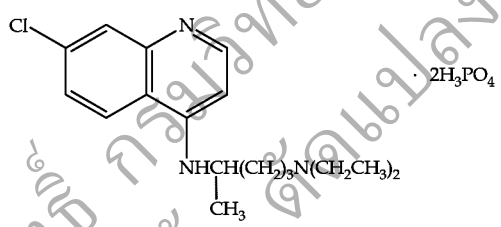
Assay preparation Constitute 1 container of Chloramphenicol Sodium Succinate for Injection as directed in the labelling. Dilute an accurately measured volume of the constituted solution quantitatively with *water* to obtain a solution having a concentration of about 20 μg of chloramphenicol per ml.

Procedure Concomitantly measure the absorbances of *Standard preparation* at the maximum at about 278 nm, and *Assay preparation* at the maximum at about 276 nm, using *water* as the blank (Appendix 2.2).

Calculation Calculate the content of $C_{11}H_{12}Cl_2N_2O_5$ in one container of the Injection taken, using the declared potency of Chloramphenicol RS.

Other requirements Complies with the requirements described under "Parenteral Preparations" (Appendix 1.16).

CHLOROQUINE PHOSPHATE



$C_{18}H_{26}ClN_3.2H_3PO_4$ 515.86 *50-63-5*

1,4-Pentanediamine, N^4-(7-chloro-4-quinolinyl)-N^1,N^1-diethyl-, phosphate (1:2).

Category Antiprotozoal (antimalarial).

Chloroquine Phosphate contains not less than 98.5 per cent and not more than 101.0 per cent of $C_{18}H_{26}ClN_3.2H_3PO_4$, calculated on the dried basis.

Description White or almost white, crystalline powder; hygroscopic.

Solubility Freely soluble in *water*; very slightly soluble in *ethanol* and in *methanol*.

Stability It discolours slowly on exposure to light.

Contra-indication

1. It is contra-indicated in patients with retinal or visual field changes and hypersensitivity to 4-aminoquinolone compounds.
2. It is contra-indicated for long-term therapy in children.

Warning

1. Children and infants are especially sensitive to the 4-aminoquinolone compounds. Fatalities following accidental ingestion of relatively small doses and sudden death after intramuscular injections have been reported.
2. It should not be administered parenterally in children because of the narrow therapeutic range in this age group.
3. Certain strains of *Plasmodium falciparum* have become resistant to 4-aminoquinolone compounds including chloroquine. To improve efficacy and delay the onset of resistance, it should always be used in combination with another effective antimalarial.
4. High dose or prolonged therapy can cause irreversible retinal or auditory damage, muscular weakness and blood dyscrasias. Acute overdosage can cause acute circulatory failure, convulsions, respiratory and cardiac arrest, and death.
5. Rapid intravenous injection causes dizziness, nausea, disturbance of vision, and a transient fall of blood pressure.
6. It should be used with caution in patients with severe hematopoietic, hepatic, neurological, or gastrointestinal disorder, glucose-6-phosphate dehydrogenase deficiency, alcoholism, psoriasis, or porphyria.
7. Concurrent administration with potentially hemolytic drugs, 8-aminoquinolone compounds, bone-marrow suppressants, or dermatitis-causing medications, especially gold salts and phenylbutazone, should be avoided.
8. Risk-benefit should be considered if it is to be used in pregnant or nursing women since it may cause fetal abnormalities (such as damage to the CNS, congenital deafness).

Precaution Prolonged therapy requires periodic determinations of visual, auditory and hematopoietic functions, and examinations of knee and ankle reflexes in order to detect muscular weakness.

Packaging and storage Chloroquine Phosphate shall be kept in tightly closed containers, protected from light.

Identification

A. Dissolve 100 mg in 10 ml of *water*, add 2 ml of 2 M *sodium hydroxide* and extract with two 20-ml portions of *chloroform*, retaining the aqueous layer. Wash the chloroform extracts with *water*, dry with *anhydrous sodium sulfate*, evaporate to dryness, and dissolve the residue in 2 ml of *chloroform*. The infrared absorption spectrum of the chloroform solution is concordant with the spectrum obtained from Chloroquine Phosphate RS similarly treated (Appendix 2.1) or with the reference spectrum of Chloroquine.

B. The ultraviolet absorption spectrum of a 0.0015 per cent w/v solution in 0.01 M *hydrochloric acid*, when observed between 240 and 350 nm, exhibits three maxima, at 257 nm, 329 nm and 343 nm; the absorbances of a 1-cm layer at these wavelengths are about 0.44, 0.48 and 0.55, respectively (Appendix 2.2).

C. Dissolve 25 mg in 20 ml of *water* and add 8 ml of *trinitrophenol TS*: the precipitate, after washing successively with *water*, *ethanol* and *ether*, melts at about 207° (Appendix 4.3).

D. Neutralize with 2 M *nitric acid* the aqueous layer obtained in Test A, add an equal volume of a 10 per cent w/v solution of *ammonium molybdate*, and warm: a yellow precipitate is produced.

Melting range It exists in 2 forms, one of which melts at about 195° and the other at about 218° (Appendix 4.3).

pH 3.8 to 4.3, in a 0.1 per cent w/v solution (Appendix 4.11).

Loss on drying Not more than 2.0 per cent w/w after drying at 105° to constant weight (Appendix 4.15).

Heavy metals Not more than 20 ppm. Dissolve 2.0 g in 10 ml of *water*. Add 5 ml of *strong ammonia solution* and shake with 40 ml of *dichloromethane*. Filter the aqueous layer and neutralize the filtrate with *glacial acetic acid*. Heat on a water-bath to eliminate dichloromethane, allow to cool and dilute to 20.0 ml with *water*. A 12.0-ml portion of the resulting solution complies with the "Limit Test for Heavy Metals" (Method II, Appendix 5.2). For the standard preparation, use *lead standard solution (2 ppm Pb)*.

Related substances Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel GF254* as the coating substance.

Test solution Dissolve 500 mg of the test substance in *water* and dilute to 10.0 ml with the same solvent.

Reference solution 1 Dilute 1.0 ml of the *Test solution* to 100.0 ml with *water*.

Reference solution 2 Dilute 5.0 ml of *Reference solution 1* to 10.0 ml with *water*.

Mobile phase Prepare a mixture of 10 volumes of *diethylamine*, 40 volumes of *cyclohexane* and 50 volumes of *chloroform*.

Procedure Apply separately to the plate, 2 µl of each of the solutions. After removal of the plate, allow it to dry in air and examine under ultraviolet light (254 nm). Any spot in the chromatogram obtained from *Test solution*, apart from the principal spot, is not more intense than the spot in the chromatogram obtained from *Reference solution 1* (1.0 per cent) and not more than one such spot is more intense than the spot in the chromatogram obtained from *Reference solution 2* (0.5 per cent).

Assay Dissolve about 200 mg of Chloroquine Phosphate, accurately weighed, in 50 ml of *anhydrous glacial acetic acid*. Titrate with 0.1 M *perchloric acid VS*, using *crystal violet TS* as indicator, to a blue end-point, or determining the end-point potentiometrically (Appendix 6.1). Perform a blank determination, and make any necessary correction. Each ml of 0.1 M *perchloric acid* is equivalent to 25.79 mg of $C_{18}H_{26}ClN_3.2H_3PO_4$.

CHLOROQUINE PHOSPHATE TABLETS

Category Antiprotozoal (antimalarial).

Chloroquine Phosphate Tablets contain not less than 92.5 per cent and not more than 107.5 per cent of the labelled amount of $C_{18}H_{26}ClN_3.2H_3PO_4$.

Strengths available 150 and 300 mg (base).

Dose

Suppressive treatment—Adults: 300 mg once weekly starting 1 week before and continuing until 4 weeks after leaving a malarial area.

Children: 5 mg per kg of body weight, not to exceed the adult dose, once weekly.

Therapeutic treatment—Adults: 600 mg initially followed by 300 mg in 6 to 8 hours, and 300 mg once a day on the second and third days.

Contra-indication; Warning; Precaution See under *Chloroquine Phosphate*, p. 70.

Additional information

1. The medication should be taken with or after meals to minimize gastro-intestinal irritation.

2. Oral chloroquine can also be used in the treatment of acute and chronic rheumatoid arthritis. The daily dose is up to 2.4 mg per kg of lean body weight.

Packaging and storage Chloroquine Phosphate Tablets shall be protected from light.

Labelling The label on the container states the quantity equivalent to the amount of chloroquine.

Identification

A. Dissolve a portion of the powdered tablets, containing 100 mg of chloroquine phosphate, in a mixture of 10 ml of *water* and 2 ml of 2 M *sodium hydroxide* and extract with two 20-ml portions of *chloroform*. Wash the chloroform extracts with *water*, dry with *anhydrous sodium sulfate*, evaporate to dryness, and dissolve the residue in 2 ml of *chloroform*: the infrared absorption spectrum of the resulting solution is concordant with the spectrum obtained from Chloroquine Phosphate RS, similarly treated (Appendix 2.1), or with the reference spectrum of Chloroquine.

B. Extract a portion of the powdered tablets, containing 25 mg of chloroquine phosphate, with 20 ml of *water*, filter and add 8 ml of *trinitrophenol TS* to the filtrate: the precipitate, after washing successively with *water*, *ethanol* and *ether*, melts at about 207° (Appendix 4.3).

C. Extract a portion of the powdered tablets, containing 500 mg of chloroquine phosphate, with 25 ml of *water* and filter. To the filtrate add 2.5 ml of 5 M *sodium hydroxide* and extract with three 10-ml portions of *ether*: the aqueous layer yields the *reactions* characteristic of phosphates (Appendix 5.1).

Related substances Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel GF254* as the coating substance.

Test solution Shake a quantity of the powdered tablets containing 1 g of chloroquine phosphate with 20.0 ml of *water* for 30 minutes, centrifuge and use the supernatant liquid; if necessary, filter through a glass fibre paper.

Reference solution 1 Dilute 1.0 ml of *Test solution* to 100.0 ml with *water*.

Reference solution 2 Dilute 5.0 ml of *Reference solution 1* to 10.0 ml with *water*.

Mobile phase Prepare a mixture of 10 volumes of *diethylamine*, 40 volumes of *cyclohexane* and 50 volumes of *chloroform*.

Procedure Apply separately to the plate, 2 µl of each of the solutions. After removal of the plate, allow it to dry in air and examine under ultraviolet light (254 nm). Any secondary spot in the chromatogram obtained from *Test solution* is not more intense than the spot in the chromatogram obtained from *Reference solution 1* (1.0 per cent) and not more than one such spot is more intense than the spot in the chromatogram obtained from *Reference solution 2* (0.5 per cent).

Dissolution Carry out the test as described in the "Dissolution Test" (Appendix 4.24).

Dissolution medium: *water*; 900 ml.

Apparatus 2: 100 rpm.

Time: 45 minutes.

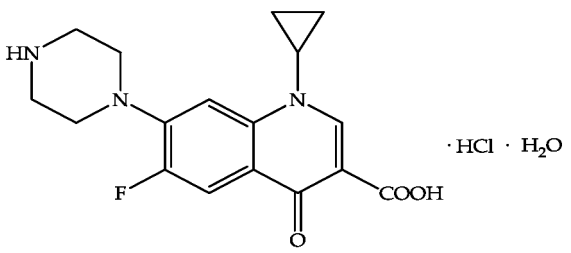
Procedure Determine the amount of $C_{18}H_{26}ClN_3.2H_3PO_4$ dissolved from absorbances at the maximum at about 343 nm of filtered portions of the test solution, suitably diluted with *Dissolution medium*, if necessary, in comparison with a standard solution having a known concentration of Chloroquine Phosphate RS in the same medium (Appendix 2.2).

Tolerances Not less than 75 per cent (*Q*) of the labelled amount of $C_{18}H_{26}ClN_3.2H_3PO_4$ is dissolved in 45 minutes.

Assay Weigh and finely powder not less than 20 Chloroquine Phosphate Tablets. Dissolve an accurately weighed portion of the powder, containing about 500 mg of chloroquine phosphate, in 20 ml of 1 M *sodium hydroxide* and extract with four 25-ml portions of *chloroform*. Combine the chloroform extracts and evaporate to a volume of about 10 ml. Add 40 ml of *anhydrous glacial acetic acid*. Titrate with 0.1 M *perchloric acid VS*, determining the end-point potentiometrically (Appendix 6.1). Each ml of 0.1 M *perchloric acid* is equivalent to 25.79 mg of $C_{18}H_{26}ClN_3.2H_3PO_4$.

Other requirements Comply with the requirements described under "Tablets" (Appendix 1.16).

CIPROFLOXACIN HYDROCHLORIDE



$C_{17}H_{18}FN_3O_3.HCl.H_2O$ 385.82 *86393-32-0*

3-Quinolinecarboxylic acid, 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-, monohydrochloride, monohydrate.

Category Antibacterial.

Ciprofloxacin Hydrochloride contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{17}H_{18}FN_3O_3$.HCl, calculated on the anhydrous basis.

Description Faintly yellowish to light yellow crystals.

Solubility Sparingly soluble in *water*; slightly soluble in *acetic acid* and in *methanol*; very slightly soluble in *absolute ethanol*; practically insoluble in *acetone*, in *acetonitrile*, in *ethyl acetate*, in *hexane*, and in *dichloromethane*.

Contra-indication; Warning; Precaution; Additional information See under *Norfloxacin*, p. 133.

Packaging and storage Ciprofloxacin Hydrochloride shall be kept in tightly closed containers and protected from light.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Ciprofloxacin Hydrochloride RS (Appendix 2.1) or with the reference spectrum of Ciprofloxacin Hydrochloride.

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

C. It yields the *reactions* characteristic of chlorides (Appendix 5.1).

pH 3.0 to 4.5, in a 2.5 per cent w/v solution (Appendix 4.11).

Water Not less than 4.7 per cent w/w and not more than 6.7 per cent w/w (Karl Fischer Method, Appendix 4.12).

Sulfated ash Not more than 0.10 per cent w/w (Appendix 5.3), determined in a platinum crucible.

Sulfate Not more than 0.04 per cent w/w (Appendix 5.2). A 375-mg sample shows no more sulfate than that corresponds to 0.15 ml of 0.010 M *sulfuric acid*.

Heavy metals Not more than 20 ppm (Method II, Appendix 5.2). Use 1.0 g; for the Standard Preparation, use 2 ml of *lead standard solution (10 ppm Pb)*.

Fluoroquinolonic acid Not more than 0.2 per cent w/w. Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel GF254* as the coating substance and a mixture of 4 volumes of *dichloromethane*, 4 volumes of *methanol*, 2 volumes of *strong ammonia solution* and 1 volume of *acetonitrile* as the mobile phase. Apply separately to the plate, 5 l of each of the following solutions. For Test solution, dissolve a quantity of the test substance in *water* to obtain a test solution containing 10.0 mg/ml. For Standard solution, transfer 5.0 mg of Fluoroquinolonic Acid RS to a 50-ml volumetric flask containing 0.05 ml of 6 M *ammonia*, add *water* to volume, and mix. Transfer 2.0 ml of this solution to a 10-ml volumetric flask, dilute with *water* to volume, and mix. Place the plate in a suitable chamber in which is placed a beaker containing 50 ml of *strong ammonia solution*. After 15 minutes, transfer the plate to a suitable chromatographic chamber, and develop the chromatogram. After removal of the plate, allow it to dry in air for about 15 minutes. Examine under ultraviolet light (254 nm): any spot from the Test solution, at an R_f value corresponding to the principal spot from the Standard solution, is not greater in size or intensity than that obtained from the Standard solution.

Chromatographic purity Carry out the test as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase, Standard preparation, Resolution solution, Assay preparation, Chromatographic system, and **Procedure** Proceed as directed in the *Assay*.

Calculation Calculate the percentage of each impurity peak in the chromatogram obtained from the Assay preparation taken by the expression:

$$100 r_i / r_t ,$$

in which r_i is the response of each impurity peak, and r_t is the sum of the responses of all the peaks: not more than 0.2 per cent of ciprofloxacin ethylenediamine analog or of any other individual impurity peak is found, and the sum of all the impurity peaks is not more than 0.5 per cent.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Prepare a mixture of 87 volumes of 0.025 M *phosphoric acid*, previously adjusted with *triethylamine* to a pH of 3.0±0.1, and 13 volumes of *acetonitrile*. Make adjustments if necessary.

Resolution solution Dissolve a quantity of Ciprofloxacin Ethylenediamine Analog RS in *Standard preparation* to obtain a solution containing 500 μg per ml.

Standard preparation Dissolve an accurately weighed quantity of Ciprofloxacin Hydrochloride RS in *Mobile phase* to obtain a solution having a known concentration of about 500 μg per ml.

Assay preparation Transfer about 25 mg of Ciprofloxacin Hydrochloride, accurately weighed, to a 50-ml volumetric flask, dissolve in, dilute with *Mobile phase* to volume, and mix.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (25 cm × 4 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles (3 to 10 μm) maintained at 30°±1°, (b) *Mobile phase* at a flow rate of about 1.5 ml per minute and (c) an ultraviolet photometer set at 278 nm.

To determine the suitability of the chromatographic system, chromatograph *Resolution solution*, and record the peak responses as directed under *Procedure*: the retention time for ciprofloxacin is between 6.4 and 10.8 minutes, the relative retention times are about 0.7 for ciprofloxacin ethylenediamine analog and 1.0 for ciprofloxacin and the resolution factor between the ciprofloxacin ethylenediamine analog and ciprofloxacin peaks is not less than 6. Chromatograph *Standard preparation*, and record the peak responses as directed under *Procedure*: the symmetry factor for the ciprofloxacin peak is not more than 4.0 and the relative standard deviation for replicate injections is not more than 1.5 per cent.

Procedure Separately inject equal volumes (about 10 μl) of *Standard preparation* and *Assay preparation* into the chromatograph, record the chromatograms and measure the areas for the major peaks.

Calculation Calculate the content of $C_{17}H_{18}FN_3O_3$.HCl in the Ciprofloxacin Hydrochloride taken, using the declared content of $C_{17}H_{18}FN_3O_3$.HCl in Ciprofloxacin Hydrochloride RS.

CIPROFLOXACIN HYDROCHLORIDE TABLETS

Category Antibacterial.

Ciprofloxacin Hydrochloride Tablets contain Ciprofloxacin Hydrochloride equivalent to not less than 90.0 per cent and not more than 110.0 per cent of the labelled amount of $C_{17}H_{18}FN_3O_3$.

Strengths available 100, 250, 500, 750, and 1000 mg (base).

Dose Adults—Anthrax: 500 mg every 12 hours.

Bone and joint infections; lower respiratory tract infection; skin and skin structure infection: 500 mg every 12 hours for 7 to 14 days. Severe or complicated infections: 750 mg every 12 hours for 7 to 14 days.

Diarrhea, bacterial: 500 mg every 12 hours for 5 to 7 days.

Traveler's diarrhea: 500 mg every 12 hours for 1 to 3 days

Gonorrhea, endocervical and urethral: 250 to 500 mg as a single dose.

Typhoid fever: 500 mg every 12 hours for 10 days.

Urinary tract infection, acute and uncomplicated: 250 mg every 12 hours for 3 days.

Urinary tract infection, complicated and pyelonephritis: 250 mg every 12 hours for 7 to 14 days. Severe complication: 500 mg every 12 hours for 7 to 14 days.

The maximum total dose should not exceed 1.5 g daily.

Adolescents and children: when alternative therapy could not be used, 10 to 20 mg per kg of body weight every 12 hours.

Anthrax: 10 to 15 mg per kg of body weight every 12 hours.

The maximum total dose should not exceed 1 g daily.

Contra-indication; Warning; Precaution; Additional information See under *Norfloxacin*, p. 133.

Labelling The label on the container states the quantity equivalent to the amount of ciprofloxacin.

Identification

A. Carry out the test as described in the "Thin-layer chromatography" (Appendix 3.1), using *silica gel GF254* as the coating substance and a mixture of 4 volumes of *dichloromethane*, 4 volumes of *methanol*, 2 volumes of *strong ammonia solution*, and 1 volume of *acetonitrile* as the mobile phase. Place the plate in an atmosphere of ammonia for 15 minutes before use. Apply separately to the plate, 10 μl of each of the following solutions. For solution (A), place a number of the tablets, equivalent to 1.5 g of ciprofloxacin, in a suitable flask containing 750 ml of *water*, and sonicate for 20 minutes. Dilute with *water* to 1000.0 ml and mix. Centrifuge a portion of this suspension, and use the clear supernatant obtained as the test solution. Solution (B) is 1.5 mg per ml of Ciprofloxacin Hydrochloride RS in *water*. After removal of the plate, allow it to dry in air for 15 minutes and examine under ultraviolet light (254 nm and 366 nm). The principal spot in the chromatogram obtained from solution (A) corresponds to that obtained from solution (B).

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

Dissolution Carry out the test as described in the "Dissolution Test" (Appendix 4.24).

Dissolution medium: 0.01 M *hydrochloric acid*; 900 ml.

Apparatus 2: 50 rpm.

Time: 30 minutes.

Procedure Determine the amount of $C_{17}H_{18}FN_3O_3$.HCl dissolved from absorbances at the maximum at about 276 nm of filtered portions of the test solution, suitably diluted with *Dissolution medium*, if necessary, in comparison with a standard solution having a known concentration of Ciprofloxacin Hydrochloride RS in the same medium (Appendix 2.2).

Tolerances Not less than 80 per cent (*Q*) of the labelled amount of $C_{17}H_{18}FN_3O_3$ is dissolved in 30 minutes.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Diluent Prepare a mixture of 87 volumes of 0.025 M *phosphoric acid*, previously adjusted with *triethylamine* to a pH of 2.0±0.1, and 13 volumes of *acetonitrile*.

Mobile phase Prepare a mixture of 87 volumes of 0.025 M *phosphoric acid*, previously adjusted with *triethylamine* to a pH of 3.0±0.1, and 13 volumes of *acetonitrile*. Make adjustments if necessary.

Resolution solution Dissolve a quantity of Ciprofloxacin Ethylenediamine Analog RS in *Standard preparation* to obtain a solution containing 50 µg per ml.

Standard preparation Dissolve an accurately weighed quantity of Ciprofloxacin Hydrochloride RS in *Diluent* to obtain a solution having a known concentration of about 200 µg per ml.

Assay preparation Transfer 5 Ciprofloxacin Hydrochloride Tablets to a 500-ml volumetric flask, add 400 ml of *Diluent* and sonicate for 20 minutes. Dilute with *Diluent* to volume, mix and filter. Dilute an accurately measured volume of this solution with *Diluent* to obtain a solution containing about 200 µg of ciprofloxacin per ml.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (25 cm × 4.6 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles (3 to 10 µm) maintained at 30°±1°, (b) *Mobile phase* at a flow rate of about 1.5 ml per minute and (c) an ultraviolet photometer set at 278 nm.

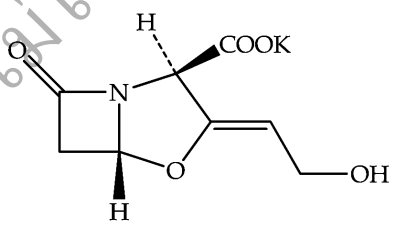
To determine the suitability of the chromatographic system, chromatograph *Resolution solution*, and record the peak responses as directed under *Procedure*: the retention time for ciprofloxacin is between 6.4 and 10.8 minutes, the relative retention times are about 0.7 for ciprofloxacin ethylenediamine analog and 1.0 for ciprofloxacin and the resolution factor between the ciprofloxacin ethylenediamine analog and ciprofloxacin peaks is not less than 6. Chromatograph *Standard preparation*, and record the peak responses as directed under *Procedure*: the symmetry factor for the ciprofloxacin peak is not more than 2.0 and the relative standard deviation for replicate injections is not more than 1.5 per cent.

Procedure Separately inject equal volumes (about 10 µl) of *Standard preparation* and *Assay preparation* into the chromatograph, record the chromatograms, and measure the responses for the major peaks.

Calculation Calculate the content of $C_{17}H_{18}FN_3O_3$ in the portion of the Tablets taken, using the declared content of $C_{17}H_{18}FN_3O_3$ in Ciprofloxacin Hydrochloride RS. Each mg of $C_{17}H_{18}FN_3O_3$ is equivalent to 1.1101 mg of $C_{17}H_{18}FN_3O_3$.HCl.

Other requirements Comply with the requirements described under "Tablets" (Appendix 1.16).

CLAVULANATE POTASSIUM



$C_8H_8KNO_5$ 237.25 *61177-45-5*

4-Oxa-1-azabicyclo[3.2.0]heptane-2-carboxylic acid, 3-(2-hydroxyethylidene)-7-oxo-, monopotassium salt, 2*R*-(2α,3*Z*,5α)-.

Category Beta-lactamase inhibitor.

Clavulanate Potassium contains the equivalent of not less than 75.5 per cent and not more than 92.0 per cent of $C_8H_9NO_5$, calculated on the anhydrous basis.

Packaging and storage Clavulanate Potassium shall be kept in tightly closed containers and stored at a temperature between 2° and 8°.

Labelling The label on the container states (1) storage condition; (2) parenteral or non-parenteral grade.

Identification

A. The chromatogram of the Assay preparation obtained as directed in the *Assay* exhibits a major peak for clavulanic acid, the retention time of which corresponds to that exhibited in the chromatogram of the Standard preparation obtained as directed in the *Assay*.

B. It yields the *reactions* characteristic of potassium salts (Appendix 5.1).

pH 5.5 to 8.0, in a 1.0 per cent w/v solution (Appendix 4.11).

Water Not more than 1.5 per cent w/w (Karl Fischer Method, Appendix 4.12).

Limit of clavam-2-carboxylate potassium Not more than 0.01 per cent. Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Prepare 0.1 M *sodium dihydrogenphosphate*, adjust with *phosphoric acid* to a pH of 4.0±0.1, and filter through a membrane filter of 0.5 μm or finer porosity. Make adjustments if necessary.

Standard solution Dissolve Clavam-2-Carboxylate Potassium RS in *water* to obtain a solution having a known concentration of about 40 μg per ml.

Test solution Transfer about 100 mg of Clavulanate Potassium, accurately weighed, to a 10-ml volumetric flask, dissolve in and dilute with *water* to volume, and mix.

Resolution solution Dissolve a suitable quantity of Clavulanate Potassium in *Standard solution* to obtain a solution containing about 1 mg of clavulanate potassium and 30 μg of clavam-2-carboxylate potassium per ml.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (30 cm × 4 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles (3 to 10 μm) (b) *Mobile phase* at a flow rate of 0.5 ml per minute, and (c) an ultraviolet photometer set at 210 nm.

To determine the suitability of the chromatographic system, chromatograph *Standard solution*, and record the peak responses as directed under *Procedure*: the symmetry factor is not more than 1.5 and the relative standard deviation for replicate injections is not more than 2.0 per cent. Chromatograph *Resolution solution*, and record the peak responses as directed under *Procedure*: the resolution factor between the clavam-2-carboxylic acid and clavulanic acid peaks is not less than 1.0.

Procedure Separately inject equal volumes (about 20 μl) of *Standard solution* and *Test solution* into the chromatograph, record the chromatograms, and measure the responses for the major peaks. The relative retention times are about 0.7 for clavam-2-carboxylic acid and 1.0 for clavulanic acid. Calculate the percentage of clavam-2-carboxylate potassium

Limit of methanol and *tert*-butylamine Not more than 0.1 per cent w/w of methanol or not more than 0.2 per cent w/w of *tert*-butylamine. Carry out the determination as described in the "Gas Chromatography" (Appendix 3.4).

Standard solution To a 100-ml volumetric flask containing 50.0 ml of 3 M *sodium hydroxide* add about 6 ml of *methanol* and 12 ml of tert-*butylamine*, both accurately weighed, dilute with 3 M *sodium hydroxide* to volume, and mix. Transfer 10.0 ml of this stock solution to a second 100-ml volumetric flask, dilute with 3 M *sodium hydroxide* to volume, and mix. Transfer 10.0 ml of this solution to a third 100-ml volumetric flask, dilute with 3 M *sodium hydroxide* to volume, and mix. Transfer 7.0 ml of this solution to a fourth 100-ml volumetric flask, add 3.0 ml of 3 M *sodium hydroxide*, dilute with *4-methyl-2-pentanone* to volume, and mix. Allow the phases to separate, and use the clear methyl isobutyl ketone layer as the *Standard solution*. This solution contains about 36.6 μg of methanol and 63.8 μg of *tert*-butylamine per ml. Use this solution within 5 hours.

Test solution Transfer about 3 g of Clavulanate Potassium, accurately weighed, to a 100-ml volumetric flask, add 10.0 ml of 3 M *sodium hydroxide*, and shake until dissolved. Allow to cool in a cold water-bath, dilute with *4-methyl-2-pentanone* to volume, stopper the flask, and shake vigorously for 3 minutes, with occasional venting. Allow the phases to separate, and use the clear methyl isobutyl ketone layer as the *Test solution*. Use this solution within 5 hours.

Chromatographic system The chromatographic procedure may be carried out using (a) a glass column (30 m × 0.32 mm) coated with dimethylpolysiloxane oil stationary phase and is maintained at 40°, then programmed to increase at a rate of 55° per minute to 200°, and held at that temperature for 4 minutes, (b) the injection port and the detector block are maintained at 150°, (c) *nitrogen* as the carrier gas, and (d) a flame ionization detector. To determine the suitability of the chromatographic system, chromatograph *Standard solution*, and record the methanol, *tert*-butylamine, and methyl isobutyl ketone peak responses as directed for *Procedure*: the symmetry factor is not more than 1.6 for the methanol and *tert*-butylamine peaks; the column efficiency is not less than 25,000 theoretical plates; the resolution factor between the methanol peak and the *tert*-butylamine peak is not less than 20 and between the *tert*-butylamine peak and the 4-methyl-2-pentanone peak is not less than 10; and the relative standard deviation for replicate injections is not more than 10 per cent.

Procedure Separately inject equal volumes (about 1 µl) of *Standard solution* and *Test solution* into the chromatograph, record the chromatograms, and measure the areas of the responses of the methanol and *tert*-butylamine peaks.

Calculation Calculate the percentages of methanol and of *tert*-butylamine in the Clavulanate Potassium.

Chromatographic purity Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Solution A Prepare a solution of 0.05 M *sodium dihydrogenphosphate*, adjust with *phosphoric acid* to a pH of 4.0±0.1, and filter through a filter of 0.5-µm or finer porosity.

Solution B Prepare a mixture of equal volumes of *Solution A* and *methanol*.

Mobile phase Use variable mixtures of *Solution A* and *Solution B* as directed for *Chromatographic system*. Make adjustments to either solution as necessary.

Standard solution Prepare a solution of Clavulanate Lithium RS in *Solution A* having a known concentration of about 0.1 mg per ml.

Test solution Prepare a solution of Clavulanate Potassium in *Solution A* containing 10.0 mg per ml.

Resolution solution Prepare a solution of Clavulanate Lithium RS and amoxicillin in *Solution A* containing 0.1 mg of each per ml.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (10 cm × 4.6 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles (3 to 10 µm) and is maintained at a constant temperature of about 40°, (b) *Mobile phase* at a flow rate of about 1 ml per minute, and (c) an ultraviolet photometer set at 230 nm.

The system is programmed to provide a mobile phase consisting of variable mixtures of *Solution A* and *Solution B*. The system is equilibrated for 15 minutes with 100 per cent *Solution A*, and held at that composition for 4 minutes after injection of the solution under test, after which the proportion of *Solution B* is increased linearly from 0 to 50 per cent over a period of 11 minutes. The system is held at that composition for 3 minutes, and is then changed to 100 per cent *Solution A* for 6 minutes. To determine the suitability of the chromatographic system, chromatograph the *Resolution solution*, and record the peak responses as directed for *Procedure*: the relative retention times are about 2.5 for amoxicillin and 1.0 for clavulanic acid; the symmetry factor for the clavulanic acid peak is not more than 2.0; the column efficiency determined from the clavulanic acid peak is not less than 2000 theoretical plates; and the resolution factor between the clavulanic acid peak and the amoxicillin peak is not less than 13. Chromatograph *Standard solution*, and record the peak responses as directed for *Procedure*: the relative standard deviation for replicate injections is not more than 2 per cent.

Procedure Separately inject equal volumes (about 20 µl) of *Standard solution* and *Test solution* into the chromatograph, record the chromatograms, and measure the peak responses.

Calculation Calculate the percentage, in terms of clavulanate potassium equivalent, of each impurity in the Clavulanate Potassium taken by the expression:

$$10(237.3/205.1)(C)(r_i/r_S),$$

in which 237.3 is the molecular weight of clavulanate potassium; 205.1 is the molecular weight of clavulanate lithium; C is the concentration, in mg per ml, of Clavulanate Potassium RS in *Standard solution*; r_i is the peak response of an individual impurity peak in the chromatogram obtained from *Test solution*; and r_S is the clavulanic acid peak response in the chromatogram obtained from *Standard solution*. The sum of all the impurity peaks is not more than 2 per cent.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

pH 4.4 Sodium phosphate buffer Dissolve 7.8 g of *sodium dihydrogenphosphate* in 900 ml of *water*, adjust with *phosphoric acid* or 10 M *sodium hydroxide* to a pH of 4.4±0.1, dilute with *water* to make 1000 ml, and mix.

Mobile phase Prepare a suitable mixture of 95 volumes of *pH 4.4 sodium phosphate buffer* and 5 volumes of *methanol*. Make adjustments if necessary.

Standard preparation Dissolve an accurately weighed quantities of Clavulanate Lithium RS in *water* to obtain a solution having a known concentration of about 250 µg per ml.

Assay preparation Transfer about 50 mg of Clavulanate Potassium, accurately weighed, to a 200-ml volumetric flask, dissolve in and dilute with *water* to volume, and mix.

Resolution Solution Dissolve a suitable quantity of amoxicillin in *Standard preparation* to obtain a solution containing about 0.5 mg of amoxicillin and 250 µg of clavulanate lithium per ml.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (30 cm × 4 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles (3 to 10 µm), (b) *Mobile phase* at a flow rate of

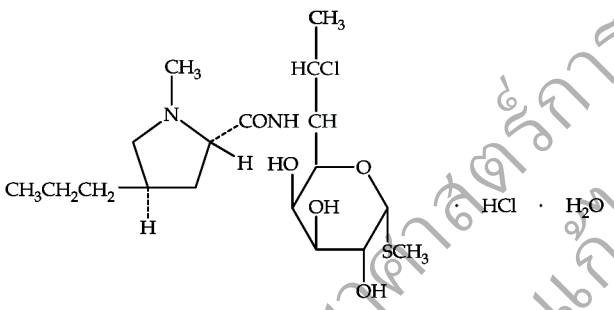
about 2 ml per minute, and (c) an ultraviolet photometer set at 220 nm.

To determine the suitability of the chromatographic system, chromatograph *Resolution solution*, and record the peak responses as directed under *Procedure*: the resolution factor between the amoxicillin and clavulanic acid peaks is not less than 3.5 and the relative retention times are about 0.5 for clavulanic acid and 1.0 for amoxicillin. Chromatograph *Standard preparation*, and record the peak responses as directed under *Procedure*: the symmetry factor for clavulanic acid peak is not more than 1.5 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Procedure Separately inject equal volumes (about 20 µl) of *Standard preparation* and *Assay preparation* into the chromatograph, record the chromatograms, and measure the responses for the major peaks.

Calculation Calculate the content of $C_8H_9NO_5$ in the Clavulanate Potassium taken, using the declared content of $C_8H_9NO_5$ in Clavulanate Lithium RS.

CLINDAMYCIN HYDROCHLORIDE



$C_{18}H_{33}ClN_2O_5S.HCl.H_2O$ 479.47 *58207-19-5*
L-*threo*-α-D-*galacto*-Octopyranoside, methyl 7-chloro-6,7,8-trideoxy-6-[[(1-methyl-4-propyl-2-pyrrolidinyl)-carbonyl]amino]-1-thio-, (2*S*-*trans*)-, monohydrochloride, monohydrate.
Anhydrous 461.44 *21462-39-5*

Category Antibacterial.

Clindamycin Hydrochloride is the hydrated hydrochloride salt of clindamycin, a substance produced by the chlorination of lincomycin. It has a potency equivalent to not less than 800 µg of $C_{18}H_{33}ClN_2O_5S$ per mg.

Description White or almost white, crystalline powder.

Solubility Freely soluble in *water*, in *dimethylformamide* and in *methanol*; soluble in *ethanol*; practically insoluble in *acetone*.

Contra-indication It is contra-indicated in patients with a history of hypersensitivity to lincosamides or in patients with diarrheal states.

Warning

1. It may cause severe potentially fatal *Clostridium difficile*-associate diarrhea and colitis.

2. It may cause nausea, vomiting, skin rashes or urticaria, transient elevation of serum alkaline phosphatase and transaminases, jaundice, or blood dyscrasia.

3. It should be used with extreme caution in patients with impaired hepatic and renal functions, in pregnant women, newborns or infants, or in patients who are receiving erythromycin, chloramphenicol, neuromuscular blocking agents, or antiperistaltic antidiarrheals.

4. Risk-benefit should be considered if it is to be used in pregnant or nursing women.

Precaution Complete blood counts and hepatic function determinations are required at periodic intervals during prolonged therapy. Discontinue medication and/or check with a physician immediately if signs of pseudomembranous colitis occur during or after therapy.

Additional information

1. Since the drug does not adequately diffuse into the cerebrospinal fluid, it is not recommended for the treatment of meningitis.

2. Cross-sensitivity between clindamycin and doxorubicin may be possible.

Packaging and storage Clindamycin Hydrochloride shall be kept in tightly closed containers and stored at a temperature not exceeding 30°.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Clindamycin Hydrochloride RS (Appendix 2.1) or with the reference spectrum of Clindamycin Hydrochloride.

B. Dissolve 10 mg in 2 ml of dilute *hydrochloric acid* and heat on a water-bath for 3 minutes. Add 3 ml of *sodium carbonate TS* and 1 ml of a 2 per cent w/v solution of *sodium nitroferricyanide*: a violet-red colour develops.

C. It yields the *reactions* characteristic of chlorides (Appendix 5.1).

Crystallinity It is crystalline (Appendix 4.14).

pH 3.0 to 5.5, in a 10.0 per cent w/v solution (Appendix 4.11).

Specific rotation +135° to +150°, calculated on the anhydrous basis, determined in a 4.0 per cent w/v solution (Appendix 4.8).

Water Not less than 3.0 per cent w/w and not more than 6.0 per cent w/w (Karl Fischer Method, Appendix 4.12).

Sulfated ash Not more than 0.5 per cent w/w (Appendix 5.3).

Related substances Not more than 4.0 per cent w/w of 7-epiclindamycin and not more than 2.0 per cent w/w of clindamycin B; not more than 1.0 per cent w/w of any other individual related substance, and not more than 6.0 per cent w/w of the total of all related substances, including lincomycin. Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase and **Chromatographic system** Proceed as directed in the *Assay*.

Standard solution Dissolve accurately weighed quantities of Lincomycin Hydrochloride RS and Clindamycin Hydrochloride RS quantitatively in *Mobile phase* to obtain a solution having known concentrations of about 0.5 mg per ml of Lincomycin Hydrochloride RS and 1 mg per ml of Clindamycin Hydrochloride RS per ml. Transfer 10.0 ml of this solution to a 100-ml volumetric flask, dilute with *Mobile phase* to volume, and mix.

Test solution Transfer about 125 mg of the test substance, accurately weighed, to a 25-ml volumetric flask, dissolve in and dilute with *Mobile phase* to volume, and mix.

To determine the suitability of the chromatographic system, chromatograph *Standard solution*, and record the peak responses as directed under *Procedure*: the relative retention times are about 0.4 for lincomycin, 0.65 for clindamycin B, 0.8 for 7-epiclindamycin, and 1.0 for clindamycin.

Procedure Separately inject equal volumes (about 10 µl) of *Test solution* and *Standard solution*, and record the chromatograms for a period of time that is six times the retention time of the clindamycin peak.

Calculations Calculate the percentage of lincomycin in the test substance taken by the expression:

$$2.5(C_L P_L / W)(r_U / r_S),$$

in which C_L is the concentration, in mg per ml, of Lincomycin Hydrochloride RS in *Standard solution*; P_L is the potency in µg of lincomycin ($C_{18}H_{34}N_2O_6S$) per mg, of Lincomycin Hydrochloride RS; W is the weight, in mg, of the test substance taken to prepare *Test solution*; and r_U and r_S are the lincomycin peak responses obtained from *Test solution* and *Standard solution*, respectively. Calculate the percentage of all other related substance in the Clindamycin Hydrochloride taken by the expression:

$$2.5(CP / W)(r_i / r_C),$$

in which C is the concentration, in mg per ml, of Clindamycin Hydrochloride RS in *Standard solution*; P is the potency, in µg, of clindamycin ($C_{18}H_{33}ClN_2O_5S$) per mg, of Clindamycin Hydrochloride RS; W is the weight, in mg, of the test substance taken to prepare *Test solution*; r_i is the response of an individual related substance, other than lincomycin, in the chromatogram obtained from *Test solution* and r_C is the clindamycin peak response in the chromatogram obtained from *Standard solution*.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Mix 55 volumes of buffer solution prepared by dissolving 6.8 g of *potassium dihydrogen phosphate* in 1000 ml of *water*, and adjusting with 8 M *potassium hydroxide* to a pH of 7.5, and 45 vol-umes of *acetonitrile*. Make adjustments if necessary. (**Note** Increasing the proportion of *acetonitrile* in the *Mobile phase* decreases the retention time, and decreasing it increases the resolution between 7-epiclindamycin and clindamycin.)

Standard preparation Dissolve an accurately weighed quantity of Clindamycin Hydrochloride RS quantitatively in *Mobile phase* to obtain a solution having a known concentration of about 1 mg per ml.

Assay preparation Transfer about 125 mg of Clindamycin Hydrochloride, accurately weighed, to a 25-ml volumetric flask, dissolve in and dilute with *Mobile phase* to volume, and mix. Transfer 5.0 ml of this solution to a 25-ml volumetric flask, dilute with *Mobile phase* to volume, and mix.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (25 cm × 4.6 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles (5 µm), (b) *Mobile phase* at a flow rate of about 1 ml per minute, and (c) an ultraviolet photometer set at 210 nm.

To determine the suitability of the chromatographic system, chromatograph *Standard preparation*, and record the peak responses as directed under *Procedure*: the resolution factor between clindamycin B and 7-epiclindamycin is not less than 2.4, the resolution factor between clindamycin and 7-epiclindamycin is not less

than 3.0, the symmetry factor for the clindamycin peak is not more than 1.2, and the relative standard deviation for the clindamycin peak is not more than 1.0 per cent.

Procedure Separately inject equal volumes (about 10 µl) of *Standard preparation* and *Assay preparation* into the chromatograph, record the chromatograms for a period of time that is about twice the retention time of the clindamycin peak and measure the major peaks.

Calculation Calculate the content, in µg, of $C_{18}H_{33}ClN_2O_5S$ in each mg of the Clindamycin Hydrochloride taken by the expression:

$$125(CP/W)(r_u/r_s),$$

in which C is the concentration, in mg per ml, of Clindamycin Hydrochloride RS in *Standard preparation*; P is the potency, in µg of clindamycin per mg, of Clindamycin Hydrochloride RS; W is the weight, in mg, of Clindamycin Hydrochloride taken to prepare *Assay preparation*; and r_u and r_s, are the clindamycin peak responses obtained from *Assay preparation* and *Standard preparation*, respectively.

CLINDAMYCIN HYDROCHLORIDE CAPSULES

Category Antibacterial.

Clindamycin Hydrochloride Capsules contain the equivalent of not less than 90.0 per cent and not more than 120.0 per cent of the labelled amount of $C_{18}H_{33}ClN_2O_5S$.

Strengths available 75, 150 and 300 mg (base).

Dose Adults: 150 to 450 mg four times a day or up to 1.8 g daily.

Children: 8 to 20 mg per kg of body weight daily in three or four divided doses.

In children weighing 10 kg or less, the minimum recommended dose is 37.5 mg every 8 hours.

Contra-indication; Warning; Precaution; Additional information See under *Clindamycin Hydrochloride*, p. 78.

Packaging and storage Clindamycin Hydrochloride Capsules shall be kept in tightly closed containers, protected from light.

Labelling The label on the container states the quantity equivalent to the amount of clindamycin.

Identification

A. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

B. Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel GF254* as the coating substance and a mixture of 1.5 volumes of *strong ammonia solution*, 30 volumes of *toluene* and 70 volumes of *methanol* as the mobile phase. Apply separately to the plate, 10 µl of each of the following solutions. For solution (A), shake a portion of the capsules contents, equivalent to 50 mg of clindamycin, with 10 ml of *methanol* and filter. Solution (B) contains 5 mg per ml of Clindamycin Hydrochloride RS in *methanol*. After removal of the plate, allow it to dry in air and spray with *dilute potassium iodobismuthate TS*: the principal spot in the chromatogram obtained from solution (A) corresponds to that obtained from solution (B).

Water Not more than 7.0 per cent w/w (Karl Fischer Method, Appendix 4.12).

Dissolution Carry out the test as described in the "Dissolution Test" (Appendix 4.12).

Dissolution medium: *pH 6.8 phosphate buffer*; 900 ml.

Apparatus 1: 100 rpm.

Time: 30 minutes.

Procedure Determine the amount of clindamycin hydrochloride dissolved using the following method.

Mobile phase Dissolve 16 g of dl-*10-camphorsulfonic acid*, 8 g of *ammonium acetate*, and 8 ml of *glacial acetic acid* in 1600 ml of *water*, and mix. Add 2400 ml of *methanol* to this solution, mix and adjust with *dilute hydrochloric acid* or 5 M *sodium hydroxide* to a pH of 6.0±0.05.

Standard solution Prepare a solution of Clindamycin Hydrochloride RS in *water* having an accurately known concentration similar to that expected in *Test solution*.

Test solution Use a filtered portion of the solution under test, diluted with *water* if necessary.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (30 cm × 4 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles (3 µm), (b) *Mobile phase* at a flow rate of about 2 ml per minute, and (c) a refractive index detector.

To determine the suitability of the chromatographic system, chromatograph *Standard solution*, and record the peak responses as directed under *Procedure*: the symmetry factor for the clindamycin peak is not more than 2.0

and the relative standard deviation for replicate injections is not more than 3.0 per cent

Procedure Separately inject equal volumes (about 50 µl) of *Standard solution* and *Test solution* into the chromatograph, record the chromatograms, and measure the responses for the major peaks. Calculate the amount of $C_{18}H_{33}ClN_2O_5S$ dissolved.

Tolerances Not less than 80 per cent (*Q*) of the labelled amount of $C_{18}H_{33}ClN_2O_5S$ is dissolved in 30 minutes.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Add 2 g of dl-*10-camphorsulfonic acid*, 1 g of *ammonium acetate*, and 1 ml of *glacial acetic acid* to 200 ml of *water* in a 500-ml volumetric flask, and mix to dissolve. Dilute with *methanol* to volume, and mix. Adjust, if necessary, with *hydrochloric acid* or a 50 per cent w/v solution of *sodium hydroxide* to a pH of 6.0±0.1. Make adjustments, if necessary.

Internal standard solution Add 0.5 ml of *phenylethyl alcohol* to a 100-ml volumetric flask, dilute with *Mobile phase* to volume, and mix.

Standard preparation Transfer about 90 mg of Clindamycin Hydrochloride RS, accurately weighed, to a suitable container. Add 5.0 ml of *Internal standard solution*, and swirl to dissolve.

Assay preparation Remove, as completely as possible, the contents of not less than 20 Clindamycin Hydrochloride Capsules, and weigh accurately. Mix the combined contents, and transfer an accurately weighed portion, equivalent to about 75 mg of clindamycin, to a suitable container. Add 5.0 ml of *Internal standard solution*, and shake for about 30 minutes. Centrifuge or filter, if necessary, to obtain a clear solution.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (30 cm × 4 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles (5 µm), (b) *Mobile phase* at a flow rate of about 1 ml per minute, and (c) a refractive index detector.

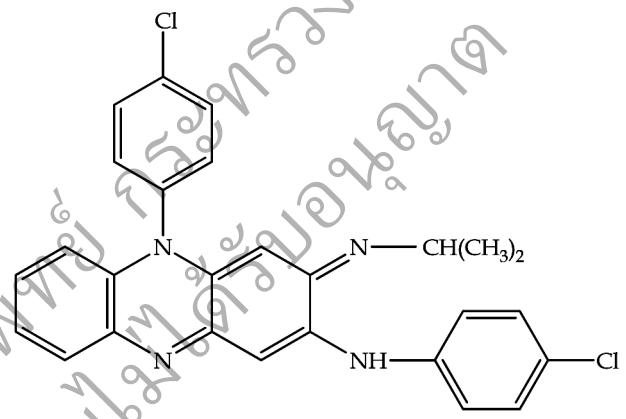
To determine the suitability of the chromatographic system, chromatograph *Standard preparation*, and record the peak responses as directed under *Procedure*: the relative retention times are about 1.7 for clindamycin and 1.0 for phenylethyl alcohol, the resolution factor between the clindamycin hydrochloride and the phenylethyl alcohol peaks is not less than 5.0, and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Procedure Separately inject equal volumes (about 25 µl) of *Standard preparation* and *Assay preparation* into the chromatograph, record the chromatograms and measure the responses for the major peaks.

Calculation Calculate the content of $C_{18}H_{33}ClN_2O_5S$ in the portion of the Capsules taken, using the declared content of $C_{18}H_{33}ClN_2O_5S$ in Clindamycin Hydrochloride RS.

Other requirements Comply with the requirements described under "Capsules" (Appendix 1.16).

CLOFAZIMINE



$C_{27}H_{22}Cl_2N_4$ 473.40 *2030-63-9*

2-Phenazinamine, *N*,5-bis(4-chlorophenyl)-3,5-dihydro-3-[(1-methylethyl)imino]-.

Category Antibacterial (leprostatic).

Clofazimine contains not less than 98.5 per cent and not more than 101.5 per cent of $C_{27}H_{22}Cl_2N_4$, calculated on the dried basis.

Description Dark red crystals.

Solubility Practically insoluble in *water*; soluble in *chloroform* and in *benzene*; sparingly soluble in *ethanol*, in *acetone* and in *ethyl acetate*.

Warning

1. It should be used with caution in patients with hepatic function impairment, gastro-intestinal problems such as abdominal pain and diarrhea.

2. It should not be used in pregnant or nursing women unless clearly indicated since skin discoloration can occur in infants.

3. It may cause splenic infarction, gastro-intestinal disturbances, bowel obstruction and bleeding, ichthyosis and dryness of the skin, rash, pruritus, and pigmentation of skin and conjunctiva and cornea.

Additional information The urine, skin and other tissues may be temporarily discoloured.

Packaging and storage Clofazimine shall be kept in tightly closed containers, protected from light.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Clofazimine RS (Appendix 2.1) or with the reference spectrum of Clofazimine.

B. The principal spot in the chromatogram obtained from the Test preparation is similar in position and size to that in the chromatogram of the Standard preparation A as obtained in the test for *Chromatographic purity*.

C. Dissolve 2 mg in 3 ml of *acetone* and add 2 drops of *hydrochloric acid*: an intense violet colour is produced. Add 0.5 ml of 5 M *sodium hydroxide*: the colour changes to orange-red.

Melting temperature 217° (Appendix 4.3).

Loss on drying Not more than 0.5 per cent w/w after drying at 105° to constant weight (Appendix 4.15).

Sulfated ash Not more than 0.1 per cent w/w (Appendix 5.3).

Chromatographic purity Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel GF254* as the coating substance and a mixture of 10 volumes of *dichloromethane* and 1 volume of *1-propanol* as the mobile phase. Immediately before use, expose the plate to ammonia vapours for 30 minutes by suspending the plate in a tank containing a shallow layer of approximately 25 ml of *Ammonia solution*. (**Note** Prevent the plate from coming into contact with the liquid.)

Standard solutions Dissolve an accurately weighed quantity of Clofazimine RS in *dichloromethane*, and mix to obtain the Standard solution A having a known concentration of about 0.5 mg per ml. Dilute portions of the Standard solution A quantitatively with *dichloromethane* to obtain the Standard solutions B and C having known concentrations of 250 and 100 µg per ml, respectively.

Test solution Dissolve an accurately weighed quantity of the test substance in *dichloromethane* to obtain a solution having a known concentration of about 50 mg per ml.

Ammonia solution Transfer 1.0 ml of *strong ammonia solution* to a 100-ml volumetric flask, dilute with *water* to volume, and mix. (**Note** Use freshly prepared solution.)

Procedure Apply separately to the plate, 5 µl of each of *Test solution* and *Standard solutions*. After removal of the plate, allow it to dry in air and examine under ultraviolet light (254 nm). Compare the intensities of any secondary spots observed in the chromatogram of *Test solution* with those of the principal spots in the chromatograms of *Standard solution*: no secondary spot is larger or more intense than the principal spot obtained from *Standard solution A* (1.0 per cent), and the sum of the intensities of all secondary spots obtained from *Test solution* corresponds to not more than 2.0 per cent.

Assay Dissolve about 300 mg of Clofazimine, accurately weighed, in 5 ml of *chloroform*, with the aid of heat if necessary. Add 20 ml of *acetone* and 5 ml of *anhydrous glacial acetic acid*, and titrate with 0.1 M *perchloric acid VS*, determining the end-point potentiometrically (Appendix 6.1). Perform a blank determination, and make any necessary correction. Each ml of 0.1 M *perchloric acid* is equivalent to 47.34 mg of $C_{27}H_{22}Cl_2N_4$.

CLOFAZIMINE CAPSULES

Category Antibacterial (leprostatic).

Clofazimine Capsules contain not less than 90.0 per cent and not more than 110.0 per cent of the labelled amount of $C_{27}H_{22}Cl_2N_4$.

Strengths available 50 and 100 mg.

Dose Adults: In combination with one or more other antileprosy agents, 50 to 100 mg a day; with recurring leprotic erythema nodosum, up to 300 mg a day may be required.

Warning; Additional information See under *Clofazimine*, p 81.

Identification

A. The principal spot in the chromatogram obtained from the Test preparation is similar in position and size to that in the chromatogram of the Standard preparation A as obtained in the test for *Chromatographic purity*.

B. Dissolve a portion of the capsule contents, containing 2 mg of clofazimine, in 3 ml of *acetone* and add 2 drops of *hydrochloric acid*: an intense violet colour is produced. Add 0.5 ml of 5 M *sodium hydroxide*: the colour changes to orange-red.

Disintegration Carry out the test as described in the "Disintegration Test for Tablets and Capsules" (Appen-

dix 4.23). Disintegration occurs in not more than 15 minutes with discs, *simulated gastric fluid TS* being used.

Chromatographic purity Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1).

Ammonia solution, Chromatographic system and **Procedure** Proceed as directed in the Chromatographic purity under *Clofazimine*, p. 82.

Standard solutions Dissolve an accurately weighed quantity of Clofazimine RS in *dichloromethane*, and mix to obtain the Standard solution A having a known concentration of about 0.5 mg per ml. Dilute portions of the Standard solution A quantitatively with *dichloromethane* to obtain the Standard solutions B and C having known concentrations of 100 and 40 μg per ml, respectively.

Test solution To a portion of the capsule content, containing 500 mg of clofazimine, add 25 ml of *dichloromethane* and 25 ml of 0.1 M *sodium hydroxide*, and sonicate for 30 minutes. Withdraw the dichloromethane layer, and filter through *anhydrous sodium sulfate*.

Assay

0.1 M Methanolic hydrochloric acid Pipette 10 ml of *hydrochloric acid* into a 1000-ml volumetric flask containing about 500 ml of *methanol*, mix and dilute with *methanol* to volume.

Reference solution Pipette 5 ml of *dichloromethane* into a 50-ml volumetric flask, dilute with *0.1 M Methanolic hydrochloric acid* to volume and mix.

Standard preparation Dissolve an accurately weighed quantity of Clofazimine RS in *dichloromethane* and dilute quantitatively and stepwise, if necessary, with *dichloromethane* to obtain a solution having a known concentration of about 75 μg per ml. Transfer 5.0 ml of this solution to a 50-ml volumetric flask, dilute with *0.1 M Methanolic hydrochloric acid* to volume and mix.

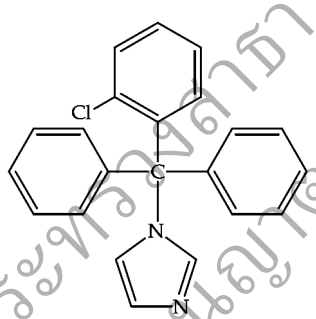
Assay preparation Remove, as completely as possible, the contents of not less than 20 Clofazimine Capsules, and weigh accurately. Mix the combined contents, dissolve an accurately weighed portion of the powder in *dichloromethane*, filter the solution through a pledget of cotton, and dilute quantitatively and stepwise, if necessary, with *dichloromethane* to obtain a solution having a concentration of about 75 μg per ml. Transfer 5.0 ml of this solution to a 50-ml volumetric flask, dilute with *0.1 M Methanolic hydrochloric acid* to volume and mix.

Procedure Concomitantly measure the absorbances of *Standard preparation* and *Assay preparation* at the maximum at about 491 nm, using *Reference solution* as the blank (Appendix 2.2).

Calculation Calculate the content of $C_{27}H_{22}Cl_2N_4$ in the portion of the Capsules taken, using the declared content of $C_{27}H_{22}Cl_2N_4$ in Clofazimine RS.

Other requirements Comply with the requirements described under "Capsules" (Appendix 1.16).

CLOTRIMAZOLE



$C_{22}H_{17}ClN_2$ 344.84 *23593-75-1*

1*H*-Imidazole, 1-[(2-chlorophenyl)diphenylmethyl]-.

Category Antifungal (topical).

Clotrimazole contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{22}H_{17}ClN_2$, calculated on the dried basis.

Description White or pale yellow, crystalline powder.

Solubility Practically insoluble in *water*; soluble in *chloroform* and in *ethanol*; slightly soluble in *ether*.

Contra-indication It is contra-indicated in patients who are hypersensitive to imidazole derivatives.

Warning

1. Clotrimazole is not for ophthalmic use and should be used with caution around the eyes.

2. It may cause blistering, erythema, edema, pruritus, burning, stinging, peeling, urticaria, skin fissures, and general irritation of the skin.

3. Mild burning, skin rash, itching, vulval irritation, lower abdominal cramps, bloating, slight cramping, vaginal soreness, dyspareunia, and slight urinary frequency may occasionally occur in patients receiving clotrimazole vaginal tablets.

4. Risk-benefit should be considered if it is to be used in pregnant women, especially during the first trimester.

Precaution

1. Clotrimazole should be discontinued upon the appearance of any symptoms suggesting sensitivity or irritation, and the appropriate treatment should be instituted.

2. When this medication is used in the treatment of candidiasis, occlusive dressings should be avoided since they provide conditions which favour growth of yeast and release of its irritating endotoxin.

3. Periodic determinations of liver function should be performed, particularly in patients with pre-existing hepatic impairment.

Additional information It is advisable to continue medicine for full time of treatment.

Packaging and storage Clotrimazole shall be kept in well-closed containers, protected from light.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Clotrimazole RS (Appendix 2.1) or with the reference spectrum of Clotrimazole.

B. Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel GF254* as the coating substance and a mixture of 1 volume of *strong ammonia solution*, 20 volumes of *1-propanol* and 180 volumes of *toluene* as the mobile phase. Apply separately to the plate, 10 µl of each of the following solutions. For solution (A), dissolve 500 mg of the test substance in *ethanol* and dilute to 5 ml with the same solvent. For solution (B) dilute 1 ml of solution (A) to 10 ml with *ethanol*. Solution (C) contains 10 mg per ml of Clotrimazole RS in *ethanol*. After removal of the plate, allow it to dry in air and examine under ultraviolet light (254 nm): the principal spot in the chromatogram obtained from solution (B) is similar in position and size to that obtained from solution (C).

C. Dissolve 10 mg in 3 ml of *sulfuric acid*: the solution is pale yellow. Add 10 mg of *yellow mercury(II) oxide* and 20 mg of *sodium nitrite*. Allow to stand with occasional shaking: an orange colour develops, becoming orange-brown.

Melting range 141° to 145° (Appendix 4.3).

Loss on drying Not more than 0.5 per cent w/w after drying at 105° for 2 hours (Appendix 4.15).

Sulfated ash Not more than 0.1 per cent w/w (Appendix 5.3).

Heavy metals Not more than 10 ppm (Method II, Appendix 5.2). Use 2.0 g; for the Standard preparation, use *lead standard solution (1 ppm Pb)*.

(2-Chlorophenyl)diphenylmethanol Not more than 0.2 per cent. Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel G* as the coating substance and a mixture of 90 volumes of *toluene*, 10 volumes of *1-propanol* and 0.5 volume of *strong ammonia solution* as the mobile phase.

Reference solution (a) Dissolve 50 mg of Clotrimazole RS in *ethanol* and dilute to 5 ml with the same solvent.

Reference solution (b) Dissolve 10 mg of (2-Chlorophenyl)diphenylmethanol RS in *ethanol* and dilute to 5 ml with the same solvent. Dilute 1 ml of the solution to 10 ml with *ethanol*.

Test solution (a) Dissolve 500 mg of the test substance in *ethanol* and dilute to 5 ml with the same solvent.

Test solution (b) Dilute 1 ml of *Test solution (a)* to 10 ml with *ethanol*.

Apply separately to the plate 10 µl of each solution. After removal of the plate, allow it to dry in air. Spray with a 10 per cent v/v solution of *sulfuric acid* in *ethanol* and heat at 100° to 105° for 30 minutes. Any spot corresponding to (2-chlorophenyl)diphenylmethanol in the chromatogram obtained from *Test solution (a)* is not more intense than the spot in the chromatogram obtained from *Reference solution (b)*.

Imidazole Not more than 0.2 per cent. Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel G* as the coating substance and a mixture of 90 volumes of *toluene*, 10 volumes of *1-propanol* and 0.5 volume of *strong ammonia solution* as the mobile phase.

Reference solution Dissolve 10 mg of Imidazole RS in *ethanol* and dilute to 10 ml with the same solvent. Dilute 1 ml of the solution to 10 ml with *ethanol*.

Test solution Dissolve 500 mg of the test substance in *ethanol* and dilute to 10 ml with the same solvent.

Apply separately to the plate 10 µl of each solution. After removal of the plate, allow it to dry in air. At the bottom of a chromatographic tank, place an evaporating dish containing a mixture of 1 volume of *hydrochloric acid*, 1 volume of *water* and 2 volumes of a 1.5 per cent w/v solution of *potassium permanganate*, close the tank and allow to stand for 15 minutes. Place the dried plate in the tank and close the tank. Leave the plate in contact with the chlorine vapour for 5 minutes. Withdraw the plate and place it in a current of cold air until the excess of chlorine is removed and an area of coating below points of application does not give a blue colour with a drop of *potassium iodide* and *starch TS*. Spray with *potassium iodide* and *starch TS*. Any spot corresponding to imidazole in the chromatogram obtained from *Test solution* is not more intense than the spot in the chromatogram obtained from *Reference solution*.

Assay Dissolve about 170 mg of Clotrimazole, accurately weighed, in 50 ml of *anhydrous glacial acetic acid*. Titrate with 0.1 M *perchloric acid VS*, using 0.5 ml of *1-naphtholbenzein TS* as indicator or determining the endpoint potentiometrically (Appendix 6.1). Perform a blank determination, and make any necessary correction. Each ml of 0.1 M *perchloric acid* is equivalent to 34.48 mg of $C_{22}H_{17}ClN_2$.

CLOTRIMAZOLE CREAM

Category Antifungal (topical).

Clotrimazole Cream contains not less than 90.0 per cent and not more than 110.0 per cent of the labelled amount of $C_{22}H_{17}ClN_2$.

Strength available 1 per cent w/w.

Dose *Topical*, to the affected area of the skin and surrounding areas, twice a day, morning and evening.

Contra-indication; Warning; Precaution; Additional information See under *Clotrimazole*, p. 81.

Packaging and storage Clotrimazole Cream shall be kept in tightly closed containers.

Identification

A. Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel GF254* as the coating substance and 200 ml of *ether* as the mobile phase. Place a beaker, containing 25 ml of *strong ammonia solution*, in the chromatographic chamber, cover the chamber, and allow to equilibrate for 2 hours. Apply separately to the plate, 20 µl of each of the following solutions. For solution (A), transfer a portion of the cream, containing 5 mg of clotrimazole, to a 50-ml centrifuge tube. Add 5 ml of *chloroform*, mix and centrifuge to obtain a clear chloroform phase. Solution (B) contains a 0.1 per cent w/v solution of Clotrimazole RS in *chloroform*. After removal of the plate, allow it to dry in air and examine under ultraviolet light (254 nm): the principal spot in the chromatogram obtained from solution (A) corresponds to that obtained from solution (B). Dissolve 3 g of *bismuth oxynitrate* and 30 g of *potassium iodide* in 10 ml of diluted *hydrochloric acid* (1 in 4), dilute with *water* to 100 ml, mix, and prepare a spraying reagent by diluting 10 ml of this solution and 5 ml of diluted *hydrochloric acid* (1 in 4) with *water* to 200 ml, and mixing. Spray the plate evenly with this spraying reagent: the principal spots from solutions (A) and (B) are orange.

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

(2-Chlorophenyl)diphenylmethanol Not more than 1.0 per cent. Carry out the test as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase and **Chromatographic system** Proceed as directed in the *Assay*.

Diluting solution A mixture of 7 volumes of *methanol* and 3 volumes of 0.02 M *phosphoric acid*.

Reference solution Dissolve an accurately weighed quantity of (2-Chlorophenyl)diphenylmethanol RS in *Diluting solution* to obtain a solution having a known concentration of about 2 µg per ml.

Test solution (a) Extract a portion of the cream, containing 20 mg of clotrimazole by warming with 20 ml of *methanol* in a water-bath at 50° for 5 minutes, shaking occasionally. Remove from the water-bath, shake the mixture vigorously while cooling to room temperature, cool in ice for 15 minutes, centrifuge for 5 minutes and decant the supernatant liquid. Repeat the extraction with two further 20-ml quantities of *methanol*. To the combined methanol extracts add 10 ml of *methanol* and dilute to 100.0 ml with 0.02 M *phosphoric acid*. Cool in ice and filter.

Test solution (b) Dilute 1 volume of *Test solution (a)* to 50 volumes with *Diluting solution*.

To determine the suitability of the chromatographic system, chromatograph *Test solution (b)*, and record the peak responses as directed under *Procedure*: the column efficiency, determined from the clotrimazole peak, is not less than 1800 theoretical plates and the relative standard deviation for replicate injections is not more than 2.0.

Procedure Separately inject equal volumes (about 20 µl) of *Reference solution* and *Test solution (a)* into the chromatograph, and record the chromatograms for *Test solution (a)* for 1.5 times the retention time of the principal peak. The area of any peak corresponding to (2-chlorophenyl)diphenylmethanol in the chromatogram obtained from *Test solution (a)* is not greater than the area of the peak in the chromatogram obtained from *Reference solution*.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Prepare a mixture of 30 volumes of 0.02 M *phosphoric acid* and 70 volumes of *methanol*, the pH of the mixture being adjusted to 7.5 with a 10 per cent v/v solution of *triethylamine* in *methanol*. Make adjustments if necessary.

Standard preparation Dissolve about 20 mg of Clotrimazole RS, accurately weighed, in 70 ml of *methanol*, add sufficient 0.02 M *phosphoric acid* to produce 100.0 ml and dilute 10.0 ml of the resulting solution to 50.0 ml with a mixture of 70 volumes of *methanol* and 30 volumes of 0.02 M *phosphoric acid*.

Assay preparation Extract an accurately weighed portion of Clotrimazole Cream, containing about 20 mg of clotrimazole, by warming with 20 ml of *methanol* in a water-bath at 50° for 5 minutes, shaking occasionally. Remove from the water-bath, shake the mixture vigorously while cooling to room temperature, cool in ice for 15 minutes, centrifuge for 5 minutes and decant the supernatant liquid. Repeat the extraction with two further 20-ml quantities of *methanol*. To the combined methanol extracts add 10 ml of *methanol* and dilute to 100.0 ml with 0.02 M *phosphoric acid*. Cool in ice and filter. Dilute 10.0 ml of the filtrate to 50.0 ml with a mixture of 70 volumes of *methanol* and 30 volumes of 0.02 M *phosphoric acid*.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (20 cm × 4.6 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles (5 µm), (b) *Mobile phase* at a flow rate of about 1.5 ml per minute, and (c) an ultraviolet photometer set at 215 nm.

To determine the suitability of the chromatographic system, chromatograph *Standard preparation*, and record the peak responses as directed under *Procedure*: the relative standard deviation for replicate injections is not more than 2.0.

Procedure Separately inject equal volumes (about 20 µl) of *Standard preparation* and *Assay preparation* into the chromatograph, record the chromatograms, and measure the responses for the major peaks.

Calculation Calculate the content of $C_{22}H_{17}ClN_2$ in the portion of the Cream taken, using the declared content of $C_{22}H_{17}ClN_2$ in Clotrimazole RS.

Other requirements Complies with the requirements described under "Topical Semi-solid Preparations (Appendix 1.16).

CLOTRIMAZOLE VAGINAL TABLETS

Category Antifungal (vaginal).

Clotrimazole Vaginal Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the labelled amount of $C_{22}H_{17}ClN_2$.

Strengths available 100 and 500 mg.

Dose Adults: Insert one 100-mg tablet intravaginally at bedtime for 7 nights or two 100-mg tablets intravaginally at bedtime for 3 nights or insert one 500-mg tablet intravaginally once only, preferably at bedtime.

Contra-indication; Warning; Precaution; Additional information See under *Clotrimazole*, p. 83.

Identification

A. Comply with the tests for Identification A described under *Clotrimazole Cream*, p. 67. For solution (A), transfer a portion of the powdered tablets, containing 50 mg of clotrimazole, in a screw-capped 50-ml centrifuge tube. Add 10 ml of *chloroform*, shake vigorously for about 2 minutes and centrifuge to obtain a clear chloroform phase. (**Note** The supernatant liquid may remain slightly turbid.)

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

(2-Chlorophenyl)diphenylmethanol Not more than 1 per cent. Carry out the test as described in the High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase, Diluting solution, Reference solution and **Procedure** Proceed as directed in the (2-Chlorophenyl)diphenylmethanol under *Clotrimazole Cream*, p. 85.

Test solution (a) Transfer a portion of the powdered vaginal tablets, containing 100 mg of clotrimazole to a 100-ml volumetric flask. Add 50 ml of *methanol* and shake for 20 minutes. Dilute to volume with *methanol* and filter. To 20.0 ml of the filtrate add 50 ml of *methanol* and sufficient 0.02 M *phosphoric acid* to produce 100.0 ml.

Test solution (b) Dilute 1 volume of *Test solution (a)* to 50 volumes with *Diluting solution*.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase, **Standard preparation, Chromatographic system** and **Procedure** Proceed as directed in the Assay under *Clotrimazole Cream*, p. 85.

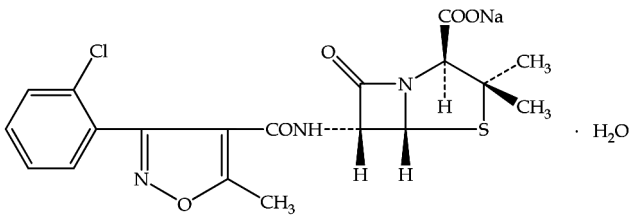
Assay preparation Weigh and finely powder not less than 20 Clotrimazole Vaginal Tablets. Transfer an

accurately weighed portion of the powder, containing about 100 mg of clotrimazole, to a 250-ml volumetric flask. Add 50 ml of *methanol* and shake for 20 minutes. Dilute to volume with *methanol* and filter. To 10.0 ml of the filtrate add 60 ml of *methanol* and sufficient 0.02 M *phosphoric acid* to produce 100.0 ml.

Calculation Calculate the content of $C_{22}H_{17}ClN_2$ in the portion of the Tablets taken, using the declared content of $C_{22}H_{17}ClN_2$ in Clotrimazole RS.

Other requirements Comply with the requirements described under "Tablets" (Appendix 1.16).

CLOXACILLIN SODIUM



$C_{19}H_{17}ClN_3O_5S.Na.H_2O$ 475.88 *7081-44-9*
4-Thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid, 6-[[[3-(2-chlorophenyl)-5-methyl-4-isoxazolyl]carbonyl] amino] -3,3-dimethyl-7-oxo-, [2*S*-(2α,5α,6β)]-, monosodium salt, monohydrate.
Anhydrous 457.86 *642-78-4*

Category Antibacterial.

Cloxacillin Sodium contains not less than 90.0 per cent and not more than 96.0 per cent of $C_{19}H_{18}ClN_3O_5S$, calculated on the anhydrous basis.

Description White, crystalline powder.

Solubility Freely soluble in *water* and in *methanol*; soluble in *ethanol*; slightly soluble in *acetone* and in *chloroform*.

Stability It is hygroscopic. Even in the absence of light, Cloxacillin Sodium is gradually degraded on exposure to a humid atmosphere, the decomposition being faster at higher temperatures.

Contra-indication; Warning; Precaution See under *Penicillin V Potassium*, p. 140.

Additional information It is a preferred drug for the treatment of mild to moderate Staphylococcal infections of the skin and soft tissue, respiratory and genitourinary tracts, and joints.

See also under *Penicillin V Potassium*, p. 140.

Packaging and storage Cloxacillin Sodium shall be kept in tightly closed containers and stored at a temperature not exceeding 25°. If it is intended for parenteral administration, it shall also be kept under sterile condition.

Labelling The label on the container states (1) storage condition; (2) parenteral or non-parenteral grade.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Cloxacillin Sodium RS (Appendix 2.1) or with the reference spectrum of Cloxacillin Sodium. It yields the *reactions* characteristic of penicillins (Appendix 5.1).

B. Ignite 20 mg and dissolve the residue in *dilute acetic acid*. The solution yields the *reactions* characteristic of sodium salts (Appendix 5.1).

D. Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silanized silica gel H* as the coating substance and a mixture of 30 volumes of *acetone* and 70 volumes of a 15.4 per cent w/v of solution of *ammonium acetate*, adjusted to pH 5.0 with *glacial acetic acid* as the mobile phase. Apply separately to the plate, 1 µl of each of the following solutions. For solution (A) dissolve 25 mg of Cloxacillin Sodium in 5 ml of *water*. Solution (B) contains 5 mg per ml of Cloxacillin Sodium RS and solution (C) contains 5 mg per ml of each of Cloxacillin Sodium RS, Dicloxacillin Sodium RS and Flucloxacillin Sodium RS. After removal of the plate, allow it to dry in air, expose it to iodine vapour until the spots appear. The principal spot in the chromatogram obtained from solution (A) corresponds to that in the chromatogram obtained from solution (B). The test is not valid unless the chromatogram obtained from solution (C) shows three clearly separated spots.

Crystallinity It is crystalline (Method I, Appendix 4.14).

pH 4.5 to 7.5, in a 1.0 per cent w/v solution (Appendix 4.11).

Specific rotation +160° to +169°, calculated on the anhydrous basis, determined in a 1.0 per cent w/v solution (Appendix 4.8).

Water Not less than 3.0 per cent w/w and not more than 5.0 per cent w/w (Karl Fischer Method, Appendix 4.12).

***N,N*-Dimethylaniline** Not more than 20 ppm. (Appendix 5.16).

2-Ethylhexanoic acid Not more than 0.8 per cent w/w. If manufactured by a process that may leave residues of 2-ethylhexanoic acid in the product. Carry out the determination as described in the "Gas Chromatography" (Appendix 3.4), using a suitable validated method.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Prepare a mixture of 25 volumes of *acetonitrile* and 75 volumes of a 0.27 per cent w/v solution of *potassium dihydrogenphosphate* adjusted to pH 5.0 with 2 M *sodium hydroxide*. Make adjustments if necessary.

Resolution solution Dissolve an accurately weighed quantity of each of Cloxacillin Sodium RS and Flucloxacillin Sodium RS in *Mobile phase* and dilute quantitatively to obtain a solution having known concentrations of about 100 µg per ml.

Standard preparation Dissolve an accurately weighed quantity of Cloxacillin Sodium RS in *Mobile phase* to obtain a solution having a known concentration of about 1 mg per ml. Dilute 5.0 ml of the solution to 50.0 ml with *Mobile phase*.

Assay preparation A Dissolve about 50 mg of Cloxacillin Sodium in *Mobile phase* and dilute to 50.0 ml with *Mobile phase*.

Assay preparation B Dilute 5.0 ml of *Assay preparation A* to 50.0 ml with *Mobile phase*.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (25 cm × 4 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles (5 µm), (b) *Mobile phase* at a flow rate of about 1 ml per minute, and (c) an ultraviolet photometer set at 225 nm.

To determine the suitability of the chromatographic system, chromatograph *Resolution solution*, and record the peak responses as directed under *Procedure*: the resolution factor between cloxacillin (the first peak) and flucloxacillin (the second peak) is not less than 2.5. Chromatograph *Standard preparation*, and record the peak responses as directed under *Procedure*: the relative standard deviation for six replicate injections is not more than 1.0 per cent.

Procedure Separately inject equal volumes (about 20 µl) of *Standard preparation* and *Assay preparation B* into the chromatograph, record the chromatograms, and measure the responses for the major peaks.

Calculation Calculate the content of $C_{19}H_{18}ClN_3O_5S$ in the Cloxacillin Sodium taken, using the declared content of $C_{19}H_{18}ClN_3O_5S$ in Cloxacillin Sodium RS.

Other requirements Cloxacillin Sodium intended for parenteral administration complies with the following additional requirements.

Bacterial endotoxins When test as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains not more than 0.40 Endotoxin Unit per mg of cloxacillin.

Sterility Complies with the "Sterility Test" (Method I, Appendix 10.1).

CLOXACILLIN SODIUM CAPSULES

Category Antibacterial.

Cloxacillin Sodium Capsules contain the equivalent of not less than 92.5 per cent and not more than 107.5 per cent of the labelled amount of $C_{19}H_{18}ClN_3O_5S$.

Strengths available 250 and 500 mg (base).

Dose Adults and children weighing more than 20 kg: 250 to 500 mg every 6 hours taken on an empty stomach.

Children up to 20 kg of body weight: 6.25 to 12.5 mg per kg of body weight every 6 hours taken on an empty stomach.

Contra-indication; Warning; Precaution See under *Penicillin V Potassium*, p. 140.

Additional information It is a preferred drug for the treatment of mild to moderate Staphylococcal infections of the skin and soft tissue, respiratory and genitourinary tracts, and joints.

See also under *Penicillin V Potassium*, p. 140.

Packaging and storage Cloxacillin Sodium Capsules shall be kept in tightly closed containers.

Labelling The label on the container states the quantity equivalent to the amount of cloxacillin.

Identification

A. The infrared absorption spectrum of the capsule contents is concordant with the spectrum obtained from Cloxacillin Sodium RS (Appendix 2.1) or with the reference spectrum of Cloxacillin Sodium.

B. Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using a *silanized silica gel H* as the coating substance and a mixture of 3 volumes of *acetone* and 7 volumes of a 15.4 per cent w/v solution of *ammonium acetate*, adjusted to pH 5.0 with *glacial acetic acid* as the mobile phase. Apply separately to the plate, 1 µl of each of the following solutions. For solution (A), shake a quantity of the capsule contents, containing 250 mg of cloxacillin sodium, with 50 ml of *water*, filter and use the clear filtrate. For solution (B), dissolve 25 mg of Cloxacillin Sodium RS in 5 ml of *water* and for solution (C), dissolve 25 mg of each of Cloxacillin Sodium RS, Dicloxacillin

Sodium RS and Flucloxacillin Sodium RS together in 5 ml of *water*. After removal of the plate, allow it to dry in air and expose it to iodine vapour until the spots appear. Examine the chromatogram in daylight. The principal spot in the chromatogram obtained from solution (A) corresponds to that in the chromatogram obtained from solution (B). The test is valid only if the chromatogram obtained from solution (C) shows three distinctly separated spots.

C. Capsule contents yield the *reactions* characteristic of sodium salts (Appendix 5.1).

Water Not more than 5.0 per cent w/w (Karl Fischer Method, Appendix 4.12).

Dissolution Carry out the test as described in the "Dissolution Test" (Appendix 4.24).

Dissolution medium: *water*; 900 ml.

Apparatus 1: 100 rpm.

Time: 45 minutes.

Procedure Determine the amount of cloxacillin ($C_{19}H_{18}ClN_3O_5S$) of a filtered portion of the test solution, suitably diluted with *Dissolution medium*, if necessary, as described in the *Assay* in comparison with a standard solution having a known concentration of Cloxacillin Sodium RS in the same medium.

Tolerances Not less than 75 per cent (*Q*) of the labelled amount of $C_{19}H_{18}ClN_3O_5S$ is dissolved in 45 minutes.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Prepare a mixture of 25 volumes of *acetonitrile* and 75 volumes of a 0.27 per cent w/v solution of *potassium dihydrogenphosphate* adjusted to pH 5.0 with *dilute sodium hydroxide TS*. Make adjustments if necessary.

Resolution solution Dissolve an accurately weighed quantity of each of Cloxacillin Sodium RS and Flucoxacillin Sodium RS in *Mobile phase* and dilute quantitatively to obtain a solution having known concentrations of about 100 μg per ml.

Standard preparation Dissolve an accurately weighed quantity of Cloxacillin Sodium RS in *Mobile phase* and dilute quantitatively to obtain a solution having a known concentration of about 1.1 mg per ml.

Assay preparation Remove as completely as possible, the contents of not less than 20 Cloxacillin Sodium Capsules and weigh accurately. Mix the combined contents, and transfer an accurately weighed quantity, containing about 50 mg of cloxacillin, to a 50-ml volumetric flask. Add 40 ml of *Mobile phase*, shake for 15 minutes and add *Mobile phase* to volume. Filter and dilute 5.0 ml of the solution to 50.0 ml with *Mobile phase*.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (25 cm × 4.6 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles (5 μm), (b) *Mobile phase* at a flow rate of about 1.0 ml per minute, and (c) an ultraviolet photometer set at 225 nm.

To determine the suitability of the chromatographic system, chromatograph *Standard preparation*, and record the peak responses as directed under *Procedure*: the resolution factor between cloxacillin (the first peak) and flucloxacillin (the second peak) is not less than 2.5.

Procedure Separately inject equal volumes (about 20 μl) of *Standard preparation* and *Assay preparation* into the chromatograph, record the chromatograms, and measure the responses for the major peaks.

Calculation Calculate the content of $C_{19}H_{18}ClN_3O_5S$ in the portion of the Capsules taken, using the declared content of $C_{19}H_{18}ClN_3O_5S$ in Cloxacillin Sodium RS.

Other requirements Comply with the requirements described under "Capsules" (Appendix 1.16).

CLOXACILLIN SODIUM FOR INJECTION

Category Antibacterial.

Cloxacillin Sodium for Injection is a sterile material prepared from cloxacillin sodium with or without excipients. It contains the equivalent of not less than 95.0 per cent and not more than 105.0 per cent of the labelled amount of $C_{19}H_{18}ClN_3O_5S$.

Strengths available 250, 500, 1000, and 2000 mg (base).

Dose Adults and children weighing more than 20 kg: *Intravenous*, 250 to 500 mg every 6 hours.

Children up to 20 kg: *Intravenous*, 6.25 to 12.5 mg per kg of body weight every 6 hours.

Contra-indication; Warning; Precaution See under *Penicillin V Potassium*, p. 140.

Additional information It is a preferred drug for the treatment of mild to moderate Staphylococcal infections of the skin and soft tissue, respiratory and genitourinary tracts, and joints.

See also under *Penicillin V Potassium*, p. 140.

Packaging and storage Cloxacillin Sodium for Injection shall be kept in Containers for Sterile Solids as described under "Parenteral Preparations" (Appendix

1.16), protected from light, and stored at a temperature not exceeding 30°.

Labelling The label on the container states the quantity equivalent to the amount of cloxacillin.

Identification Complies with the tests described under *Cloxacillin Sodium Capsules*, p. 88.

pH 5.0 to 7.0, in a 10.0 per cent w/v solution (Appendix 4.11).

Water Not more than 4.5 per cent w/w (Karl Fischer Method, Appendix 4.12). Use 0.3 g.

Iodine-absorbing substances Not more than 5 per cent, calculated on the anhydrous basis, when determined by the following method. Dissolve about 115 mg, accurately weighed, in sufficient *mixed phosphate buffer pH 7.0* to produce 25.0 ml. To 10.0 ml add 10 ml of *mixed phosphate buffer pH 4.0* and 10.0 ml of 0.01 M *iodine VS* and titrate immediately with 0.01 M *sodium thiosulfate VS* using *starch mucilage*, added towards the end of the titration, as indicator. Perform a blank determination (Residual Titrations, Appendix 6.17). The difference between the titrations represents the amount of iodine-absorbing substances present. Each ml of 0.01 M *sodium thiosulfate* is equivalent to 0.5039 mg of iodine-absorbing substances.

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains not more than 0.4 Endotoxin Unit per mg of cloxacillin.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase, Standard preparation, and **Chromatographic system** Proceed as directed in the Assay under *Cloxacillin Sodium Capsules*, p. 88.

Assay preparation Dissolve an accurately weighed quantity of the mixed contents of 10 containers of Cloxacilin Sodium for Injection containing about 50 mg of cloxacillin in sufficient of *Mobile phase* to produce 50.0 ml. Dilute 5.0 ml of the solution to 50.0 ml with *Mobile phase*.

Procedure Proceed as directed under Procedure in the Assay under *Cloxacillin Sodium Capsules*, p. 88.

Calculation Calculate the content of $C_{19}H_{18}ClN_3O_5S$ in a container of average content weight of the Injection taken, using the declared content of $C_{19}H_{18}ClN_3O_5S$ in Cloxacillin Sodium RS.

Other requirements Complies with the requirements described under "Parenteral Preparations" (Appendix 1.16).

CLOXACILLIN SODIUM FOR ORAL SOLUTION

Category Antibacterial.

Cloxacillin Sodium for Oral Solution is a dry mixture of Cloxacillin Sodium and one or more suitable buffers, colours, flavours, and preservatives. It contains the equivalent of not less than 90.0 per cent and not more than 120.0 per cent of the labelled amount of $C_{19}H_{18}ClN_3O_5S$ when constituted as directed.

Strength available 125 mg (base) per 5 ml.

Dose Adults and children weighing more than 20 kg: 250 to 500 mg every 6 hours taken on empty stomach.

Children up to 20 kg of body weight: 6.25 to 12.5 mg per kg of body weight every 6 hours taken on empty stomach.

Contra-indication; Warning; Precaution See under *Penicillin V Potassium*, p. 140.

Additional information It is a preferred drug for the treatment of mild to moderate Staphylococcal infections of the skin and soft tissue, respiratory and genitourinary tracts, and joints.

See also under *Penicillin V Potassium*, p. 140.

Packaging and storage Cloxacillin Sodium for Oral Solution shall be kept in tightly closed containers and stored at a temperature not exceeding 30°. After constitution, it should be used within the period and stored as stated on the label.

Labelling The label on the container states the quantity equivalent to the amount of cloxacillin.

Identification Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using a silanized *silica gel GF254* as the coating substance and a mixture of 70 volumes of 0.05 M *potassium hydrogenphthalate*, 30 volumes of *acetone* and 1 volume of *formic acid* that has been adjusted first to pH 6.0 with 5 M *sodium hydroxide* and then to pH 9.0 with 0.1 M *sodium hydroxide* as the mobile phase. Apply separately to the plate, 1 µl of each of the following solutions. For solution (A), dilute a quantity of the oral solution, equivalent to 50 mg of cloxacillin, to 20 ml with *phosphate buffer pH 7.0* and for solution (B), dissolve 25 mg of Cloxacillin Sodium RS in 5 ml of *water*. After removal of the plate, allow it to dry in air and spray with a mixture of 100 volumes of *starch mucilage*, 6 volumes of *glacial acetic acid* and 2 volumes of a 1 per cent w/v solution of *iodine* in a 4 per cent w/v solution of *potassium iodide*. The principal spot in the chromatogram obtained from

solution (A) corresponds to that obtained from solution (B).

pH 4.0 to 7.5, in the solution constituted as directed in the labelling (Appendix 4.11).

Water Not more than 1.0 per cent w/w (Karl Fischer Method, Appendix 4.12).

Deliverable volume Complies with the requirements described under "Deliverable Volume" (Appendix 4.21).

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase, Standard preparation, and **Chromatographic system** Proceed as directed in the Assay under *Cloxacillin Sodium Capsules*, p. 88.

Assay preparation Transfer an accurately measured volume of Cloxacillin Sodium for Oral Solution, constituted as directed in the labelling, equivalent to about 50 mg of cloxacillin, to a 50-ml volumetric flask, dilute with *Mobile phase* to volume, and mix. Filter a portion of this solution through a suitable filter of 0.5 μm or finer porosity, and use the filtrate as the Assay preparation.

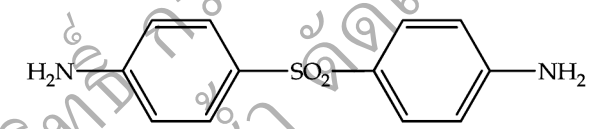
Procedure Proceed as directed under Procedure in the Assay under *Cloxacillin Sodium Capsules*, p. 88.

Calculation Calculate the quantity of $C_{19}H_{18}ClN_3O_5S$, in each ml of the Constituted Oral Solution taken, using the declared content of $C_{19}H_{18}ClN_3O_5S$ in Cloxacillin Sodium RS.

Other requirements Complies with the requirements described under "Oral Liquids" (Appendix 1.16).

DAPSONE

Diaphenylsulfone



$C_{12}H_{12}N_2O_2S$ 248.30 *80-08-0*

Benzenamine, 4,4′-sulfonylbis-.

Category Antibacterial (antileprosy); dermatitis herpetiformis suppressant.

Dapsone contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{12}H_{12}N_2O_2S$, calculated on the dried basis.

Description White or creamy white, crystalline powder; odourless.

Solubility Very slightly soluble in *water*; freely soluble in *ethanol*; soluble in *acetone* and in dilute mineral acids.

Contra-indication It is contra-indicated in patients with severe anemia; the anemia should be treated prior to initiation of dapsone therapy.

Warning

1. It should be used with caution in patients with cardiac or pulmonary disease, with glucose-6-phosphate dehydrogenase deficiency and in pregnant women.

2. It may cause hemolytic anemia, methemoglobinemia, leukopenia, gastro-intestinal upset, headache, nervousness, motor neuropathy, blurred vision, paresthesias and pruritus, hematuria, liver damage, jaundice, and rash.

3. It should be used with caution in patients with methemoglobin reductase deficiency, hemoglobin M, or hepatic function impairment, and in those who are exposed to other drugs or agents that are capable of inducing hemolysis.

4. Deaths from agranulocytosis, aplastic anemia, and other blood dyscrasias have been reported.

Precaution

1. Periodic hemograms as well as liver function tests are recommended during the course of treatment.

2. Discontinue the treatment when a lepra reaction appears, and consult the physician immediately.

3. Dapsone therapy should be promptly discontinued if new or toxic dermatologic reactions, e.g. exfoliative dermatitis, toxic erythema, erythema-multiforme, toxic epidermal necrolysis, morbiliform and scarlatiniform eruptions, dapsone syndrome (hypersensitivity to sulfone including rash, fever, jaundice and eosinophilia), or Stevens-Johnson syndrome occur.

4. Nursing mothers should discontinue nursing since it may potentially be tumorigenic and may cause drug-induced hemolytic anemia in neonates.

Additional information Although dapsone crosses the placenta, studies in humans have not shown that dapsone causes adverse effects on reproductive capacity or on the fetus.

Packaging and storage Dapsone shall be kept in tightly closed containers, protected from light.

Stability It is gradually degraded on exposure to a humid atmosphere, the decomposition being faster at

higher temperatures. It becomes discoloured on exposure to light.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Dapsone RS (Appendix 2.1) or with the reference spectrum of Dapsone.

B. The ultraviolet absorption spectrum of a 0.0005 per cent w/v solution in *methanol*, when observed between 230 and 350 nm, exhibits two maxima at 260 nm and 295 nm; the absorbances of a 1-cm layer at these wavelengths are about 0.36 and 0.60, respectively (Appendix 2.2).

C. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

D. A 2-ml portion of a 0.005 per cent w/v solution in 0.1 M *hydrochloric acid* yields the *reaction* characteristic of primary aromatic amines (Appendix 5.1).

Melting range 175° to 181° (Appendix 4.3).

Loss on drying Not more than 1.5 per cent w/w after drying at 105° for 3 hours (Appendix 4.15).

Sulfated ash Not more than 0.1 per cent w/w (Appendix 5.3).

Selenium Not more than 30 ppm (Appendix 5.2). Use 100 mg and mix with 100 mg of *magnesium oxide*.

Chromatographic purity Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using a suitable high-performance thin-layer chromatographic plate coated with a 150- to 200-µm layer of chromatographic silica gel as the coating substance and a mixture of 60 volumes of *chloroform*, 15 volumes of *acetone*, 15 volumes of *1-butanol* and 10 volumes of *formic acid* as the mobile phase.

Standard solution A Dissolve Dapsone RS in *methanol* and mix to obtain a solution having a known concentration of about 12.5 mg per ml.

Standard solution B Dilute *Standard solution A* quantitatively with *methanol* to obtain a solution having a known concentration of about 125 µg per ml.

Standard solution C Dilute *Standard solution B* quantitatively with *methanol* to obtain a solution having a known concentration of about 62.5 µg per ml.

Test solution Dissolve an accurately weighed quantity of the test substance in *methanol* to obtain a solution containing 12.5 mg per ml.

Procedure (**Note** Prepare the solvent system fresh daily.) Apply separately 4 µl of each of the solutions to the plate. After removal of the plate, allow it to dry in air and spray with a 0.1 per cent w/v solution of *4-dimethylaminocinnamaldehyde* in a mixture of equal volumes of *glacial acetic acid* and *water*. Examine the spots that are developed immediately, and compare the intensities of any secondary spots observed in the chromatogram of *Test solution* with those of the principal spot in the chromatogram of *Standard solutions*: no secondary spot from the chromatogram of *Test solution* is larger or more intense than the principal spot obtained from *Standard solution C* (0.5 per cent), and the sum of the intensities of all the secondary spots obtained from *Test solution* corresponds to not more than 1.0 per cent.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Transfer 100 ml of *2-propanol*, 100 ml of *acetonitrile*, and 100 ml of *ethyl acetate* to a 1000-ml volumetric flask. Add *pentane* to volume without mixing. Mix and allow the mixture to cool to room temperature.

Standard preparation Dissolve an accurately weighed quantity of Dapsone RS in *methanol* to obtain a solution having a known concentration of about 250 µg per ml. Pipette 5 ml of this solution into a 50-ml volumetric flask, dilute with *Mobile phase* to volume, and mix to obtain a solution having a known concentration of about 25 µg per ml.

Assay preparation Transfer about 50 mg of Dapsone, accurately weighed, to a 200-ml volumetric flask. Dissolve in *methanol*, dilute with *methanol* to volume, and mix. Pipette 5 ml of this solution into a 50-ml volumetric flask, dilute with *Mobile phase* to volume, and mix.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (30 cm × 3.9 mm) packed with porous silica microparticles (10 µm), (b) *Mobile phase* at a flow rate capable of giving the required resolution and a suitable elution time, and (c) an ultraviolet photometer set at 254 nm.

To determine the suitability of the chromatographic system, chromatograph *Standard preparation*, and record the peak responses as directed under *Procedure*: the relative standard deviation is not more than 2 per cent.

Procedure Separately inject equal volumes (about 10 µl) of *Standard preparation* and *Assay preparation* into the chromatograph, record the chromatograms, and measure the responses of the major peaks.

Calculation Calculate the content of $C_{12}H_{12}N_2O_2S$ in the Dapsone taken, using the declared content of $C_{12}H_{12}N_2O_2S$ in Dapsone RS.

DAPSONE TABLETS

Category Antibacterial (antileprosy); dermatitis herpetiformis suppressant.

Dapsone Tablets contain not less than 92.5 per cent and not more than 107.5 per cent of the labelled amount of $C_{12}H_{12}N_2O_2S$.

Strengths available 25 and 100 mg.

Dose Leprosy—Adults: In combination with one or more other antileprosy agents, 50 to 100 mg once a day; or 1.4 mg per kg of body weight once a day.

Children: In combination with one or more other antileprosy agents, 1.4 mg per kg of body weight once a day.

Dermatitis herpetiformis suppressant—Adults: Initially 50 mg daily. Doses may be increased up to 300 mg daily if symptoms are not completely controlled. The dose should then be reduced to the lowest effective maintenance dose as soon as possible.

Children: Initially 2 mg per kg of body weight daily. Doses may be increased if symptoms are not completely controlled. The dose should then be reduced to the lowest effective maintenance dose as soon as possible.

Contra-indication; Warning; Precaution; Additional information See under *Dapsone*, p. 91.

Packaging and storage Dapsone Tablets shall be protected from light.

Identification

A. Shake a portion of the powdered tablets, containing 100 mg of dapsone, with 20 ml of *acetone*, filter and evaporate the filtrate to dryness: the infrared absorption spectrum of the residue is concordant with the spectrum obtained from Dapsone RS (Appendix 2.1) or with the reference spectrum of Dapsone.

B. Triturate a portion of the powdered tablets, containing 100 mg of dapsone, with 50 ml of *methanol*, and filter. Dilute a portion of the filtrate with *methanol* to make approximately a 0.0005 per cent w/v solution: the ultraviolet absorption spectrum of the resulting solution, when observed between 230 and 350 nm, exhibits two maxima at 260 nm and 295 nm (Appendix 2.2).

C. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the Assay.

Dissolution Carry out the test as described in the "Dissolution Test" Appendix 4.24).

Dissolution medium: diluted *hydrochloric acid* (2 in 100); 1000 ml.

Apparatus 1: 100 rpm.

Time: 60 minutes.

Procedure Withdraw and filter a portion of the test solution. Transfer an accurately measured portion of the filtrate, estimated to contain about 200 µg of dapsone, to a 25-ml volumetric flask, add 5 ml of 1 M *sodium hydroxide*, dilute with *water* to volume, and mix. Determine the amount of $C_{12}H_{12}N_2O_2S$ dissolved from absorbances at the maximum at about 290 nm of the solutions so obtained from the test solution in comparison with a standard solution having a known concentration of Dapsone RS in the same medium (Appendix 2.2).

Tolerances Not less than 75 per cent (*Q*) of the labelled amount of $C_{12}H_5N_2O_2S$ is dissolved in 60 minutes.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

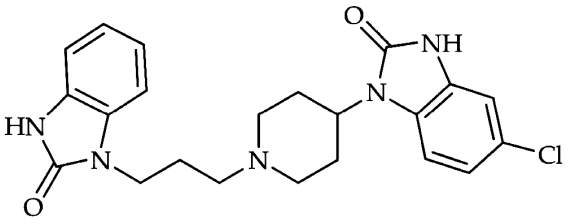
Mobile phase, Standard preparation, Chromatographic system, and **Procedure** Proceed as directed in the Assay under *Dapsone*, p. 92.

Assay preparation Weigh and finely powder not less than 20 Dapsone Tablets. Transfer an accurately weighed portion of the powder, containing about 50 mg of dapsone, to a 200-ml volumetric flask. Add 150 ml of *methanol* and sonicate at a temperature of 35° for 15 minutes, with occasional shaking. Allow to cool to room temperature, add *methanol* to volume and mix. Centrifuge a portion of the mixture until clear. Pipette 5 ml of the clear supernatant liquid to a 50-ml volumetric flask, dilute with *Mobile phase* to volume and mix.

Calculation Calculate the content of $C_{12}H_{12}N_2O_2S$ in the portion of the Tablets taken, using the declared content of $C_{12}H_{12}N_2O_2S$ in Dapsone RS.

Other requirements Comply with the requirements described under "Tablets" (Appendix 1.16).

DOMPERIDONE



$C_{22}H_{24}ClN_5O_2$ 425.91 *57808-66-9*

2*H*-Benzimidazol-2-one, 5-chloro-1-[1-[3-(2,3-dihydro-2-oxo-1*H*-benzimidazol-1-yl)propyl]-4-piperidinyl]-1,3-dihydro-.

Category Anti-emetic; dopaminergic blocking agent.

Domperidone contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{22}H_{24}ClN_5O_2$, calculated on the dried basis.

Description White or almost white powder.

Solubility Practically insoluble in *water*; soluble in *dimethylformamide*; slightly soluble in *ethanol* and in *methanol*.

Contra-indication

1. It is contra-indicated in patients with a prolactin-releasing pituitary tumour (prolactinoma).

2. It should not be used whenever stimulation of gastric motility is to be avoided or could be harmful, e.g. in the presence of gastro-intestinal hemorrhage, obstruction or perforation.

Warning

1. It should be used with caution in patients with hepatic impairment.

2. It may cause allergic reactions such as rash or urticaria, abdominal cramps, dystonic reactions (extrapyramidal phenomena), reversible raised serum prolactin levels which may lead to galactorrhea and gynecomastia, or hypertensive crises in patients with pheochromocytoma. Neurological side-effects may occur in young babies or in patients with impaired blood brain barrier.

3. Caution should be exercised if it is to be used concomitantly with anticholinergic, antimuscarinic agents, opioid analgesics, CYP3A4 inhibitors (e.g., azole antifungals, macrolide antibiotics, HIV protease inhibitors and nefazodone) or MAO inhibitors.

4. Risk-benefit should be considered if it is to be used in pregnant or nursing women.

Additional information

1. As the drug interferes with serum prolactin levels, it may interfere with other hypoprolactinemic agents and with some diagnostic tests.

2. Antacids and antisecretory agents lower the oral bioavailability of domperidone. They should not be taken simultaneously with this medicine.

Packaging and storage Domperidone shall be kept in well-closed containers, protected from light.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Domperidone RS (Appendix 2.1) or with the reference spectrum of Domperidone.

B. Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using suitable *octadecylsilyl silica gel* as the coating substance and a mixture of 20 volumes of *ammonium acetate TS*, 40 volumes of *dioxane* and 40 volumes of *methanol* as the mobile phase. Apply separately to the plate, 5 µl of each of the following solutions. For solution (A) dissolve 20 mg of the test substance in *methanol* and dilute to 10 ml with the same solvent. For solution (B) dissolve 20 mg of Domperidone RS in *methanol* and dilute to 10 ml with the same solvent. For solution (C) dissolve 20 mg of Domperidone RS and 20 mg of Droperidol RS in *methanol* and dilute to 10 ml with the same solvent. After removal of the plate, dry it for 15 minutes, expose it to iodine vapour until the spots appear and examine in daylight: the principal spot in the chromatogram obtained from solution (A) is similar in position and size to the principal spot in the chromatogram obtained from solution (B). The test is not valid unless the chromatogram obtained from solution (C) shows two clearly separated spots.

C. Dissolve 5 mg of the test substance in 3 ml of *methanol*, add 2 drops of a solution containing 10 per cent w/v of *cobalt(II) nitrate* and 10 per cent w/v of *calcium chloride*, mix and add, with shaking, 2 drops of 2 M *sodium hydroxide*: a violet-blue colour and precipitate are produced.

Clarity of solution A 1.0 per cent w/v solution in *dimethylformamide* is clear (Appendix 4.1).

Loss on drying Not more than 0.5 per cent w/w after drying at 105° to constant weight (Appendix 4.15).

Heavy metals Not more than 20 ppm (Method II, Appendix 5.2). Use 1.0 g; for the Standard Preparation, use 2 ml of *lead standard solution (10 ppm Pb)*.

Sulfated ash Not more than 0.1 per cent w/w (Appendix 5.3).

Related substances Carry out the test as described in the "High-pressure Liquid Chromatography" (Appendix 3.5). Prepare the solutions immediately before use.

Mobile phase Mix 3 volumes of *methanol* and 7 volumes of a 0.5 per cent w/v solution of *ammonium acetate*.

Reference solution (a) Dissolve 10.0 mg of Domperidone RS and 15.0 mg of Droperidol RS in *dimethylformamide* and dilute to 100.0 ml with the same solvent.

Reference solution (b) Dilute 1.0 ml of *Test solution* to 100.0 ml with *dimethylformamide*. Dilute 5.0 ml of this solution to 20.0 ml with the same solvent.

Test solution Dissolve 100 mg of the test substance in *dimethylformamide* and dilute to 10.0 ml with the same solvent.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (10 cm × 4.6 mm) packed with base-deactivated octadecylsilyl silica gel for chromatography (3 µm), (b) *Mobile phase* at a flow rate of 1.5 ml per minute, changing by linear gradient to *methanol* over 10 minutes, followed by elution with *methanol* for 2 minutes, and (c) an ultraviolet photometer set at 280 nm.

Procedure Inject 10 µl of *Reference solution (a)*. When the chromatogram is recorded in the prescribed conditions, the retention times are: domperidone, about 6.5 minutes and droperidol, about 7 minutes. The test is not valid unless the resolution between the peaks due to domperidone and droperidol is at least 2.0. If necessary, adjust the concentration of methanol in the mobile phase or adjust the time program for the linear gradient.

Separately inject equal volumes (about 10 µl) of *dimethylformamide* as a blank, *Test solution* and *Reference solution (b)*.

In the chromatogram obtained from *Test solution*: the area of any peak, apart from the principal peak, is not more than the area of the principal peak in the chromatogram obtained from *Reference solution (b)* (0.25 per cent); the sum of the areas of all peaks, apart from the principal peak, is not more than twice the area of the principal peak in the chromatogram obtained from *Reference solution (b)* (0.5 per cent). Disregard any peak in the chromatogram obtained from the blank run and any peak with an area less than 0.2 times that of the principal peak in the chromatogram obtained from *Reference solution (b)*.

Assay Dissolve about 300 mg of Domperidone, accurately weighed, in 50 ml of a mixture of 1 volume of *anhydrous glacial acetic acid* and 7 volumes of *2-butanone*. Titrate with 0.1 M *perchloric acid VS* until the colour changes from orange-yellow to green, using *naphtholbenzein TS* as indicator (Appendix 6.1). Perform a blank determination, and make any necessary correction. Each ml of 0.1 M *perchloric acid* is equivalent to 42.59 mg of $C_{22}H_{24}ClN_5O_2$.

DOMPERIDONE ORAL SUSPENSION

Category Anti-emetic; dopaminergic blocking agent.

Domperidone Oral Suspension contains not less than 90.0 per cent and not more than 110.0 per cent of the labelled amount of $C_{22}H_{24}ClN_5O_2$. It may contain one or more suitable buffers, colours, flavours, preservatives and suspending agents.

Strength available 1 mg per ml.

Dose Adults: 10 to 20 mg three times a day, 15 to 30 minutes before meals. If necessary, it may be taken again at bedtime.

Children: 250 µg per kg of body weight three times a day, 15 to 30 minutes before meals. If necessary, it may be taken again at bedtime.

Contra-indication; Warning; Additional information See under *Domperidone*, p. 94.

Packaging and storage Domperidone Oral Suspension shall be protected from light.

Identification

A. Complies with the test for Identification B described under *Domperidone*, p. 94.

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

pH 5.0 to 7.5 (Appendix 4.11).

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

pH 3.0 Buffer solution Dissolve 2.72 g of *potassium dihydrogenphosphate* in 900 ml of *water* and adjust with *phosphoric acid* to a pH of 3.0±0.1. Dilute with *water* to make 1000.0 ml.

Mobile phase Prepare a mixture of 53 volumes of *pH 3.0 Buffer solution*, 30 volumes of *methanol* and 17 volumes of *acetonitrile*. Make adjustments if necessary.

Internal standard solution Transfer 100 mg of *propranolol* to a 50-ml volumetric flask, dissolve in and dilute with *methanol* to volume, and mix.

Standard preparation 1 Dissolve about 60 mg of Domperidone RS, accurately weighed, in 1 ml of *acetonitrile*, dilute with *Mobile phase* to 25.0 ml and mix.

Standard preparation 2 Transfer 2.0 ml of *Standard preparation 1* to a 50-ml volumetric flask, add 5.0 ml of *Internal standard solution*, dilute with *Mobile phase* to volume, and mix.

Resolution solution Dissolve about 20 mg of Droperidol RS, accurately weighed, in 0.5 ml of *acetonitrile*, dilute with *Mobile phase* to 20.0 ml and mix. Transfer 4.0 ml of this solution to a 50-ml volumetric flask, add 2.0 ml of *Standard preparation 1*, 5.0 ml of *Internal standard solution* and dilute with *Mobile phase* to volume.

Assay preparation Transfer an accurately weighed portion of Domperidone Oral Suspension containing about 5 mg of domperidone, to a 50-ml volumetric flask. Add 0.1 ml of *acetonitrile* and 5.0 ml of *Internal standard solution*, dilute with *Mobile phase* to volume and mix.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (25 cm × 4.6 mm) packed with base-deactivated octadecylsilane chemically bonded to porous silica or ceramic microparticles (5 µm), (b) *Mobile phase* at a flow rate of about 1 ml per minute, and (c) an ultraviolet photometer set at 280 nm.

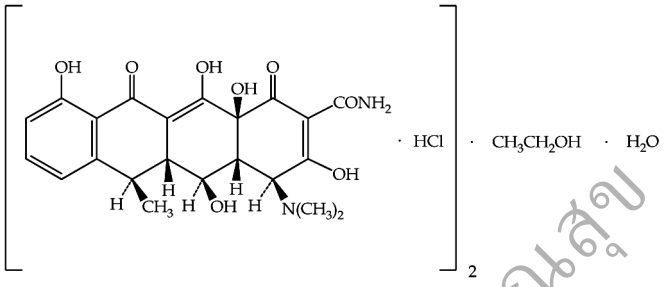
Equilibrate the column for not less than 3 hours before determining the suitability of the chromatographic system, chromatograph *Resolution solution* and record the peak responses as directed under *Procedure*: the resolution factor between domperidone and droperidol peaks is not less than 1.5, and the symmetry factor for the domperidone peak is not more than 2.0. The relative retention times are about 0.69 for domperidone, 0.75 for droperidol and 1.0 for propranolol. Chromatograph *Standard preparation 2*, and record the peak responses as directed under *Procedure*: the relative standard deviation for replicate injections is not more than 2.0 per cent.

Procedure Separately inject equal volumes (about 10 µl) of *Standard preparation 2* and *Assay preparation* into the chromatograph, record the chromatograms, and measure the responses for the major peaks.

Calculation Calculate the content of $C_{22}H_{24}ClN_5O_2$ in the portion of the Oral Suspension taken, using the declared content of $C_{22}H_{24}ClN_5O_2$ in Domperidone RS.

Other requirements Complies with the requirements described under "Oral Liquids" (Appendix 1.16).

DOXYCYCLINE HYCLATE



$(C_{22}H_{24}N_2O_8.HCl)_2.C_2H_6O.H_2O$ 1025.89 *24390-14-5*

2-Naphthacenecarboxamide, 4-(dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,5,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-, [4*S*-(4α,4aα,5α,5aα,6α,12aα)]-, monohydrochloride, compound with ethanol (2:1), monohydrate.

Category Antibacterial; antiprotozoal.

Doxycycline Hyclate contains not less than 800 µg and not more than 920 µg of $C_{22}H_{24}N_2O_8$ per mg.

Description Yellow, crystalline powder; hygroscopic.

Solubility Freely soluble in *water* and in *methanol*; sparingly soluble in *ethanol*. It dissolves in solutions of alkali hydroxides and carbonates.

Contra-indication It is contra-indicated in patients with history of hypersensitivity reactions to any member of tetracyclines.

Warning

1. It should not be used in children under 8 years of age, in pregnant women or in nursing mothers or in patients with systemic lupus erythematosus.
2. It should be used with caution in patients with impaired liver function or myasthenia gravis.
3. It may cause discolouration of infants' or children's teeth, photosensitivity, benign intracranial hypertension, hepatotoxicity, and pancreatitis.
4. Concomitant therapy with antacids, iron preparations, anticonvulsants including carbamazepine, phenobarbitone, and phenytoin; and rifampicin should be avoided.

Precaution See under *Tetracycline Hydrochloride*, p. 157.

Additional information

1. It may be taken with food, milk, or carbonated beverage if gastro-intestinal irritation occurs.
2. Doxycycline has been reported to be relatively free of renal side effects, and is therefore preferred to other tetracyclines in case of renal dysfunction.
3. Darkened or discoloured tongue may be alarming to patients although medically insignificant.

4. Gastro-intestinal disturbances are reported to be less frequent than with tetracycline, and doxycycline may also cause less tooth discolouration.

Packaging and storage Doxycycline Hyclate shall be kept in tightly closed containers, protected from light.

Labelling The label on the container states (1) storage condition; (2) parenteral or non-parenteral grade.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Doxycycline Hyclate RS (Appendix 2.1) or with the reference spectrum of Doxycycline Hyclate.

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

C. To 0.5 mg add 2 ml of *sulfuric acid*: a yellow colour is produced.

D. It yields the *reactions* characteristic of chlorides (Appendix 5.1).

Crystallinity It is crystalline (Method I, Appendix 4.14).

pH 2.0 to 3.0, in a 1.0 per cent w/v solution (Appendix 4.11).

Specific rotation –105° to –120°, calculated on the anhydrous basis, determined in a 1.0 per cent w/v solution of a mixture of 1 volume of 1 M *hydrochloric acid* and 99 volumes of *methanol* (Appendix 4.8).

Water Not less than 1.4 per cent w/w and not more than 2.8 per cent w/w (Karl Fischer Method, Appendix 4.12).

Related substances Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile, Diluent, and **Chromatographic system** Proceed as directed in the *Assay*.

System suitability solution Prepare as directed for the Resolution solution in the *Assay*.

Methacycline standard stock solution Dissolve an accurately weighed quantity of Methacycline Hydrochloride RS in *Diluent*, and dilute quantitatively, and stepwise if necessary, to obtain a solution having a known concentration of about 1.2 mg per ml.

Standard solution 1 Prepare as directed for the Standard preparation in the *Assay*.

Standard solution 2 Transfer 2.0 ml of *Standard solution 1* and 2.0 ml of *Methacycline standard stock solution* to a 100-ml volumetric flask, dilute with *Diluent* to volume, and mix. This solution contains about 0.024 mg each of Doxycycline Hyclate RS and Methacycline Hydrochloride RS per ml.

Test solution Prepare as directed for the Assay preparation in the *Assay*.

Procedure Separately inject equal volumes (about 20 µl) of *Standard solution 2* and *Test solution* into the chromatograph, record the chromatograms for a period of time that is 1.7 times the retention time of doxycycline, and measure the peak areas.

Calculation Calculate the percentage of methacycline in the portion of Doxycycline Hyclate taken by the expression:

$$10{,}000(C_M/W)(r_u/r_M),$$

in which C_M is the concentration, in mg per ml, of Methacycline Hydrochloride RS in *Standard solution 2*; W is the weight, in mg, of Doxycycline Hyclate taken to prepare *Test solution*; and r_u and r_M are the methacycline peak responses obtained from *Test solution* and *Standard solution 2*, respectively. Not more than 2 per cent of methacycline is found. Calculate the percentage of each related compound, other than methacycline, in the portion of Doxycycline Hyclate taken by the expression:

$$10{,}000(C_s/W)(r_i/r_s),$$

in which C_s is the concentration, in mg per ml, of Doxycycline Hyclate RS in *Standard solution 2*; W is the weight, in mg, of Doxycycline Hyclate taken to prepare the *Test solution*; r_i is the peak response for each impurity obtained from *Test solution*; and r_s is the doxycycline peak response obtained from *Standard solution 2*. Not more than 0.5 per cent of any impurity eluting before methacycline is found; not more than 2 per cent of 6-epidoxycycline is found; and not more than 0.5 per cent of any impurity eluting after the main doxycycline peak is found.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Transfer 2.72 g of *potassium dihydrogenphosphate*, 740 mg of *sodium hydroxide*, 500 mg of *tetrabutylammonium hydrogensulfate*, and 400 mg of *disodium edetate* to a 1000-ml volumetric flask. Add about 850 ml of *water*, and stir to dissolve. Add 60 g of *2-methyl-2-propanol* with the aid of *water*, dilute with *water* to volume, adjust with 1 M *sodium hydroxide* to a pH of 8.0±0.1 and filter. Make any necessary adjustments. Decreasing the proportion of *2-methyl-2-propanol* results in a longer retention time of doxycycline and improved separation of doxycycline from the related substances.

Diluent Prepare 0.01 M *hydrochloric acid*.

Resolution solution Prepare a solution of Doxycycline Hyclate RS in *Diluent* containing about 6 mg of doxycycline per ml. Transfer 5.0 ml of this solution to a 25-ml volumetric flask, heat on a water-bath for 60 minutes, and evaporate to dryness on a hot plate, taking care not to char the residue. Dissolve the residue in *Diluent*, dilute with *Diluent* to volume, mix and filter. This solution contains a mixture of 4-epidoxycycline, methacycline, 6-epidoxycycline and doxycycline. When stored in a refrigerator, this solution may be used for 14 days. (**Note** Throughout the following sections, protect the Standard preparation and the Assay preparation from light.)

Standard preparation Transfer about 12 mg of Doxycycline Hyclate RS, accurately weighed, to a 10-ml volumetric flask, add about 6 ml of *Diluent*, sonicate for about 5 minutes or until dissolved, dilute with *Diluent* to volume, and mix.

Assay preparation Transfer about 120 mg of Doxycycline Hyclate, accurately weighed, to a 100-ml volumetric flask, dissolve and dilute with *Diluent* to volume, mix, and filter.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (25 cm × 4.6 mm) packed with rigid spherical styrene-divinylbenzene copolymer maintained at 60°±1°, (b) *Mobile phase* at a flow rate of about 1 ml per minute and (c) an ultraviolet photometer set at 270 nm.

To determine the suitability of the chromatographic system, chromatograph *Resolution solution*, and record the peak responses as directed under *Procedure*: the relative retention times are about 0.4 for 4-epidoxycycline (the main degradation product), 0.6 for methacycline, 0.7 for 6-epidoxycycline, and 1.0 for doxycycline, the resolution factor between the 4-epidoxycycline and doxycycline peaks is not less than 3.0, and the symmetry factor for the doxycycline peak is not more than 2.0. Chromatograph *Standard preparation*, and record the peak responses as directed under *Procedure*: the relative standard deviation for replicate injections is not more than 2.0 per cent.

Procedure Separately inject equal volumes (about 20 µl) of *Standard preparation* and *Assay preparation* into the chromatograph, record the chromatograms and measure the responses for the major peaks.

Calculation Calculate the content of $C_{22}H_{24}N_2O_8$ in the Doxycycline Hyclate taken, using the declared content of $C_{22}H_{24}N_2O_8$ in Doxycycline Hyclate RS.

DOXYCYCLINE HYCLATE CAPSULES

Category Antibacterial; antiprotozoal.

Doxycycline Hyclate Capsules contain the equivalent of not less than 90.0 per cent and not more than 120.0 per cent of the labelled amount of $C_{22}H_{24}N_2O_8$.

Strength available 100 mg (base).

Dose Adults and children over 8 years of age weighing over 45 kg: 100 mg twice a day for one day, then 100 mg daily.

Children over 8 years of age weighing 45 kg and under: 2.2 mg per kg of body weight twice a day for one day, then 2.2 mg per kg of body weight daily.

Contra-indication; Warning; Additional information See under *Doxycycline Hyclate*, p. 96.

Precaution See under *Tetracycline Hydrochloride*, p. 139.

Packaging and storage Doxycycline Hyclate Capsules shall be kept in tightly closed containers, protected from light.

Labelling The label on the container states the quantity equivalent to the amount of doxycycline.

Identification The capsule contents comply with the tests for *Identification B*, *C* and *D* described under *Doxycycline Hyclate*, p. 96.

Water Not more than 8.5 per cent w/w (Karl Fischer Method, Appendix 4.12).

Dissolution Carry out the test as described in the "Dissolution Test" (Appendix 4.24).

Dissolution medium: *water*; 900 ml.

Apparatus 2: 75 rpm. Maintain a distance of 45±5 mm between the blade and the inside bottom of the vessel.

Time: 30 minutes.

Procedure Determine the amount of $C_{22}H_{24}N_2O_8$ dissolved from absorbances at the maximum at about 276 nm of filtered portions of the test solution, suitably diluted with *Dissolution medium*, if necessary, in comparison with a standard solution having a known concentration of Doxycycline Hyclate RS in the same medium (Appendix 2.2).

Tolerances Not less than 80 per cent (*Q*) of the labelled amount of $C_{22}H_{24}N_2O_8$ is dissolved in 30 minutes.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

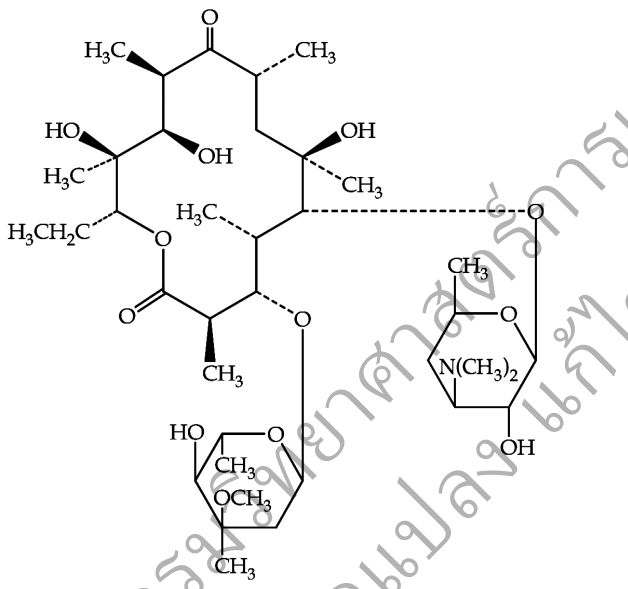
Mobile phase, Diluent, Resolution solution, Standard preparation, Chromatographic system, and **Procedure** Proceed as directed in the Assay described under *Doxycycline Hyclate*, p. 96.

Assay preparation Remove, as completely as possible, the contents of not less than 20 Doxycycline Hyclate Capsules, and weigh accurately. Mix the combined contents, and transfer an accurately weighed portion, containing about 100 mg of doxycycline, to a 100-ml volumetric flask, add 75 ml of *Diluent*, sonicate for 5 minutes, shake for 15 minutes, dilute with *Diluent* to volume, mix, and filter.

Calculation Calculate the content of $C_{22}H_{24}N_2O_8$ in the portion of the Capsules taken, using the declared content of $C_{22}H_{24}N_2O_8$ in Doxycycline Hyclate RS.

Other requirements Comply with the requirements described under "Capsules" (Appendix 1.16).

ERYTHROMYCIN



$C_{37}H_{67}NO_{13}$ 733.94 *114-07-8*

(3*R**,4*S**,5*S**,6*R**,7*R**,9*R**,11*R**,12*R**,13*S**,14*R**)-4-[(2,6-Dideoxy-3-*C*-methyl-3-*O*-methyl-*α*-L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-3,5,7,9,11,13-hexamethyl-6-[[3,4,6-trideoxy-3-(dimethylamino)-*β*-D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione.

Category Antibacterial.

Erythromycin consists primarily of erythromycin A ($C_{37}H_{67}NO_{13}$). The sum of the percentages of erythromycin A, erythromycin B, and erythromycin C is not less than 85.0 per cent and not more than 100.5 per cent, calculated on the anhydrous basis.

Description White or slightly yellow powder or colourless or slightly yellow crystals.

Solubility Slightly soluble in *water* (the solubility decreases as the temperature rises); freely soluble in *ethanol*; soluble in *methanol*.

Stability It is slightly hygroscopic but relatively stable in dry state. In aqueous solutions, it retains its activity during prolonged storage under refrigeration or when frozen, but slowly loses activity over several days at room temperature or higher.

Contra-indication It is contra-indicated in patients with acute porphyria and in those with hypersensitive to erythromycin and other macrolides.

Warning

1. It should be used with extreme caution in patients with a history of cardiac arrhythmias or QT prolongation interval especially in high doses of erythromycin or in those with hepatic function impairment or in elderly patients with reversible hearing loss.
2. Caution should be exercised when it is to be used concomitantly with hepatotoxic medications, alfentanil, astemizole, carbamazepine, chloramphenicol, clindamycin, cisapride, dispyramide, lincomycin, cyclosporine, warfarin, xanthine derivatives (e.g., aminophylline, theophylline, caffeine, oxtriphylline (except dyphylline)), pimozide, probenecid or alkalinizing agents (e.g., sodium bicarbonate, acetazolamide).
3. It may cause abdominal pain and cramping, nausea, vomiting and diarrhea.
4. Superinfection with resistant organisms and pseudo-membranous colitis associated with erythromycin use may occur.
5. Oral candidiasis may occur following prolonged or repeated erythromycin therapy.
6. Drug-induced rhabdomyolysis should be considered in patients receiving erythromycin concomitantly with lovastatin or another 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor.
7. Risk-benefit should be considered if it is to be used in pregnant or nursing women.

Precaution

1. Hepatic function should be determined periodically.
2. Group A beta-hemolytic streptococcal infections should be treated for at least 10 days to prevent the

development of acute rheumatic fever or acute glomerulonephritis.

Packaging and storage Erythromycin shall be kept in tightly closed containers, protected from light, and stored at a temperature not exceeding 30°.

Labelling The label on the container states (1) storage condition; (2) parenteral or non-parenteral grade.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Erythromycin RS (Appendix 2.1) or with the reference spectrum of Erythromycin.

B. To 5 mg add 2 ml of *sulfuric acid* and shake gently: a reddish brown colour is produced.

C. Dissolve 3 mg in 2 ml of *acetone* and add 2 ml of *hydrochloric acid*: an orange colour is produced, which changes to red and then to deep purplish red. Add 2 ml of *chloroform* and shake: the chloroform layer becomes purple.

D. To 5 mg add 5 ml of a 0.02 per cent w/v solution of *xanthydrol* in a mixture of 1 volume of *hydrochloric acid* and 99 volumes of 5 M *acetic acid* and heat on a water-bath: a red colour is produced.

Crystallinity It is crystalline (Appendix 4.14).

Specific rotation –71° to –78°, calculated on the anhydrous basis, determined in a 2.0 per cent w/v solution in *ethanol*. Measure the angle of rotation 30 minutes after preparing the solution (Appendix 4.8).

Water Not more than 10.0 per cent w/w (Karl Fischer Method, Appendix 4.12). Use 20 ml of *methanol* containing 10 per cent of *imidazole* in place of *methanol* in the titration vessel.

Sulfated ash Not more than 0.2 per cent w/w (Appendix 5.3).

Thiocyanate Not more than 0.3 per cent w/v. **(Note** Prepare all solutions in low-actinic volumetric flasks and use within 30 minutes.) Carry out the determination as described in the "Ultraviolet and Visible Spectrophotometry" (Appendix 2.2).

Standard solutions Transfer about 100 mg of *potassium thiocyanate*, previously dried at 105° for 1 hour, cooled, and accurately weighed, to each of two 50-ml volumetric flasks. Add about 20 ml of *methanol* to each flask, swirl to dissolve, dilute with *methanol* to volume, and mix. Transfer 5.0 ml of each of these stock solutions to separate 50-ml volumetric flasks, dilute with *methanol* to volume, and mix. Transfer 5.0 ml of each of these intermediate solutions to separate 50-ml volumetric flasks. To each flask add 1.0 ml of *iron(III) chloride TS*, dilute with *methanol* to volume, and mix.

Test solution Transfer about 100 mg of the test substance, accurately weighed, to a 50-ml volumetric flask. Add 20 ml of *methanol*, and swirl to dissolve. Add 1.0 ml of *iron(III) chloride TS*, dilute with *methanol* to volume, and mix.

Blank solution Transfer 1.0 ml of *iron(III) chloride TS* to a 50-ml volumetric flask, dilute with *methanol* to volume, and mix.

Procedure Measure the absorbances of each *Standard solution* and *Test solution* at the maximum at about 492 nm, using *Blank solution* as the blank.

Calculation Calculate the suitability value, *S*, by the expression:

$$(A_1/W_1)(W_2/A_2),$$

in which A_1 and A_2 are the absorbance values obtained from the respective Standard solutions, and W_1 and W_2 are the weights, in mg, of the potassium thiocyanate taken to prepare the corresponding Standard solutions. In a suitable determination, the value, *S*, is not less than 0.985 and not more than 1.015. Calculate the percentage of thiocyanate in the Erythromycin taken by the expression:

$$(58.08/97.18)(A_U/W_U)(0.5)[(W_1/A_1) + (W_2/A_2)],$$

in which 58.08 and 97.18 are the molecular weights of the thiocyanate moiety and of potassium thiocyanate, respectively, A_U is the absorbance of the Test solution, W_U is the weight, in mg, of Erythromycin taken to prepare the Test solution, and the other terms are as defined above.

Related substances Carry out the test as described in the "High-pressure Liquid Chromatography" (Appendix 3.5) under *Assay*, using, the chromatograms of the Assay preparation and the Diluted standard preparation obtained in the *Assay*, calculate the percentage of any individual related substance observed having the greatest response, other than erythromycin A, erythromycin B, erythromycin C, and erythromycin A enol ether, in the Erythromycin taken by the expression:

$$25(CP/W)(r_i/r_S),$$

in which *C* is the concentration, in mg per ml, of Erythromycin RS in *Diluted standard preparation*; *P* is the designated percentage of erythromycin A in the Erythromycin RS; *W* is the weight, in mg, of Erythromycin taken to prepare the Assay preparation; r_i is the peak response of an individual related substance, other than erythromycin A, erythromycin B, erythromycin C, or erythromycin A enol ether, observed in the chromato-

gram obtained from *Assay preparation*; and r_S is the erythromycin A peak response in the chromatogram obtained from *Diluted standard preparation*: not more than 3.0 per cent w/v of any individual related substance is found. Calculate the percentage of erythromycin A enol ether in the Erythromycin taken by the expression:

$$(25/11)(CP/W)(r_E/r_S),$$

in which 11 is the response factor for erythromycin A enol ether in relation to that of erythromycin A; r_E is the peak response of the erythromycin A enol ether peak observed in the chromatogram obtained from *Assay preparation*; and the other terms are as defined above: not more than 3.0 per cent w/v of erythromycin A enol ether is found. The percentage of erythromycin B obtained in the *Assay* is not more than 12.0 per cent w/v; and the percentage of erythromycin C obtained in the *Assay* is not more than 5.0 per cent w/v.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Solution A Dissolve 1.75 g of *dipotassium hydrogenphosphate* in 50 ml of *water*, adjust with diluted *phosphoric acid* (1 in 10) or 0.2 M *sodium hydroxide* to a pH of 9.0, add 400 ml of *water*, 165 ml of *2-methyl-2-propanol*, and 30 ml of *acetonitrile*. Dilute with *water* to 1000 ml, and mix.

pH 3.5 buffer Adjust 20 ml of *phosphate buffer pH 7.0* with *phosphoric acid* to a pH of 3.5.

Mobile phase Prepare a mixture of 5 volumes of *Solution A*, 2 volumes of *acetonitrile*, and 1 volume of *water*. Make adjustments if necessary.

Diluent Prepare a mixture of 15 volumes of *phosphate buffer pH 7.0* and 1 volume of *methanol*.

(**Note** Use the following solutions promptly, or within 1 day if stored in a refrigerator.)

Standard preparation Transfer about 100 mg of Erythromycin RS, accurately weighed, to a 25-ml volumetric flask, add 5 ml of *methanol*, swirl to dissolve, dilute with *Diluent* to volume, and mix.

Diluted standard preparation Transfer 3.0 ml of *Standard preparation* to a 100-ml volumetric flask, dilute with *Diluent* to volume, and mix. This solution contains about 0.12 mg of Erythromycin RS per ml.

Erythromycins B and C standard preparation Transfer about 5 mg each of Erythromycin B RS and Erythromycin C RS, both accurately weighed, to a 25-ml volumetric flask, add 5 ml of *methanol*, swirl to dissolve, dilute with *Diluent* to volume, and mix.

Resolution solution Transfer about 2 mg of *N*-Demethylerythromycin A RS to a 10-ml volumetric flask, add 0.4 ml of *Standard preparation*, dilute with *Erythromycin B and C standard preparation* to volume, and mix.

Erythromycin A enol ether retention time solution Dissolve about 10 mg of Erythromycin RS in 2 ml of *methanol*. Add 10 ml of *pH 3.5 buffer*, mix, and allow to stand for about 30 minutes.

Assay preparation Transfer about 100 mg of Erythromycin, accurately weighed, to a 25-ml volumetric flask, add 5 ml of *methanol*, and swirl to dissolve. Dilute with *Diluent* to volume, and mix.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (25 cm × 4.6 mm) packed with a rigid, spherical styrene-divinylbenzene copolymer (1000 Å), maintained at a constant temperature of about 65°, (b) *Mobile phase* at a flow rate of about 2 ml per minute, and (c) an ultraviolet photometer set at 215 nm.

To determine the suitability of the chromatographic system, chromatograph *Resolution solution*, and record the peak responses as directed under *Procedure*: the relative retention times are about 0.56 for *N*-demethylerythromycin A, 0.61 for erythromycin C, 1.0 for erythromycin A, and 1.6 for erythromycin B, and resolution factor between *N*-demethylerythromycin A and erythromycin C is not less than 0.8 and between erythromycin related compound N and erythromycin A not less than 5.5. Chromatograph *Erythromycin A enol ether retention time solution*, and record the peak responses as directed under *Procedure*: the retention time of the erythromycin A enol ether peak is about 3.2 relative to that of the erythromycin A peak as observed in the chromatogram obtained from *Resolution solution*. Chromatograph *Standard preparation* and record the peak responses as directed under *Procedure*: the relative standard deviation for replicate injections is not more than 2.0 per cent.

Procedure Separately inject equal volumes (about 100 µl) of *Standard preparation*, *Diluted Standard preparation*, *Erythromycins B and C standard solution* and *Assay preparation* into the chromatograph, record the chromatograms for a period of time that is adequate to include the erythromycin A enol ether peak, if present, and measure the responses for the major peaks.

Calculation Calculate the content of erythromycin A in the portion of Erythromycin taken by the expression:

$$5(C_AP/W)(r_u/r_A),$$

in which C_A is the concentration, in mg per ml, of Erythromycin RS in *Standard preparation*, P is the designated percentage of erythromycin A in Erythromycin RS, W is the quantity, in mg, of Erythromycin taken to prepare *Assay preparation*, and r_u and r_A are the erythromycin A peak responses in the chromatograms obtained from *Assay preparation* and *Standard preparation*, respectively. Calculate the content of erythromycin B and erythromycin C in the portion of Erythromycin taken by the expression:

$$25\ (C_s P / W)(r_u / r_s),$$

in which C_s is the concentration, in mg per ml, of the relevant Reference Substance in *Erythromycins B* and *C standard solution*, P is the designated percentage of erythromycin B or erythromycin C in the relevant Reference Substance, W is the quantity, in mg, of Erythromycin taken to prepare *Assay preparation*, and r_u and r_s are the peak responses of the relevant analyte in the chromatograms obtained from *Assay preparation* and *Erythromycins B and C standard solution*, respectively.

ERYTHROMYCIN DELAYED-RELEASE TABLETS

Category Antibacterial.

Erythromycin Delayed-release Tablets contain not less than 90.0 per cent and not more than 120.0 per cent of the labelled amount of $C_{37}H_{67}NO_{13}$.

Strength available 250 mg.

Dose Adults: 250 mg every 6 hours. In severe infections, dosage may be increased up to 4 g daily.

Children: 30 to 50 mg per kg of body weight or 0.9 to 3 g per m^2 of body surface area (Appendix 1.17) daily given in 4 equally divided doses and may be doubled in severe infections.

Contra-indication; Warning; Precaution See under *Erythromycin*, p. 99.

Packaging and storage Erythromycin Delayed-release Tablets shall be kept in tightly closed containers, protected from light.

Identification

A. Shake a portion of the powdered tablets containing 100 mg of erythromycin with 5 ml of *chloroform*, decolourize, if necessary, with *decolorizing charcoal*, filter, and evaporate the filtrate to dryness. The infrared absorption spectrum of the residue, after drying at a pressure not exceeding 0.7 kPa (about 5 Torr), is concordant with the spectrum obtained from Erythromycin RS (Appendix 2.1) or with the reference spectrum of Erythromycin.

B. Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel G* as the coating substance and a mixture of 17 volumes of *methanol* and 3 volumes of *chloroform* as the mobile phase but developing the chromatogram in an unlined chromatographic chamber. Apply separately to the plate, 10 µl of each of the following solutions. For solution (A), shake a portion of the powdered tablets with *methanol* to obtain a concentration of 2.5 mg of erythromycin per ml. Solution (B) contains 2.5 mg/ml of Erythromycin RS in *methanol*. After removal of the plate, allow it to dry in air. Spray the plate with a mixture of 18 volumes of *absolute ethanol*, 1 volume of *anisaldehye* and 1 volume of *sulfuric acid*. Heat the plate at 100° for 10 minutes. Erythromycin appears as black-to-purple spots. The principal spots in the chromatogram obtained from solution (A) correspond to those obtained from solution (B).

C. Dissolve a portion of the powdered tablets containing 3 mg of erythromycin as completely as possible in 2 ml of *acetone* and add 2 ml of *hydrochloric acid*: an orange colour is produced which changes to red and then to deep purplish red. Add 2 ml of *chloroform* and shake: the chloroform layer becomes purple.

Water Not more than 10.0 per cent w/w (Karl Fischer Method, Appendix 4.12). Use 20 ml of *methanol* containing 10 per cent of *imidazole* in place of *methanol* in the titration vessel.

Dissolution Carry out Method B described in the "Dissolution" (Appendix 4.24).

Apparatus 1: 100 rpm.

Times: 60 minutes, stage 1.
60 minutes, stage 2.

Acid stage Using 900 ml of *simulated gastric fluid TS* (prepared without pepsin) in place of 0.1 M *hydrochloric acid*, conduct this stage of the test for 60 minutes, and do not perform an analysis of the medium.

Buffer stage Using 900 ml of *phosphate buffer pH 6.8*, conduct this stage of the test for 60 minutes.

Test solution If necessary, dilute a filtered portion of the test solution with *Dissolution medium* to obtain a solution having a concentration of about 0.28 mg of erythromycin per ml, and mix.

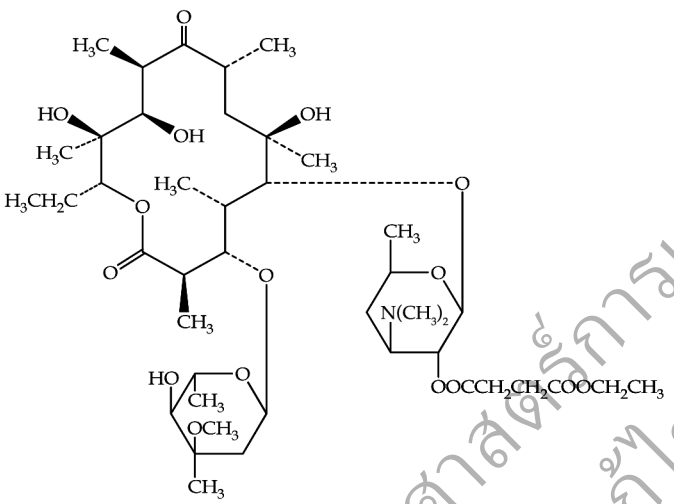
Procedure Transfer a 2.0-ml portion of the Test solution to a suitable separator. Add 6 ml of pH 1.2 buffer, and 8 ml of a solution of bromocresol purple, prepared by dissolving 1 g of *bromocresol purple* in 100 ml of *phosphate buffer pH 4.5* and mix. Extract with 40.0 ml of *chloroform*. Determine the amount of

$C_{37}H_{67}NO_{13}$ dissolved from absorbances at a maximum at about 410 nm using the chloroform extracts (Appendix 2.2). Similarly prepare a standard solution having a known concentration of Erythromycin RS and treat similarly.

Assay Place not less than four Erythromycin Tablets in a high-speed glass blender jar with 200 ml of *methanol* and blend for 3 minutes. Add 300 ml of Buffer 2 and blend for 3 minutes. Proceed as directed under the microbiological assay of Erythromycin according to the "Microbiological Assay of Antibiotics" (Appendix 6.10).

Other requirements Comply with the requirements described under "Tablets" (Appendix 1.16).

ERYTHROMYCIN ETHYLSUCCINATE



$C_{43}H_{75}NO_{16}$ 862.07 *1264-62-6*

Erythromycin, 2′-(ethyl butanedioate).

Category Antibacterial.

Erythromycin Ethylsuccinate consists primarily of the 2′- ethylsuccinate ester of erythromycin A. The sum of the percentages of erythromycin A, erythromycin B, and erythromycin C is not less than 76.5 per cent, calculated on the anhydrous basis.

Description White, crystalline powder; hygroscopic.

Solubility Practically insoluble in *water*; freely soluble in *acetone*, in *ethanol* and in *methanol*.

Stability It is hygroscopic.

Contra-indication; Warning; Precaution See under *Erythromycin*, p. 99.

Additional information

1. Erythromycin ethylsuccinate is better absorbed when taken with meals.

2. In pediatric patients, equivalent doses of erythromycin ethylsuccinate and erythromycin base produce comparable blood levels.

Packaging and storage Erythromycin Ethylsuccinate shall be kept in tightly closed containers, protected from light.

Labelling The label on the container states (1) number of μg per mg; (2) storage condition.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Erythromycin Ethylsuccinate RS (Appendix) or with the reference spectrum of Erythromycin Ethylsuccinate.

B. To 5 mg add 2 ml of *sulfuric acid* and shake gently: a reddish brown colour is produced.

C. Dissolve 3 mg in 2 ml of *acetone* and add 2 ml of *hydrochloric acid*: an orange colour is produced, which changes to red and then to deep purplish red. Add 2 ml of *chloroform* and shake: the chloroform layer becomes purple.

Crystallinity It is crystalline (Appendix 4.14); except that when it is labelled as being in the amorphous state it does not meet the requirements.

Specific rotation –70° to –82°, calculated on the anhydrous basis, determined in a 1.0 per cent w/v solution in *acetone* (Appendix 4.8). The specific rotation is determined at least 30 minutes after preparing the solution.

Water Not more than 3.0 per cent w/w (Karl Fischer Method, Appendix 4.12). Use 20 ml of *methanol* containing 10 per cent of *imidazole* in place of *methanol* in the titration vessel.

Sulfated ash Not more than 1.0 per cent after ignition at 600°, the charred residue being moistened with 2 ml of *nitric acid* and 5 drops of *sulfuric acid* (Method II, Appendix 5.3).

Related substances Complies with the test described under *Assay*, begin peak integration after the two peaks for succinates that elute just after the solvent front. Measure the areas of the peak responses.

Calculation Calculate the percentage of each related substance having the greatest response, other than erythromycin A, erythromycin B, erythromycin C, erythromycin A enol ether, and erythromycin *N*-ethylsuccinate (retention time relative to erythromycin A peak is about 1.3), in the portion of Erythromycin Ethylsuccinate taken by the expression:

$$50(C_{S2}P/W)(r_i/r_{S2}),$$

in which C_{S2} is the concentration, in mg per ml, of Erythromycin RS in *Standard preparation 2*, P is the designated percentage of erythromycin A in Erythromycin RS, W is the quantity, in mg, of Erythromycin Ethylsuccinate taken to prepare the Assay substance, r_i is the peak response of each related substance, other than erythromycin A, erythromycin B, erythromycin C, erythromycin A enol ether, and erythromycin *N*-ethylsuccinate, in the chromatogram obtained from *Assay preparation*, and r_{S2} is the erythromycin A peak response in the chromatogram obtained from *Standard preparation 2*: not more than 3.0 per cent of any individual related substance is found.

Calculate the percentage of erythromycin A enol ether in the portion of Erythromycin Ethylsuccinate taken by the expression:

$$(50/11)(C_{S2}P/W)(r_E/r_{S2}),$$

in which 11 is the response factor for erythromycin A enol ether in relation to that of erythromycin A; r_E is the peak response of the erythromycin A enol ether observed in the chromatogram obtained from *Assay preparation*; and the other terms are as defined above: not more than 3.0 per cent of erythromycin A enol ether is found.

Calculate the percentage of erythromycin *N* ethylsuccinate in the portion of Erythromycin Ethylsuccinate taken by the expression:

$$(50/7.4)(C_{S2}P/W)(r_N/r_{S2}),$$

in which 7.4 is the response factor for erythromycin *N*-ethylsuccinate in relation to that of erythromycin A, r_N is the peak response of the erythromycin *N*-ethylsuccinate (retention time relative to the erythromycin A peak is about 1.3) observed in the chromatogram obtained from *Assay preparation*; andthe other terms are as defined above: not more than 3.0 per cent of erythromycin *N*-ethylsuccinate is found.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Hydrolysis reagent Prepare a solution of *potassium dihydrogenphosphate* (2 in 100), and adjust with *phosphoric acid* to a pH of 8.0.

pH 8.0 buffer Prepare a solution of *potassium dihydrogenphosphate* (3.5 in 100), and adjust with *phosphoric acid* to a pH of 8.0.

pH 3.5 buffer Adjust 20 ml of *pH 8.0 buffer* with *phosphoric acid* to a pH of 3.5.

Mobile phase Mix 50 ml of *pH 8.0 buffer* with 400 ml of *water*, add 175 ml of *2-methyl-2-propanol* and 30 ml of *acetonitrile*, dilute with *water* to 1000 ml, and mix. Make adjustments if necessary.

(**Note** Use the following solutions promptly, or within 1 day if stored in a refrigerator.)

Standard preparation 1 Transfer about 50 mg of Erythromycin RS, accurately weighed, to a 25-ml volumetric flask, add 12.5 ml of *methanol*, and swirl to dissolve. Dilute with *Hydrolysis reagent* to volume, and mix.

Standard preparation 2 Transfer about 5 mg each of Erythromycin B RS and Erythromycin C RS, both accurately weighed, to a 50-ml volumetric flask, add 25.0 ml of *methanol*, and swirl to dissolve. Add 2.5 ml of *Standard preparation 1*, dilute with *Hydrolysis reagent* to volume, and mix.

System suitability solution Dissolve 2 mg of *N*-Demethylerythromycin A RS in 20 ml of *Standard preparation 2*, and mix.

Erythromycin A enol ether solution Dissolve 10 mg of Erythromycin RS in 2 ml of *methanol*. Add 10 ml of *pH 3.5 buffer*, mix, and allow to stand for about 30 minutes. Refrigerate this solution until used, and discard 8 hours after preparation.

Assay preparation Transfer about 115 mg of Erythromycin Ethylsuccinate, accurately weighed, to a 50-ml volumetric flask, add 25.0 ml of *methanol*, and swirl to dissolve. Add 20 ml of *Hydrolysis reagent*, mix, and allow to stand at room temperature for about 12 hours to effect hydrolysis. Dilute with *Hydrolysis reagent* to volume, and mix.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (25 cm × 4.6 mm) packed with a rigid spherical styrene-divinylbenzene copolymer (5 to 10 μm) maintained at a constant temperature of about 70°, (b) *Mobile phase* at a flow rate of about 2 ml per minute and (c) an ultraviolet photometer set at 215 nm.

To determine the suitability of the chromatographic system, chromatograph *System suitability solution*, and record the peak responses as directed under *Procedure*: the order of elution of the components is *N*-demethylerythromycin A, erythromycin C, erythromycin A, and erythromycin B; and the resolution, R, between *N*-demethylerythromycin A and erythromycin C is not less than 0.8 and between *N*-demethylerythromycin A and erythromycin A not less than 5.5. Chromatograph *Erythromycin A enol ether solution*, and record the peak responses as directed under *Procedure*: adjust the duration of chromatography to include the erythromycin A enol ether peak, which has a retention time of about 4.3 to 4.7 times that of erythromycin A. Chro-

matograph *Standard preparation 1*, and record the peak responses as directed under *Procedure*: the relative standard deviation for replicate injections is not more than 1.0 per cent.

Procedure Separately inject equal volumes (about 200 µl) of *Standard preparation 1*, *Standard preparation 2*, and *Assay preparation* into the chromatograph; record the chromatograms for a period of time that is adequate to include the erythromycin A enol ether peak, if present. For assay preparation, begin peak integration after the two peaks for succinates that elute just after the solvent front. Measure the areas of the peak responses.

Calculation Calculate the percentage of erythromycin A in the portion of the Erythromycin Ethylsuccinate taken by the expression:

$$50(C_{S1}P/W)(r_U/r_{S1}),$$

in which C_{S1} is the concentration, in mg per ml, of Erythromycin RS in *Standard preparation 1*; P is the designated percentage of erythromycin A in Erythromycin RS; W is the quantity, in mg, of Erythromycin Ethylsuccinate taken to prepare *Assay preparation*; and r_u and r_{s1} are the erythromycin A peak responses in the chromatograms obtained from *Assay preparation* and *Standard preparation 1*, respectively.

Calculate the percentage of erythromycin B and erythromycin C in the portion of the Erythromycin Ethylsuccinate taken by the expression:

$$50(C_{S2}P/W)(r_U/r_{S2}),$$

in which C_{S2} is the concentration, in mg per ml, of Erythromycin RS in *Standard preparation 2*; P is the designated percentage of erythromycin B or erythromycin C in Erythromycin B RS or Erythromycin C RS, respectively; W is the quantity, in mg, of Erythromycin Ethylsuccinate taken to prepare *Assay preparation*; and r_U and r_{S2} are the erythromycin B or erythromycin C peak responses in the chromatograms obtained from *Assay preparation* and *Standard preparation 2*, respectively.

ERYTHROMYCIN ETHYLSUCCINATE FOR ORAL SUSPENSION

Category Antibacterial.

Erythromycin Ethylsuccinate for Oral Suspension is a dry mixture of Erythromycin Ethylsuccinate with one or more suitable buffers, colour, diluents, dispersants, and flavours. It contains the equivalent of not less than 90.0 per cent and not more than 120.0 per cent of the labelled amount of $C_{37}H_{67}NO_{13}$.

Strengths available 200 and 400 mg per 5 ml (base).

Dose Adults: 400 mg every 6 hours or 800 mg every 12 hours. The maximum total dose should not exceed 4 g per day.

Children: 7.5 to 12.5 mg per kg of body weight every 6 hours or 15 to 25 mg per kg of body weight every 12 hours.

Severe infections: 15 to 25 mg per kg of body weight every 6 hours.

Contra-indication; Warning; Precaution See under *Erythromycin*, p. 99.

Additional information After constitution, Erythromycin Ethylsuccinate for Oral Suspension does not require refrigeration if used within 14 days.

See also under *Erythromycin Ethylsuccinate*, p. 103.

Packaging and storage Erythromycin Ethylsuccinate for Oral Suspension shall be kept in tightly closed containers. After constitution, it should be used within the period stated on the label.

Labelling The label on the container states the quantity equivalent to the amount of erythromycin.

Identification

A. Dilute a quantity of the oral suspension containing the equivalent of 100 mg of erythromycin to 25 ml with *water* and extract with two 10-ml portions of *dichloromethane*. Wash the combined extracts with five 10-ml quantities of *water*, filter using silicone-treated filter paper (Phase Separator paper is suitable) and evaporate to dryness. The infrared absorption spectrum of the residue after drying at 105° for 15 minutes is concordant with the spectrum obtained from Erythromycin Ethylsuccinate RS (Appendix 2.1) or with the reference spectrum of Erythromycin Ethylsuccinate.

B. Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel G* as the coating substance and a mixture of 17 volumes of *methanol* and 3 volumes of *chloroform* as the mobile phase but developing the chromatogram in an unlined chromatographic chamber. Apply separately to the

plate, 10 µl of each of the following solutions. For solution (A), add a volume of *methanol* to a portion of the oral suspension, sufficient to yield a solution containing the equivalent of 2.5 mg of erythromycin per ml, and stir for 30 minutes. Centrifuge a portion of this mixture, and use the clear supernatant liquid. Solution (B) contains 3 mg per ml of Erythromycin Ethylsuccinate RS in *methanol*. After removal of the plate, allow it to dry in air. Spray the plate with a mixture of 18 volumes of *absolute ethanol*, 1 volume of *4-methoxybenzaldehyde* and 1 volume of *sulfuric acid*. Heat the plate at 100° for 10 minutes. Erythromycin and succinic acid moieties appear as black-to-purple spots. The principal spots in the chromatogram obtained from solution (A) correspond to those obtained from solution (B).

pH 6.5 to 9.5, in the suspension constituted as directed in the labelling (Appendix 4.15).

Loss on drying Not more than 1.0 per cent w/w after drying about 100 mg in a capillary-stoppered bottle at 60° at a pressure not exceeding 2.7 kPa (about 20 Torr) for 3 hours (Appendix 4.15).

Assay Constitute Erythromycin Ethylsuccinate for Oral Suspension as directed in the labelling. Proceed as directed in the microbiological assay of Erythromycin according to the "Microbiological Assay of Antibiotics" (Appendix 6.10), using an accurately measured volume of the oral suspension, freshly mixed and free from air bubbles, blended for 4±1 minutes in a high-speed glass blender jar with sufficient *methanol* to give a stock solution containing the equivalent of about 1 mg of erythromycin per ml.

Other requirements Comply with the requirements described under "Oral Liquids" (Appendix 1.16).

ERYTHROMYCIN ETHYLSUCCINATE TABLETS

Category Antibacterial.

Erythromycin Ethylsuccinate Tablets contain the equivalent of not less than 90.0 per cent and not more than 120.0 per cent of the labelled amount of $C_{37}H_{67}NO_{13}$.

Strength available 400 mg (base).

Dose Adults: 400 mg every 6 hours or 800 mg every 12 hours. The maximum total dose should not exceed 4 g per day.

Children: 7.5 to 12.5 mg per kg of body weight every 6 hours or 15 to 25 mg per kg of body weight every 12 hours.

Severe infections: 15 to 25 mg per kg of body weight every 6 hours.

Contra-indication; Warning; Precaution See under *Erythromycin*, p. 99.

Additional information See under *Erythromycin Ethylsuccinate*, p. 99.

Packaging and storage Erythromycin Ethylsuccinate Tablets shall be kept in tightly closed containers, protected from light.

Labelling The label on the container states the quantity equivalent to the amount of erythromycin.

Identification

A. Shake a portion of the powdered tablets equivalent to 100 mg of erythromycin with 5 ml of *chloroform*, decolourize, if necessary, with *decolorizing charcoal*, filter and evaporate the filtrate to dryness. The infrared absorption spectrum of the residue, after drying at a pressure not exceeding 0.7 kPa (about 5 Torr), is concordant with the spectrum obtained from Erythromycin Ethylsuccinate RS (Appendix 2.1) or with the reference spectrum of Erythromycin Ethylsuccinate.

B. Comply with the test for Identification B described under *Erythromycin Ethylsuccinate for Oral Suspension*, p. 105. For solution (A), add a volume of *methanol* to yield a solution containing the equivalent of 2.5 mg of erythromycin per ml and shake by mechanical means for 30 minutes. Centrifuge a portion of this mixture, and use the clear supernatant liquid.

C. Comply with the test for Identification C described under *Erythromycin Delayed-release Tablets*, p. 102.

Loss on drying Not more than 4.0 per cent w/w after drying about 100 mg at 60° at a pressure not exceeding 0.7 kPa (about 5 Torr) for 3 hours (Appendix 4.15).

Dissolution Carry out the test as described in the "Dissolution Test" (Appendix 4.24).

Dissolution medium: 0.01 M *hydrochloric acid*; 900 ml.

Apparatus 2: 50 rpm.

Time: 45 minutes.

Procedure Determine the amount of $C_{37}H_{67}NO_{13}$ dissolved by employing the following method.

Colour reagent Slowly, and with constant mechanical stirring, add 325 ml of *sulfuric acid* to 173 ml of cold *water*. Allow the solution to cool, add 2 ml of a 2.5 per cent w/v solution of *iron(III) chloride* and 1 g of *4-*

dimethylaminobenzaldehyde, and stir to dissolve. Store in a low actinic flask. (**Note** Prepare this colour reagent on the day of use.)

Standard solution Dissolve an accurately weighed quantity of Erythromycin RS in *Dissolution medium* to obtain a solution having a known concentration of about 0.44 mg per ml, using sonication if necessary to dissolve. (**Note** Use this Standard solution within 5.5 hours.)

Test solution Filter and use the filtrate.

Procedure To three separate 50-ml glass-stoppered conical flasks add 2.0 ml of *Standard solution*, 2.0 ml of *Test solution*, and 2.0 ml of *Dissolution medium* (to serve as the Blank), respectively. Place the flasks in an ice-bath for about 15 minutes. At precise 1-minute intervals add 10.0 ml of *Colour reagent* to *Standard solution*, *Test solution* and *Blank*, in turn. Immediately after adding *Colour reagent*, remove each flask from the ice bath, insert the stopper, mix, and allow to stand at room temperature for exactly 30 minutes. Sequentially measure the absorbances of *Standard solution* and *Test solution* at precise 1-minute intervals at the maximum at about 480 nm (Appendix 2.2), against the blank.

Calculation Calculate the quantity, in mg, of $C_{37}H_{67}NO_{13}$ equivalent dissolved by the expression:

$$0.9CP(A_U/A_S),$$

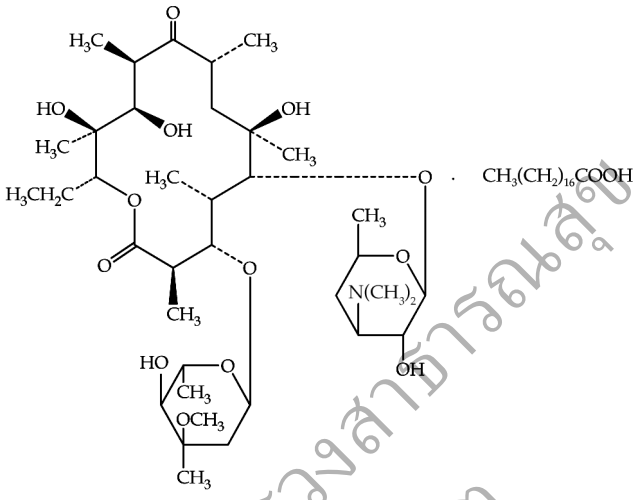
in which C is the concentration, in mg per ml, of Erythromycin RS in *Standard solution*, *P* is the designated content, in µg per mg, of erythromycin in Erythromycin RS, and A_U and A_S are the absorbances of *Test solution* and *Standard solution*, respectively.

Tolerances Not less than 75 per cent (*Q*) of the labelled amount of $C_{37}H_{67}NO_{13}$ is dissolved in 45 minutes.

Assay Blend not less than four Erythromycin Ethylsuccinate Tablets, accurately counted, for 4±1 minutes in a high-speed glass blender jar with a sufficient and accurately measured volume of *methanol* to give a stock solution containing the equivalent of not more than 5 mg of erythromycin per ml. Proceed as directed under the microbiological assay of erythromycin according to the "Microbiological Assay of Antibiotics" (Appendix 6.10).

Other requirements Comply with the requirements described under "Tablets" (Appendix 1.16).

ERYTHROMYCIN STEARATE



$C_{37}H_{67}NO_{13}.C_{18}H_{36}O_2$ 1018.42 *643-22-1*

Erythromycin, octadecanoate (salt).

Category Antibacterial.

Erythromycin Stearate is the stearic acid salt of Erythromycin, with an excess of Stearic acid. The sum of the percentages of erythromycin A, erythromycin B, and erythromycin C is not less than 55.0 per cent, calculated on the anhydrous basis.

Description White, crystalline powder.

Solubility Practically insoluble in *water*; soluble in *acetone* and in *methanol*. Solutions may be opalescent.

Contra-indication; Warning; Precaution; Additional information See under *Erythromycin*, p. 99.

Packaging and storage Erythromycin Stearate shall be kept in tightly closed containers, protected from light.

Labelling The label on the container states (1) number of µg per mg; (2) storage condition.

Identification

A. The infrared absorption spectrum of the contents is concordant with the spectrum obtained from Erythromycin Stearate RS (Appendix 2.1) or with the reference spectrum of Erythromycin Stearate.

B. Complies with the tests for Identification B and C described under *Erythromycin Ethylsuccinate*, p. 103.

C. Heat gently 100 mg with 5 ml of 2 M *hydrochloric acid* and 10 ml of *water* until the solution boils: oily globules rise to the surface. Cool, remove the fatty layer, heat it with 3 ml of 0.1 M *sodium hydroxide*, and allow to cool: the solution sets to a gel. Add 10 ml of hot *water* and shake: the solution froths. To 1 ml of the solution add a 10 per cent w/v solution of *calcium*

chloride: a granular precipitate, insoluble in *hydrochloric acid*, is produced.

Crystallinity It is crystalline (Appendix 4.14).

Water Not more than 4.0 per cent w/w (Karl Fischer Method, Appendix 4.12). Use 20 ml of *methanol* containing 10 per cent of *imidazole* in place of *methanol* in the titration vessel. Use 300 mg.

Sulfated ash Not more than 1.0 per cent after ignition at 600°, the charred residue being moistened with 2 ml of *nitric acid* and 5 drops of *sulfuric acid* (Method II, Appendix 5.3).

Related substances Carry out the test as described under *Assay*, using *Assay preparation* and *Standard preparation 2*.

Calculation Calculate the percentage of each related substance having the greatest response, other than erythromycin A, erythromycin B, erythromycin C, erythromycin A enol ether, and pseudoerythromycin A enol ether (retention time relative to erythromycin A peak is about 1.5), in the portion of Erythromycin Stearate taken by the expression:

$$30(C_{S2}P/W)(r_i/r_{S2}),$$

in which C_{S2} is the concentration, in mg per ml, of Erythromycin RS in *Standard preparation 2*; P is the designated percentage of erythromycin A in Erythromycin RS; W is the quantity, in mg, of Erythromycin Stearate taken to prepare the Assay preparation; r_i is the peak response of each related compound, other than erythromycin A, erythromycin B, erythromycin C, erythromycin A enol ether, and pseudoerythromycin A enol ether, observed in the chromatogram obtained from *Assay preparation*; and r_{S2} is the erythromycin A peak response in the chromatogram obtained from *Standard preparation 2*: not more than 3.0 per cent of any individual related compound is found.

Calculate the percentage of erythromycin A enol ether in the portion of Erythromycin Stearate taken by the expression:

$$(30/11)(C_{S2}P/W)(r_E/r_{S2}),$$

in which 11 is the response factor for erythromycin A enol ether in relation to that of erythromycin A, r_E is the peak response of the erythromycin A enol ether observed in the chromatogram obtained from *Assay preparation*, and the other terms are as defined above: not more than 3.0 per cent of erythromycin A enol ether is found.

Calculate the percentage of pseudoerythromycin A enol ether in the portion of Erythromycin Stearate taken by the expression:

$$(30/6.6)(C_{S2}P/W)(r_P/r_{S2}),$$

in which 6.6 is the response factor for pseudoerythromycin A enol ether in relation to that of erythromycin *A*, r_P is the peak response of the pseudoerythromycin A enol ether (retention time relative to the erythromycin A peak is about 1.5) observed in the chromatogram obtained from *Assay preparation*, and the other terms are as defined above: not more than 3.0 per cent of pseudoerythromycin A enol ether is found.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

pH 8.0 buffer Prepare a 2.0 per cent w/v solution of *dipotassium hydrogenphosphate*, and adjust with *phosphoric acid* to a pH of 8.0.

pH 9.0 buffer Prepare a 3.5 per cent w/v solution of *dipotassium hydrogenphosphate*, and adjust with *potassium hydroxide TS* or a 10 per cent w/v solution of *phosphoric acid*, as appropriate, to a pH of 9.0.

pH 3.5 buffer Adjust 20 ml of pH *8.0 buffer* with *phosphoric acid* to a pH of 3.5.

Mobile phase Prepare a mixture of 50 volumes of *pH 9.0 buffer*, 400 volumes of *water*, 175 volumes of 2-*methyl*-2-*propanol* and 30 volumes of *acetonitrile*, dilute with *water* to 1000 ml, and mix. Make adjustments if necessary. (**Note** Use the following solutions promptly, or within 1 day if stored in a refrigerator.)

Standard preparation 1 Transfer about 40 mg of Erythromycin RS, accurately weighed, to a conical flask, add 5.0 ml of *methanol*, and swirl to dissolve. Add 5.0 ml of *pH 8.0 buffer*, and mix.

Standard preparation 2 Transfer about 6 mg each of Erythromycin RS, Erythromycin B RS, Erythromycin C RS and N-Demethylerythromycin A, all accurately weighed, to a 50-ml conical flask, add 15.0 ml of *methanol*, and swirl to dissolve. Add 15.0 ml of *pH 8.0 buffer*, and mix.

Erythromycin A enol ether solution Dissolve about 5 mg of Erythromycin RS in 1 ml of *methanol*. Add 5 ml of *pH 3.5 buffer*, mix, and allow to stand for about 30 minutes

Assay preparation Transfer about 165 mg of Erythromycin stearate, accurately weighed, to a 100-ml conical flask, add 15.0 ml of *methanol*, and swirl to dissolve. Add 15.0 ml of *pH 8.0 buffer*, and mix. Allow the resulting suspension to settle, and pass a portion of the supernatant through a filter having a 0.2-μm or finer porosity. Use the clear filtrate.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (25 cm × 4.6 mm) packed with a rigid, spherical styrene-divinylbenzene copolymer (1000 Å), maintained at a constant temperature of about 70°, (b) *Mobile phase* at a flow rate of about 2 ml per minute, and (c) an ultraviolet photometer set at 215 nm.

To determine the suitability of the chromatographic system, chromatograph *Standard preparation 2*, and record the peak responses as directed under *Procedure*: the order of elution of the components is *N*-demethylerythromycin A, erythromycin C, erythromycin A, and erythromycin B; and the resolution factor between *N*-demethylerythromycin A and erythromycin C peaks is not less than 0.8 and between *N*-demethylerythromycin A and erythromycin A not less than 5.5. Chromatograph *Erythromycin A enol ether solution*, and adjust the duration of chromatography to include the erythromycin A enol ether peak, which has a retention time of about 4.3 to 4.7 times that of erythromycin A. Chromatograph *Standard preparation 1*, and record the peak responses as directed under *Procedure*: the relative standard deviation for replicate injections is not more than 2.0 per cent.

Procedure Separately inject equal volumes (about 100 µl) of *Standard preparation 1*, *Standard preparation 2* and *Assay preparation* into the chromatograph, record the chromatograms for a period of time that is adequate to include the erythromycin A enol ether peak, if present, and measure the responses for the major peaks.

Calculation Calculate the content of erythromycin A in the portion of Erythromycin Stearate taken by the expression:

$$30(C_{S1}P/W)(r_U/r_{S1}),$$

in which C_{S1} is the concentration, in mg per ml, of Erythromycin RS in *Standard preparation 1*, P is the designated percentage of erythromycin A in Erythromycin RS, W is the quantity, in mg, of Erythromycin Stearate taken to prepare *Assay preparation*, and r_U and r_{S1}, are the erythromycin A peak responses in the chromatograms obtained from *Assay preparation* and *Standard preparation 1*, respectively.

Calculate the content of erythromycin B and erythromycin C in the portion of Erythromycin Stearate taken by the expression:

$$30(C_{S2}P/W)(r_U/r_{S2}),$$

in which C_{S2} is the concentration, in mg per ml, of relevant Reference Substance in *Standard preparation 2*, P is the designated percentage of erythromycin B in Erythromycin C in the relevant Reference Standard, W is the quantity, in mg, of Erythromycin Stearate taken to prepare *Assay preparation*, and r_U and r_{S2} are the peak responses of the relevant analyte in the chromatograms obtained from *Assay preparation* and *Standard preparation 2*, respectively. The percentage of erythromycin B is not more than 12.0 per cent; and the percentage of erythromycin C is not more than 5.0 per cent.

ERYTHROMYCIN STEARATE TABLETS

Category Antibacterial.

Erythromycin Stearate Tablets contain the equivalent of not less than 90.0 per cent and not more than 120.0 per cent of the labelled amount of $C_{37}H_{67}NO_{13}$.

Strengths available 250 and 500 mg (base).

Dose Adults: 250 mg every 6 hours or 500 mg every 12 hours. The maximum total dose should not exceed 4 g per day.

Children: 7.5 to 12.5 mg per kg of body weight every 6 hours or 15 to 25 mg per kg of body weight every 12 hours.

Severe infections: 15 to 25 mg per kg of body weight every 6 hours.

Contra-indication; Warning; Precaution See under *Erythromycin*, p. 99.

Packaging and storage Erythromycin Stearate Tablets shall be kept in tightly closed containers, protected from light.

Labelling The label on the container states the quantity equivalent to the amount of erythromycin.

Identification

A. Shake a portion of the powdered tablets equivalent to 100 mg of erythromycin with 10 ml of *water*. Decant the supernatant liquid and discard. Extract the residue by shaking with 10 ml of *methanol*, filter the extract and evaporate to dryness. The infrared absorption spectrum of the residue, after drying at a pressure not exceeding 0.7 kPa (about 5 Torr), is concordant with the spectrum obtained from Erythromycin Stearate RS (Appendix 2.1) or with the reference spectrum of Erythromycin Stearate.

B. Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel G* as the coating substance and a mixture of 17 volumes of *methanol* and 3 volumes of *chloroform* as the mobile phase but developing the chromatogram in an unlined chromatographic chamber. Apply separately to the plate, 20 µl of each of the following solutions. For

solution (A), add a volume of *methanol* to a portion of the powdered tablets, sufficient to yield a solution containing the equivalent of 5 mg of erythromycin per ml. Shake this mixture by mechanical means for 30 minutes. Centrifuge a portion of this mixture, and use the clear supernatant liquid. Solution (B) contains 8 mg/ml of Erythromycin Stearate RS in *methanol*. After removal of the plate, allow it to dry in air, spray with a 0.2 per cent w/v solution of *2,7-dichlorofluorescein* in *methanol*, and examine the plate under ultraviolet light (366 nm). The principal fluorescent spots in the chromatogram obtained from solution (A) correspond to those obtained from solution (B). Then spray the plate with a mixture of 18 volumes of *absolute ethanol*, 1 volume of *4-methoxybenzaldehyde* and 1 volume of *sulfuric acid*. Heat the plate at 100° for 10 minutes. Erythromycin appears as a black-to-purple spot: the principal spot obtained from solution (A) corresponds to that obtained from solution (B).

C. Dissolve a portion of the powdered tablets containing 3 mg of erythromycin as completely as possible in 2 ml of *acetone* and add 2 ml of *hydrochloric acid*: an orange colour is produced which changes to red and then to deep purplish red. Add 2 ml of *chloroform* and shake: the chloroform layer becomes purple.

D. Extract a portion of the powdered tablets equivalent to 50 mg of erythromycin with 10 ml of *chloroform*, filter and evaporate to dryness. Heat 100 mg of the residue gently with 5 ml of 2 M *hydrochloric acid* and 10 ml of *water* until the solution boil: oily globules rise to the surface. Cool, remove the fatty layer, heat it with 3 ml of 0.1 M *sodium hydroxide*, and allow to cool: the solution sets to a gel. Add 10 ml of hot *water* and shake: the solution froths. To 1 ml add a 10 per cent w/v solution of *calcium chloride*: a granular precipitate is produced which is insoluble in *hydrochloric acid*.

Loss on drying Not more than 5.0 per cent w/w after drying about 100 mg in a capillary-stoppered bottle at 60° at a pressure not exceeding 2.7 kPa (about 20 Torr) for 3 hours (Appendix 4.15).

Dissolution Carry out the test as described in the "Dissolution Test" (Appendix 4.24).

Dissolution medium: *pH 6.8 phosphate buffer*; 900 ml.

Apparatus 2: 100 rpm.

Time: 120 minutes.

Standard solution Dissolve an accurately weighed quantity of Erythromycin Stearate RS in *methanol* (not more than 1 ml of *methanol* for each 14 mg of the Reference Substance), and dilute with *water*, quantitatively and with mixing, to obtain a stock solution containing about 0.56 mg per ml. Immediately prior to use, dilute the stock solution quantitatively with *water* to obtain a solution having a known concentration of about 280 µg per ml.

Test solution If necessary, dilute a filtered portion of the test solution with *Dissolution medium* to obtain a solution having a concentration of about 0.28 mg of erythromycin per ml, and mix.

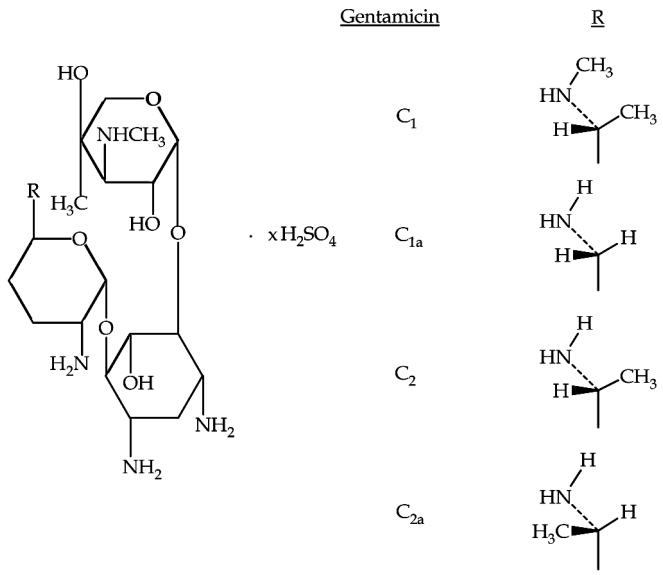
Procedure Transfer 5.0-ml portions of *Standard solution* and *Test solution* to separate 25-ml volumetric flasks, and treat each as follows. Add 2.0 ml of *water*, and allow to stand for 5 minutes with intermittent swirling. Add 15.0 ml of 0.25 M *sodium hydroxide*, dilute with *Dissolution medium* to volume, and mix. Heat to 60° for 5 minutes, and allow to cool. Concomitantly measure the absorbances of these solutions at the maximum at about 236 nm (Appendix 2.2), using a blank solution similarly prepared, except that 2.0 ml of 0.25 M *sulfuric acid* is substituted for the 2.0 ml of *water*. Calculate the amount of $C_{37}H_{67}NO_{13}$ dissolved.

Tolerances Not less than 75 per cent *(Q)* of the labelled amount $C_{37}H_{67}NO_{13}$ is dissolved in 120 minutes.

Assay Place not less than four Erythromycin Stearate Tablets in a high-speed glass blend jar with 200 ml of *methanol* and blend for 3 minutes. Add 300 ml of Buffer 2 and blend for 3 minutes. Proceed as directed under the microbiological assay of erythromycin according to the "Microbiological Assay of Antibiotics" (Appendix 6.10).

Other requirements Comply with the requirements described under "Tablets" (Appendix 1.16).

GENTAMICIN SULFATE



Gentamicin sulfate *1405-41-0*

Category Antibacterial.

Gentamicin Sulfate is the sulfate salt, or a mixture of such salts, of the antibiotic substances produced by the growth of *Micromonospora purpurea*. It has a potency equivalent to not less than 590 µg of gentamicin per mg, calculated on the dried basis.

Description White or almost white powder; hygroscopic.

Solubility Freely soluble in *water*; insoluble in *acetone*, in *chloroform* and in *ether*.

Contra-indication It is contra-indicated in patients with a history of hypersensitivity reactions to any member of aminoglycosides.

Warning

1. It should not be used in pregnant women.
2. It is potentially ototoxic and nephrotoxic, especially in elderly patients, infants and neonates or in patients with renal function impairment.
3. It should be used with caution in patients with myasthenia gravis, parkinsonism, renal function impairment or eighth-cranial nerve impairment.
4. Concurrent and/or sequential use of two or more aminoglycosides by any routes; or concurrent use of capreomycin, methoxyflurane, parenteral polymyxins, other nephrotoxic drugs, or neuromuscular blocking agents with aminoglycosides should be avoided.
5. It may cause neuromuscular blockade.
6. Hypersensitivity reactions of different degrees may occur.

Precaution

1. Renal and vestibular function determinations, and audiogram may be required prior to, periodically during, and following prolonged therapy.
2. Serum aminoglycoside concentrations should be monitored in all patients.

Additional information

1. Subcutaneous administration is not recommended and may be painful.
2. The initial symptoms of ototoxicity may be reversible; however, deafness may occur several weeks after therapy is discontinued.

Packaging and storage Gentamicin Sulfate shall be kept in tightly closed containers, protected from light.

Labelling The label on the container states (1) the number of µg of activity per mg; (2) storage condition; (3) parenteral or non-parenteral grade.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Gentamicin Sulfate RS or with the reference spectrum of Gentamicin Sulfate (Appendix 2.1).

B. Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel G* as the coating substance and as the mobile phase the lower layer obtained by shaking together 1 volume of *chloroform*, 1 volume of *methanol* and 1 volume of *strong ammonia solution* and allowing to separate. Apply separately to the plate, 1 µl of each of two solutions containing (A) 20 mg per ml of the test substance and (B) 20 mg per ml of Gentamicin Sulfate RS. After removal of the plate, allow to dry in air, spray with a 0.25 per cent w/v solution of *ninhydrin* in a mixture of equal volumes of *pyridine* and *acetone*, and heat at 105° for 2 minutes: the three principal spots in the chromatogram obtained from solution (A) correspond with those obtained from solution (B).

C. It yields the *reactions* characteristic of sulfates (Appendix 5.1).

pH 3.5 to 4.5, in a 4.0 per cent w/v solution (Appendix 4.11).

Specific rotation +107° to +121°, calculated on the dried basis, determined in a 10.0 per cent w/v solution (Appendix 4.8).

Loss on drying Not more than 18.0 per cent w/w after drying at 110° at a pressure not exceeding 0.7 kPa (about 5 Torr) for 3 hours (Appendix 4.15).

Sulfate Not less than 32.0 per cent w/w and not more than 35.0 per cent w/w (Appendix 5.2). Dissolve about 250 mg of the test substance, accurately weighed, in 100 ml of *distilled water* and adjust the solution to pH 11 using *strong ammonia solution*. Add 10.0 ml of 0.1 M *barium chloride VS* and about 0.5 mg of *phthalein purple*. Titrate with 0.1 M *sodium edetate VS*, adding 50 ml of *ethanol* when the colour of the solution begins to change and continue the titration until the violet-blue colour disappears. Perform a blank determination (Residual Titration, Appendix 6.17). Each ml of 0.1 M *barium chloride* is equivalent to 9.606 mg of SO_4.

Sulfated ash Not more than 1.0 per cent w/w (Appendix 5.3).

Methanol Not more than 1.0 per cent. Carry out the test as described in the "Gas Chromatography" (Appendix 3.4).

Internal standard solution Transfer 2.5 ml of *1-propanol* to a 500-ml volumetric flask, dilute with *water* to volume, and mix. This solution contains 0.50 per cent v/v of *1-propanol*.

Standard solution Transfer 1.25 ml of *methanol* and 1.25 ml of *1-propanol* to a 500-ml volumetric flask, dilute with *water* to volume, and mix to obtain a *Standard solution* containing 0.25 per cent v/v of *methanol* and 0.25 per cent v/v of *1-propanol*.

Control solution Dissolve 0.50 g of the test substance in 2.0 ml of *water*.

Test solution Dissolve 0.50 g of the test substance in 1.0 ml of *Internal standard solution*, add 1.0 ml of *water*, and mix.

Chromatographic system The chromatographic procedure may be carried out using (a) a glass column (1.5 m × 4 mm) packed with ethylvinylbenzene divinylbenzene copolymer having a nominal surface area of 500 to 600 m^2 per g and an average pore diameter of 0.0075 µm (b) *nitrogen* as the carrier gas at a constant flow rate of 30 ml to 40 ml per minute, and (c) a flame-ionization detector. Maintain the column at a constant temperature between 120° and 140° and the injection port and the detector at a temperature at least 50° higher than that of the column.

Chromatograph *Standard solution*, and measure the peak responses as directed under *Procedure*: the resolution factor between the 1-propanol and methanol peaks is not less than 1.0.

Chromatograph *Control solution*, measure the peak responses as directed under *Procedure*, and examine the chromatogram: if any peak is observed at a retention time corresponding to that of 1-propanol, use the response of that peak to correct the 1-propanol peak response in the chromatogram obtained from *Test solution*.

Procedure (**Note** Use peak areas where peak responses are indicated.) Using a syringe with a polytef-tipped plunger, separately inject equal volumes (about 2 µl) of *Standard solution* and *Test solution* into the chromatograph, record the chromatograms, and measure the 1-propanol and the methanol peak responses.

Calculation Calculate the percentage of methanol in the test substance taken by the expression:

$$1.58(P/M)(R_U/R_S),$$

in which P is the percentage (v/v) of methanol in *Standard solution*, M is the quantity, in g, of the test substance taken to prepare *Test solution*, R_U is the ratio of the methanol peak response to the 1-propanol peak response (corrected, if necessary, by subtracting the response of any peak at the locus of the 1-propanol peak observed in the chromatogram of *Control solution*) in the chromatogram obtained from *Test solution*, and R_S is the ratio of the methanol peak response to the 1-propanol peak response in the chromatogram obtained from *Standard solution*.

Content of gentamicins Carry out the test as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

***o*-Phthalaldehyde solution** Dissolve 1.0 g of *o-phthalaldehyde* in 5 ml of *methanol*, and add 95 ml of 0.4 M *boric acid*, previously adjusted with 8 M *potassium hydroxide* to a pH of 10.4, and 2 ml of *thioglycolic acid*. Adjust the resulting solution with 8 M *potassium hydroxide* to a pH of 10.4.

Mobile phase Mix 700 ml of *methanol*, 250 ml of *water*, and 50 ml of *glacial acetic acid*. Dissolve 5 g of *sodium 1-heptanesulfonate* in this solution. Make adjustments if necessary.

Standard solution Prepare a solution of Gentamicin Sulfate RS in *water* containing about 0.65 mg per ml. Transfer 10 ml of this solution to a suitable test-tube, add 5 ml of *2-propanol* and 4 ml of o-*phthalaldehyde solution*, mix, and add *2-propanol* to obtain 25 ml of solution. Heat at 60° in a water-bath for 15 minutes, and cool.

Test solution Using the test substance, proceed as directed for *Standard solution*.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (10 cm × 5 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles (5 µm), (b) *Mobile phase* at a flow rate of 1.5 ml per minute, and (c) an ultraviolet photometer set at

330 nm. Chromatograph *Standard solution*, and record the peak responses as directed under *Procedure*: the capacity factor determined from the gentamicin C_1 peak is between 2 and 7, the column efficiency determined from the gentamicin C_2 peak is not less than 1200 theoretical plates, the resolution factor between any two peaks is not less than 1.25, and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Procedure (**Note** Use peak areas where peak responses are indicated.) Separately inject equal volume (about 20 µl) of *Standard solution* and *Test solution* into the chromatograph, record the chromatograms, and measure the responses for the major peaks. The elution order is gentamicin C_1, gentamicin C_{1a}, gentamicin C_{2a}, and gentamicin C_2.

Calculation Calculate the percentage contents of gentamicin C_1, gentamicin C_{1a}, gentamicin C_{2a}, and gentamicin C_2 taken by the expression:

$$100r_f / r_s,$$

in which r_f is the peak response corresponding to the particular gentamicin, and r_s is the sum of the responses of all four peaks: the content of gentamicin C_1 is between 25 and 50 per cent, the content of gentamicin C_{1a} is between 10 and 35 per cent, and the sum of the contents of gentamicin C_{2a} and gentamicin C_2 is between 25 and 55 per cent.

Assay Carry out the microbiological assay of Gentamicin Sulfate according to the "Microbiological Assay of Antibiotics" (Appendix 6.10).

Other requirements Gentamicin Sulfate intended for parenteral administration complies with the following additional requirements.

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains not more than 0.71 Endotoxin Unit per mg of gentamicin.

Sterility Complies with the "Sterility Test" (Method I, Appendix 10.1).

GENTAMICIN SULFATE CREAM

Category Antibacterial.

Gentamicin Sulfate Cream contains the equivalent of not less than 90.0 per cent and not more than 135.0 per cent of the labelled amount of gentamicin.

Strength available 0.1 per cent w/w (base).

Dose *Topical*, to the skin, three or four times a day.

Warning It may cause hypersensitivity e.g. itching, redness, swelling, or other signs of irritation.

Additional information Water-washable cream of gentamicin sulfate may be useful in treating wet, oozing primary infections and greasy, secondary infections of the skin such as pustular acne or infected seborrheic dermatitis.

Packaging and storage Gentamicin Sulfate Cream shall be stored at a temperature not exceeding 30°.

Labelling The label on the container states the quantity equivalent to the amount of gentamicin.

Identification Complies with the test for Identification C described under *Gentamicin Sulfate*, p. 111. Apply separately to the plate, 20 µl of each of the following solutions. For solution (A), disperse a portion of the cream, equivalent to 7.5 mg of gentamicin, in 20 ml of *chloroform*, extract with 10 ml of *water* and use the aqueous layer. Solution (B) is a solution of Gentamicin Sulfate RS containing the equivalent of 750 µg of gentamicin per ml.

Assay Shake an accurately weighed portion of Gentamicin Sulfate Cream, equivalent to about 1 mg of gentamicin, with 50 ml of *ether*, and extract with four 20-ml portions of Buffer 2. Combine the aqueous extracts, dilute quantitatively and stepwise with Buffer 2, and proceed as directed under the microbiological assay of Gentamicin Sulfate according to the "Microbiological Assay of Antibiotics" (Appendix 6.10).

Other requirements Comply with the requirements described under "Topical Semi-solid Preparations" (Appendix 1.16).

ลิขสิทธิ์ กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข
ห้ามทำซ้ำ ดัดแปลง และใช้เพื่อการค้า

GENTAMICIN SULFATE EYE DROPS

Category Antibacterial (ophthalmic).

Gentamicin Sulfate Eye Drops are a sterile, buffered solution of Gentamicin Sulfate with preservatives. They contain the equivalent of not less than 90.0 per cent and not more than 135.0 per cent of the labelled amount of gentamicin.

Strength available 0.3 per cent w/v (base).

Dose *Topical*, to the conjunctiva, 1 to 2 drops into the affected eye every 4 hours for mild to moderate infections or as often as once every hour for severe infections.

Warning See under *Gentamicin Sulfate Cream*, p. 113.

Packaging and storage Gentamicin Sulfate Eye Drops shall be stored at a temperature not exceeding 30°.

Labelling The label on the container states the quantity equivalent to the amount of gentamicin.

Identification Comply with the test for Identification C described under *Gentamicin Sulfate*, p. 113. Apply separately to the plate, (A) a volume of the eye drops equivalent to 30 μg of gentamicin and (B) 100 μg of Gentamicin Sulfate RS dissolved in a volume of *water* equal to the volume of the eye drops used.

pH 6.5 to 7.5 (Appendix 4.11).

Assay Proceed as directed under the microbiological assay of Gentamicin Sulfate according to the "Microbiological Assay of Antibiotics" (Appendix 6.10), using an accurately measured volume of Gentamicin Sulfate Eye Drops.

Other requirements Comply with the requirements described under "Eye Preparations" (Appendix 1.16).

GENTAMICIN SULFATE INJECTION

Category Antibacterial.

Gentamicin Sulfate Injection is a sterile solution of Gentamicin Sulfate in Water for Injection. It contains the equivalent of not less than 90.0 per cent and not more than 125.0 per cent of the labelled amount of gentamicin. It may contain suitable buffers, preservatives and sequestering agents, unless it is intended for intrathecal use, in which case it contains only suitable tonicity agents.

Strengths available 10 and 40 mg (base) per ml.

Dose Adults: *Intramuscular* or slow *intravenous infusion* (over 30 to 60 minutes), 1 to 1.7 mg per kg of body weight every 8 hours for 7 to 10 days or more, or up to 8 mg per kg of body weight daily in severe, life-threatening infections. (**Note** Doses up to 15 mg per kg of body weight daily have been used in the treatment of intraocular infections.)

Children: *Intramuscular* or slow *intravenous infusion* (over 30 to 60 minutes), 2 to 2.5 mg per kg of body weight every 8 hours for 7 to 10 days or more.

Older neonates and infants: *Intramuscular* or slow *intravenous infusion* (over 30 to 60 minutes), 2.5 mg per kg of body weight every 8 to 16 hours for 7 to 10 days or more.

Premature or full-term neonates up to 1 week of age: *Intramuscular* or slow *intravenous infusion* (over 30 to 60 minutes) 2.5 mg per kg of body weight every 12 to 24 hours for 7 to 10 days or more.

Contra-indication; Warning; Precaution See under *Gentamicin Sulfate*, p. 111.

Additional information

1. In patients with impaired renal function, intramuscular and intravenous doses and/or frequency of administration of gentamicin must be modified in response to serum concentrations of the drug and the degree of renal impairment.

2. Extemporaneous admixtures with some drugs, especially betalactam antibacterials, should be avoided.

See also under *Gentamicin Sulfate*, p. 111.

Packaging and storage Gentamicin Sulfate Injection shall be kept in single-dose or in multiple-dose containers, preferably of Type I glass, and stored at a temperature not exceeding 30°.

Labelling The label on the container states the quantity equivalent to the amount of gentamicin.

Identification Complies with the test for Identification C described under *Gentamicin Sulfate*, p. 111.

pH 3.0 to 5.5 (Appendix 4.11).

Particulate matter Complies with the requirements described under "Particulate Matter in Injections" (Small-volume Injections, Appendix 4.27).

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains not more than 0.71 Endotoxin Unit per mg of gentamicin.

Assay Proceed as directed under the microbiological assay of Gentamicin Sulfate according to the "Microbiological Assay of Antibiotics" (Appendix 6.10), using an accurately measured volume of Gentamicin Sulfate Injection.

Other requirements Complies with the requirements described under "Parenteral Preparations" (Appendix 1.16).

GENTAMICIN SULFATE OINTMENT

Category Antibacterial.

Gentamicin Sulfate Ointment contains the equivalent of not less than 90.0 per cent and not more than 135.0 per cent of the labelled amount of gentamicin.

Strength available 0.1 per cent w/w (base).

Dose *Topical*, to the skin, three or four times a day.

Warning See under *Gentamicin Sulfate Cream*, p. 113.

Packaging and storage Gentamicin Sulfate Ointment shall be stored at a temperature not exceeding 30°.

Labelling The label on the container states the quantity equivalent to the amount of gentamicin.

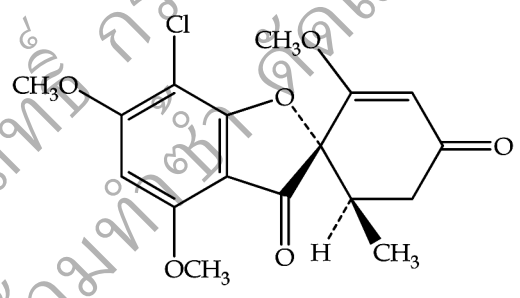
Identification Complies with the test for Identification described under *Gentamicin Sulfate Cream*, p. 113.

Water Not more than 1.0 per cent w/w, 20 ml of a mixture of 7 volumes of *toluene* and 3 volumes of *methanol* used in place of *methanol* in the titration vessel (Karl Fischer Method, Appendix 4.12).

Assay Carry out the Assay described under *Gentamicin Sulfate Cream*, p. 113.

Other requirements Comply with the requirements described under "Topical Semi-solid Preparations" (Appendix 1.16).

GRISEOFULVIN



$C_{17}H_{17}ClO_6$ 352.77 *126-07-8*

Spiro[benzofuran-[2](3*H*),1′-2cyclohexene]-3,4′-dione, 7-chloro-2′,4,6-trimethoxy-6′-methyl-, (1′*S-trans*)-.

Category Antifungal.

Griseofulvin contains not less than 97.0 per cent and not more than 102.0 per cent of $C_{17}H_{17}ClO_6$, calculated on the dried basis.

Description White to creamy white powder, in which particles of the order of 4 μm in diameter predominate; odourless.

Solubility Very slightly soluble in *water*; soluble in *acetone*, in *dimethylformamide* and in *chloroform*; sparingly soluble in *methanol*.

Contra-indication It is contra-indicated in pregnancy or in patients with porphyria, systemic lupus erythematosus or liver failure.

Warning

1. It may cause headache, fatigue, dizziness, insomnia, gastro-intestinal distress, blood dyscrasias, renal insufficiency, hepatotoxicity, skin rash, urticaria, oral thrush, photosensitization, allergic reactions, and estrogenic-like effects in children.
2. Concurrent use with alcohol, barbiturates or oral anticoagulants should be avoided.
3. Overgrowth of yeasts or bacteria may accompany or follow long-term or too intensive treatment with griseofulvin.

Precaution

1. Determination of complete blood count, serum creatinine concentration, hepatic function and urinalysis should be performed at periodic interval.
2. Patients who are under treatment with griseofulvin should be warned to avoid exposure to intense natural or artificial sunlight.

Packaging and storage Griseofluvin shall be kept in well-closed containers.

Labelling The label on the container states that griseofulvin is in microsize form.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Griseofulvin RS (Appendix 2.1) or with the reference spectrum of Griseofulvin.

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

C. Dissolve 5 mg in 1 ml of *sulfuric acid* and add 5 mg of powdered *potassium dichromate*: a wine-red colour is produced.

Melting range 217° to 224°, after being dried (Appendix 4.3).

Clarity of solution A 7.5 per cent w/v solution in *dimethylformamide* is clear (Method II A, Appendix 4.1).

Particle size In a mortar, grind 10 mg with 10 drops of *hydroxyethylcellulose TS*. Add a further 3.50 ml of *hydroxyethylcellulose TS* and grind again. Transfer a drop of the suspension to a suitable counting chamber, 0.10 mm deep, place a cover glass over it, and examine under a microscope 10 fields of vision, each of 0.04-mm^2 area, using a magnification of 600°: not more than 30 crystals larger than 5 μm are visible in any field of vision.

Specific rotation +348° to +364°, calculated on the dried basis, determined in a 1 per cent w/v solution in *dimethylformamide* (Appendix 4.8).

Loss on drying Not more than 1.0 per cent w/w after drying about 100 mg accurately weighed, in a capillary-stoppered bottle at a pressure not exceeding 0.7 kPa (about 5 Torr) at 60° for 3 hours (Appendix 4.15).

Sulfated ash Not more than 0.2 per cent w/w (Method II, Appendix 5.3).

Heavy metals Not more than 25 ppm (Method II, Appendix 5.2). Use 0.80 g; for the Standard Preparation, use *lead standard solution (1 ppm Pb)*.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Prepare a mixture of 60 volumes of *water*, 35 volumes of *acetonitrile* and 5 volumes of *tetrahydrofuran*. Make adjustments if necessary.

Standard preparation Dissolve an accurately weighed quantity of Griseofulvin RS in *methanol* to obtain a solution having a known concentration of about 1.25 mg per ml. Transfer 5.0 ml of this solution to a 50-ml volumetric flask, dilute with *Mobile phase* to volume, and mix. This solution contains about 125 μg of Griseofulvin RS in each ml.

Assay preparation Transfer about 62 mg of Griseofulvin, accurately weighed, to a 50-ml volumetric flask, dissolve in and dilute with *methanol* to volume, and mix. Transfer 5.0 ml of this solution to a 50-ml volumetric flask, dilute with *Mobile phase* to volume, and mix.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (25 cm × 4.6 mm) packed with nitrile groups chemically bonded to porous silica particles (3 to 10 μm), (b) *Mobile phase* at a flow rate of about 1 ml per minute and (c) an ultraviolet photometer set at 254 nm.

To determine the suitability of the chromatographic system, chromatograph *Standard preparation*, and record the peak responses as directed under *Procedure*: the relative standard deviation for replicate injections is not more than 2.0 per cent.

Procedure Separately inject equal volumes (about 20 μl) of *Standard preparation* and *Assay preparation* into the chromatograph, record the chromtograms and measure the responses for the major peaks.

Calculation Calculate the content of $C_{17}H_{17}ClO_6$ in the Griseofulvin taken, using the declared content of $C_{17}H_{17}ClO_6$ in Griseofulvin RS.

GRISEOFULVIN TABLETS

Category Antifungal.

Griseofulvin Tablets contain not less than 90.0 per cent and not more than 115.0 per cent of the labelled amount of $C_{17}H_{17}ClO_6$.

Strengths available 125, 250 and 500 mg.

Dose Adults: 500 mg to 1 g daily in single or divided doses.

Children: 10 mg per kg of body weight daily in single or divided doses.

Contra-indication; Warning See under *Griseofulvin*, p. 115.

Precaution If fever, sore throat or skin rash occurs, notify the physician.

See also under *Griseofulvin*, p. 115.

Additional information

1. It should be taken with or after meals or milk, preferably taken after fatty meals to increase absorption.

2. Griseofulvin may be available in ultramicrosize form (less than 1 μm in diameter). The dose should be reduced accordingly.

Packaging and storage Griseofulvin Tablets shall be kept in tightly closed containers.

Labelling The label on the container states that griseofulvin is in microsize form.

Identification

A. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

B. Dissolve 5 mg of the tablet contents in 1 ml of *sulfuric acid* and add 5 mg of powdered *potassium*

dichromate: a red colour is produced.

Dissolution Carry out the test as described in the "Dissolution Test" (Appendix 4.24).

Dissolution medium: *water* containing 40.0 mg of *sodium dodecyl sulfate* per ml; 1000 ml.

Apparatus 2: 75 rpm.

Time: 90 minutes

Procedure Determine the amount of $C_{17}H_{17}ClO_6$ dissolved from absorbances at about 291 nm of filtered portions of the test solution, suitably diluted with a mixture of 4 volumes of *methanol* and 1 volume of *water*, if necessary, in comparison with a standard solution having a known concentration of Griseofulvin RS in the same medium (Appendix 2.2).

Tolerances Not less than 75 per cent (*Q*) of the labelled amount of $C_{17}H_{17}ClO_6$ is dissolved in 90 minutes.

Loss on drying Not more than 5.0 per cent w/w after drying about 100 mg of finely ground tablets, accurately weighed, in a capillary-stoppered bottle *in vacuum* at a pressure not exceeding 0.7 kPa (about 5 Torr) at 60° for 3 hours (Appendix 4.15).

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase, Standard preparation, Chromatographic system, and **Procedure** Proceed as directed in the Assay under *Griseofulvin*, p. 116.

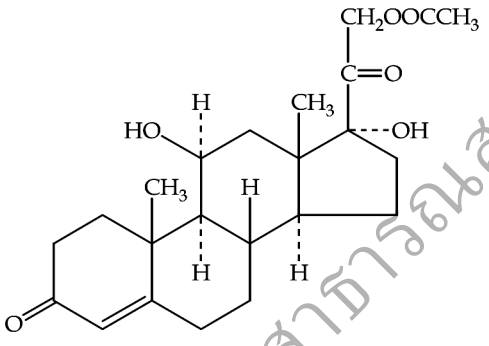
Assay preparation Weigh and finely powder not less than 20 Griseofulvin Tablets. Transfer an accurately weighed portion of the powder, containing about 125 mg of griseofulvin, to a 100-ml volumetric flask. Add about 70 ml of *methanol*, shake by mechanical means for 30 minutes, and dilute with *methanol* to volume. Mix and filter. Transfer 5.0 ml of the clear filtrate to a 50-ml volumetric flask, dilute with *Mobile phase* to volume, and mix.

Calculation Calculate the content of $C_{17}H_{17}ClO_6$ in the portion of the Tablets taken, using the declared content of $C_{17}H_{17}ClO_6$ in Griseofulvin RS.

Other requirements Comply with the requirements described under "Tablets" (Appendix 1.16).

HYDROCORTISONE ACETATE

Cortisol Acetate



$C_{23}H_{32}O_6$ 404.50 *50-03-3*

Pregn-4-ene-3,20-dione,21-(acetyloxy)-11,17-dihydroxy-, (11*β*)-.

Category Corticosteroid (topical); anti-inflammatory (steroidal).

Hydrocortisone Acetate contains not less than 96.0 per cent and not more than 104.0 per cent of $C_{23}H_{32}O_6$, calculated on the dried basis.

Description White to practically white, crystalline powder; odourless.

Solubility Insoluble in *water*; slightly soluble in *ethanol* and in *chloroform*.

Contra-indication It is contra-indicated in vaccinia and varicella in the presence of skin infections, acute purulent infections of the eye, fungal infection of the ear, or perforation of the tympanic membrane, herpes simplex of the eye, infectious diseases without anti-infective coverage, active peptic ulcer, myasthenia gravis, or idiopathic thrombocytopenic purpura.

Warning

1. Topical corticosteroids should not be used in the treatment of acne.
2. It may mask signs of infection and new infections may appear during its use.
3. Prolonged use may cause skin atrophy, increased intra-ocular pressure and cataracts, or retard corneal healing.
4. It may be absorbed in sufficient amount to cause systemic effects especially when applied to large areas, to broken skin, or under occlusive dressing.
5. It should not be applied to the breasts prior to nursing.
6. Bandaging or wrapping the treated skin area should be avoided unless directed to do so by a physician.

7. Tight-fitting diapers or plastic pants should not be used on a child if the diaper area is being treated.

8. Risk-benefit should be considered if it is to be used in pregnant women.

Packaging and kept storage Hydrocortisone Acetate shall be kept in well-closed containers, protected from light.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Hydrocortisone Acetate RS (Appendix 2.1) or with the reference spectrum of Hydrocortisone Acetate.

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation as obtained in the *Assay*.

C. It yields the *reactions* characteristic of acetyl groups (Appendix 5.1).

Melting temperature About 200°, with decomposition (Appendix 4.3).

Specific rotation +158° to +165°, determined in a 0.5 per cent w/v solution in *dioxane* (Appendix 4.8).

Loss on drying Not more than 0.5 per cent w/w after drying about 500 mg at 105° for 3 hours (Appendix 4.15).

Sulfated ash Negligible, from 100 mg (Appendix 5.3).

Chromatographic purity Carry out the test as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Solution A Prepare a mixture of 80 volumes of *water* and 20 volumes of *acetonitrile*. Make adjustments if necessary.

Solution B Prepare a mixture of 70 volumes of *acetonitrile* and 30 volumes of *water*. Make adjustments if necessary.

Mobile phase Use variable mixtures of *Solution A* and *Solution B* as directed for *Chromatographic system*.

Diluting solution Prepare a mixture of 700 volumes of *acetonitrile*, 300 volumes of *water*, and 1 volume of *glacial acetic acid*.

Standard solution Dissolve an accurately weighed quantity of Hydrocortisone Acetate RS in *Diluting solution*, and dilute quantitatively, and stepwise if necessary, with *Diluting solution* to obtain a solution having a known concentration of about 5 µg per ml.

Test solution Transfer about 10 mg of the test substance, accurately weighed, to a 10-ml volumetric flask, dissolve in and dilute with *Diluting solution* to volume, and mix.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (15 cm × 4.6 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles (3 to 10 µm), (b) *Mobile phase* at a flow rate of about 1 ml per minute and (c) an ultraviolet photometer set at 254 nm.

Time (minutes)	*Solution A* (%)	*Solution B* (%)	Elution
0	90	10	equilibration
0-5	90	10	isocratic
5-25	90→10	10→90	linear gradient
25-30	10	90	isocratic
30-35	10→90	90→10	linear gradient
35-40	90	10	re-equilibration

Chromatograph *Standard solution*, and record the peak responses as directed for *Procedure*: the relative standard deviation for replicate injections is not more than 5.0 per cent.

Procedure Separately inject equal volumes (about 10 µl) of *Standard solution* and *Test solution* into the chromatograph, record the chromatograms, and measure the peak responses.

Calculation Calculate the percentage of each impurity in the portion of the test substance taken by the expression:

$$0.5(r_i / r_s),$$

in which r_i is the peak response for each impurity, and r_s is the peak response of the *Standard solution*: not more than 1.0 per cent of any individual impurity is found, and not more than 2.0 per cent of total impurities is found.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Prepare a mixture of 475 volumes of *butyl chloride*, 475 volumes of *water-saturated butyl chloride*, 70 volumes of *tetrahydrofuran*, 35 volumes of *methanol* and 30 volumes of *glacial acetic acid*. Make adjustments if necessary.

Standard preparation Dissolve an accurately weighed quantity of Hydrocortisone Acetate RS in *Mobile phase*, and dilute quantitatively, and stepwise if necessary, with *Mobile phase* to obtain a solution having a known concentration of about 100 µg per ml.

Assay preparation Transfer about 10 mg of Hydrocortisone Acetate, accurately weighed, to a 100-ml volumetric flask, add *Mobile phase* to volume, and mix.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (30 cm × 4 mm) packed with porous silica particles (10 µm), (b) *Mobile phase* at a flow rate of about 1 ml per minute and (c) an ultraviolet photometer set at 254 nm.

To determine the suitability of the chromatographic system, chromatograph *Standard preparation*, and record the peak responses as directed under *Procedure*: the symmetry factor is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Procedure Separately inject equal volumes (about 10 µl) of *Standard preparation* and *Assay preparation* into the chromatograph, record the chromatograms and measure the responses for the major peaks.

Calculation Calculate the content of $C_{23}H_{32}O_6$ in the Hydrocortisone Acetate taken, using the declared content of $C_{23}H_{32}O_6$ in Hydrocortisone Acetate RS.

HYDROCORTISONE ACETATE AND NEOMYCIN SULFATE EYE OINTMENT

Category Corticosteroid (ophthalmic); anti-inflammatory (steroidal); antibacterial.

Hydrocortisone Acetate and Neomycin Sulfate Eye Ointment contains not less than 90.0 per cent and not more than 110.0 per cent of the labelled amount of $C_{23}H_{32}O_6$, and the equivalent of not less than 90.0 per cent and not more than 135.0 per cent of the labelled amount of neomycin.

Strength available 1.5 per cent w/w of Hydrocortisone Acetate and 0.5 per cent w/w of Neomycin Sulfate (equivalent to 0.35 per cent w/w of neomycin base).

Dose *Topical*, to the conjunctiva, three or four times a day initially, with frequency of application gradually being decreased as inflammation subsides.

Contra-indication; Warning; Precaution; Additional information See under *Hydrocortisone Acetate*, p. 117 and *Neomycin Sulfate*, p. 132.

Labelling The label on the container states the quantity equivalent to the amount of neomycin.

Identification

A. Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel G* as the coating substance and a mixture of 4 volumes of *methanol*, 2 volumes of *2-propanol*, 2 volumes of *dichloromethane*, 2 volumes of *strong ammonia solution*, and 1.5 volumes of *water* as the mobile phase. Apply separately to the plate, 10 µl of each of the following solutions. For solution (A) transfer a portion of the ointment containing 3.5 mg of neomycin to a 15-ml centrifuge tube. Add 4 ml of *chloroform* to the centrifuge tube, and shake well to disperse the ointment. Add 1 ml of 0.1 M *hydrochloric acid*, vortex for 4 minutes, centrifuge and use the clear supernatant. For solution (B) dissolve a portion of Neomycin Sulfate RS in 0.1 M *hydrochloric acid* to obtain a solution containing the equivalent of 3.5 mg of neomycin per ml. After removal of the plate, dry at 105° for 10 minutes, spray with a 0.2 per cent w/v solution of *ninhydrin* in *1-butanol*, and heat at 105° for 5 minutes: the principal spot in the chromatogram obtained from solution (A) corresponds to that obtained from solution (B).

B. The retention time of the major peak for hydrocortisone acetate in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay for hydrocortisone acetate*.

Water Not more than 1.0 per cent w/w, 20 ml of a mixture of 7 volumes of *toluene* and 3 volumes of *methanol* being used in place of methanol in the titration vessel (Karl Fischer Method, Appendix 4.12).

Assay

FOR HYDROCORTISONE ACETATE Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Prepare a mixture of 475 volumes of *butyl chloride*, 475 volumes of *water-saturated butyl chloride*, 70 volumes of *tetrahydrofuran*, 35 volumes of *methanol* and 30 volumes of *glacial acetic acid*. Make adjustments if necessary.

Standard preparation Dissolve an accurately weighed quantity of Hydrocortisone Acetate RS in *water-saturated chloroform* to obtain a solution having a known concentration of about 100 µg per ml.

Assay preparation Transfer an accurately weighed quantity of Hydrocortisone Acetate and Neomycin Sulfate Eye Ointment, containing about 2.5 mg of hydrocortisone acetate, to a stoppered centrifuge tube. Add 25.0 ml of *water-saturated chloroform* and 10 glass beads. Securely close the container, and shake vigorously for approximately 15 minutes. Centrifuge, and use the clear, lower chloroform layer.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (30 cm × 4 mm) packed with porous silica particles (10 µm), (b) *Mobile phase* at a flow rate of about 1 ml per minute and (c) an ultraviolet photometer set at 254 nm.

To determine the suitability of the chromatographic system, chromatograph *Standard preparation*, and record the peak responses as directed under *Procedure*: the relative standard deviation for replicate injections is not more than 2.0 per cent.

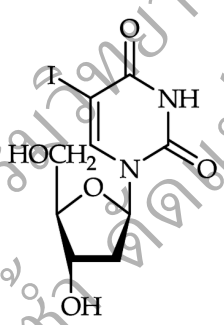
Procedure Separately inject equal volumes (about 10 µl) of *Standard preparation* and *Assay preparation* into the chromatograph, record the chromatograms and measure the responses for the major peaks.

Calculation Calculate the content of $C_{23}H_{32}O_6$ in the portion of the Ointment taken, using the declared content of $C_{23}H_{32}O_6$ in Hydrocortisone Acetate RS.

FOR NEOMYCIN SULFATE Transfer an accurately weighed portion of Hydrocortisone Acetate and Neomycin Sulfate Eye Ointment, containing about 3.5 mg of neomycin, to a separator, add 50 ml of *ether*, shake and extract with four 20-ml portions of Buffer 2. Combine the aqueous extracts, and dilute with Buffer 2 to an appropriate volume to obtain a stock solution of convenient concentration. Dilute this stock solution quantitatively and stepwise with Buffer 2 to obtain a solution having a concentration assumed to the median dose level of the standard and proceed as directed under the microbiological assay of Neomycin Sulfate according to the "Microbiological Assay of Antibiotics"(Appendix 6.10).

Other requirements Comply with the requirements described under "Eye Preparations" (Appendix 1.16).

IDOXURIDINE



$C_9H_{11}IN_2O_5$ 354.10 *54-42-2*

Uridine, 2′-deoxy-5-iodo-.

Category Antiviral.

Idoxuridine contains not less than 98.0 per cent and not more than 101.0 per cent of $C_9H_{11}IN_2O_5$, calculated on the dried basis.

Description White or almost white, crystalline powder.

Solubility Slightly soluble in *water* and in *ethanol*; practically insoluble in *ether*.

Stability Aqueous solutions are most stable when the pH is adjusted to between 2º and 6º; they should be freshly prepared and kept in a refrigerator. Some decomposition products are more toxic than idoxuridine and reduce its antiviral activity.

Warning

1. It should be used with caution in conditions when there is deep ulceration involving the stromal layers of the cornea, as delayed healing has resulted in corneal perforation. Prolonged topical use should be avoided.

2. It may cause hypersensitivity i.e. itching, redness, swelling, pain, or other signs of irritation not present before therapy, allergic reactions, corneal clouding, or punctate defects in the corneal epithelium; or increase sensitivity of eyes to light. Prolonged use may cause follicular conjunctivitis, punctal occlusion or conjunctival scarring.

3. Idoxuridine has been reported to be cytotoxic and potentially mutagenic, carcinogenic and teratogenic; its systemic use is thus not recommended.

4. Boric acid preparations should not be applied to the eye in patients also receiving ocular preparations of idoxuridine as irritation ensues.

5. Since safety for topical use in pregnancy or in the nursing mothers has not been established, risk-benefit should be considered before use.

Precaution

1. Periodic ophthalmologic, including slit-lamp, examinations are recommended during therapy.

2. The medication should be continued for 5 to 7 days more after the lesion is apparently healed, otherwise recurrence may occur.

Additional information

1. Patients sensitive to iodine or iodine-containing preparations may be sensitive to this medication also.

2. Corticosteroids can accelerate the spread of viral infections and are usually contra-indicated in superficial herpes simplex virus keratitis. However, steroids may be used concurrently with idoxuridine in the treatment of herpes simplex infections with stromal lesions, corneal edema, or iritis. Prolonged administration with corticosteroids may be required. Idoxuridine should be continued for a few days after the steroid has been discontinued.

Packaging and storage Idoxuridine shall be kept in well-closed containers, protected from light.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Idoxuridine RS (Ap-

pendix 2.1) or with the reference spectrum of Idoxuridine.

B. Examine the chromatograms obtained in the test for *Related substances*: the principal spot in the chromatogram obtained from Test solution (b) is similar in position and size to the principal spot in the chromatogram obtained from Reference solution (c).

C. Heat 5 mg in a test-tube over a naked flame: violet vapour is evolved.

D. Disperse 2 mg in 1 ml of *water* and add 2 ml of *diphenylamine TS*. Heat in a water-bath for 10 minutes: a persistent light-blue colour develops.

pH 5.5 to 6.5, in a 0.10 per cent w/v solution (Appendix 4.11).

Loss on drying Not more than 1.0 per cent w/w after drying at 60° at a pressure not exceeding 2.7 kPa (about 20 Torr) over *phosphorus pentoxide desiccant* for 2 hours (Appendix 4.15).

Specific rotation +28° to +32°, calculated on the dried basis, determined in a 1.0 per cent w/v solution in 1 M *sodium hydroxide* (Appendix 4.8).

Sulfated ash Not more than 0.1 per cent w/v (Appendix 5.3).

Related substances Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel GF254* as the coating substance and a mixture of 10 volumes of *strong ammonia solution*, 40 volumes of *chloroform* and 50 volumes of *2-propanol* as the mobile phase.

Solvent mixture Mix 1 volume of *strong ammonia solution* and 5 volumes of *methanol*.

Test solution (a) Dissolve 200 mg of the test substance and dilute to 5 ml with *Solvent mixture*.

Test solution (b) Dilute 1.0 ml of *Test solution (a)* to 10 ml with *Solvent mixture*.

Reference solution (a) Dissolve 20 mg of *5-iodouracil*, 20 mg of *2′-deoxyuridine* and 20 mg of *5-bromo-2′-deoxyuridine* and dilute to 100 ml with *Solvent mixture*.

Reference solution (b) Dissolve 200 mg of the test substance in 5.0 ml of *Reference solution (a)*.

Reference solution (c) Dissolve 20 mg of Idoxuridine RS and dilute to 5 ml with *Solvent mixture*.

Reference solution (d) Dilute 1.0 ml of *Test solution (b)* to 20.0 ml with *Solvent mixture*.

Procedure Apply separately to the plate 5 µl of each solution. After removal of the plate, allow it to dry in a current of cold air and examine in ultraviolet light (254 nm). In the chromatogram obtained from *Test solution (a)*: any spots corresponding to 5-iodouracil, 2′-deoxyuridine and 5-bromo-2′-deoxyuridine are not more intense than the corresponding spots in the chromatogram obtained from *Reference solution (a)* (0.5 per cent); any spot, apart from the principal spot and the spots corresponding to 5-iodouracil, 2′-deoxyuridine and 5-bromo-2′-deoxyuridine, is not more intense than the spot in the chromatogram obtained from *Reference solution (d)* (0.5 per cent). The test is not valid unless the chromatogram obtained from *Reference solution (b)* shows four clearly separated spots.

Inorganic iodide Not more than 0.1 per cent w/w. Dissolve 250 mg of the test substance in 25 ml of 0.1 M *sodium hydroxide*, add 5 ml of *dilute hydrochloric acid* and dilute to 50 ml with *water*. Allow to stand for 10 minutes and filter. To 25.0 ml of the filtrate, add 5 ml of *hydrogen peroxide TS (10 volumes)* and extract with 10.0 ml of *chloroform*. Any pink colour in the organic layer is not more intense than that in a standard prepared at the same time in the same manner using 1.0 ml of a 0.033 per cent w/v solution of *potassium iodide* instead of the test substance.

Assay Dissolve about 300 mg of Idoxuridine, accurately weighed, in 20 ml of *dimethylformamide*. Titrate with 0.1 M *tetrabutylammonium hydroxide VS*, determining the end-point potentiometrically (Appendix 6.1). Perform a blank determination, and make any necessary correction. Each ml of 0.1 M *tetrabutylammonium hydroxide* is equivalent to 35.41 mg of $C_9H_{11}IN_2O_5$.

IDOXURIDINE EYE DROPS

Category Antiviral (ophthalmic).

Idoxuridine Eye Drops are a sterile solution of Idoxuridine in Purified Water. They contain not less than 90.0 per cent and not more than 110.0 per cent of the labelled amount of $C_9H_{11}IN_2O_5$.

Strength available 0.1 per cent w/v.

Dose Keratitis, herpes simplex virus: *Topical*, to the conjunctiva, 1 drop every hour during the day and every 2 hours during the night; or 1 drop every minute for 5 minutes with the dosage schedule repeated every 4 hours day and night. Treatment should be continued until definite improvement occurs, as demonstrated by loss of staining with fluorescein. Dose may then be reduced to 1 drop every 2 hours during the day and every 4 hours during the night.

Warning; Precaution; Additional information See under *Idoxuridine*, p. 120.

Packaging and storage Idoxuridine Eye Drops shall be stored at a temperature between 2° and 8°, protected from light.

Labelling The label on the container states that they should not be used for continuous periods of treatment exceeding 21 days.

Identification

A. Dilute a suitable volume of the eye drops with 0.01 M *sodium hydroxide* to produce a solution containing 0.004 per cent w/v of idoxuridine: the ultraviolet absorption spectrum of the resulting solution, when observed between 230 nm and 350 nm, exhibits a maximum only at 279 nm (Appendix 2.2).

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

pH 4.5 to 7.0 (Appendix 4.11).

Related substances Carry out the test as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Chromatographic system Proceed as directed in the *Assay*.

Mobile phase Prepare a mixture of 24 volumes of *water* and 1 volume of *methanol*.

Reference solution Prepare the solution containing 4.0 µg per ml of *2′-deoxyuridine*, 8.0 µg per ml of *5-iodouracil*, 4.0 µg per ml of *5-bromo-2′-deoxyuridine* and 1 µg per ml of *sulfanilamide* (internal standard).

Test solution (a) Dilute a suitable volume of the eye drops with *water* to give a final concentration of 800 µg per ml of idoxuridine.

Test solution (b) Dilute a suitable volume of the eye drops with sufficient of a suitable solution of *sulfanilamide* to give a final concentration of 800 µg per ml of idoxuridine and 1 µg per ml of *sulfanilamide* (internal standard).

The order of elution of the peaks following the internal standard is deoxyuridine, iodouracil, bromodeoxyuridine, and idoxuridine. Several peaks due to excipients may appear in the chromatogram obtained from *Test solution (b)* before the peak due to the internal standard.

In the chromatogram obtained from *Test solution* (b), the ratios of the areas of any peaks due to 2′-deoxyuridine, 5-iodouracil and 5-bromo-2′-deoxyuridine to the area of the peak due to sulfanilamide are not greater than the ratios of the areas of the corresponding peaks in the chromatogram obtained from *Reference solution*.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Prepare a mixture of 13 volumes of *methanol* and 87 volumes of *water*. Make adjustments if necessary.

Internal standard solution Prepare a solution containing about 120 mg of *sulfathiazole* in 10.0 ml of *ethanol*, warming if necessary, and dilute to 100.0 ml with *water*.

Standard preparation Shake about 100 mg of Idoxuridine RS, accurately weighed, with 50 ml of *water* until dissolved and then dilute to 100.0 ml with *water* and mix. To 15.0 ml of this solution add 2.0 ml of *Internal standard solution*, dilute to 20.0 ml with *water*, and mix.

Assay preparation Dilute an accurately measured volume of Idoxuridine Eye Drops with *water* to obtain a solution containing about 1 mg per ml of idoxuridine. To 15.0 ml of this solution add 2.0 ml of *Internal standard solution*, dilute to 20.0 ml with *water* and mix.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (30 cm × 4 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles, (b) *Mobile phase* at a flow rate of about 1.7 ml per minute, and (c) an ultraviolet photometer set at 254 nm.

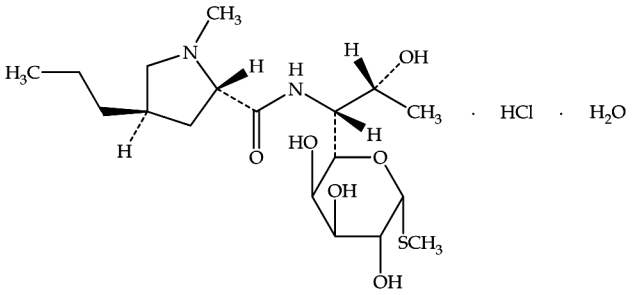
To determine the suitability of the chromatographic system, chromatograph *Standard preparation* and record the peak responses as directed under *Procedure*: the relative standard deviation for replicate injections is not more than 2.0 per cent.

Procedure Separately inject equal volumes (about 10 µl) of *Standard preparation* and *Assay preparation* into the chromatograph, record the chromatograms and measure the responses for the major peaks.

Calculation Calculate the content of $C_9H_{11}IN_2O_5$ in each ml of the Eye Drops taken, using the declared content of $C_9H_{11}IN_2O_5$ in Idoxuridine RS.

Other requirements Comply with the requirements described under "Eye Preparations" (Appendix 1.16).

LINCOMYCIN HYDROCHLORIDE



$C_{18}H_{34}N_2O_6S.HCl.H_2O$ 461.01 *7179-49-9*

D-*erythro*-α-D-*galacto*-Octopyranoside, methyl 6, 8-dideoxy-6-[[(1-methyl-4-propyl-2-pyrrolidinyl) carbonyl]amino]-1-thio-, monohydrochloride, monohydrate, (2*S*-*trans*)-.

Anhydrous 443.00 *859-18-7*

Category Antibacterial.

Lincomycin Hydrochloride has a potency equivalent to not less than 790 µg of $C_{18}H_{34}N_2O_6S$ per mg.

Description White or almost white, crystalline powder; odourless or faint odour.

Solubility Freely soluble in *water*; soluble in *dimethylformamide*; very slightly soluble in *acetone*.

Contra-indication It is contra-indicated in patients with a history of hypersensitivity reactions to clindamycin.

Warning

1. It may cause pseudomembranous colitis, gastro-intestinal disturbances, hypersensitivity reactions, thrombophlebitis, erythema, or pain and swelling at the injection site.

2. It should be used with caution in patients with a history of gastro-intestinal disease, particularly colitis, severe renal impairment, in atopic individuals or in those receiving drugs having neuromuscular blocking action.

3. Rapid intravenous administration may cause hypotension, syncope and cardiac arrest.

4. Due to antagonizing effect, concomitant use with chloramphenicol or erythromycin should be avoided.

5. Risk-benefit should be considered if it is to be used in pregnant or nursing women.

Precaution

1. If clinically important or persistent diarrhea occurs during lincomycin therapy, the drug should be discontinued.

2. Liver and kidney function tests and blood cell counts should be performed periodically.

Packaging and storage Lincomycin Hydrochloride shall be kept in tightly closed containers, protected from light. It shall also be kept under sterile condition.

Labelling The label on the container states (1) storage condition; (2) parenteral grade.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Lincomycin RS (Appendix 2.1) or with the reference spectrum of Lincomycin.

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the Assay.

C. Dissolve about 10 mg in 2 ml of *dilute hydrochloric acid* and heat in a water-bath for 3 minutes. Add 3 ml of *sodium carbonate TS* and 1 ml of a 2 per cent w/v solution of *sodium nitroferricyanide*: a violet-red colour develops.

D. It yields the *reactions* characteristic of chlorides (Appendix 5.1).

Crystallinity It is crystalline (Method I, Appendix 4.14).

pH 3.0 to 5.5, in a 10 per cent w/v solution (Appendix 4.11).

Specific rotation +135° to +150°, calculated on the anhydrous, determined in a 4.0 per cent w/v solution (Appendix 4.8).

Water Not less than 3.0 per cent and not more than 6.0 per cent w/w (Karl Fischer Method, Appendix 4.12).

Lincomycin B Use the chromatogram obtained from Assay preparation in the *Assay*: the area of the lincomycin B peak is not greater than 5.0 per cent of the sum of the areas of the lincomycin B peak and the lincomycin peak.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Add 13.5 ml of *phosphoric acid* to 1000 ml of *water*, and adjust with *strong ammonia solution* to a pH of 6.0. Prepare a mixture of 78 volumes of this solution, 15 volumes of *acetonitrile*, and 15 volumes of *methanol*. Make adjustments if necessary.

Standard preparation Dissolve an accurately weighed quantity of Lincomycin Hydrochloride RS in *Mobile phase* to obtain a solution having a known concentration of about 1.2 mg per ml, using sonication if necessary to effect solution.

Assay preparation To about 12 mg of Lincomycin Hydrochloride, accurately weighed, add 10.0 ml of *Mobile phase*. Shake by mechanical means for 5 minutes, and sonicate if necessary to effect solution.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (25 cm × 4.6 mm) packed with octylsilane chemically bonded to totally porous silica particles (3 to 10 μm), (b) *Mobile phase* at a flow rate of about 1 ml per minute, and (c) an ultraviolet photometer set at 210 nm.

To determine the suitability of the chromatographic system, chromatograph *Standard preparation*, and record the peak responses as directed under *Procedure*: the symmetry factor for the main lincomycin peak is not more than 1.3 and the relative standard deviation for replicate injections is not more than 2.0 per cent. The relative retention times are about 0.5 for lincomycin B and 1.0 for lincomycin.

Procedure Separately inject equal volumes (about 10 μl) of *Standard preparation* and *Assay preparation* into the chromatograph, record the chromatograms and measure the responses for the major peaks.

Calculation Calculate the potency of $C_{18}H_{34}N_2O_6S$ in the Lincomycin Hydrochloride taken, using the declared potency of $C_{18}H_{34}N_2O_6S$ in Lincomycin Hydrochloride RS.

Other requirements Lincomycin Hydrochloride intended for parenteral administration complies with the following additional requirements.

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains not more than 0.5 Endotoxin Unit per mg of lincomycin.

Sterility Complies with the "Sterility Test" (Method I, Appendix 10.1).

LINCOMYCIN HYDROCHLORIDE INJECTION

Category Antibacterial.

Lincomycin Hydrochloride Injection is a sterile solution of Lincomycin Hydrochloride in Water for Injection. It contains an amount of Lincomycin Hydrochloride equivalent to not less than 90.0 per cent and not more than 120.0 per cent of the labelled amount of $C_{18}H_{34}N_2O_6S$.

Description Clear, colourless to slightly yellow solution, having a slight odour.

Strength available 300 mg (base) per ml.

Dose Adults: *Intramuscular*, 600 mg every 12 to 24 hours or *intravenous* 600 mg to 1 g, administered over at least 1 hour, every 8 to 12 hours.

Subconjunctival, 75 mg.

The maximum total dose should not exceed 8 g daily.

Children and infants: *Intramuscular*, 10 mg per kg of body weight every 12 to 24 hours. *Intravenous*, administered over at least 1 hour, 3.3 to 6.7 mg per kg of body weight every 8 hours; or 5 to 10 mg per kg of body weight every 12 hours.

Contra-indication; Warning; Precaution See under *Lincomycin Hydrochloride*, p. 123.

Packaging and storage Lincomycin Hydrochloride Injection shall be kept in single-dose or in multiple-dose containers, preferably of Type I glass, protected from light and stored at a temperature not exceeding 30°.

Labelling The label on the container states the quantity equivalent to the amount of lincomycin.

Identification

A. Add *acetone* to a volume of the injection containing 200 mg of lincomycin hydrochloride until precipitation begins and add a further 20 ml of *acetone*. Filter the precipitate, wash with two 10-ml quantities of *acetone*, dissolve the residue in the minimum of a mixture of 4 volumes of *chloroform* and 1 volume of *methanol*, evaporate to dryness and dry at 60° at a pressure not exceeding 2 kPa (about 15 Torr) for 4 hours: the infrared absorption spectrum of the residue is concordant with the spectrum obtained from Lincomycin RS (Appendix 2.1) or with the reference spectrum of Lincomycin.

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that of the Standard preparation, as obtained in the *Assay*.

pH 3.0 to 5.5 (Appendix 4.11).

Particulate matter Complies with the requirements described under "Particulate Matter in Injections (Small-volume Injections, Appendix 4.27).

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains not more than 0.5 Endotoxin Unit per mg of lincomycin.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

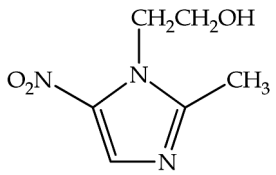
Mobile phase, Standard preparation, Chromatographic system and **Procedure** Proceed as directed in the Assay under *Lincomycin Hydrochloride*, p. 123.

Assay preparation Transfer an accurately measured volume of Lincomycin Hydrochloride Injection, equivalent to about 600 mg of lincomycin, to a 50-ml volumetric flask, dilute with *Mobile phase* to volume, and mix. Transfer 2.0 ml of this solution to a 25-ml volumetric flask, dilute with *Mobile phase* to volume, and mix.

Calculation Calculate the potency of $C_{18}H_{34}N_2O_6S$ in each ml of the Injection taken, using the declared potency of $C_{18}H_{34}N_2O_6S$ in Lincomycin Hydrochloride RS.

Other requirements Complies with the requirements described under "Parenteral Preparations" (Appendix 1.16).

METRONIDAZOLE



$C_6H_9N_3O_3$ 171.15 *443-48-1*

1*H*-Imidazole-1-ethanol, 2-methyl-5-nitro-.

Category Antiprotozoal; antibacterial.

Metronidazole contains not less than 99.0 per cent and not more than 101.0 per cent of $C_6H_9N_3O_3$, calculated on the dried basis.

Description White or pale yellow crystalline powder; odourless.

Solubilily Sparingly soluble in *water* and in *ethanol*; slightly soluble in *chloroform*, and in *ether*.

Stability It darkens on exposure to light.

Contra-indication It is contra-indicated in patients with evidence or a history of blood dyscrasias and with active organic disease of CNS.

Warning

1. It may cause nausea, dizziness, headache, anorexia, dry mouth, a sharp or unpleasant metallic taste, and stomach pain or cramps.

2. It may enhance the activity of warfarin and other anticoagulants.

3. Ingestion of alcohol may precipitate a disulfiram-like reaction.

4. High doses or prolonged use may cause peripheral neuropathy (numbness, tingling, pain, or weakness in hands or feet).

5. Risk-benefit should be considered if it is to be used in pregnant or nursing women.

Precaution

1. Mild leukopenia has been reported. Total and differential leukocyte counts should therefore be made before and after treatment, especially if a second course of therapy is necessary.

2. If abnormal neurological signs appear, treatment with metronidazole should be discontinued promptly.

Additional information

1. To prevent reinfection in trichomoniasis, the sexual partner should receive concurrent therapy.

2. It may cause dark urine without medical significance.

Packaging and storage Metronidazole shall be kept in well-closed containers, protected from light.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Metronidazole RS (Appendix 2.1) or with the reference spectrum of Metronidazole.

B. The ultraviolet absorption spectrum of a 0.001 per cent w/v solution in 0.1 M *hydrochloric acid*, when observed between 230 and 350 nm, exhibits a maximum only at 277 nm; the absorbance of a 1-cm layer at this wavelength is about 0.38 (Appendix 2.2).

C. Dissolve 100 mg in 4 ml of 0.5 M *sulfuric acid*, add 10 ml of *trinitrophenol TS*, and allow to stand: the precipitate, after washing with *water* and drying at 105°, melts at about 150° (Appendix 4.3).

D. Heat 10 mg in a water-bath for 5 minutes with 10 mg of *zinc powder*, 1 ml of *water*, and 5 drops of *hydrochloric acid*, cool in ice, add 0.5 ml of *sodium nitrite TS*, and remove the excess of nitrite with *sulfamic acid*. Add 0.5 ml of the product to a mixture of 0.5 ml of 2-*naphthol TS* and 2 ml of 5 M *sodium hydroxide*: an orange-red colour is produced.

Melting range 159° to 163° (Appendix 4.3).

Loss on drying Not more than 0.5 per cent w/w after drying at 105° for 2 hours (Appendix 4.15).

Heavy metals Not more than 20 ppm (Method III, Appendix 5.2). Use 1 g; for the Standard Preparation, use 2 ml of *lead standard solution (10 ppm Pb)*.

Sulfated ash Not more than 0.1 per cent w/w (Appendix 5.3).

Related substances Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel HF254* as the coating substance and a mixture

of 9 volumes of *chloroform* and 1 volume of *diethylamine* as the mobile phase.

Test solution A Dissolve an accurately weighed quantity of the test substance in *acetone* to obtain a solution containing 20 mg per ml.

Test solution B Dilute *Test solution A* quantitatively with *acetone* to obtain a solution containing 100 μg per ml.

Procedure Apply separately to the plate, 5 μl of each of the solutions. After removal of the plate, allow it to dry in air and examine under ultraviolet light (254 nm). Any spot obtained from *Test solution A*, other than the principal spot, is not more intense than that obtained from *Test solution B*.

Non-basic substances Dissolve 1 g of the test substance in 10 ml of diluted *hydrochloric acid* (1 in 2). The solution is clear.

Assay Dissolve about 450 mg of Metronidazole, accurately weighed, in 10 ml of *anhydrous glacial acetic acid*, warming slightly and cooling if necessary to effect solution. Titrate with 0.1 M *perchloric acid VS*, using *1-naphtholbenzein TS* as indicator or determining the end-point potentiometrically (Appendix 6.1). Perform a blank determination, and make any necessary correction. Each ml of 0.1 M *perchloric acid* is equivalent to 17.12 mg of $C_6H_9N_3O_3$.

METRONIDAZOLE TABLETS

Category Antiprotozoal; antibacterial.

Metronidazole Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the labelled amount of $C_6H_9N_3O_3$.

Strengths available 200, 250, 400, and 500 mg.

Dose Adults—Amebiasis: 500 to 750 mg three times a day for 5 to 10 days.

Trichomoniasis: 2 g as a single dose; 1 g twice a day for 1 day; or 250 mg three times a day for 7 days.

Anaerobic infection: Initially 800 mg, followed by 400 mg every 8 hours for 7 days.

Ulcer, duodenal, *Helicobacter pylori*-associated: 500 mg three times a day, in conjunction with other suitable drugs, for one to two weeks.

Children—Amebiasis: 11.6 to 16.7 mg per kg of body weight three times a day for 5 to 10 days.

Trichomoniasis: 5 mg per kg of body weight three times a day for 7 days.

Anaerobic infection: 7.5 to 10 mg per kg of body weight every 8 hours.

Contra-indication; Warning; Precaution; Additional information See under *Metronidazole*, p. 125.

Packaging and storage Metronidazole Tablets shall be protected from light.

Identification

A. To a portion of the powdered tablets, containing 300 mg of metronidazole, add 20 ml of diluted *hydrochloric acid* (1 in 100), shake for several minutes, and filter: the ultraviolet absorption spectrum of the suitable aliquots of the filtrate in a mixture 1 volume of *sulfuric acid* in 350 volumes of *methanol* when observed between 230 and 350 nm, exhibits a maximum at 278 nm.

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

Dissolution Carry out the test as described in the "Dissolution Test" (Appendix 4.24).

Dissolution medium: 0.1 M *hydrochloric acid*; 900 ml.

Apparatus 1: 100 rpm.

Time: 60 minutes.

Procedure Determine the amount of $C_6H_9N_3O_3$ dissolved from absorbances at the maximum at about 278 nm of filtered portions of the test solution, suitably diluted with *Dissolution medium*, if necessary, in comparison with a standard solution having a known concentration of Metronidazole RS in the same medium (Appendix 2.2).

Tolerances Not less than 85 per cent (*Q*) of the labelled amount of $C_6H_9N_3O_3$ is dissolved in 60 minutes.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Prepare a mixture of 4 volumes of *water* and 1 volume of *methanol*.

Standard preparation Dissolve an accurately weighed quantity of Metronidazole RS in *Mobile phase*, and dilute quantitatively and stepwise with the same solvent to obtain a solution having a known concentration of about 500 μg per ml.

Assay preparation Transfer to a suitable size volumetric flask not less than 10 Metronidazole Tablets, whole or ground, which when diluted with *methanol* will yield a solution having a known concentration of about 10 mg per ml. Add *methanol*, and shake by mechanical means for 30 minutes or until the Tablets are disintegrated. Dilute with *methanol* to volume, and allow the solution to stand until the insoluble material

has settled. Pipette 5.0 ml of the clear supernatant liquid into a 100-ml volumetric flask, dilute with *Mobile phase* to volume, and mix. Filter the solution.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (15 cm × 4.6 mm) packed with octylsilane chemically bonded to totally porous microsilica particles (5 to 10 μm), (b) *Mobile phase* at a flow rate of about 1 ml per minute, and (c) an ultraviolet photometer set at 254 nm.

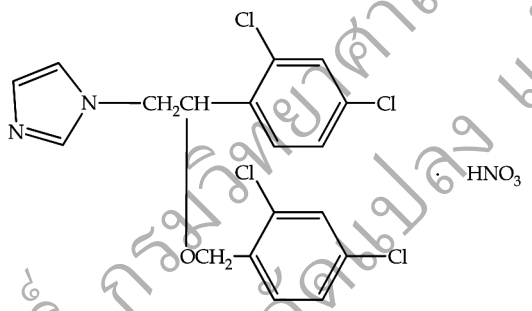
To determine the suitability of the chromatographic system, chromatograph *Standard preparation*, and record the peak responses as directed under *Procedure*: the relative standard deviation for replicate injections is not more than 2.0 per cent and the symmetry factor is not more than 2.0.

Procedure Separately inject equal volumes (about 10 μl) of *Standard preparation* and *Assay preparation* into the chromatograph, record the chromatograms and measure the responses for the major peaks.

Calculation Calculate the content of $C_6H_9N_3O_3$ in the portion of the Tablets taken, using the declared content of $C_6H_9N_3O_3$ in Metronidazole RS.

Other requirements Comply with the requirements described under "Tablets" (Appendix 1.16).

MICONAZOLE NITRATE



$C_{18}H_{14}Cl_4N_2O.HNO_3$ 479.15 *22832-87-7*

1*H*-Imidazole, 1-[2-(2,4-dichlorophenyl)-2-[(2,4-dichlorophenyl)methoxy]ethyl]-, mononitrate.

Category Antifungal (topical).

Miconazole Nitrate contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{18}H_{14}Cl_4N_2O.HNO_3$, calculated on the dried basis.

Description White or almost white, crystalline or microcrystalline powder; odourless or almost odourless.

Solubility Very slightly soluble in *water* and in *ether*; slightly soluble in *chloroform* and in *ethanol*.

Contra-indication See under *Clotrimazole*, p. 83.

Warning

1. It should not be used in children under 2 years of age.
2. It should be used cautiously around the eyes.
3. It may cause skin rash, blistering, burning, redness, or other signs of skin irritation.
4. Risk-benefit should be considered if it is to be used in pregnancy, especially during the first trimester.

Precaution

1. Miconazole nitrate should be discontinued upon the appearance of any symptoms suggesting sensitivity or irritation.
2. When this medication is used in the treatment of candidiasis, occlusive dressing should be avoided since they provide conditions which favour growth of yeast and release of its irritating endotoxin.

Packaging and storage Miconazole Nitrate shall be kept in well-closed containers, protected from light.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Miconazole Nitrate RS (Appendix 2.1) or with the reference spectrum of Miconazole Nitrate.

B. The ultraviolet absorption spectrum of a 0.04 per cent w/v solution in a mixture of 9 volumes of *methanol* and 1 volume of 0.1 M *hydrochloric acid*, when observed between 230 and 350 nm, exhibits three maxima at 264 nm, 272 nm and 280 nm; the absorbances of a 1-cm layer at these wavelengths are about 0.40, 0.58 and 0.48, respectively (Appendix 2.2).

C. Shake 10 mg with 5 ml of *water* and cool in an ice-bath. Keeping the suspension cool throughout, add 8 drops of a 10 per cent w/v solution of *potassium chloride*, 2 drops of *diphenylamine TS* and dropwise, with shaking, 5 ml of *sulfuric acid*: an intense blue colour is produced.

Melting range 178° to 184° (Appendix 4.3).

Clarity of solution A 1.0 per cent w/v solution in *methanol* is clear (Appendix 4.1).

Specific rotation –0.10° to +0.10°, determined in a 1.0 per cent w/v solution in *methanol* (Appendix 4.8).

Loss on drying Not more than 0.5 per cent w/w after drying at 105° for 2 hours (Appendix 4.15).

Sulfated ash Not more than 0.2 per cent w/w (Appendix 5.3).

Related substances Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Prepare a solution of 6.0 g of *ammonium acetate* in a mixture of 300 ml of *acetonitrile*, 320 ml of *methanol* and 380 ml of *water*. Make adjustments if necessary.

Resolution solution Dissolve 2.5 mg of Miconazole Nitrate RS and 2.5 mg of Econazole Nitrate RS in *Mobile phase* and dilute to 100.0 ml with the same solvent.

Test solution (a) Dissolve 100 mg of the test substance in *Mobile phase* and dilute to 10.0 ml with the same solvent.

Test solution (b) Dilute 1.0 ml of *Test solution (a)* to 100.0 ml with *Mobile phase*. Dilute 5.0 ml of this solution to 20.0 ml with the same solvent.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (10 cm × 4.6 mm) packed with octadecylsilane chemically bonded to porous silica particles (3 µm), (b) *Mobile phase* at a flow rate of about 2 ml per minute and (c) an ultraviolet photometer set at 235 nm.

To determine the suitability of the chromatographic system, chromatograph *Resolution solution*, and record the peak responses as directed under *Procedure*: the resolution factor between the miconazole and econazole peaks is not less than 10. Chromatograph *Test solution (b)*, and record the peak responses as directed under *Procedure*: the relative standard deviation for replicate injections is not more than 2.0 per cent.

Procedure Separately inject equal volumes (about 10 µl) of *Test solution (a)* and *Test solution (b)* into the chromatograph, record the chromatograms and measure the responses for the major peaks. Continue the chromatography for 1.2 times the retention time of the principal peak. In the chromatogram obtained from *Test solution (a)*, the area of any peak apart from the principal peak is not greater than the area of the principal peak in the chromatogram obtained from *Test solution (b)* (0.25 per cent); the sum of the areas of the peaks apart from the principal peak is not greater than twice the area of the principal peak in the chromatogram obtained from *Test solution (b)* (0.5 per cent). Disregard any peak due to the nitrate ion and any peak with an area less than 0.2 times the area of the principal peak in the chromatogram obtained from *Test solution (b)*.

Assay Dissolve about 350 mg of Miconazole Nitrate, accurately weighed, in 50 ml of *anhydrous glacial acetic acid*. Titrate with 0.1 M *perchloric acid VS*, determining the end-point potentiometrically (Appendix 6.4). Perform a blank determination, and make any necessary correction. Each ml of 0.1 M *perchloric acid* is equivalent to 47.91 mg of $C_{18}H_{14}C_{l4}N_2O.HNO_3$.

MICONAZOLE NITRATE CREAM

Category Antifungal (topical).

Miconazole Nitrate Cream contains not less than 90.0 per cent and not more than 110.0 per cent of the labelled amount of $C_{18}H_{14}C_{l4}N_2O.HNO_3$.

Strength available 2 per cent w/w.

Dose *Topical*, to the skin, once or twice a day.

Contra-indication See under *Clotrimazole*, p. 83.

Warning; Precaution See under *Miconazole Nitrate*, p. 127.

Packaging and storage Miconazole Nitrate Cream shall be kept in tightly closed containers.

Identification

A. Mix a quantity containing 40 mg of miconazole nitrate with 20 ml of a mixture of 1 volume of 1 M *sulfuric acid* and 4 volumes of *methanol* and shake with two 50-ml quantities of *hexane UV*, discarding the organic layers. Make the aqueous phase alkaline with 2 M *ammonia* and extract with two 40-ml quantities of *chloroform*. Combine the chloroform extracts, shake with 5 g of *anhydrous sodium sulfate*, filter and dilute the filtrate to 100 ml with *chloroform*. Evaporate 50 ml to dryness and dissolve the residue in 50 ml of a mixture of 1 volume of 0.1 M *hydrochloric acid* and 9 volumes of *methanol*. The ultraviolet absorption spectrum of the resulting solution, when observed between 230 and 350 nm, exhibits three maxima at 264 nm, 272 nm and 280 nm (Appendix 2.2).

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

Related substance Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase, Resolution solution and **Chromatographic system** Proceed as directed in the *Assay*.

Test solution (a) Use the *Assay preparation*.

Test solution (b) Dilute 5 volumes of *Test solution (a)* to 100 volumes with a mixture of equal volumes of *methanol* and *tetrahydrofuran* and dilute 5 volumes of the resulting solution to 100 volumes with the same solvent mixture.

Procedure Separately inject equal volumes (about 10 µl) of *Test solution (a)* and *Test solution (b)* into the chromatograph, record the chromatograms and measure the responses for the major peaks. In the chromatogram obtained from *Test solution (a)*, the area of any secondary peak is not more than area of the principal peak in the chromatogram obtained from *Test solution (b)* (0.25 per cent); the sum of the areas of any secondary peaks is not more than twice the area of the principal peak in the chromatogram obtained from *Test solution (b)* (0.5 per cent).

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Prepare a solution of 6.0 g of *ammonium acetate* in a mixture of 300 ml of *acetonitrile*, 320 ml of *methanol* and 380 ml of *water*. Make adjustments if necessary.

Standard preparation Dissolve about 50 mg of Miconazole Nitrate RS, accurately weighted, in a sufficient amount of a mixture of equal volumes of *methanol* and *tetrahydrofuran* and dilute to 50.0 ml with the same solvent.

Resolution solution Dissolve 2.5 mg of Miconazole Nitrate RS and 2.5 mg of Econazole Nitrate RS in a mixture of equal volumes of *methanol* and *tetrahydrofuran* and dilute to 100.0 ml with the same solvent.

Assay preparation Shake an accurately measured quantity of Miconazole Nitrate Cream containing about 50 mg of miconazole nitrate with 30 ml of a mixture of equal volumes of *methanol* and *tetrahydrofuran* for 30 minutes, add sufficient of the same solvent mixture to produce 50.0 ml and filter.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (10 cm × 4.6 mm) packed with octadecylsilane chemically bonded to porous silica particles (3 µm), (b) *Mobile phase* at a flow rate of about 2 ml per minute and (c) an ultraviolet photometer set at 235 nm.

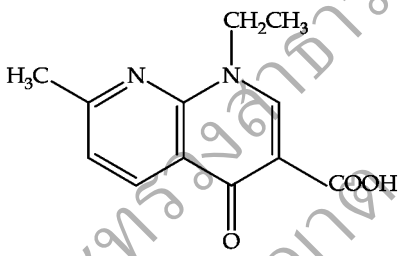
To determine the suitability of the chromatographic system, chromatograph *Resolution solution*, and record the peak responses as directed under *Procedure*: the resolution factor between the miconazole and econazole peaks is not less than 10. Chromatograph *Standard preparation*, and record the peak responses as directed under *Procedure*: the relative standard deviation for replicate injections is not more than 2.0 per cent.

Procedure Separately inject equal volumes (about 10 µl) of *Standard preparation* and *Assay preparation* into the chromatograph, record the chromatograms and measure the responses for the major peaks.

Calculation Calculate the content of $C_{18}H_{14}C_{14}N_2O.HNO_3$ in the portion of the Cream taken, using the declared content of $C_{18}H_{14}Cl_4N_2O.HNO_3$ in Miconazole Nitrate RS.

Other requirements Comply with the requirements described under "Topical Semi-solid Preparations" (Appendix 1.16).

NALIDIXIC ACID



$C_{12}H_{12}N_2O_3$ 232.24 *389-08-2*

1,8-Naphthyridine-3-carboxylic acid, 1-ethyl-1,4-dihydro-7-methyl-4-oxo-.

Category Antibacterial.

Nalidixic Acid contains not less than 98.0 per cent and not more than 102.0 per cent of $C_{12}H_{12}N_2O_3$, calculated on the dried basis.

Description White to very pale yellow, crystalline powder; odourless.

Solubility Very slightly soluble in *water* and in *ether*; soluble in *chloroform*, in *dichloromethane* and in solutions of fixed alkali hydroxides and carbonates; slightly soluble in *acetone*, in *ethanol*, in *methanol*, and in *toluene*.

Contra-indication; Warning; Precaution See under *Norfloxacin*, p. 133.

Additional information

1. The drug should not be used in the treatment of systemic infections where high serum and tissue concentrations of antibacterial agents are desirable.

2. To minimize the development of bacterial resistance, patients should be carefully instructed not to omit doses of the drug, especially during the first few days of therapy.

See also under *Norfloxacin*, p. 133.

Packaging and storage Nalidixic acid shall be kept in tightly closed containers, protected from light.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Nalidixic Acid RS (Appendix 2.1) or with the reference spectrum of Nalidixic Acid.

B. The ultraviolet absorption spectrum of a 0.0005 per cent w/v solution in 0.1 M *sodium hydroxide*, when observed between 230 and 350 nm, exhibits two maxima at 258 and 334 nm. The ratio of the absorbances measured at 258 nm to that measured at 334 nm is 2.2 to 2.4 (Appendix 2.2).

Melting range 225° to 231° (Appendix 4.3).

Loss on drying Not more than 0.5 per cent w/w after drying at 105° for 2 hours (Appendix 4.15).

Sulfated ash Not more than 0.10 per cent w/w (Appendix 5.3).

Heavy metals Not more than 20 ppm (Method II, Appendix 5.2). Use 1.0 g; for the Standard Preparation, use 2 ml of *lead standard solution (10 ppm Pb)*.

Chromatographic purity Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using precoated high-performance thin-layer chromatography *silica gel GF254* as the stationary phase and a mixture of 70 volumes of *ethanol*, 20 volumes of *chloroform* and 10 volumes of 5 M *ammonia* as the mobile phase.

Standard preparations Prepare a solution of Nalidixic Acid RS in *chloroform* containing 1.0 mg per ml. Dilute quantitatively with *chloroform* to obtain *Standard preparations* having the following composition:

Standard preparation	Dilution	Concentration (mg per ml)	Percentage (per cent, for comparison with test substance)
A	1 in 10	0.10	0.5
B	1 in 25	0.04	0.2
C	1 in 50	0.02	0.1

Test preparation Dissolve an accurately weighed quantity of the test substance in *chloroform* to obtain a solution containing 20 mg per ml.

Procedure Apply separately to the plate, 10 µl of each of *Test preparation* and *Standard preparations*. After removal of the plate, allow it to dry with the aid of warm circulating air and examine under ultraviolet light (254 nm). Compare the intensities of any secondary spots observed in the chromatogram of the Test preparation with those of the principal spots in the chromatograms of the Standard preparations: no secondary spot is more intense than the principal spot obtained from the Standard preparation A (0.5 per cent), and the sum of the intensities of all secondary spots obtained from the Test preparation does not exceed 1.0 per cent.

Assay Dissolve about 150 mg of Nalidixic Acid, accurately weighed, in 25 ml of *dimethylformamide*. Protect the contents against atmospheric carbon dioxide by using a cover. With the aid of a magnetic stirrer, titrate with 0.1 M *tetrabutylammonium hydroxide VS*, using *thymolphthalein TS* as indicator or determining the endpoint potentiometrically (Appendix 6.1). Perform a blank determination, and make any necessary correction. Each ml of 0.1 M *tetrabutylammonium hydroxide* is equivalent to 23.22 mg of $C_{12}H_{12}N_2O_3$.

NALIDIXIC ACID TABLETS

Category Antibacterial.

Nalidixic Acid Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the labelled amount of $C_{12}H_{12}N_2O_3$.

Strengths available 250, 500 and 1000 mg.

Dose Adults: 1 g four times daily for 1 to 2 weeks.

Children and infants 3 months of age and over: 55 mg per kg of body weight daily, administered in four equally divided doses.

Contra-indication; Warning; Precaution See under *Norfloxacin*, p. 133.

Additional information

1. The drug should not be used in the treatment of systemic infections where high serum and tissue concentrations of antibacterial agents are desirable.

2. To minimize the development of bacterial resistance, patients should be carefully instructed not to omit doses of the drug, especially during the first few days of therapy.

See also under *Norfloxacin*, p. 133.

Packaging and storage Nalidixic acid Tablets shall be kept in tightly closed containers, protected from light.

Identification To a portion of the powdered tablets, containing 1 g of nalidixic acid, add 50 ml of *chloroform*, shake for 15 minutes, filter, and evaporate the filtrate to dryness. The residue, after drying at 105°, complies with the following tests.

A. The infrared absorption spectrum of the residue is concordant with the spectrum obtained from Nalidixic Acid RS (Appendix 2.1) or with the reference spectrum of Nalidixic Acid.

B. The ultraviolet absorption spectrum of a 0.0008 per cent w/v solution of the residue in 0.1 M *sodium hydroxide*, when observed between 230 and

350 nm, exhibits two maxima at 258 nm and 334 nm (Appendix 2.2).

C. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

D. The residue melts at about 228° (Appendix 4.3).

Dissolution Carry out the test as described in the "Dissolution Test" (Appendix 4.24).

Dissolution medium: pH 8.60 buffer, prepared by mixing 23 volumes of 0.2 M *sodium hydroxide* with 25 volumes of 0.2 M *potassium dihydrogenphosphate* and 20 volumes of *methanol*, cooling, mixing with *water* to obtain 100 volumes of solution, adjusting, if necessary, by the addition of 1 M *sodium hydroxide* to a pH of 8.60±0.05. The initial volume for the test is 900 ml.

Apparatus 2: 60 rpm.

Time: 30 minutes.

Procedure Determine the amount of $C_{12}H_{12}N_2O_3$ dissolved from absorbances at the maximum at about 258 nm of filtered portion of the test solution, suitably diluted with 0.01 M *sodium hydroxide*, if necessary, in comparison with a standard solution having a known concentration of Nalidixic Acid RS in 0.01 M *sodium hydroxide*, using as the blank a mixture of *Dissolution medium* and 0.01 M *sodium hydroxide* in the same proportions as present in the test solution.

Tolerances Not less than 80 per cent (*Q*) of the labelled amount of $C_{12}H_{12}N_2O_3$ is dissolved in 30 minutes.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Prepare a solution of 784 mg of *dipotassium hydrogenphosphate* in 325 ml of *water*. To this solution add a solution of 2.62 g of *hexadecyltrimethylammonium bromide* in 350 ml of *methanol*. To the combined solution add 325 ml of *methanol*, mix and filter. This solution has an apparent pH of about 10. Make adjustments if necessary.

Internal standard solution Prepare a solution of *sulfanilic acid* in *Mobile phase* containing 800 µg per ml.

Standard preparation Dissolve an accurately weighed quantity of Nalidixic Acid RS in *methanol* to obtain a solution having a known concentration of about 180 µg per ml. Transfer 5.0 ml of this solution and 1.0 ml of *Internal standard solution* to a 25-ml volumetric flask, dilute with *methanol* to volume and mix.

Assay preparation Weigh and finely powder not less than 20 Nalidixic Acid Tablets. Transfer an accurately weighed portion of the powder containing about 150 mg of nalidixic acid, to a 500-ml volumetric flask. Add 400 ml of *methanol* and sonicate for 30 minutes. Shake by mechanical means for 30 minutes, sonicate again for 30 minutes, dilute with *methanol* to volume, mix, and filter. Transfer 3.0 ml of the clear filtrate and 1.0 ml of *Internal standard solution* to a 25-ml volumetric flask, dilute with *methanol* to volume and mix.

Procedure Separately inject equal volumes (about 20 µl) of *Standard preparation* and *Assay preparation* into the chromatograph, record the chromatograms, and measure the responses for the major peaks.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (30 cm × 3.9 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles (3 to 10 µm), (b) *Mobile phase* at a flow rate of about 1.5 ml per minute and (c) an ultraviolet photometer set at 254 nm.

To determine the suitability of the chromatographic system, chromatograph *Standard preparation*, and record the peak responses as directed under *Procedure*: the relative retention times are 1.0 for sulfanilic acid and 1.4 for nalidixic acid, the resolution factor between sulfanilic acid and nalidixic acid peaks is more than 1, and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Calculation Calculate the content of $C_{12}H_{12}N_2O_3$ in the portion of the Tablets taken, using the declared content of $C_{12}H_{12}N_2O_3$ in Nalidixic Acid RS.

Other requirements Comply with the requirements described under "Tablets" (Appendix 1.16).

NEOMYCIN SULFATE

Neomycin sulfate *1405-10-3*

Category Antibacterial.

Neomycin Sulfate is a mixture of the sulfates of substances produced by the growth of certain selected strains of *Streptomyces fradiae*. It contains not less than 600 µg of neomycin per mg, calculated on the dried basis.

Description White or yellowish white powder; hygroscopic.

Solubility Freely soluble in *water*; very slightly soluble in *ethanol*; insoluble in *acetone*, in *chloroform* and in *ether*.

Stability It is hygroscopic, but it is relatively stable in the dry state. In aqueous solutions, it may become darkened when stored at room temperature, but its antibacterial potency is still retained for several months.

Therefore, its aqueous solutions should be refrigerated.

Contra-indication It is contra-indicated in patients with a history of hypersensitivity reactions to any member of aminoglycosides.

Warning See under *Gentamicin Sulfate*, p. 111.

Precaution Because of the potential risk of the increased absorption of neomycin sulfate from the inflamed or ulcerated gastro-intestinal tract, it should be used with caution in such conditions.

See also under *Gentamicin Sulfate*, p. 111.

Additional information

1. Because of its potential toxicity, the parenteral use of neomycin is not recommended for any indication.

2. Local application should be limited to the treatment of neomycin-sensitive staphylococcal infections and prolonged topical use should be avoided as it leads to skin sensitization.

Packaging and storage Neomycin Sulfate shall be kept in tightly closed containers, protected from light, and stored at a temperature not exceeding 30°.

Labelling The label on the container states (1) the number of µg of activity per mg; (2) storage condition; (3) sterile or non-sterile grade.

Identification

A. Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel G* as the coating substance and as the mobile phase a mixture of 1 volume of *chloroform*, 3 volumes of *methanol* and 2 volumes of *strong ammonia solution*. Apply separately to the plate, 1 µl of each of two solutions containing (A) 1 mg per ml of the test substance and (B) 1 mg per ml of Neomycin Sulfate RS and at a third point, apply 1 µl of a mixture of equal volumes of solutions (A) and (B). After removal of the plate, allow it to dry in air, spray with a 1 per cent w/v solution of *ninhydrin* in *1-butanol*, and heat at 105° for 2 minutes: the principal red spot in the chromatogram obtained from solution (A) corresponds with that in the chromatogram obtained from solution (B) and the principal red spot in the third chromatogram appears as a single compact spot.

B. Dissolve 10 mg in 5 ml of *water*, add 2 drops of *pyridine* and 2 ml of a 0.1 per cent w/v solution of *ninhydrin* and heat on a water-bath at temperature between 65° and 70° for 10 minutes: a deep violet colour is produced.

C. It yields the *reactions* characteristic of sulfates (Appendix 5.1).

pH 5.0 to 7.5, in a 1.0 per cent w/v solution (Appendix 4.11).

Specific rotation +53.5° to +59.0°, calculated on the dried basis, determined in a 10.0 per cent w/v solution (Appendix 4.8).

Loss on drying Not more than 8.0 per cent w/w after drying at 60° at a pressure not exceeding 0.7 kPa (about 5 Torr) for 3 hours (Appendix 4.15).

Sulfated ash Not more than 1.0 per cent w/w (Appendix 5.3).

Assay Carry out the microbiological assay of Neomycin Sulfate according to the "Microbiological Assay of Antibiotics" (Appendix 6.10).

NEOMYCIN SULFATE TABLETS

Category Antibacterial.

Neomycin Sulfate Tablets contain the equivalent of not less than 90.0 per cent and not more than 125.0 per cent of the labelled amount of neomycin.

Strength available 500 mg.

Dose Adults—Diarrhea, enteropathogenic *Escherichia coli*: 50 mg per kg of body weight daily in four divided doses for 2 to 3 days. A dosage of 3 g daily is usually adequate.

Encephalopathy, hepatic (adjunct): 4 to 12 g daily in four divided doses for 5 to 7 days.

Contra-indication It is contra-indicated in cases of intestinal obstruction or in patients with history of hypersensitivity reactions to any member of aminoglycosides.

Warning See under *Gentamicin Sulfate*, p. 111.

Precaution Because of the potential risk of the increased absorption of neomycin sulfate from the inflamed or ulcerated gastro-intestinal tract, it should be used with caution in such conditions.

See also under *Gentamicin Sulfate*, p. 111.

Additional information See under *Neomycin Sulfate*, p. 132.

Packaging and storage Neomycin Sulfate Tablets shall be kept in tightly closed containers.

Labelling The label on the container states the quantity equivalent to the amount of neomycin.

Identification

A. Comply with test for Identification B described under *Neomycin Sulfate*, p. 132. Apply separately to the

plate, 2 µl of each of the following solutions. For solution (A) shake a portion of the powdered tablets containing 100 mg of neomycin with 25 ml of *water* and filter. Solution (B) is a 4 mg per ml solution of Neomycin Sulfate RS in *water*. At a third point, apply 2 µl of a mixture of equal volumes of solutions (A) and (B).

B. The powdered tablets yield the *reactions* characteristic of sulfates (Appendix 5.1).

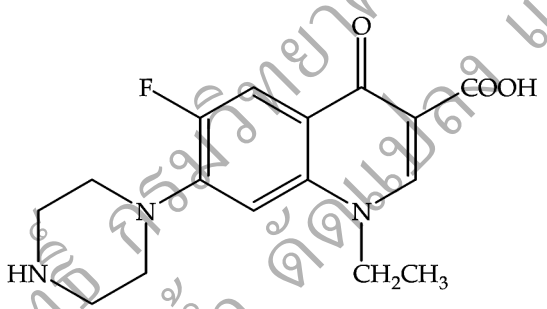
Loss on drying Not more than 10.0 per cent w/w after drying at 60° at a pressure not exceeding 0.7 kPa (about 5 Torr) for 3 hours (Appendix 4.15).

Disintegration Carry out the test as described in the "Disintegration Test for Tablets and Capsules" (Appendix 4.23). Disintegration occurs in not more than 60 minutes.

Assay Weigh and finely powder not less than 20 Neomycin Sulfate Tablets. Shake an accurately weighed portion of the powder with a sufficient accurately measured volume of Buffer 2 to obtain a stock solution having a convenient concentration. Dilute this stock solution quantitatively and stepwise with Buffer 2, and proceed as directed under the microbiological assay of Neomycin according to the "Microbiological Assay of Antibiotics" (Appendix 6.10).

Other requirements Comply with the requirements described under "Tablets" (Appendix 1.16).

NORFLOXACIN



$C_{16}H_{18}FN_3O_3$ 319.33 *70458-96-7*

3-Quinolinecarboxylic acid, 1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-.

Category Antibacterial.

Norfloxacin contains not less than 99.0 per cent and not more than 101.0 per cent of $C_{16}H_{18}FN_3O_3$, calculated on the dried basis.

Description White to pale yellow, crystalline powder.

Solubility Slightly soluble in *water*, in *acetone* and in *ethanol*; freely soluble in *acetic acid*; sparingly soluble in *chloroform*; very slightly soluble in *methanol* and in *ethyl acetate*; insoluble in *ether*.

Stability It is sensitive to light and moisture.

Contra-indication It is contra-indicated in patients with hypersensitivity to quinolone derivatives, with a history of tendinitis or tendon rupture with norfloxacin or any quinolone and during the first trimester of pregnancy, nursing mothers and infants under 3 months.

Warning

1. It should be used with caution in patients with known or suspected CNS disorders (e.g., severe cerebral arterosclerosis, seizure disorders), hepatic or renal function impairment.

2. It may cause nausea, vomiting, fever, headache, drowsiness, malaise, vertigo, visual disturbances, abdominal pain, cholestasis, thrombocytopenia, leukopenia, hemolytic anemia, prolonged QT interval, asthenia, myalgia, arthropathy, myasthenia gravis exacerbation, tendinitis and tendon rupture, angio-edema, allergic skin reactions, photosensitivity, toxic epidermal necrolysis, exfoliative dermatitis, vasculitis, erythema multiform and Stevens-Johnson Syndrome.

3. Caution should be exercised if it is to be used concomitantly with antacids containing magnesium, aluminium or calcium; oral multivitamin and mineral supplements containing divalent or trivalent cations; probenecid; theophylline, caffeine-containing products; sucralfate; oral anticoagulants and drugs that prolong QT interval.

4. Risk-benefit should be considered if it is to be used in prepubertal children and pregnant women during the second and third trimesters.

Precaution

1. Periodic determinations of hepatic, renal and hematopoietic functions are recommended during prolonged therapy.

2. Patients should be cautioned to avoid unnecessary exposure to direct sunlight.

3. Patients who become drowsy when taking this medicine should be cautioned against engaging in activities requiring alertness and skill such as driving a car or operating hazardous machinery or appliances.

4. Patients should be advised to discontinue the drug and inform their physician if they experience pain, swelling, inflammation or rupture of a tendon and to rest and refrain from exercise.

5. Careful monitoring of the patient and periodic *in vitro* susceptibility tests are essential to protect superinfection.

Additional information

1. Patients should be instructed to drink sufficient quantities of fluids to ensure proper hydration and adequate urinary output during therapy.

2. Cross-resistance occurs among the fluoroquinolones.

Packaging and storage Norfloxacin shall be kept in tightly closed containers, protected from light.

Labelling The label on the container states storage condition.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Norfloxacin RS (Appendix 2.1) or with the reference spectrum of Norfloxacin.

B. The ultraviolet absorption spectrum of a 0.0005 per cent w/v in 0.1 M *sodium hydroxide* exhibits a maximum and a minimum at the same wavelengths as that of a similar solution of Norfloxacin RS, concomitantly measured, and the respective absorptivities at about 273 nm, calculated on the dried basis, do not differ by more than 3 per cent. (**Note** Use low-actinic glassware.)

Loss on drying Not more than 1.0 per cent w/w after drying at 100° at a pressure not exceeding 0.7 kPa (about 5 Torr) to constant weight (Appendix 4.15).

Sulfated ash Not more than 0.10 per cent w/w (Appendix 5.3), determined in a platinum crucible.

Heavy metals Not more than 15 ppm (Method II, Appendix 5.2). Use 2.0 g; for the Standard Preparation, use 3 ml of *lead standard solution (10 ppm Pb)*.

Chromatographic purity Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using precoated high-performance thin-layer chromatographic *silica gel GF254* plate (0.25-mm layer) as the stationary phase and a mixture of 40 volumes of *chloroform*, 40 volumes of *methanol*, 20 volumes of *toluene*, 14 volumes of *diethylamine* and 8 volumes of *water* as the mobile phase.

Test solution Dissolve a quantity of the test substance in a mixture of 1 volume of *methanol* and 1 volume of *dichloromethane* to obtain a test solution containing 8.0 mg per ml.

Comparison solution A Dissolve 4.0 mg of Norfloxacin RS in 1 ml of *glacial acetic acid*, add 4 ml of *methanol*, and mix. To 1 ml of this Standard stock solution add 9 ml of the mixture of 1 volume of *methanol* and 1 volume of *dichloromethane* to obtain *Comparison solution A*.

Comparison solution B Dilute a portion of *Comparison solution A* with an equal volume of the mixture of 1 volume of *methanol* and 1 volume of *dichloromethane* to obtain *Comparison solution B*.

Procedure Apply separately to the plate, previously washed with *methanol* and air-dried, 5 µl of *Test solution*, 1, 1.5 and 2 µl of *Comparison solution A* and 5 µl of *Comparison solution B*. The spots of *Comparison solutions A* and *B* are equivalent to 0.2, 0.3, 0.4, and 0.5 per cent of impurities, respectively. Allow the solvent front to move about nine-tenths of the length of the plate. After removal of the plate, allow it to dry in air. Examine under ultraviolet light (254 and 366 nm). Compare the intensities of any secondary spots observed in the chromatogram of the Test solution with those of the principal spots in the chromatograms of the Comparison solutions *A* and *B*: the sum of the intensities of secondary spots obtained from the Test solution corresponds to not more than 0.5 per cent of impurities.

Assay Dissolve about 460 mg of Norfloxacin, accurately weighed, in 100 ml of *anhydrous glacial acetic acid*. Titrate with 0.1 M *perchloric acid VS*, determining the end-point potentiometrically (Appendix 6.1). Perform a blank determination, and make any necessary correction. Each ml of 0.1 M *perchloric acid* is equivalent to 31.93 mg of $C_{16}H_{18}FN_3O_3$.

NORFLOXACIN TABLETS

Category Antibacterial.

Norfloxacin Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the labelled amount of $C_{16}H_{18}FN_3O_3$.

Strengths available 100, 200 and 400 mg.

Dose It should be taken on an empty stomach.

Adults—Gastro-intestinal infection: 400 mg every 12 hours for 3 to 5 days

Traveler's diarrhea: 400 mg every 12 hours for 1 to 3 days.

Gonorrhea or urethritis, gonococcal: 800 mg as a single dose.

Prostatitis: 400 mg every 12 hours for 28 days.

Urinary tract infections, uncomplicated: 400 mg every 12 hours for 3 days.

Urinary tract infections, complicated: 400 mg every 12 hours for 10 to 21 days.

Contra-indication; Warning; Precaution; Additional information See under *Norfloxacin*, p. 133.

Identification

A. Carry out the test as described in the "Thin-layer chromatography" (Appendix 3.1), using *silica gel GF254* as the coating substance and a mixture of 40 volumes of *chloroform*, 40 volumes of *methanol*, 20 volumes of *toluene*, 14 volumes of *diethylamine*, and 8 volumes of *water* as the mobile phase. Apply separately to the plate, 50 µl of each of the following solutions. For solution (A), shake a quantity of finely powdered tablets, equivalent to 75 mg of norfloxacin, with 50 ml of a mixture of an equal volumes of acidic methanol, prepared by mixing 1000 ml of *methanol* and 9 ml of *hydrochloric acid*, and *dichloromethane*. Centrifuge a portion of this suspension, and use the clear supernatant obtained as the test solution. Solution (B) is 1.5 mg per ml of Norfloxacin RS in the same solvent. After removal of the plate, allow it to dry in air for 15 minutes and examine under ultraviolet light (254 nm). The principal spot in the chromatogram obtained from solution (A) corresponds to that obtained from solution (B).

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

Dissolution Carry out the test as described in the "Dissolution Test" (Appendix 4.24).

pH 4.0 buffer To 900 ml of *water* in a 1000-ml volumetric flask add 2.86 ml of *glacial acetic acid* and 1.0 ml of a 50 per cent w/v solution of *sodium hydroxide*, dilute with *water* to volume, and mix. If necessary, adjust with *glacial acetic acid* or the sodium hydroxide solution to a pH of 4.0.

Dissolution medium: *pH 4.0 buffer*; 750 ml.

Apparatus 2: 50 rpm.

Time: 30 minutes.

Procedure Determine the amount of $C_{16}H_{18}FN_3O_3$ dissolved from absorbances at the maximum at about 278 nm of filtered portions of the test solution, suitably diluted with *Dissolution medium*, if necessary, in comparison with a standard solution having a known concentration of Norfloxacin RS in the same medium (Appendix 2.2).

Tolerances Not less than 80 per cent (*Q*) of the labelled amount of $C_{16}H_{18}FN_3O_3$ is dissolved in 30 minutes.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Prepare a mixture of 85 volumes of diluted *phosphoric acid* (1 in 1000), and 15 volumes of *acetonitrile*. Make adjustments if necessary.

Standard preparation Dissolve an accurately weighed quantity of Norfloxacin RS in *Mobile phase* and dilute quantitatively, and stepwise if necessary, with *Mobile phase* to obtain a solution having a known concentration of about 200 µg per ml.

Assay preparation Weigh and finely powder not less than 20 Norfloxacin Tablets. Transfer an accurately weighed portion of the powder containing about 100 mg of norfloxacin, to a 200-ml volumetric flask. Add 80 ml of *Mobile phase*, sonicate for 10 minutes, dilute with diluted *phosphoric acid* (1 in 1000) to volume and mix. Transfer 10.0 ml of this solution to a 25-ml volumetric flask, dilute with *Mobile phase* to volume, mix and filter.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (30 cm × 3.9 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles (3 to 10 µm) maintained at 40°±1°, (b) *Mobile phase* at a flow rate of about 2.0 ml per minute and (c) an ultraviolet photometer set at 275 nm.

To determine the suitability of the chromatographic system, precondition the column with 0.01 M *sodium dihydrogenphosphate* adjusted with *phosphoric acid* to a pH of 4.0, flowing at a rate of 0.5 ml per minute for 8 hours. Chromatograph *Standard preparation*, and record the peak responses as directed under *Procedure*: the capacity factor is not less than 2, the symmetry factor for the norfloxacin peak is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Procedure Separately inject equal volumes (about 10 µl) of *Standard preparation* and *Assay preparation* into the chromatograph, record the chromatograms, and measure the responses for the major peaks.

Calculation Calculate the content of $C_{16}H_{18}FN_3O_3$ in the portion of the Tablets taken, using the declared content of $C_{16}H_{18}FN_3O_3$ in Norfloxacin RS.

Other requirements Comply with the requirements described under "Tablets" (Appendix 1.16).

NYSTATIN

Nystatin *1400-61-9*

Category Antifungal.

Nystatin is a mixture of antifungal polyenes produced by the growth of certain strains of *Streptomyces noursei*. It consists largely of nystatin A_1. It contains not less than 4400 Units per mg and not more than 5000 Units per mg.

Description Yellow to light brown powder; hygroscopic.

Solubility Very slightly soluble in *water*; sparingly soluble in *chloroform*, in *ethanol*, in *ether*, and in *methanol*.

Warning

1. High dose may cause nausea, vomiting, gastrointestinal distress, diarrhea, urticaria and rarely the Stevens-Johnson syndrome.

2. Because it is not absorbed from the gastrointestinal tract, it is not suitable for the treatment of systemic fungal infections.

Packaging and storage Nystatin shall be kept in tightly closed containers, protected from light, and stored at a temperature between 2° and 8°.

Labelling The label on the container states (1) number of Units per mg; (2) storage condition.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Nystatin RS (Appendix 2.1) or with the reference spectrum of Nystatin.

B. Dissolve 100 mg in a mixture of 5.0 ml of *glacial acetic acid* and 50 ml of *methanol* and dilute to 100 ml with *methanol*, dilute 1 ml of this solution to 100 ml with *methanol*. The ultraviolet absorption spectrum of the resulting solution, when immediately observed between 220 and 350 nm, exhibits four maxima at 230 nm, 291 nm, 305 nm and 319 nm, and a shoulder at 280 nm. The ratios of the absorbance at the maxima at 291 nm and 319 nm to the absorbance at the absorption maximum at 305 nm are 0.61 to 0.73 and 0.83 to 0.96, respectively. The ratio of the absorbance measured at the maximum at 230 nm to that measured at the shoulder at 280 nm is 0.83 to 1.25 (Appendix 2.2).

C. To 2 mg add 2 drops of *hydrochloric acid*: a brown colour develops.

pH 6.5 to 8.0, in a 3.0 per cent w/v suspension in *water* (Appendix 4.11).

Loss on drying Not more than 5.0 per cent w/w after drying at 60° at a pressure not exceeding 0.7 kPa (about 5 Torr) for 3 hours (Appendix 4.15).

Sulfated ash Not more than 3.5 per cent w/w (Appendix 5.3).

Assay Carry out the microbiological assay of Nystatin according to the "Microbiological Assay of Antibiotics" (Appendix 6.10).

NYSTATIN ORAL SUSPENSION

Category Antifungal (oral-local).

Nystatin Oral Suspension contains not less than 90.0 per cent and not more than 130.0 per cent of the labelled amount of nystatin. It contains suitable dispersants, flavours, preservatives, and suspending agents.

Strength available 100,000 Units per ml

Dose Adults and children: 400,000 to 600,000 Units four times a day.

Infants: 200,000 Units four times a day.

Premature and low-birth-weight infants: 100,000 Units four times a day.

Warning See under *Nystatin*, p. 136.

Additional information

1. For oral candidiasis, oral suspension should be retained in each side of the mouth as long as possible before swallowing.

2. The drug should be continued for at least two days after symptoms have subsided.

Packaging and storage Nystatin Oral Suspension shall be protected from light and stored at a temperature not exceeding 30°.

Labelling The label on the container states the number of Units per ml.

Identification Shake a portion of the suspension containing 300,000 Units with a mixture of 5.0 ml of *glacial acetic acid* and 50 ml of *methanol*. Add sufficient *methanol* to produce 100 ml, filter and dilute 1 ml of the filtrate to 100 ml with *methanol*. The ultraviolet absorption spectrum of the resulting solution, when immediately observed between 250 and 350 nm, exhibits three maxima at 291 nm, 305 nm and 319 nm. The ratios of the absorbances at the maxima at 291 nm and 319 nm to the absorbance at the maximum at 305 nm are 0.61 to

0.73 and 0.83 to 0.96, respectively. Use in the reference cell a solution prepared in exactly the same manner but omitting the preparation being examined.

pH 4.5 to 6.0, or if it contains glycerol, 5.3 to 7.5 (Appendix 4.11).

Uniformity of dosage units For suspension packaged in single-unit containers, it complies with the test described in the "Uniformity of Dosage Units" (Appendix 4.28).

PROCEDURE FOR CONTENT UNIFORMITY (**Note** Use low-actinic glassware. The correction factor, *F*, calculated as directed in section (4) of Content Uniformity under "Uniformity of Dosage Units" (Appendix 4.28), is invalid if the value obtained by the formula in the second sentence is greater than 25; follow sections (5) and (6), except to substitute 0.750 for 0.9000.) Transfer the well-shaken contents of one container of Nystatin Oral Suspension to a 100-ml volumetric flask, dissolve in *methanol*, dilute with *methanol* to volume, and mix. Dilute an accurately measured volume of this solution quantitatively, and stepwise if necessary, with *methanol* to obtain a concentration of about 25 Units per ml. Measure the absorbance of the resulting solution at the maximum at about 304 nm (Appendix 2.2). Calculate the content, in Units, in the container by comparison with Nystatin RS similarly treated and concurrently examined.

Assay (**Note** Do not use a plastic blender or cover since dimethylformamide can dissolve plastic material.) Carry out the following procedure protected from light. Blend an accurately measured volume of Nystatin Oral Suspension, freshly mixed and free from air bubbles, for 3 to 5 minutes in a high-speed blender with a sufficient accurately measured volume of *dimethylformamide* to obtain a solution of convenient concentration. Dilute an accurately measured portion of this solution quantitatively with *dimethylformamide* to obtain a solution containing about 400 Units per ml. Dilute this stock solution quantitatively and stepwise with Buffer 4, and proceed as directed under the microbiological assay of Nystatin according to the "Microbiological Assay of Antibiotics" (Appendix 6.10).

Other requirements Comply with the requirements described under "Oral Liquids" (Appendix 1.16).

NYSTATIN TABLETS

Category Antifungal.

Nystatin Tablets contain not less than 90.0 per cent and not more than 130.0 per cent of the labelled amount of nystatin.

Strength available 500,000 Units.

Dose Intestinal candiasis—Adults: 500,000 to 1,000,000 Units three times a day.

Children 5 years of age and over: 500,000 Units four times a day.

Warning See under *Nystatin*, p. 136.

Additional information The drug should be continued for at least two days after symptoms have subsided.

Packaging and storage Nystatin Tablets shall be protected from light.

Labelling The label on the container states (1) the number of Units; (2) that they are intended for oral use only.

Identification Comply with the test for Identification described under *Nystatin Oral Suspension*, p. 136. Shake a portion of the powdered tablets containing 300,000 Units with a mixture of 5.0 ml of *glacial acetic acid* and 50 ml of *methanol*. Add sufficient *methanol* to produce 100 ml, filter and dilute 1 ml of the filtrate to 100 ml with *methanol*.

Loss on drying Not more than 5.0 per cent w/w after drying the powdered tablets at 60° at a pressure not exceeding 0.7 kPa (about 5 Torr) for 3 hours (Appendix 4.15).

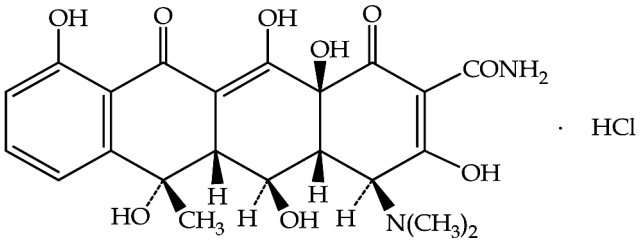
Disintegration Carry out the test described in the "Disintegration Test for Tablets and Capsules" (Appendix 4.23), but using a 0.6 per cent v/v solution of *hydrochloric acid* in place of *water*. If the tablets fail to disintegrate, wash them rapidly by immersion in *water* and continue the test using *phosphate buffer pH 6.8*: the tablets then disintegrate within a further 30 minutes.

Assay (**Note** Do not use a plastic blender or cover since dimethylformamide can dissolve plastic material.) Carry out the following procedure protected from light. Blend not less than five Nystatin Tablets for 3 to 5 minutes in a high-speed blender with a sufficient accurately measured volume of *dimethylformamide* to obtain a solution of convenient concentration. Dilute an accurately measured portion of this solution quantitatively with *dimethylformamide* to obtain a stock solution containing about 400 Units per ml. Dilute this stock solution quantitatively and stepwise with Buffer 4, and

proceed as directed under the microbiological assay of Nystatin according to the "Microbiological Assay of Antibiotics" (Appendix 6.10).

Other requirements Comply with the requirements described under "Tablets" (Appendix 1.16).

OXYTETRACYCLINE HYDROCHLORIDE



$C_{22}H_{24}N_2O_9.HCl$ 496.90 *2058-46-0*

2-Naphthacenecarboxamide, 4-(dimethylamino)-1,4, 4a,5,5a,6,11,12a-octahydro-3,5,6,10,12,12a-hexahydroxy-6-methyl-1,11-dioxo-, [4*S*-(4α,4aα,5α,5aα,6β,12aα)]-, monohydrochloride.

Category Antibacterial; antiprotozoal.

Oxytetracycline Hydrochloride contains not less than 835 µg of $C_{22}H_{24}N_2O_9$ per mg, calculated on the dried basis.

Description Yellow, crystalline powder; taste, bitter; odourless; hygroscopic.

Solubility Freely soluble in *water*, but crystals of oxytetracycline base separate as a result of partial hydrolysis of the hydrochloride. Sparingly soluble in *ethanol* and in *methanol*, and even less soluble in *absolute ethanol*; insoluble in *chloroform* and in *ether*.

Stability It is hygroscopic. In solutions, it is destroyed when pH is less than 2 or more than 7.

Contra-indication; Warning; Precaution; Additional information See under *Tetracycline Hydrochloride*, p. 157.

Packaging and storage Oxytetracycline Hydrochloride shall be kept in tightly closed containers, protected from light.

Labelling The label on the container states parenteral or non-parenteral grade.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Oxytetracycline Hydrochloride RS (Appendix 2.1) or with the reference spectrum of Oxytetracycline Hydrochloride.

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

C. To 0.5 mg add 2 ml of *sulfuric acid*: a deep crimson colour is produced. Add 1 ml of *water*: the colour changes to yellow.

D. It yields the *reactions* characteristic of chlorides (Appendix 5.1).

Crystallinity It is crystalline (Method I, Appendix 4.14).

pH 2.0 to 3.0, in a 10.0 per cent w/v solution (Appendix 4.11).

Loss on drying Not more than 2.0 per cent w/w after drying at 60° at a pressure not exceeding 0.7 kPa (about 5 Torr) for 3 hours (Appendix 4.15).

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Tetrabutylammonium hydrogensulfate solution Dissolve 1 g of *tetrabutylammonium hydrogen sulfate* in 100 ml of *water*. Adjust with 1 M *sodium hydroxide* to a pH of 7.5.

Disodium edetate solution Dissolve 40 mg of *disodium edetate* in 100 ml of *water*. Adjust with 1 M *sodium hydroxide* to a pH of 7.5.

Phosphate buffer pH 7.5 Prepare a mixture of 85 volumes of 0.33 M *dipotassium hydrogenphosphate* and 15 volumes of 0.33 M *sodium dihydrogenphosphate*. Adjust, if necessary, by adding more of the appropriate component to a pH of 7.5.

Mobile phase Transfer, with the aid of 200 ml of *water*, 50 g of *2-methyl-2-propanol* to a 1000-ml volumetric flask. Add 60 ml of *Phosphate buffer pH 7.5*, 50 ml of *Tetrabutylammonium hydrogensulfate solution*, and 10 ml of *Disodium edetate solution*, and dilute with *water* to volume. Make adjustments if necessary.

Standard preparation Dissolve an accurately weighed quantity of Oxytetracycline RS in 0.01 M *hydrochloric acid* to obtain a solution having a known concentration of about 220 µg per ml.

System suitability preparation Dissolve a suitable quantity of *tetracycline hydrochloride* in 0.01 M *hydrochloric acid* to obtain a solution containing about 200 µg per ml. To 3.0 ml of this solution add 1.5 ml of *Standard preparation*, and dilute with *water* to 25.0 ml.

Assay preparation Transfer about 44 mg of Oxytetracycline Hydrochloride, accurately weighed, to a 200-ml volumetric flask, add 25 ml of 0.01 M *hydrochloric acid*, swirl to dissolve, dilute with 0.01 M *hydrochloric acid* to volume, and mix.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (25 cm × 4.6 mm) packed with rigid spherical styrene-divinylbenzene copolymer maintained at 60°±2°, (b) *Mobile phase* at a flow rate of about 1 ml per minute and (c) an ultraviolet photometer set at 254 nm.

To determine the suitability of the chromatographic system, chromatograph *System suitability preparation*, and record the peak responses as directed under *Procedure*: the relative retention times are about 0.6 for oxytetracycline and 1.0 for tetracycline, and the resolution factor between the oxytetracycline and tetracycline peaks is not less than 5. Chromatograph *Standard preparation*, and record the peak responses as directed under *Procedure*: the symmetry factor is not more than 1.25 and the relative standard deviation for replicate injections is not more than 1.0 per cent.

Procedure Separately inject equal volumes (about 20 µl) of *Standard preparation* and *Assay preparation* into the chromatograph, record the chromatograms and measure the responses for the major peaks.

Calculation Calculate the content of $C_{22}H_{24}N_2O_9$ in the Oxytetracycline Hydrochloride taken, using the declared content of $C_{22}H_{24}N_2O_9$ in Oxytetracycline Hydrochloride RS.

OXYTETRACYCLINE HYDROCHLORIDE CAPSULES

Category Antibacterial; antiprotozoal.

Oxytetracycline Hydrochloride Capsules contain the equivalent of not less than 90.0 per cent and not more than 120.0 per cent of the labelled amount of $C_{22}H_{24}N_2O_9$.

Strength available 250 mg (base).

Dose Adults: 250 to 500 mg every 6 hours.

Children over 8 years of age: 6.25 to 12.5 mg per kg of body weight every 6 hours.

Contra-indication; Warning; Precaution; Additional information See under *Tetracycline Hydrochloride*, p. 157.

Packaging and storage Oxytetracycline Hydrochloride Capsules shall be kept in tightly closed containers, protected from light.

Labelling The label on the container states the quantity equivalent to the amount of oxytetracycline.

Identification

A. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

B. To 0.5 mg of the capsule contents add 2 ml of *sulfuric acid*: a deep crimson colour is produced. Add 1 ml of *water*: the colour changes to yellow.

C. Dissolve 2 mg of the capsule contents in 5 ml of a 1 per cent w/v solution of *sodium carbonate* and add 2 ml of *diazobenzenesulfonic acid TS*: a light brown colour is produced.

D. It yields the *reactions* characteristic of chlorides (Appendix 5.1).

Loss on drying Not more than 5.0 per cent w/w after drying about 100 mg of the capsule contents in a capillary-stoppered bottle at 60° at a pressure not exceeding 0.7 kPa (about 5 Torr) for 3 hours (Appendix 4.15).

Dissolution Carry out the test as described in the "Dissolution Test" (Appendix 4.24).

Dissolution medium: *water*; 900 ml.

Apparatus 2: 75 rpm.

Time: 60 minutes.

Procedure Determine the amount of $C_{22}H_{24}N_2O_9$ dissolved from absorbances at the maximum at about 273 nm of filtered portions of the test solution, suitably diluted with *water*, in comparison with a standard solution having a known concentration of Oxytetracycline RS in the same medium, using 5 ml of 0.1 M *hydrochloric acid* to dissolve the standard (Appendix 2.2).

Tolerances Not less than 80 per cent (*Q*) of the labelled amount of $C_{22}H_{24}N_2O_9$ is dissolved in 60 minutes.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Tetrabutylammonium hydrogensulfate solution, Disodium edetate solution, Phosphate buffer pH 7.5, Mobile phase, Standard preparation, System suitability preparation, Chromatographic system, and **Procedure** Proceed as directed in the Assay described under *Oxytetracycline Hydrochloride*, p. 138.

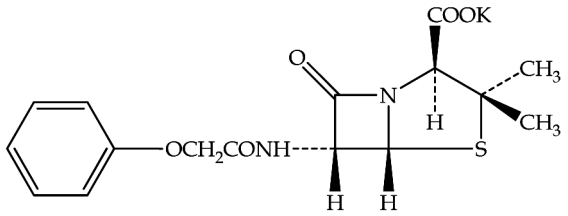
Assay preparation Remove, as completely as possible, the contents of not less than 20 Oxytetracycline Hydrochloride Capsules, and weigh accurately. Mix the combined contents, and transfer an accurately weighed portion, containing about 100 mg of oxytetracycline, to a 500 ml-volumetric flask, add 50 ml of 0.01 M *hydrochloric acid*, and swirl to dissolve. Dilute with 0.01 M S*hydrochloric acid* to volume, mix and filter.

Calculation Calculate the content of $C_{22}H_{24}N_2O_9$ in the portion of the Capsules taken, using the declared content of $C_{22}H_{24}N_2O_9$ in Oxytetracycline Hydrochloride RS.

Other requirements Comply with the requirements described under "Capsules" (Appendix 1.16).

PENICILLIN V POTASSIUM

Phenoxymethylpenicillin Potassium



$C_{16}H_{17}N_2O_5S.K$ 388.48 *132-98-9*

4-Thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid, 3,3-dimethyl-7-oxo-6-[(phenoxyacetyl)amino]-, [2*S*-(2α, 5α,6β)]-, monopotassium salt.

Category Antibacterial.

Penicillin V Potassium contains not less than 85.0 per cent and not more than 91.0 per cent of $C_{16}H_{18}N_2O_5$ S, calculated on the anhydrous basis.

Description White or almost white, crystalline powder; odourless or with a faint characteristic odour.

Solubility Soluble in 1.5 parts of *water*; slightly soluble in *ethanol*; practically insoluble in *chloroform* and in *ether*.

Contra-indication It is contra-indicated in patients who have shown hypersensitivity to any member of the penicillins or of the cephalosporins or other related allergens.

Warning

1. Hypersensitivity reactions are the most common adverse effects noted with the penicillins. Serious and occasionally fatal hypersensitivity (anaphylactoid) reactions may occur, regardless of the route of administration or the dose.

2. Serious superinfections with resistant organisms, especially gram-negative bacteria (e.g., Pseudomonas, Proteus) and Candida, may occur following long-term therapy with penicillins.

3. Massive doses of penicillins may cause hematologic abnormalities (including anemia, thrombocytopenia, leukopenia, neutropenia, and eosinophillia).

4. *Clostridium difficile* colitis may develop in some patients.

5. Concurrent use with bacteriostatic drugs (e.g., tetracyclines, chloramphenicol, etc.) is not recommended.

Precaution Periodic assessment of renal, hepatic and hematologic systems is recommended during long-term therapy.

Additional information Group A beta-hemolytic streptococcal infections should be treated for at least 10 days to prevent the development of acute rheumatic fever or acute glomerulonephritis.

Packaging and storage Penicillin V Potassium shall be kept in tightly closed containers.

Labelling The label on the container states storage condition.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Penicillin V Potassium RS (Appendix 2.1) or with the reference spectrum of Penicillin V Potassium.

B. To 2 mg in a test-tube, add 1 drop of *water* followed by 2 ml of *sulfuric acid* and mix: the solution is colourless. Immerse the test-tube in a water-bath for 1 minute: the solution remains colourless.

C. Place 2 mg in a test-tube, add 1 drop of *water* and 2 ml of *formaldehyde-sulfuric acid TS* and mix: the solution is red. Immerse the test-tube in a water-bath for 1 minute: a red-brown colour is produced.

D. Ignite a small quantity. Dissolve the residue in *water* and filter. To the filtrate, add 2 ml of an 8 per cent w/v solution of *sodium hydroxide*: it yields the *reactions* characteristic of potassium salts (Appendix 5.1).

E. The retention time of the major peak in the chromatogram obtained from Assay preparation corresponds to that in the chromatogram of Standard preparation, as obtained in the *Assay*.

Crystallinity It is crystalline (Method I, Appendix 4.14).

pH 4.0 to 7.5, in a 3.0 per cent w/v solution (Appendix 4.11).

Specific rotation +220.0° to +235.0°, calculated on the dried basis, determined in a 1.0 per cent w/v solution (Appendix 4.8).

Loss on drying Not more than 1.5 per cent w/w after drying at 105° to constant weight (Appendix 4.15)

4-Hydroxypenicillin V Not more than 5.0 per cent w/w. Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5). Using the chromatogram of the Assay preparation obtained as directed in the *Assay*, calculate the percentage of 4-hydroxypenicillin V in the portion of Penicillin V Potassium taken by the formula:

$$100r_p/r_U,$$

in which r_p is the 4-hydroxypenicillin V peak response, and r_U is the sum of the 4-hydroxypenicillin V and penicillin V peak response.

Phenoxyacetic acid Not more than 0.5 per cent w/w. Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Prepare a mixture of 65 volumes of *water*, 35 volumes of *acetonitrile* and 1 volume of *glacial acetic acid*. Make adjustments if necessary.

Diluent Use *pH 6.6 phosphate buffer*.

Standard solution Dissolve an accurately weighed quantity of *phenoxyacetic acid* in *Diluent* to obtain a solution having a known concentration of about 100 µg per ml.

Test solution Dissolve an accurately weighed quantity of the test substance quantitatively in *Diluent* to obtain a solution containing 20.0 mg per ml. (**Note** Use this solution on the day prepared.)

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (25 cm × 4.6 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles (3 to 10 µm), (b) *Mobile phase* at a flow rate of about 1 ml per minute, (c) an ultraviolet photometer set at 254 nm. Chromatograph *Standard solution*, and record the responses as directed under *Procedure*: the symmetry factor is not more than 1.5, and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Procedure (**Note** Use peak areas where peak responses are indicated.) Separately inject equal volumes (about 20 µl) of *Standard solution* and *Test solution* into the chromatograph, record the chromatograms, and measure the responses for the phenoxyacetic acid peaks. Calculate the percentage of phenoxyacetic acid in the portion of Penicillin V taken by the formula:

$$5C(r_U/r_S),$$

in which C is the concentration, in mg per ml, of phenoxyacetic acid in *Standard solution*, and r_U and r_S are the phenoxyacetic acid peak responses obtained from *Test solution* and *Standard solution*, respectively.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Prepare a mixture of 650 volumes of *water*, 350 volumes of *acetonitrile* and 5.75 volumes of *glacial acetic acid*. Make adjustments if necessary.

Resolution solution Prepare a solution in *Mobile phase* containing about 2.5 mg of penicillin G potassium and 2.5 mg of penicillin V potassium per ml.

Standard preparation Dissolve an accurately weighed quantity of Penicillin V Potassium RS, in *Mobile phase*, to obtain a solution having a known concentration of about 2.5 mg per ml.

Assay preparation Transfer about 125 mg of Penicillin V Potassium, accurately weighed, to a 50-ml volumetric flask, dilute with *Mobile phase* to volume, and mix.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (30 cm × 4 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles, (b) *Mobile phase* at a flow rate of about 1.5 ml per minute, and (c) an ultraviolet photometer set at 254 nm.

To determine the suitability of the chromatographic system, chromatograph *Resolution solution*, and record the peak responses as directed under *Procedure*: the relative retention times are about 1.0 for penicillin G and 1.2 for penicillin V, the column efficiency from the penicilin V peak is not less than 1800 theoretical plates, and resolution factor between penicillin G and penicillin V peaks is not less than 3.0. Chromatograph *Standard preparation*, and record the peak response as directed under *Procedure*: the relative standard deviation for replicate injections is not more than 1.0 per cent.

Procedure Separately inject equal volumes (about 10 µl) of *Standard preparation* and *Assay preparation* into the chromatograph, record the chromatograms, and measure the responses for the major penicillin V peaks and any 4-hydroxypenicillin V peaks. The relative retention times are 1.0 for 4-hydroxypenicillin V and 2.5 for penicillin V.

Calculation Calculate the quantity of $C_{16}H_{18}N_2O_5S$, in of the Penicillin V Potassium taken, using the declared content of $C_{16}H_{18}N_2O_5S$ in Penicillin V Potassium RS.

PENICILLIN V POTASSIUM FOR ORAL SOLUTION

Category Antibacterial.

Penicillin V Potassium for Oral Solution is a dry mixture of Penicillin V Potassium and one or more suitable buffers, colours, flavours, preservatives, and suspending agents. It contains the equivalent of not less than 90.0 per cent and not more than 120.0 per cent of the labelled amount of $C_{16}H_{18}N_2O_5S$ when constituted as directed.

Strengths available 62.5 mg (100,000 Units), 125 mg (200,000 Units) and 250 mg (400,000 Units) (base) per 5 ml.

Dose Adults: 125 to 500 mg every 6 to 8 hours.

Children: 25 to 50 mg per kg of body weight in divided doses every 6 to 8 hours.

Contra-indication; Warning; Precaution; Additional information See under *Penicillin V Potassium*, p. 140.

Packaging and storage Penicillin V Potassium for Oral Solution shall be kept in tightly closed containers. After constitution, it should be used within the period stated on the label.

Labelling The label on the container states the quantity equivalent to the amount of penicillin V.

Identification The retention time of the major peak in the chromatogram obtained from Assay preparation corresponds to that in the chromatogram of Standard preparation, as obtained in the *Assay*.

pH 5.0 to 7.5, in the solution constituted as directed in the labelling (Appendix 4.11).

Water Not more than 1.0 per cent w/w (Karl Fischer Method, Appendix 4.12).

Deliverable volume Complies with the requirements described under "Deliverable Volume" (Appendix 4.21).

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase, Resolution solution, Standard preparation, Chromatographic system and **Procedure** Proceed as directed in the Assay under *Penicillin V Potassium*, p. 141.

Assay preparation Transfer an accurately measured volume of Penicillin V Potassium for Oral Solution, constituted as directed in the labelling, equivalent to about 250 mg of penicillin V potassium, to a 100-ml volumetric flask, dilute with *Mobile phase* to volume, and mix. Filter a portion of this solution through a suitable filter of 0.5 μm or finer porosity, and use the filtrate as the Assay preparation.

Calculation Calculate the content of $C_{16}H_{18}N_2O_5S$ in each ml of the constituted Oral Solution taken, using the declared content of $C_{16}H_8N_2O_5S$ in Penicillin V Potassium RS.

Other requirements Complies with the requirements described under "Oral Liquids" (Appendix 1.16).

PENICILLIN V POTASSIUM TABLETS

Category Antibacterial.

Penicillin V Potassium Tablets contain the equivalent of not less than 90.0 per cent and not more than 110.0 per cent of the labelled amount of $C_{16}H_{18}N_2O_5S$.

Strengths available 125 mg (200,000 Units), 250 mg (400,000 Units) and 500 mg (800,000 Units) (base).

Dose Adults: 125 to 500 mg every 6 to 8 hours.

Children: 25 to 50 mg per kg of body weight in divided doses every 6 to 8 hours.

Contra-indication; Warning; Precaution; Additional information See under *Penicillin V Potassium*, p. 140.

Packaging and storage Penicillin V Potassium Tablets shall be kept in tightly closed containers.

Labelling The label on the container states the quantity equivalent to the amount of penicillin V.

Identification The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the standard preparation, as obtained in the *Assay*.

Dissolution Carry out the test as described in the "Dissolution Test" (Appendix 4.24).

Dissolution medium: Use phosphate buffer pH 6.0 prepared as directed in *phosphate buffers*; 900 ml.

Apparatus 2: 50 rpm.

Time: 45 minutes.

Procedure determine the amount of $C_{16}H_{18}N_2O_5S$ of filtered portions of the test solution, suitably diluted with *Dissolution medium*, if necessary, as described in the Assay in comparison with a standard solution having a known concentration of Penicillin V Potassium RS in the same medium.

Tolerances Not less than 75 per cent (*Q*) of the labelled amount of $C_{16}H_{18}N_2O_5S$ is dissolved in 45 minutes.

Loss on drying Not more than 1.5 per cent w/w after drying at 60° at a pressure not exceeding 0.7 kPa (about 5 Torr) for 3 hours (Appendix 4.15).

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase, **Resolution solution, Standard preparation, Chromatographic system** and **Procedure** Proceed as directed in the Assay under *Penicillin V Potassium*, p. 141.

Assay preparation Weigh and finely powder not less than 20 Penicillin V Potassium Tablets. Transfer an accurately weighed portion of the powder, containing about 250 mg of penicillin V, to a 100-ml volumetric flask, dilute with *Mobile phase* to volume, and shake for about 5 minutes. Filter a portion of this solution through a suitable filter of 0.5 μm or finer porosity, and use the filtrate as the Assay preparation.

Calculation Calculate the content of $C_{16}H_{18}N_2O_5S$ in a portion of the Tablets taken, using the declared content of $C_{16}H_{18}N_2O_5S$ in Penicillin V Potassium RS.

Other requirements Comply with the requirements described under "Tablets" (Appendix 1.16).

PURIFIED WATER

H_2O 18.02 *7732-18-5*

PURIFIED WATER IN BULK

Category Pharmaceutic aid.

Purified Water in Bulk is prepared by means of distillation, ion exchange or any other appropriate method, from water that complies with the regulations on water intended for human consumption laid down by the competent authority. It contains no added substance.
(**Note** Purified Water in Bulk is intended for use as an ingredient of official preparations and in tests and assays unless otherwise specified.)

Description Clear, colourless liquid; odourless.

Total organic carbon Complies with the "Total Organic Carbon" (Appendix 4.36). Alternatively the test method for Oxidizable substances may be performed. To 100 ml, add 10 ml of 1 M *sulfuric acid*, and heat to boiling. Add 0.20 ml of 0.020 M *potassium permanganate*, and boil for 5 minutes: the pink colour does not completely disappear.

Water conductivity Complies with the "Water Conductivity" (For Bulk Water, Appendix 4.37).

Microbial limit Total aerobic microbial count does not exceed 2×10^2 CFU per ml, determined by membrane filtration (Appendix 10.2).

PURIFIED WATER IN CONTAINERS

Category Pharmaceutic aid.

Purified Water in Containers is Purified Water in bulk that has been filled and stored in conditions designed to assure the required microbiological quality. It contains no added substances.

Description Clear, colourless liquid; odourless.

Packaging and storage Purified Water in Containers shall be kept in unreactive storage containers that are designed to prevent microbial entry.

Labelling The label on the container states (1) the method of preparation; (2) that it is not for parenteral administration.

Oxidizable substances To 100 ml, add 10 ml of 1 M *sulfuric acid*, and heat to boiling. Add 0.20 ml of 0.020 M *potassium permanganate*, and boil for 5 minutes. The pink colour does not completely disappear. Alternatively the test method for Total Organic Carbon (Appendix 4.36) may be performed.

Water conductivity Complies with the "Water Conductivity" (For Sterile Water, Appendix 4.37).

Microbial limit Total aerobic microbial count does not exceed 2×10^2 CFU per ml, determined by membrane filtration (Appendix 10.2).

PURIFIED WATER, STERILE

Category Pharmaceutic aid (solvent).

Sterile Purified Water is Purified Water sterilized and suitably packaged. It contains no antimicrobial agents.
(**Note** It is used in the preparation of nonparenteral compendial dosage forms or in analytical applications requiring Purified Water where access to a validated Purified Water system is not practical. Do not use Sterile Purified Water in preparations intended for parenteral administration. For such purposes use Water for Injection, Bacteriostatic Water for Injection, or Sterile Water for Injection.)

Description Clear, colourless liquid; odourless.

Packaging and storage Sterile Purified Water shall be kept in suitable tightly closed containers.

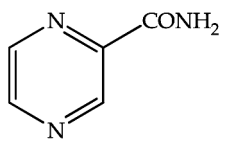
Labelling The label on the container states (1) the method of preparation; (2) that it is not for parenteral administration.

Oxidizable substances To 100 ml, add 10 ml of 1 M *sulfuric acid*, and heat to boiling. For Sterile Purified Water in containers having a fill volume of less than 50 ml, add 0.40 ml of 0.020 M *potassium permanganate*, and boil for 5 minutes; where the fill volume is 50 ml or more, add 0.20 ml of 0.020 M *potassium permanganate*, and boil for 5 minutes. If a precipitate forms, cool in an ice-bath to room temperature, and pass through a sintered-glass filter: the pink colour does not completely disappear.

Water conductivity Complies with the "Water Conductivity" (For Sterile Water, Appendix 4.37).

Sterility Complies with the "Sterility Test" (Appendix 10.1).

PYRAZINAMIDE



$C_5H_5N_3O$ 123.11 *98-96-4*

Pyrazinecarboxamide.

Category Antibacterial (antituberculosis).

Pyrazinamide contains not less than 99.0 per cent and not more than 100.5 per cent of $C_5H_5N_3O$, calculated on the anhydrous basis.

Description White or almost white, crystalline powder; odourless or almost odourless.

Solubility Sparingly soluble in *water*; slightly soluble in *chloroform*, in *ethanol*, and in *ether*.

Contra-indication It is contra-indicated in patients with severe hepatic damage and acute gout.

Warning

1. It should be used with caution in patients with diabetes mellitus, impaired hepatic or renal function and a history of out.

2. It may cause liver damage, anorexia, nausea, vomiting, arthralgia, fever, difficulty in micturition, anemia, porphyria, hyperuricemia, rashes and photosensitivity.

3. Risk-benefit should be considered if it is to be used in pregnant or nursing women.

Precaution

1. Frequent liver function tests and blood uric acid determinations should be performed during treatment.

2. The drug should be discontinued and not to be resumed if signs of hepatocellular damage or hyperuricemia accompanied by an acute gouty arthritis become evident.

Packaging and storage Pyrazinamide shall be kept in well-closed containers, protected from light.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Pyrazinamide RS (Appendix 2.1) or with the reference spectrum of Pyrazinamide.

B. The ultraviolet absorption spectrum of a 0.001 per cent w/v solution, when observed between 230 and 350 nm, exhibits a maximum at 268 nm and a small one at 310 nm; the absorbance of a 1-cm layer at 268 nm is about 0.66 (Appendix 2.2).

C. Dissolve 100 mg in 10 ml of *water* and add 1 ml of a 1.5 per cent w/v solution of *iron(II) sulfate*: an orange-red colour develops turning to blue on the addition of 1 ml of 2 M *sodium hydroxide*.

D. Boil 20 mg with 5 ml of 2 M *sodium hydroxide*: the odour of ammonia is perceptible.

Melting range 188° to 191° (Appendix 4.3).

Water Not more than 0.5 per cent w/w (Karl Fischer Method, Appendix 4.12).

Heavy metals Not more than 10 ppm. Ignite gently 1.0 g until thoroughly charred, cool, add 2 ml of *nitric acid* and 5 drops of *sulfuric acid*, heat cautiously until white fumes are evolved, and ignite until the residue is free of carbon. Cool, add 2 ml of *hydrochloric acid*, evaporate to dryness on a water-bath and dissolve the residue in 20.0 ml of *water*. A 12.0-ml portion of the resulting solution complies with the "Limit Test for Heavy Metals" (Method I, Appendix 5.2). For the Standard Preparation, use *lead standard solution (1 ppm Pb)*.

Sulfated ash Not more than 0.1 per cent w/w (Appendix 5.3); use 1.0 g.

Assay Place about 300 mg of Pyrazinamide, accurately weighed, in a 500-ml Kjeldahl flask, dissolve in 100 ml of *water*, and add 75 ml of 5 M *sodium hydroxide*. Connect the flask by means of a distillation trap to a well-

cooled condenser, the delivery tube of which dips into 20 ml of a 4 per cent w/v solution of *boric acid* contained in a suitable receiver. Boil gently for 20 minutes, avoiding insofar as possible distilling any of the liquid, and then boil vigorously to complete the distillation of the ammonia. Cool the liquid in the receiver if necessary, add *methyl red-methylene blue TS*, and titrate with 0.1 *M hydrochloric acid VS*. Perform a blank determination, and make any necessary correction. Each ml of 0.1 M *hydrochloric acid* is equivalent to 12.31 mg of $C_5H_5N_3O$.

PYRAZINAMIDE TABLETS

Category Antibacterial (antituberculosis).

Pyrazinamide Tablets contain not less than 93.0 per cent and not more than 107.0 per cent of the labelled amount of $C_5H_5N_3O$.

Strength available 500 mg.

Dose In combination with other antituberculosis drugs, 15 to 30 mg per kg of body weight once a day, or 50 to 70 mg per kg of body weight two or three times a week, depending on the treatment regimen. The maximum total dose should not exceed 2 g when taken daily, 3 g per dose for the three times a week regimen or 4 g per dose for the twice a week regimen.

Contra-indication; Warning; Precaution See under *Pyrazinamide*, p. 144.

Additional information The usual dose of pyrazinamide for patients with concomitant HIV infection is 20 to 30 mg per kg of body weight per day for the first two months of therapy.

Packaging and storage Pyrazinamide Tablets shall be protected from light.

Identification

A. Shake a portion of the powdered tablets, containing 250 mg of pyrazinamide, with 20 ml of *absolute ethanol*, filter, evaporate the filtrate to dryness, and dry the residue at 105° for 30 minutes: the infrared absorption spectrum of the residue is concordant with the spectrum obtained from Pyrazinamide RS (Appendix 2.1) or with the reference spectrum of Pyrazinamide.

B. Shake a portion of the powdered tablets, containing 50 mg of pyrazinamide, with 50 ml of *water* and filter. Dilute 1 ml of the filtrate to 100 ml with *water*: the ultraviolet absorption spectrum of the resulting solution, when observed between 230 to 350 nm, exhibits two maxima, at 268 nm and 310 nm (Appendix 2.2).

C. Boil a portion of the powdered tablets, containing 20 mg of pyrazinamide, with 5 ml of 5 M *sodium hydroxide*: ammonia, recognizable by its odour, is evolved.

D. The retention time of the major peak in the chromatogram of the *Assay* preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the Assay.

Dissolution Carry out the test as described in the "Dissolution Test" (Appendix 4.24).

Dissolution medium: *water*; 900 ml.

Apparatus 2: 50 rpm.

Time: 45 minutes.

Procedure Determine the amount of $C_5H_5N_3O$ dissolved from absorbances at the maximum at about 268 nm of filtered portions of the test solution, suitably diluted with *Dissolution medium*, if necessary, in comparison with a standard solution having a known concentration of Pyrazinamide RS in the same medium (Appendix 2.2).

Tolerances Not less than 75 per cent (*Q*) of the labelled amount of $C_5H_5N_3O$ is dissolved in 45 minutes.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Mix 10 ml of *acetonitrile* with 1000 ml of *phosphate buffer pH 8.0*. Make adjustments if necessary.

Standard preparation Transfer an accurately weighed quantity of Pyrazinamide RS to a suitable volumetric flask, dissolve in *water*, sonicating to dissolve, dilute with *water* to volume, and mix to obtain a solution having a known concentration of about 100 µg per ml. Transfer 20.0 ml of the solution to a 50-ml volumetric flask, dilute with *water* to volume, and mix.

System suitability solution Transfer 1 ml of *hydrochloric acid* to a 5-ml volumetric flask, dilute with *Standard preparation* to volume, and mix. Keep this solution on a water-bath for 5 minutes, and cool.

Assay preparation Weigh and finely powder not less than 20 Pyrazinamide Tablets. Transfer an accurately weighed portion of the powder, equivalent to about 100 mg of pyrazinamide, to a 500-ml volumetric flask, add 300 ml of *water*, and sonicate for 10 minutes. Dilute with *water* to volume, and mix. Filter a portion of this solution, discarding the first few ml of the filtrate. Transfer 20.0 ml of this filtrate to a 100-ml volumetric flask, dilute with *water* to volume, and mix.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel

column (15 cm × 3.9 mm) packed with octadecylsilane chemically bonded to totally porous silica particles, (b) *Mobile phase* at a flow rate of about 1 ml per minute, and (c) an ultraviolet photometer set at 270 nm.

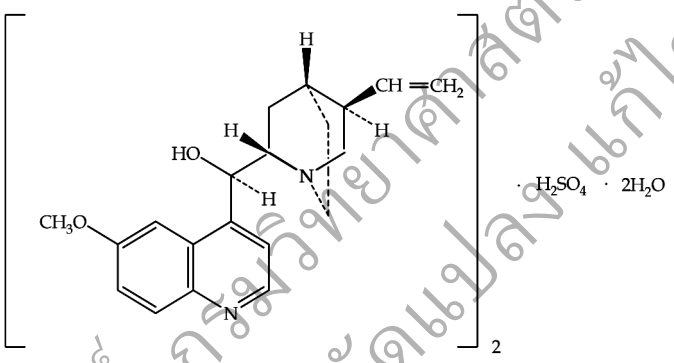
To determine the suitability of the chromatographic system, chromatograph *Standard preparation* and record the peak responses as directed under *Procedure*: the symmetry factor for the pyrazinamide peak is not more than 1.3. Chromatograph *System suitability solution*, and record the peak responses as directed for *Procedure*: the relative retention times are about 1.0 for pyrazinoic acid and 2.2 for pyrazinamide, and the resolution factor between pyrazinamide and the pyrazinoic acid peaks is not less than 6.0.

Procedure Separately inject equal volumes (about 20 µl) of *Standard preparation* and *Assay preparation* into the chromatograph, record the chromatograms and measure the responses for the major peaks.

Calculation Calculate the content of $C_5N_5N_3O$ in the portion of the Tablets taken, using the declared content of $C_5N_5N_3O$ in Pyrazinamide RS.

Other requirement Comply with the requirements described under "Tablets" (Appendix 1.16).

QUININE SULFATE



$(C_{20}H_{24}N_2O_2)_2.H_2SO_4.2H_2O$ 782.94 *6119-70-6*

Cinchonan-9-ol, 6′-methoxy-, (8α,9*R*)-, sulfate(2:1)(salt), dihydrate.

Anhydrous 746.91 *804-63-7*

Category Antiprotozoal (antimalarial).

Quinine Sulfate is the sulfate of an alkaloid, quinine, obtained from the bark of various species of *Cinchona*. It contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of total alkaloids, calculated as $(C_{20}H_{24}N_2O_2)_2.H_2SO_4$, on the dried basis.

Description White, fine, needle-like crystals, usually lusterless, making a light and readily compressible mass; odourless.

Solubility Slightly soluble in *water*, in *chloroform* and in *ethanol*; sparingly soluble in *water* at 100°; freely soluble in *ethanol* at 80°, and in a mixture of 2 volumes of *chloroform* and 1 volume of *absolute ethanol*; very slightly soluble in *ether*.

Stability It darkens on exposure to light. It is incompatible with alkalis and their carbonates, iodides and tannic acid.

Contra-indication It is contra-indicated in patients with severe glucose-6-phosphate dehydrogenase (G-6-PD) deficiency, optic neuritis, tinnitus, history of blackwater fever and thrombocytopenic purpura.

Warning

1. It should be used with caution in patients with myasthenia gravis, hypoglycemia, atrial fibrillation or other serious heart diseases.
2. Risk-benefit should be considered if it is to be used in pregnant or nursing women.

Precaution

1. Quinine should be stopped immediately if evidence of hemolysis appears. Concurrent administration with oral anticoagulants should also be avoided.
2. The repeated administration of quinine in full therapeutic doses may give rise to cinchonism, characterized by tinnitus, headache, nausea, abdominal pain, skin rashes, disturbed vision, and blindness.

Packaging and storage Quinine Sulfate shall be kept in well-closed containers, protected from light.

Identification

A. In the test for *Chromatographic purity* below, the principal spot in the chromatogram obtained from Test solution corresponds in position, colour and intensity to the principal spot in the chromatogram obtained from Standard preparation.

B. Dissolve 5 mg in 5 ml of *water* and add 4 drops of *bromine TS* and 1 ml of 2 M *ammonia*: an emerald-green colour is produced.

C. Dissolve 100 mg in 3 ml of 1 M *sulfuric acid* and dilute to 100 ml with *water*: an intense blue fluorescence develops which disappears almost completely on the addition of 0.1 ml of *hydrochloric acid*.

D. It yields the *reactions* characteristic of sulfates (Appendix 5.2).

pH 5.7 to 6.6, in a 1 per cent w/v suspension (Appendix 4.11).

Specific rotation –235° to –245°, determined in a 2.0 per cent w/v solution in 0.1 M *hydrochloric acid* (Appendix 4.8).

Sulfated ash Not more than 0.1 per cent w/w (Appendix 5.3).

Loss on drying Not less than 3.0 per cent w/w and not more than 5.0 per cent w/w after drying at 105° to constant weight (Appendix 4.15).

Heavy metals Not more than 10 ppm (Method II, Appendix 5.2). Use 2 g; for the Standard Preparation, use *lead standard solution (1 ppm Pb)*.

Chloroform-ethanol-insoluble substances Not more than 0.1 per cent w/w. Warm 2.0 g with 15 ml of a 8 mixture of 2 volumes of *chloroform* and 1 volume of *absolute ethanol* at about 50° for 10 minutes. Filter through a tared, sintered-glass filter, using gentle suction. Wash the filter with five 10-ml portions of the chloroform-ethanol mixture, dry at 105° for 1 hour, and weigh: the weight of the residue does not exceed 2 mg.

Chromatographic purity Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel G* as the coating substance.

Standard preparation Prepare a solution of Quinine Sulfate RS in *ethanol (50 per cent)* to contain 6 mg per ml.

Diluted standard preparation Dilute a portion of *Standard preparation* with *ethanol (50 per cent)* to a concentration of 0.06 mg per ml.

Related substances preparation Prepare a solution in *ethanol (50 per cent)* containing, in each ml, 0.05 mg each of Quininone RS (corresponding to 0.06 mg of quininone sulfate) and 0.10 mg of *cinchonidine* (corresponding to 0.12 mg of cinchonidine sulfate).

Test solution Prepare a solution of the test substance in *ethanol (50 per cent)* to contain 6 mg per ml.

Mobile phase Prepare a mixture of 5 volumes of *chloroform*, 4 volumes of *acetone* and 1 volume of *diethylamine*.

Procedure Apply separately to the plate, 10 µl of each of the solutions. The solvent chamber being used without previous equilibration. After removal of the plate, allow it to dry in air, spray with *glacial acetic acid* and examine under ultraviolet light (366 nm). Any spot produced by *Test solution* at the R_f value of a spot produced by *Related substances preparation* is not greater in size or intensity than that corresponding spot. Apart from these spots and from the spot appearing at the R_f value of quinine sulfate, any additional fluorescent spot is not greater in size or intensity than the spot of *Diluted standard preparation*. Spray the plate with *potassium iodoplatinate TS*. Any spot produced by *Test solution* is not greater in size or intensity than a corresponding spot from *Related substances preparation*.

Limit of dihydroquinine sulfate Not more than 10.0 per cent w/w of dihydroquinine. Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Methanesulfonic acid solution Add 35.0 ml of *methanesulfonic acid* to 20.0 ml of *glacial acetic acid*, dilute with *water* to 500 ml, and mix.

Diethylamine solution Dissolve 10.0 ml of *diethylamine* in *water* to obtain 100 ml of solution.

Mobile phase Prepare a suitable filtered and degassed mixture of 43 volumes of *water*, 5 volumes of *acetonitrile*, 1 volume of *Methanesulfonic acid solution*, and 1 volume of *Diethylamine solution*. Adjust with *Diethylamine solution* to a pH of 2.6 if found to be lower.

System suitability preparation Transfer about 10 mg each of *quinine sulfate* and *dihydroquinine* to a 50-ml volumetric flask. Dissolve in about 5 ml of *methanol*, dilute with *Mobile phase* to volume, and mix.

Test solution Transfer about 20 mg of the test substance to a 100-ml volumetric flask, dissolve in and dilute with *Mobile phase* to volume, and mix.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (30 cm × 3.9 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles (3 to 10 µm), (b) *Mobile phase* at a flow rate of about 1 ml per minute, and (c) an ultraviolet photometer set at 235 nm.

To determine the suitability of the chromatographic system, chromatograph *System suitability preparation*, and record the peak responses as directed under *Procedure*: the relative retention times are about 0.67 for quinine and 1.0 dihydroquinine. The resolution between the quinine and dihydroquinine peaks is not less than 1.2. The relative standard deviation for the peak responses of quinine is not more than 2.0 per cent.

Procedure Inject about 50 µl of the *Test solution* into a chromatograph, record the chromatogram, and measure the responses for the major peaks. The response of the dihydroquinine peak is not greater than one-ninth that of the quinine peak.

Assay Dissolve about 300 mg of Quinine Sulfate, accurately weighed, in a mixture of 10 ml of *chloroform* and 20 ml of *acetic anhydride*. Titrate with 0.1 M *perchloric acid VS*, using *crystal violet TS* as indicator, to a blue-green end-point, or determining the end-point potentio-

metrically (Appendix 6.1). Perform a blank determination, and make any necessary correction. Each ml of 0.1 M *perchloric acid* is equivalent to 24.90 mg of $(C_{20}H_{24}N_2O_2)_2.H_2SO_4$.

QUININE SULFATE TABLETS

Category Antiprotozoal (antimalarial).

Quinine Sulfate Tablets contain not less than 95.0 per cent and not more than 105.0 per cent of the labelled amount of $(C_{20}H_{24}N_2O_2)_2.H_2SO_4.2H_2O$.

Strengths available 260, 300 and 325 mg.

Dose Adults: 600 to 650 mg every 8 hours for 7 days.
Children: 25 mg per kg of body weight daily in divided doses every 8 hours for 7 days.

Contra-indication; Warning; Precaution See under *Quinine Sulfate*, p. 146.

Additional information

1. In the treatment of chloroquine-resistant *Plasmodium falciparum* malaria, quinine is given concurrently with tetracycline or clindamycin.

2. The medication should be taken with or after meals to minimize gastro-intestinal irritation.

Packaging and storage Quinine Sulfate shall be protected from light.

Identification

A. Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel G* as the coating substance and a mixture of 8 volumes of *toluene*, 2 volumes of *acetone* and 1 volume of *diethylamine* as the mobile phase. Apply separately to the plate, 2 µl of each of the following solutions. For solution (A), extract a portion of the powdered tablets, containing 100 mg of quinine sulfate, with 10 ml of a mixture of 2 volumes of *chloroform* and 1 volume of *ethanol* and filter. Solution (B) contains 10 mg per ml of Quinine Sulfate RS in the same solvent mixture. After removal of the plate, allow it to dry in air and spray with 0.05 M *ethanolic sulfuric acid* and then with *dilute potassium iodobismuthate TS*: the principal spot in the chromatogram obtained from solution (A) corresponds to that obtained from solution (B).

B. Extract a portion of the powdered tablets, containing 100 mg of quinine sulfate, with 20 ml of *water* and filter. To 5 ml add 3 drops of *bromine TS* and then 1 ml of *ammonia TS*: a green colour is produced.

C. The filtrate obtained in test B yields *reaction* A characteristic of sulfates (Appendix 5.1).

Chromatographic purity Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel G* as the coating substance.

Standard preparation, Diluted standard preparation, Related substances preparation, Mobile phase and **Procedure** Proceed as directed in the Chromato graphic purity under *Quinine Sulfate*, p. 147.

Test solution Shake a quantity of the powdered Tablets, equivalent to about 150 mg of quinine sulfate, with 25 ml of *ethanol (50 per cent)* for 10 minutes, and filter.

Dissolution Carry out the test as described in the "Dissolution Test" (Appendix 4.24).

Dissolution medium: 0.01 M *hydrochloric acid*; 900 ml.

Apparatus 1: 100 rpm.

Time: 45 minutes.

Procedure Determine the amount of $(C_{20}H_{24}N_2O_2)_2.H_2SO_4.2H_2O$ dissolved from absorbances at the maximum at about 248 nm of filtered portions of the test solution, suitably diluted with 0.01 M *hydrochloric acid*, in comparison with a standard solution having a known concentration of Quinine Sulfate RS in the same medium (Appendix 2.2).

Tolerances Not less than 75 per cent (*Q*) of the labelled amount of $(C_{20}H_{24}N_2O_2)_2.H_2SO_4.2H_2O$ is dissolved in 45 minutes.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Methanesulfonic acid solution Add 35.0 ml of *methanesulfonic acid* to 20.0 ml of *glacial acetic acid*, dilute with *water* to 500 ml, and mix.

Diethylamine solution Dissolve 10.0 ml of *diethylamine* in *water* to obtain 100 ml of solution.

Mobile phase Prepare a mixture of 86 volumes of *water*, 10 volumes of *acetonitrile*, 2 volumes of *Methanesulfonic acid solution*, and 2 volumes of *Diethylamine solution*. Adjust with *Diethylamine solution* to a pH of 2.6 if found to be lower.

System suitability preparation Transfer about 10 mg each of *quinine sulfate* and *dihydroquinine* to a 50-ml volumetric flask. Dissolve in 5.0 ml of *methanol*, dilute with *Mobile phase* to volume, and mix.

Standard preparation Transfer about 20 mg of Quinine Sulfate RS, accurately weighed, to a 100-ml volumetric flask, dissolve in and dilute with *Mobile phase* to volume, and mix.

Assay preparation Weigh and finely powder not less than 20 Quinine Sulfate Tablets. Transfer an accurately weighed portion of the powder, containing about

160 mg of quinine sulfate, to a 100-ml volumetric flask, add 80 ml of *methanol*, and shake the flask by mechanical means for 30 minutes. Dilute with *methanol* to volume, and filter, discarding the first 10 ml of the filtrate. Transfer 3.0 ml of the filtrate to a 25-ml volumetric flask, dilute with *Mobile phase* to volume and mix.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (30 cm × 3.9 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles (3 to 10 μm), (b) *Mobile phase* at a flow rate of about 1 ml per minute, and (c) an ultraviolet photometer set at 235 nm.

To determine the suitability of the chromatographic system, chromatograph *System suitability preparation*, and record the peak responses as directed under *Procedure*: the relative retention times for quinine and dihydroquinine are about 0.67 and 1.0, respectively. The resolution between the quinine and dihydroquinine peaks is not less than 1.2. The relative standard deviation for the peak response of quinine is not more than 2.0 per cent.

Procedure Separately inject equal volumes (5 μl) of *Standard preparation* and *Assay preparation* into the chromatograph, record the chromatograms, and measure the responses for the major peaks.

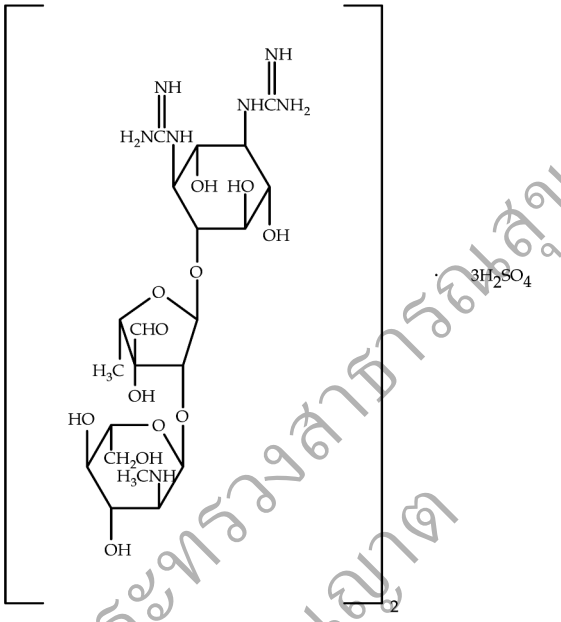
Calculation Calculate the content of $(C_{20}H_{24}N_2O_2)_2.H_2SO_4.2H_2O$ of the sum of quinine sulfate and dihydroquinine sulfate in the portion of the Tablets taken by the expression:

$$(2500/3)\, C(r_{bU} + r_{dU})/(r_{bS} + r_{dS}),$$

in which C is the concentration, in mg per ml, of Quinine Sulfate RS in *Standard preparation*, r_{bU} and r_{bS} are the peak area responses of quinine obtained from *Assay preparation* and *Standard preparation*, respectively, and r_{dU} and r_{dS} are the peak area responses of dihydroquinine obtained from *Assay preparation* and *Standard preparation*, respectively.

Other requirements Comply with the requirements described under "Tablets" (Appendix 1.16).

STREPTOMYCIN SULFATE



$(C_{21}H_{39}N_7O_{12})_2.3H_2SO_4$ 1457.38 *3810-74-0*

D-Streptamine, *O*-2-deoxy-2-(methylamino)-α-L-glucopyranosyl-(1→2)-*O*-5-deoxy-3-*C*-formyl-α-L-lyxofuranosyl-(1→4)-*N*,*N*′-bis(aminoiminomethyl)-, sulfate(2:3)(salt).

Category Antibacterial.

Streptomycin Sulfate contains not less than 650 μg and not more than 850 μg of $C_{21}H_{39}N_7O_{12}$ per mg.

Description White or almost white powder; hygroscopic.

Solubility Very soluble in *water*; practically insoluble in *ethanol* and in *ether*.

Stability It is hygroscopic, but it is relatively stable in the dry state. In aqueous solutions, it may become darkened when stored at room temperature, but its antibacterial potency is still retained for several weeks. Therefore, the aqueous solutions should be refrigerated.

Contra-indication; Warning; Precaution See under *Gentamicin Sulfate*, p. 111.

Additional information Intravenous administration should be avoided.

See also under *Gentamicin Sulfate*, p. 111.

Packaging and storage Streptomycin Sulfate shall be kept in tightly closed containers and stored at a temperature not exceeding 30°.

Labelling The label on the container states (1) the number of μg of activity per mg; (2) storage condition; (3) parenteral grade.

Identification

A. Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel G* as the coating substance and a mixture of 1 volume of *chloroform*, 3 volumes of *methanol* and 2 volumes of *strong ammonia solution* as the mobile phase. Apply separately to the plate, 1 µl of each of the two solutions containing (A) 1 mg per ml of the test substance and (B) 1 mg per ml of Streptomycin Sulfate RS and at a third point, apply 1 µl of a mixture of equal volumes of solutions (A) and (B). After removal of the plate, allow it to dry in air, spray with a 1 per cent w/v solution of *ninhydrin* in *1-butanol*, and heat at 105° for 2 minutes: the principal red spot in the chromatogram obtained from solution (A) corresponds to that in the chromatogram obtained from solution (B) and the principal red spot in the third chromatogram appears as a single compact spot.

B. Dissolve 5 to 10 mg in 4 ml of *water* and add 1 ml of 1 M *sodium hydroxide*. Heat for 4 minutes in a water-bath. Add a slight excess of 2 M *hydrochloric acid* and 2 drops of *iron(III) chloride TS*: a violet colour is produced.

C. Dissolve 100 mg in 2 ml of *water* and add 1 ml of *dilute 1-naphthol TS* and 2 ml of a solution prepared by diluting 1 ml of *sodium hypochlorite solution* to 10 ml with *water*: a red colour is produced.

D. It yields the *reactions* characteristic of sulfates (Appendix 5.1).

Clarity of solution Allow a 25 per cent w/v solution to stand protected from light, at a temperature of about 20° for 24 hours; it is not more opalescent than *reference suspension II* (Appendix 4.1).

pH 4.5 to 7.0, in a 25.0 per cent w/v solution (Appendix 4.11).

Loss on drying Not more than 5.0 per cent w/w after drying at 60° at a pressure not exceeding 0.7 kPa (about 5 Torr) for 3 hours (Appendix 4.15).

Sulfated ash Not more than 1.0 per cent w/w (Appendix 5.3).

Methanol Not more than 0.3 per cent w/w. Carry out the test as described in the "Gas Chromatography (Appendix 3.4).

Test solution Dissolve 1.00 g of the test substance in *water* and dilute to 25.0 ml with the same solvent.

Standard solution Dilute 12.0 mg of *methanol* to 100.0 ml with *water*.

Chromatographic system The chromatographic procedure may be carried out using (a) a glass column (1.5 to 2.0 m × 2 to 4 mm) packed with ethylvinylbenzene-divinylbenzene copolymer (150 to 180 µm), (b) *nitrogen* as the carrier gas at a constant flow rate of 30 to 40 ml per minute, and (c) a flame ionization detector. Maintain the column at a constant temperature between 120° and 140° and the injection port and the detector at a temperature of at least 50° higher than that of the column.

Procedure Separately inject equal volumes of *Standard solution* and *Test solution* into the chromatograph. The area of the peak corresponding to methanol in the chromatogram obtained from *Test solution* is not more than that in the chromatogram obtained from *Standard solution*.

Streptomycin B Not more than 3.0 per cent w/w. Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using silica gel 60 precoated plate and a mixture of 25 volumes of *glacial acetic acid*, 25 volumes of *methanol* and 50 volumes of *toluene* as the mobile phase.

Diluting solution A freshly prepared mixture of 3 volumes of *sulfuric acid* and 97 volumes of *methanol*.

Test solution Dissolve 200 mg of the test substance in *Diluting solution* and dilute to 5 ml with the same solvent. Heat under a reflux condenser for 1 hour, cool, rinse the condenser with *methanol*, and dilute to 20.0 ml with *Diluting solution* (1 per cent w/v solution).

Standard solution Dissolve 36 mg of *D-mannose* in *Diluting solution* and dilute to 5 ml with the same solvent. Heat under a reflux condenser for 1 hour, cool, rinse the condenser with *methanol*, and dilute to 50.0 ml with *Diluting solution*. Dilute 5.0 ml of the solution to 50.0 ml with *methanol* (0.03 per cent w/v solution expressed as streptomycin B; 1 mg of *D-mannose* is equivalent to 4.13 mg of streptomycin B).

Procedure Apply separately to the plate 10 µl of each solution. After removal of the plate, allow it to dry in air and spray with a freshly prepared mixture of equal volumes of a 0.2 per cent w/v solution of *1,3-dihydroxynaphthalene* in *ethanol* and a 20 per cent v/v solution of *sulfuric acid* and heat at 110° for 5 minutes. Any spot corresponding to streptomycin B in the chromatogram obtained from *Test solution* is not more intense than that in the chromatogram obtained from *Standard solution*.

Colourimetric test Dry the test substance and Streptomycin Sulfate RS at 60° over *phosphorus pentoxide desiccant* at a pressure not exceeding 0.1 kPa (about 0.7 Torr) for 24 hours. Dissolve 100.0 mg of the dried test substance in *water* and dilute to 100.0 ml with the same solvent. Prepare a standard solution in the same manner using 100.0 mg of the dried Streptomycin Sulfate RS. Place 5.0 ml of each solution separately in two volumetric flasks and in a third flask place 5 ml of *water*. To each flask add 5.0 ml of 2.0 M *sodium hydroxide* and heat for exactly 10 minutes in a water-bath. Cool in ice for exactly 5 minutes, add 3.0 ml of a 1.5 per cent w/v solution of *ammonium iron(III) sulfate* in 0.5 M *sulfuric acid*, dilute to 25.0 ml with *water* and mix. Exactly 20 minutes after the addition of the ammonium iron(III) sulfate solutions, measure the absorbances (Appendix 2.2) of the test solution and the standard solution in a 2-cm cell at the maximum at 525 nm, using as compensation liquid the solution prepared from 5.0 ml of *water*. The absorbance of the test solution is not less than 90.0 per cent of that of the standard solution.

Assay Carry out the microbiological assay of Streptomycin Sulfate according to the "Microbiological Assay of Antibiotics" (Appendix 6.10).

Other requirements Streptomycin Sulfate intended for parenteral administration complies with the following additional requirements.

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains not more than 0.25 Endotoxin Unit per mg of streptomycin.

Sterility Complies with the "Sterility Test" (Method I, Appendix 10.1).

STREPTOMYCIN SULFATE INJECTION

Category Antibacterial.

Streptomycin Sulfate Injection contains an amount of Streptomycin Sulfate equivalent to not less than 90.0 per cent and not more than 115.0 per cent of the labelled amount of $C_{21}H_{39}N_7O_{12}$.

Description Colourless to yellow solution.

Strength available 0.5 g (base) per ml.

Dose In combination with other antibacterials.

Adults: *Intramuscular*, 1 g daily. Dosage should be reduced to 1 g two or three times a week as soon as clinically feasible. The maximum total dose should not exceed 4 g daily.

Children: *Intramuscular*, 5 to 10 mg per kg of body weight every 6 hours, or 10 to 20 mg per kg of body weight every 12 hours.

Contra-indication; Warning; Precaution See under *Gentamicin Sulfate*, p. 111.

Packaging and storage Streptomycin Sulfate Injection shall be kept in single-dose or in multiple-dose containers, preferably of Type I glass, protected from light and stored at a temperature between 2° to 8°.

Labelling The label on the container states the quantity equivalent to the amount of streptomycin.

Identification

A. Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using a 7 per cent w/v solution of *potassium dihydrogenphosphate* as the mobile phase and the plate prepared in the following manner. Mix 300 mg of *carbomer* with 240 ml of *water*, allow to stand with moderate shaking for 1 hour, adjust to pH 7 by the gradual addition, with constant shaking, of 2 M *sodium hydroxide* and add 30 g of *silica gel H*. Spread a uniform layer of the resulting suspension 0.75 mm thick. Heat the plate at 110° for 1 hour, allow to cool and use immediately. Apply separately to the plate, 10 µl of each of the following solutions. For solution (A) dilute a suitable volume of the injection with sufficient *water* to produce a solution containing the equivalent of 0.08 per cent w/v of streptomycin. Solution (B) contains 0.1 per cent w/v of Streptomycin Sulfate RS in *water*. Solution (C) contains 0.1 per cent w/v of Streptomycin Sulfate RS, 0.1 per cent w/v of Neomycin Sulfate RS and 0.1 per cent w/v of Kanamycin Sulfate RS in *water*. After removal of the plate, allow it to dry in a current of warm air, spray with a mixture of equal volumes of a 0.2 per cent w/v solution of *1,3-naphthalenediol* in *ethanol* and a 46 per cent w/v solution of *sulfuric acid*, and heat at 150° for 5 to 10 minutes: the principal spot in the chromatogram obtained from solution (A) corresponds to that in the chromatogram obtained from solution (B). The test is not valid unless the chromatogram obtained from solution (C) shows three clearly separated principal spots.

B. Dilute 1 drop with 4 ml of *water*, add 1 ml of 1 M *sodium hydroxide* and heat in a water-bath for 4 minutes. Add a slight excess of *hydrochloric acid* and 2 drops of *iron(III) chloride TS*: a violet colour is produced.

pH 5.0 to 8.0 (Appendix 4.11).

Streptomycin B Not more than 3.0 per cent w/w. Carry out the test as described in the Streptomycin B under *Streptomycin Sulfate*, p. 149.

Test solution Dilute a volume of the injection containing the equivalent of 160 mg of the substance in *Diluting solution*.

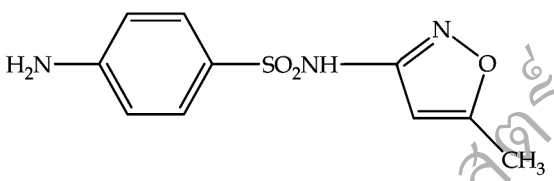
Any spot corresponding to streptomycin B in the chromatogram obtained from *Test solution* is not more intense than the spot in the chromatogram obtained from *Standard solution*.

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains not more than 0.25 Endotoxin Unit per mg of streptomycin.

Assay Dilute an accurately measured volume of Streptomycin Sulfate Injection, equivalent to about 330 mg of streptomycin, to 100 ml with *water*, and proceed as directed under the microbiological assay of Streptomycin Sulfate according to the "Microbiological Assay of Antibiotics" (Appendix 6.10).

Other requirements Complies with the requirements described under "Parenteral Preparations" (Appendix 1.16).

SULFAMETHOXAZOLE



$C_{10}H_{11}N_3O_3S$ 253.28 *723-46-6*

Benzenesulfonamide, 4-amino-*N*-(5-methyl-3-isoxazolyl)-.

Category Antibacterial; antiprotozoal.

Sulfamethoxazole contains not less than 98.5 per cent and not more than 101.0 per cent of $C_{10}H_{11}N_3O_3S$, calculated on the dried basis.

Description White or off-white, crystalline powder.

Solubility Practically insoluble in *water*, in *ether* and in *chloroform*; freely soluble in *acetone* and in dilute solutions of *sodium hydroxide*; sparingly soluble in *ethanol*.

Contra-indication It is contra-indicated in patients with history of hypersensitivity to sulfonamides or chemically related drugs (e.g., furosemide, thiazidediuretics, sulfonylureas, or carbonic anhydrase-inhibitors), in those with porphyria, severe renal or hepatic failure, intestinal and urinary obstruction, blood disorders, systemic lupus erythematosus, glucose-6-phosphate dehydrogenase deficiency (G-6-PD), in infants under 2 months of age (except in the treatment of congenital toxoplasmosis as adjunctive therapy with pyrimethamine), in pregnancy at term, and during the nursing period.

Warning

1. Deaths from hypersensitivity reactions including Stevens-Johnson Syndrome, toxic epidermal necrolysis, fulminant hepatic necrosis, agranulocytosis, aplastic anemia, and other blood dyscrasias, irreversible neuromuscular and CNS changes, and fibrosing alveolitis have been reported. Hemolytic anemia, frequently dose-related, may occur in G-6-PD individuals.

2. It should be used with caution in patients with impaired renal or hepatic function.

3. It may cause nausea, vomiting, hypersensitivity reactions, renal and hepatic impairment, porphyria, blood dyscrasias, or megaloblastic anemia.

4. Caution should be exercised if it is to be used concomitantly with *p*-aminobenzoic acid derivatives, oral anticoagulants, methotrexate, nonsteroidal anti-inflammatory drugs, anticonvulsants, or antidiabetic agents.

5. Risk-benefit must be considered before using this medicine in pregnant women.

Precaution

1. Periodic determinations of hematopoietic, hepatic and renal functions including urinalysis with careful microscopic examination are recommended during therapy with sulfonamides.

2. Therapy should be discontinued if any alteration in the hematopoietic system, a reduction in urine output, development of skin reactions, or impairment of renal or hepatic function occurs.

3. Maintain adequate fluid intake during therapy and for 2 or 3 days thereafter in order to prevent crystalluria and stone formation.

Additional information Avoid prolonged exposure to sunlight since photosensitivity may occur.

Packaging and storage Sulfamethoxazole shall be kept in well-closed containers, protected from light.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Sulfamethoxazole RS (Appendix 2.1) or with the reference spectrum of Sulfamethoxazole.

B. The ultraviolet absorption spectrum of a 0.001 per cent w/v solution in 1 M *sodium hydroxide* exhibits a maximum at 257 nm and a minimum at 224 nm; the absorbance of a 1-cm layer at 257 nm is between 0.64 and 0.69 (Appendix 2.2).

C. Dissolve 20 mg in 0.5 ml of 2 M *hydrochloric acid* and add 1 ml of *water*: the resulting solution yields the *reaction* characteristic of primary aromatic amines (Appendix 5.1), giving an orange-red precipitate.

Melting range 168° to 172° (Appendix 4.3).

Loss on drying Not more than 0.5 per cent w/w after drying at 105° for 4 hours (Appendix 4.15).

Sulfated ash Not more than 0.1 per cent w/w (Appendix 5.3).

Selenium Not more than 30 ppm (Appendix 5.2); use 200 mg.

Sulfanilamide and sulfanilic acid Not more than 0.2 per cent w/w of each. Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1).

Mobile phase Prepare a mixture of 25 volumes of *ethanol*, 25 volumes of n-*heptane*, 25 volumes of *chloroform*, and 7 volumes of *glacial acetic acid*.

Modified Ehrlich's reagent Dissolve 100 mg of *4-dimethylaminobenzaldehyde* in 1 ml of *hydrochloric acid* and dilute with *ethanol* to 100 ml.

Standard solution Dissolve 100 mg of Sulfamethoxazole RS in 0.10 ml of *strong ammonia solution*, dilute with *methanol* to 10.0 ml, and mix.

Reference solution Dissolve 20 mg of *Sulfanilamide* RS and 20 mg of *sulfanilic acid* in 10 ml of *strong ammonia solution*, and dilute with *methanol* to 100.0 ml. Transfer 5.0 ml of the solution to a 50-ml volumetric flask, add 10 ml of *strong ammonia solution*, dilute with *methanol* to volume, and mix.

Test solution Dissolve 100 mg in 0.10 ml of *strong ammonia solution*, dilute with *methanol* to 10.0 ml, and mix.

Procedure Apply separately 10 µl each of *Standard solution*, *Reference solution*, and *Test solution*, to a plate coated with *silica gel G*. Place the plate in an unsaturated chromatographic chamber. After removal of the plate, allow it to dry in air, spray with *Modified Ehrlich's reagent*, and allow the plate to stand for 15 minutes: sulfamethoxazole produces a spot at an R_f value of about 0.7, sulfanilamide at an R_f value of about 0.5, and sulfanilic acid at an R_f value of about 0.1. Any spots produced by sulfanilamide or sulfanilic acid from *Test solution* at the respective R_f values are not greater in size or intensity than spots produced by sulfanilamide or sulfanilic acid from *Reference solution*.

Assay Dissolve about 500 mg of Sulfamethoxazole, accurately weighed, in a mixture of 20 ml of *glacial acetic acid* and 40 ml of *water*, and add 15 ml of *hydrochloric acid*. Proceed as directed under the "Nitrite Titration" (Appendix 6.9), beginning with "Cool the solution...". Each ml of 0.1 M *sodium nitrite* is equivalent to 25.33 mg of $C_{10}H_{11}N_3O_3S$.

SULFAMETHOXAZOLE AND TRIMETHOPRIM ORAL SUSPENSION

Category Antibacterial; antiprotozoal.

Sulfamethoxazole and Trimethoprim Oral Suspension contains not less than 90.0 per cent and not more than 110.0 per cent of the labelled amounts of $C_{10}H_{11}N_3O_3S$ and $C_{14}H_{18}N_4O_3$.

Strength available 200 mg of sulfamethoxazole and 40 mg of trimethoprim per 5 ml.

Dose Adults and children 40 kg of body weight and over—Antibacterial (systemic): 800 mg of sulfamethoxazole and 160 mg of trimethoprim every 12 hours.

Antiprotozoal—*Pneumocystis carinii* pneumonia (PCP)—

Treatment: 18.75 to 25 mg of sulfamethoxazole and 3.75 to 5 mg of trimethoprim per kg of body weight every 6 hours for 14 to 21 days.

Prophylaxis: 800 mg of sulfamethoxazole and 160 mg of trimethoprim once a day.

Acceptable alternative dosing schedules include: 800 mg of sulfamethoxazole and 160 mg of trimethoprim three times a week (e.g., Monday, Wednesday, Friday); or 400 mg of sulfamethoxazole and 80 mg of trimethoprim once a day.

Children up to 40 kg of body weight and infants 2 months of age and over—Antibacterial (systemic): 20 to 30 mg of sulfamethoxazole and 4 to 6 mg of trimethoprim per kg of body weight every 12 hours.

Antiprotozoal—PCP—

Treatment: 18.75 to 25 mg of sulfamethoxazole and 3.75 to 5 mg of trimethoprim per kg of body weight every 6 hours for 14 to 21 days.

Prophylaxis—Children 4 weeks of age and over: 375 mg of sulfamethoxazole per m^2 of body surface area (Appendix 1.17) and 75 mg of trimethoprim per m^2 of body surface area twice a day, three times a week on consecutive days (e.g., Monday, Tuesday, Wednesday).

Acceptable alternative dosing schedules include: 750 mg of sulfamethoxazole per m^2 of body surface area and 150 mg of trimethoprim per m^2 of body surface area as a single daily dose three times a week on consecutive days (e.g., Monday, Tuesday, Wednesday); or 375 mg of sulfamethoxazole per m^2 of body surface area and

75 mg of trimethoprim per m^2 of body surface area twice a day, 7 days a week; or 375 mg of sulfamethoxazole per m^2 of body surface area and 75 mg of trimethoprim per m^2 of body surface area twice a day, three times a week on alternate days (e.g., Monday, Wednesday, Friday).
(**Note** PCP prophylaxis is recommended for all infants born to HIV-infected mothers starting at 4 weeks of age, regardless of their CD4 lymphocyte counts. However, if the infant is receiving zidovudine during the first 6 weeks of life for the prevention of perinatal HIV transmission, sulfamethoxazole and trimethoprim combination prophylaxis should be delayed until zidovudine is discontinued at 6 weeks of age, to reduce the chance of anemia that may occur if these two medications are given concurrently.)

Contra-indication; Warning; Precaution; Additional information See under *Sulfamethoxazole*, p. 152 and *Trimethoprim*, p. 166.

Packaging and storage Sulfamethoxazole and Trimethoprim Oral Suspension shall be kept in tightly closed containers, protected from light.

Identification

A. In tests A and B for *Chromatographic purity*, the respective Test preparations exhibit spots whose R_f values correspond to those spots produced by the Standard preparations of Trimethoprim RS and Sulfamethoxazole RS (R_f about 0.7).

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

pH 5.0 to 6.5 (Appendix 4.11).

Ethanol content Not more than 0.5 per cent v/v (Method II, Appendix 6.5).

Chromatographic purity

TEST A (FOR TRIMETHOPRIM DEGRADATION PRODUCT) Not more than 0.5 per cent w/w. Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1).

Solvent mixture Mix 4 volumes of *chloroform* with 1 volume of *methanol*.

Mobile phase Prepare a mixture of 80 volumes of *chloroform*, 20 volumes of *methanol* and 3 volumes of *strong ammonia solution*.

Standard solution A Prepare a solution of Trimethoprim RS in *Solvent mixture* to contain 20.0 mg per ml.

Standard solution B Dilute an accurately measured volume of *Standard solution A* quantitatively with *Solvent mixture* to obtain a solution having a known concentration of 100 μg per ml.

Test solution Transfer an accurately measured volume of the oral suspension, containing about 40 mg of trimethoprim, to a separator. Extract with three 25-ml portions of *Solvent mixture*, collecting the extracts in a 125-ml conical flask. Evaporate the combined extracts to dryness on a water-bath with the aid of a current of air. Dissolve the residue in 2.0 ml of *Solvent mixture*, and then centrifuge.

Procedure Apply separately 5 μl each of *Test solution*, *Standard solution A*, and *Standard solution B*, to a plate coated with *silica gel G*. After removal of the plate, allow it to dry in air and examine under ultraviolet light (254 nm): trimethoprim produces a spot at an R_f value of about 0.7, and the trimethoprim degradation product can be seen at an R_f value of about 0.3 to 0.5. Any spot from *Test solution* at an R_f value of about 0.3 to 0.5 is not greater in size and intensity than the spot produced by *Standard solution B* at an R_f value of about 0.7.

TEST B (FOR SULFANILAMIDE/SULFANILIC ACID, AND SULFAMETHOXAZOLE N4-GLUCOSIDE) Not more than 0.5 per cent w/w of sulfanilamide, 0.3 per cent w/w of sulfanilic acid and 3.0 per cent w/w of sulfamethoxazole N_4-glucoside. Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1).

Ethanol-methanol mixture Mix 19 volumes of *absolute ethanol* and 1 volume of *methanol*.

Mobile phase Prepare a mixture of 25 volumes of *Ethanol-methanol mixture*, 25 volumes of n-*heptane*, 25 volumes of *chloroform*, and 7 volumes of *glacial acetic acid*.

Modified Ehrlich's reagent Dissolve 100 mg of *4-dimethylaminobenzaldehyde* in 1 ml of *hydrochloric acid* and dilute with *ethanol* to 100 ml.

Standard solution A Weigh 20.0 mg of Sulfamethoxazole RS into a 10-ml volumetric flask, dissolve in 1 ml of *strong ammonia solution*, dilute with *methanol* to volume, and mix.

Standard solution B Weigh 10.0 mg of Sulfanilamide RS into a 50-ml volumetric flask, dissolve in 5 ml of *strong ammonia solution*, and dilute with *methanol* to volume. Pipette 5 ml of this solution into a 100-ml volumetric flask, add 10 ml of *strong ammonia solution*, and dilute with *methanol* to volume.

Standard solution C Weigh 10.0 mg of Sulfanilic Acid RS into a 50-ml volumetric flask, dissolve in 5 ml of *strong ammonia solution*, and dilute with *methanol* to

volume. Pipette 3 ml of this solution into a 100-ml volumetric flask, add 10 ml of *strong ammonia solution*, and dilute with *methanol* to volume.

Standard solution D Weigh 3.0 mg of Sulfamethoxazole N_4-glucoside RS into a 50-ml volumetric flask, dissolve in 5 ml of *strong ammonia solution*, and dilute with *methanol* to volume.

Test solution Transfer an accurately measured volume of the oral suspension, containing 200 mg of sulfamethoxazole, to a 100-ml volumetric flask containing 10 ml of *strong ammonia solution*, and add 50 ml of *methanol*. Shake for 3 minutes, and dilute with *methanol* to volume. Centrifuge a portion of the solution for 3 minutes.

Procedure Apply separately 50 µl each of *Test solution* and *Standard solutions A*, *B*, *C*, and *D* to a plate coated with *silica gel G*. Place the plate in an unsaturated chromatographic chamber. After removal of the plate, allow it to dry in air, spray with *Modified Ehrlich's reagent*, and allow the plate to stand for 15 minutes: sulfamethoxazole produces a spot at an R_f value of about 0.7. Any spots from *Test solution* at an R_f value of about 0.5, 0.1 and 0.3 are not greater in size and intensity than spots produced by *Standard solutions B*, C and D, respectively.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Mix 1400 ml of *water*, 400 ml of *acetonitrile*, and 2.0 ml of *triethylamine* in a 2000-ml volumetric flask. Allow to equilibrate to room temperature, and adjust with 0.2 M *sodium hydroxide* or diluted *glacial acetic acid* (1 in 100) to a pH of 5.9±0.1. Dilute with *water* to volume. Make adjustments if necessary.

Standard preparation Dissolve accurately weighed quantities of Trimethoprim RS and Sulfamethoxazole RS in *methanol*, and dilute quantitatively with *methanol* to obtain a solution containing, in each ml, about 0.32 mg and 0.32*F* mg, respectively, *F* being the ratio of the labelled amount, in mg, of sulfamethoxazole to the labelled amount, in mg, of trimethoprim in the dosage form. Transfer 5.0 ml of this solution to a 50-ml volumetric flask, dilute with *Mobile phase* to volume, and mix to obtain a Standard preparation having known concentrations of about 0.032 mg of Trimethoprim RS per ml and 0.032*F* mg of Sulfamethoxazole RS per ml.

Assay preparation Transfer an accurately measured volume of Sulfamethoxazole and Trimethoprim Oral Suspension, containing about 80 mg of sulfamethoxazole, to a 50-ml volumetric flask with the aid of about 30 ml of *methanol*. Sonicate the mixture for about 10 minutes with occasional shaking. Allow to equilibrate to room temperature, dilute with *methanol* to volume, mix, and centrifuge. Transfer 5.0 ml of the supernatant liquid to a second 50-ml volumetric flask, dilute with *Mobile phase* to volume, mix, and filter.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (30 cm × 3.9 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles (3 to 10 µm), (b) *Mobile phase* at a flow rate of about 2 ml per minute, and (c) an ultraviolet photometer set at 254 nm. Chromatograph *Standard preparation*, and record the peak responses as directed under *Procedure*: the resolution factor between the sulfamethoxazole and trimethoprim peaks is not less than 5.0, and the relative standard deviation for replicate injections is not more than 2.0 per cent. The relative retention times are about 1.8 for sulfamethoxazole and 1.0 for trimethoprim.

Procedure Separately inject equal volumes (about 20 µl) of *Standard preparation* and *Assay preparation* into the chromatograph, record the chromatograms, and measure the responses for the major peaks.

Calculation Calculate the content of $C_{10}H_{11}N_3O_3S$ and $C_{14}H_{18}N_4O_3$ in the Oral Suspension using the declared content of $C_{10}H_{11}N_3O_3S$ in Sulfamethoxazole RS and $C_{14}H_{18}N_4O_3$ in Trimethoprim RS, respectively.

Other requirements Comply with the requirements described under "Oral Liquids" (Appendix 1.16).

SULFAMETHOXAZOLE AND TRIMETHOPRIM TABLETS

Category Antibacterial; antiprotozoal.

Sulfamethoxazole and Trimethoprim Tablets contain not less than 93.0 per cent and not more than 107.0 per cent of the labelled amounts of $C_{10}H_{11}N_3O_3S$ and $C_{14}H_{18}N_4O_3$.

Strengths available 100 mg and 20 mg; 400 and 80 mg; and 800 and 160 mg of sulfamethoxazole and trimethoprim.

Dose Adults and children 40 kg of body weight and over—Antibacterial (systemic): 800 mg of sulfamethoxazole and 160 mg of trimethoprim every 12 hours.

Antiprotozoal—*Pneumocystis carinii* pneumonia (PCP)—

Treatment: 18.75 to 25 mg of sulfamethoxazole and 3.75 to 5 mg of trimethoprim per kg of body weight every 6 hours for 14 to 21 days.

Prophylaxis: 800 mg of sulfamethoxazole and 160 mg of trimethoprim once a day.

Acceptable alternative dosing schedules include: 800 mg of sulfamethoxazole and 160 mg of trimethoprim three times a week (e.g., Monday, Wednesday, Friday); or 400 mg of sulfamethoxazole and 80 mg of trimethoprim once a day.

Children up to 40 kg of body weight and infants 2 months of age and over—Antibacterial (systemic): 20 to 30 mg of sulfamethoxazole and 4 to 6 mg of trimethoprim per kg of body weight every 12 hours.

Antiprotozoal—(PCP)—

Treatment: 18.75 to 25 mg of sulfamethoxazole and 3.75 to 5 mg of trimethoprim per kg of body weight every 6 hours for 14 to 21 days.

Prophylaxis—Children 4 weeks of age and over: 375 mg of sulfamethoxazole per m^2 of body surface area (Appendix 1.17) and 75 mg of trimethoprim per m^2 of body surface area twice a day, three times a week on consecutive days (e.g., Monday, Tuesday, Wednesday).

Acceptable alternative dosing schedules include: 750 mg of sulfamethoxazole per m^2 of body surface area and 150 mg of trimethoprim per m^2 of body surface area as a single daily dose three times a week on consecutive days (e.g., Monday, Tuesday, Wednesday); or 375 mg of sulfamethoxazole per m^2 of body surface area and 75 mg of trimethoprim per m^2 of body surface area twice a day, 7 days a week; or 375 mg of sulfamethoxazole per m^2 of body surface area and 75 mg of trimethoprim per m^2 of body surface area twice a day, three times a week on alternate days (e.g., Monday, Wednesday, Friday).

(**Note** PCP prophylaxis is recommended for all infants born to HIV-infected mothers starting at 4 weeks of age, regardless of their CD4 lymphocyte counts. However, if the infant is receiving zidovudine during the first 6 weeks of life for the prevention of perinatal HIV transmission, sulfamethoxazole and trimethoprim combination prophylaxis should be delayed until zidovudine is discontinued at 6 weeks of age, to reduce the chance of anemia that may occur if these two medications are given concurrently.)

Contra-indication; Warning; Precaution; Additional information See under *Sulfamethoxazole*, p. 152 and *Trimethoprim*, p. 166.

Packaging and storage Sulfamethoxazole and Trimethoprim Tablets shall be protected from light.

Identification

A. Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel GF254* as the coating substance and a mixture of 6 volumes of *chloroform*, 5 volumes of *2-propanol*, and 1 volume of *diethylamine* as the mobile phase. Apply separately to the plate, 5 µl of each of the following solutions. For solution (A), transfer a portion of the powdered tablets, containing 4 mg of trimethoprim, to a 10-ml volumetric flask. Add 8 ml of *methanol*, warm for several minutes on a water-bath with frequent shaking and cool. Dilute with *methanol* to volume, mix and centrifuge briefly. Solution (B) contains 400 µg per ml of Trimethoprim RS in *methanol*. Solution (C) contains 2 mg per ml of Sulfamethoxazole RS in *methanol*. After removal of the plate, allow it to dry in air and examine under ultraviolet light (254 nm): the principal spots in the chromatogram obtained from solution (A) correspond to those obtained from solution (B) and solution (C).

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

Dissolution Carry out the test as described in the "Dissolution Test" (Appendix 4.24).

Dissolution medium: 0.1 M *hydrochloric acid*; 900 ml.

Apparatus 2: 75 rpm.

Time: 60 minutes.

Procedure Determine the amounts of $C_{10}H_{11}N_3O_3S$ and $C_{14}H_{18}N_4O_3$ dissolved, employing the procedure set forth in the *Assay*, making any necessary volumetric adjustments. Calculate the percentage of each active component dissolved by comparison of the peak responses obtained from filtered portions of the test solution with the peak responses from the corresponding component obtained from the Standard preparation.

Tolerances Not less than 70 per cent (*Q*) of the labelled amounts of $C_{10}H_{11}N_3O_3S$ and $C_{14}H_{18}N_4O_3$ are dissolved in 60 minutes.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase, Standard preparation, Chromatographic system, and **Procedure** Proceed as directed in the Assay under *Sulfamethoxazole* and *Trimethoprim Oral Suspension*, p. 155.

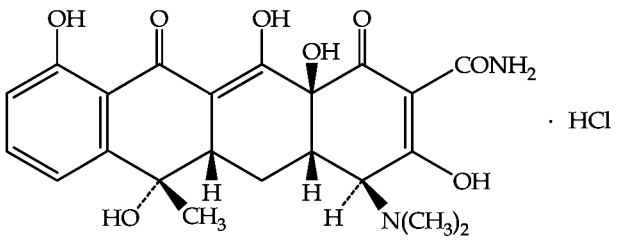
Assay preparation Weigh and finely powder not less than 20 Sulfamethoxazole and Trimethoprim Tablets. Transfer an accurately weighed portion of the powdered tablets, containing about 160 mg of sulfamethoxazole, to a 100-ml volumetric flask. Add about 50 ml of *methanol* and sonicate, with intermittent shaking, for 5 minutes. Allow to equilibrate to room

temperature, dilute with *methanol* to volume, mix, and filter. Transfer 5.0 ml of the clear filtrate to a 50-ml volumetric flask, dilute with *Mobile phase* to volume and mix.

Calculation Calculate the content of $C_{10}H_{11}N_3O_3S$ and $C_{14}H_{18}N_4O_3$ in the Tablets using the declared content of $C_{10}H_{11}N_3O_3S$ in Sulfamethoxazole RS and $C_{14}H_{18}N_4O_3$ in Trimethoprim RS, respectively.

Other requirements Comply with the requirements described under "Tablets" (Appendix 1.16).

TETRACYCLINE HYDROCHLORIDE



$C_{22}H_{24}N_2O_8.HCl$ 480.90 *64-75-5*

2-Naphthacenecarboxamide, 4-(dimethylamino)-1,4,4a, 5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-, [4*S*-(4α,4aα,5aα,6β,12aα)]-, monohydrochloride.

Category Antibacterial; antiprotozoal.

Tetracycline Hydrochloride contains not less than 900 µg of $C_{22}H_{24}N_2O_8$.HCl per mg.

Description Yellow, crystalline powder; odourless; moderately hygroscopic.

Solubility Soluble in *water* and in solutions of alkali hydroxides and carbonates; slightly soluble in *ethanol*; practically insoluble in *chloroform* and in *ether*.

Stability It is moderately hygroscopic. In solutions, it is destroyed when pH is less than 2 or more than 7.

Contra-indication It is contra-indicated in patients with history of hypersensitivity reactions to any member of tetracyclines, and in those with pre-existing renal dysfunction.

Warning

1. It should not be used in children under 8 years of age, in pregnant women or in nursing mothers.

2. It should be avoided in patients with systemic lupus erythematosus, impaired liver function and myasthenia gravis.

3. The degradation products of tetracyclines (epianhydrotetracyclines and anhydrotetracyclines) cause "Fanconi syndrome" with nausea, vomiting, proteinuria, glycosuria and amino-aciduria, commencing within 2 or 3 days of treatment.

4. It may cause discolouration of infants' or children's teeth, photosensitivity, nephrogenic diabetes insipidus (with demeclocycline), pigmentation of skin and mucous membranes (with minocycline), benign intracranial hypertension, hepatotoxicity, and pancreatitis.

5. Concomitant therapy with penicillins; antacids containing aluminium, calcium, magnesium, or other divalent or trivalent cations; calcium or iron supplements; cholestyramine; colestipol; choline; estrogen-containing oral contraceptives; magnesium salicylate; and magnesium-containing laxatives should be avoided.

6. High doses may cause nausea, vomiting, diarrhea, dry mouth, glossitis, stomatitis, and dysphagia.

Precaution

1. Prolonged therapy may result in bacterial or fungal overgrowth of non-susceptible organisms some of which may be fatal (e.g., fulminating enteritis due to resistant staphylococci).

2. Prolonged therapy requires periodic monitoring of organ system functions, including renal, hepatic and hematopoietic.

3. Group A beta-hemolytic streptococcal infections should be treated for at least 10 days to help prevent the development of acute rheumatic fever or acute glomerulonephritis.

Additional information

1. It should be taken on an empty stomach, or with food if irritation to stomach occurs, but avoid concurrent ingestion of milk or other dairy products.

2. Darkened or discoloured tongue may be alarming to patients although medically insignificant.

Packaging and storage Tetracycline Hydrochloride shall be kept in tightly closed containers, protected from light.

Labelling The label on the container states (1) storage condition; (2) parenteral or non-parenteral grade.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Tetracycline Hydrochloride RS (Appendix 2.1) or with the reference spectrum of Tetracycline Hydrochloride.

B. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

C. To 0.5 mg add 2 ml of *sulfuric acid*: a purplish red colour is produced. Add 1 ml of *water*: the colour becomes yellow.

D. It yields the *reactions* characteristic of chlorides (Appendix 5.1).

Crystallinity It is crystalline (Method I, Appendix 4.14).

pH 1.8 to 2.8, in a 1.0 per cent w/v solution (Appendix 4.11).

Specific rotation –240° to –255°, calculated on the dried basis, determined in a 0.5 per cent w/v solution in 0.1 M *hydrochloric acid* (Appendix 4.8).

Loss on drying Not more than 2.0 per cent w/w after drying at 60° at a pressure not exceeding 0.7 kPa (about 5 Torr) for 3 hours (Appendix 4.15).

Heavy metals Not more than 50 ppm (Method II, Appendix 5.2). Use 500 mg; for the Standard Preparation, use 2.5 ml of *lead standard solution (10 ppm Pb)*.

4-Epianhydrotetracycline Not more than 2.0 per cent w/w. Complies with the test as described under *Assay*, using Diluting solvent, Assay preparation, Chromatographic system and Procedure.

Standard solution Dissolve an accurately weigh quantity of 4-Epianhydrotetracycline RS in *Diluting solvent* and dilute quantitatively with *Diluting solvent* to obtain a solution having a known concentration of about 10 µg per ml.

Calculation Calculate the percentage of 4-epianhydrotetracycline hydrochloride in the Tetracycline Hydrochloride taken, using the declared content of 4-epianhydrotetracycline in 4-Epianhydrotetracycline RS.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Diluting solvent Mix 680 ml of 0.1 M *ammonium oxalate* and 270 ml of *dimethylformamide*.

Mobile phase Mix 68 volumes of 0.1 M *ammonium oxalate*, 27 volumes of *dimethylformamide* and 5 volumes of 0.2 M *diammonium hydrogenphosphate*. Adjust, if necessary, with 3 M *ammonia* or 3 M *phosphoric acid* to a pH of 7.6 to 7.7. Make any other necessary adjustments.

Resolution solution Prepare a solution in *Diluting solvent* containing 100 g of tetracycline hydrochloride and 25 µg of 4-Epianhydrotetracycline Hydrochloride RS per ml.

Standard preparation Dissolve an accurately weighed quantity of Tetracycline Hydrochloride RS, in *Diluting solvent* and dilute quantitatively with *Diluting solvent* to obtain a solution having a known concentration of about 500 µg per ml.

Assay preparation Transfer about 50 mg of Tetracycline Hydrochloride, accurately weighed, to a 100-ml volumetric flask, dissolve in, dilute with *Diluting solvent* to volume, and mix.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel guard column (3 cm × 4.6 mm) packed with octylsilane chemically bonded to totally porous silica particles (10 µm) and a stainless steel analytical column (25 cm × 4.6 mm) packed with octylsilane chemically bonded to totally porous silica particles (5 to 10 µm), (b) *Mobile phase* at a flow rate of about 2 ml per minute and (c) an ultraviolet photometer set at 280 nm.

To determine the suitability of the chromatographic system, chromatograph *Resolution solution*, and record the peak responses as directed under *Procedure*: the relative retention times are about 0.9 for 4-epianhydrotetracycline and 1.0 for tetracycline and the resolution factor between the 4-epianhydrotetracycline and tetracycline peaks is not less than 1.2. Chromatograph *Standard preparation*, and record the peak responses as directed under *Procedure*: the relative standard deviation for replicate injections is not more than 2.0 per cent.

Procedure Separately inject equal volumes (about 20 µl) of *Standard preparation* and *Assay preparation* into the chromatograph, record the chromatograms and measure the responses for the major peaks.

Calculation Calculate the content of $C_{22}H_{24}N_2O_8$.HCl in the Tetracycline Hydrochloride taken, using the declared content of $C_{22}H_{24}N_2O_8$.HCl in Tetracycline Hydrochloride RS.

TETRACYCLINE HYDROCHLORIDE CAPSULES

Category Antibacterial; antiprotozoal.

Tetracycline Hydrochloride Capsules contain not less than 90.0 per cent and not more than 125.0 per cent of the labelled amount of $C_{22}H_{24}N_2O_8$.HCl.

Strengths available 250 and 500 mg.

Dose Adults: 250 to 500 mg every 6 hours or up to 4 g daily.

Children over 8 years of age: 25 to 50 mg per kg of body weight in four divided doses.

Contra-indication; Warning; Precaution; Additional information See under *Tetracycline Hydrochloride*, p. 157.

Packaging and storage Tetracycline Hydrochloride Capsules shall be kept in tightly closed containers, protected from light.

Identification

A. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

B. To a portion of the capsule contents, containing 10 mg of tetracycline hydrochloride, add 20 ml of warm *ethanol*, allow to stand for 20 minutes, filter, and evaporate the filtrate to dryness on a water-bath. To 0.5 mg of the residue add 2 ml of *sulfuric acid*: a purplish red colour is produced. Add 1 ml of *water*: the colour changes to deep yellow.

C. The residue obtained in test B yields the *reactions* characteristic of chlorides (Appendix 5.1).

Loss on drying Not more than 4.0 per cent w/w after drying about 100 mg in a capillary-stoppered bottle at 60° at a pressure not exceeding 0.7 kPa (about 5 Torr) for 3 hours (Appendix 4.15).

4-Epianhydrotetracycline Not more than 3 per cent w/w. Proceed as directed in the 4-Epianhydrotetracycline under *Tetracycline Hydrochloride*, p. 139.

Dissolution Carry out the test as described in the "Dissolution Test" (Appendix 4.24).

Dissolution medium: *water*; 900 ml.

Apparatus 2: 75 rpm. Maintain a distance of 45±5 mm between the blade and the inside bottom of the vessel.

Time: 60 minutes for 250-mg capsules; 90 minutes for 500-mg capsules.

Procedure Determine the amount of $C_{22}H_{24}N_2O_8$.HCl dissolved from absorbances at the maximum at about 276 nm of filtered portions of the test solution, suitably diluted with *Dissolution medium*, if necessary, in comparison with a standard solution having a known concentration of Tetracycline Hydrochloride RS in the same medium (Appendix 2.2).

Tolerances Not less than 80 per cent (*Q*) of the labelled amount of $C_{22}H_{24}N_2O_8$.HCl is dissolved in 60 minutes for 250-mg capsules; 90 minutes for 500-mg capsules.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Diluting solvent, Mobile phase, Resolution solution, Standard preparation, Chromatographic system, and **Procedure** Proceed as directed in the Assay described under *Tetracycline Hydrochloride*, p. 157.

Assay preparation Remove, as completely as possible, the contents of not less than 20 Tetracycline Hydrochloride Capsules, and weigh accurately. Mix the combined contents, and transfer an accurately weighed portion of the freshly mixed powder, containing about 50 mg of tetracycline hydrochloride, to a 100-ml volumetric flask, add 50 ml of *Diluting solvent*, mix, and sonicate for 5 minutes. Allow to cool, add *Diluting solvent* to volume, mix, and filter.

Calculation Calculate the content of $C_{22}H_{24}N_2O_8$.HCl in the Capsules taken, using the declared content of $C_{22}H_{24}N_2O_8$.HCl in Tetracycline Hydrochloride RS.

Other requirements Comply with the requirements described under "Capsules" (Appendix 1.16).

TETRACYCLINE HYDROCHLORIDE TABLETS

Category Antibacterial; antiprotozoal.

Tetracycline Hydrochloride Tablets contain not less than 90.0 per cent and not more than 125.0 per cent of the labelled amount of $C_{22}H_{24}N_2O_8$.HCl.

Strengths available 250 and 500 mg.

Dose Adults: 250 to 500 mg every 6 hours or up to 4 g daily.

Children over 8 years of age: 25 to 50 mg per kg of body weight in four divided doses.

Contra-indication; Warning; Precaution; Additional information See under *Tetracycline Hydrochloride*, p. 157.

Packaging and storage See under *Tetracycline Hydrochloride Capsules*, p. 159.

Identification Comply with the tests described under *Tetracycline Hydrochloride Capsules*, p 141.

Loss on drying Not more than 3.0 per cent w/w after drying about 100 mg at 60° at a pressure not exceeding 0.7 kPa (about 5 Torr) for 3 hours (Appendix 4.15).

4-Epianhydrotetracycline Not more than 3 per cent w/w. Proceed as directed in the 4-Epianhydrotetracycline under *Tetracycline Hydrochloride*, p. 158.

Dissolution Carry out the test as described in the "Dissolution Test" (Appendix 4.24).

Dissolution medium: *water*; 900 ml.

Apparatus 2: 75 rpm. Maintain a distance of 45±5 mm between the blade and the inside bottom of the vessel.

Time: 60 minutes.

Procedure Determine the amount of $C_{22}H_{24}N_2O_8$.HCl dissolved from absorbances at the maximum at about 276 nm of filtered portions of the test solution, suitably diluted with *Dissolution medium*, if necessary, in comparison with a standard solution having a known concentration of Tetracycline Hydrochloride RS in the same medium (Appendix 2.2).

Tolerances Not less than 80 per cent (*Q*) of the labelled amount of $C_{22}H_{24}N_2O_8$.HCl is dissolved in 60 minutes.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

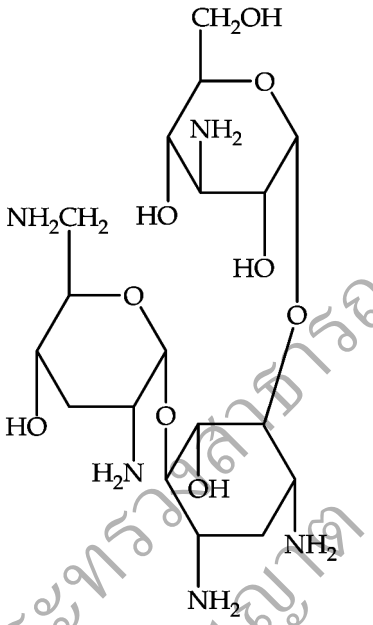
Diluting solvent, Mobile phase, Resolution solution, Standard preparation, Chromatographic system, and **Procedure** Proceed as directed in the Assay described under *Tetracycline Hydrochloride*, p. 158.

Assay preparation Weigh and finely powder not less than 20 Tetracycline Hydrochloride Tablets. Transfer an accurately weighed portion of the powder, containing about 50 mg of tetracycline hydrochloride, to a 100-ml volumetric flask, add 50 ml of *Diluting solvent*, mix, and sonicate for 5 minutes. Allow to cool, add *Diluting solvent* to volume, mix, and filter.

Calculation Calculate the content of $C_{22}H_{24}N_2O_8$.HCl in the Tablets taken, using the declared content of $C_{22}H_{24}N_2O_8$.HCl in Tetracycline Hydrochloride RS.

Other requirements Comply with the requirements described under "Tablets" (Appendix 1.16)

TOBRAMYCIN



$C_{18}H_{37}N_5O_9$ 467.52 *32986-56-4*

D-Streptamine, *O*-3-amino-3-deoxy-α-D-glucopyranosyl-(1→6)-*O*-[2,6-diamino-2,3,6-trideoxy-α-D-*ribo*-hexopyranosyl-(1→4)]-2-deoxy-.

Category Antibacterial.

Tobramycin contains not less than 900 μg of $C_{18}H_{37}N_5O_9$ per mg, calculated on the anhydrous basis.

Description White to off-white powder; hygroscopic.

Solubility Freely soluble in *water*; very slightly soluble in *ethanol*; practically insoluble in *chloroform* and in *ether*.

Contra-indication; Warning; Precaution; Additional information See under *Gentamicin Sulfate*, p. 111.

Packaging and storage Tobramycin shall be kept in tightly closed containers and stored at a temperature not exceeding 25°.

Labelling The label on the container states (1) the number of μg of activity per mg; (2) storage condition; (3) parenteral grade.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Tobramycin RS (Appendix 2.1) or with the reference spectrum of Tobramycin.

B. Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel G* as the coating substance and a mixture of 60 volumes of *methanol*, 30 volumes of *strong ammonia solution* and 25 volumes of *chloroform* as the mobile phase. Apply separately to the plate, 3 μl of each of two solutions

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containing (A) 6 mg per ml of the test substance and (B) 6 mg per ml of Tobramycin RS and at a third point, apply 3 µl of a mixture of equal volumes of solutions (A) and (B). After removal of the plate, allow to dry in air, and heat at 110° for 15 minutes. Immediately spray with a 1 per cent w/v solution of *ninhydrin* in a mixture of 100 volumes of *1-butanol* and 1 volume of *pyridine*: the principal pink spot in the chromatogram obtained from solution (A) corresponds to that in the chromatogram obtained from solution (B) and the principal red spot in the third chromatogram appears as a single compact spot.

C. The retention time of the major peak in the chromatogram of the Derivatized assay preparation corresponds to that of the Derivatized standard preparation, as obtained in the *Assay*.

pH 9.0 to 11.0, in a 10 per cent w/v solution (Appendix 4.11).

Water Not more than 8.0 per cent w/w (Karl Fischer Method, Appendix 4.12).

Heavy metals Not more than 30 ppm (Method II, Appendix 5.2). Use 0.67 g; for the Standard Preparation, use *lead standard solution (1 ppm Pb)*.

Sulfated ash Not more than 1.0 per cent w/w, the charred residue being moistened with 2 ml of *nitric acid* and 5 drops of *sulfuric acid* (Appendix 5.3).

Chromatographic purity Not more than 1.0 per cent Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel G* as the coating substance and a mixture of 50 volumes of a 29.2 per cent w/v solution of *sodium chloride*, 30 volumes of *ethanol* and 20 volumes of *water* as the mobile phase.

Dilute sodium hypochlorite solution Dilute 20 ml of *sodium hypochlorite TS* with *water* to obtain 100 ml of solution.

Starch-potassium iodide reagent Dissolve 1.1 g of *potassium iodide* in 60 ml of *water*, boil for 15 minutes, and slowly add a suspension of 1.5 g of *soluble starch* in 10 ml of *water*. Add 25 ml of *water*, and boil for 10 minutes. Allow to cool, dilute with *water* to 100 ml, and mix.

Test solution Transfer about 50 mg, accurately weighed, of the test substance to a 10-ml volumetric flask, add 7 ml of *water* to dissolve it, and adjust with 0.5 M *sulfuric acid* to a pH of 5.5±0.4. Dilute with *water* to volume and mix.

Reference solution Dilute 1 ml of *Test solution* to 10 ml with *water*.

Apply separately to the plate 1 µl of each solution. After removal of the plate, evaporate the solvent in a current of hot air, and then heat at 110° for 10 minutes. Lightly spray the hot plate with *Dilute sodium hypochlorite solution*. Dry the plate in a current of cold air until a sprayed area of the plate below the origin gives at most a faint blue colour with a drop of *Starch-potassium iodide reagent*. Then spray the plate with *Starch-potassium iodide reagent*: bluish-purple spots are immediately visible. Any spot obtained from *Test solution*, other than principal spot, is not more intense than the principal spot obtained from *Reference solution*.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Dissolve 2.0 g of *tris(hydroxymethyl) aminomethane* in 800 ml of *water*. To this solution add 20 ml of 0.5 M *sulfuric acid*, dilute with *acetonitrile* to obtain 2000 ml of solution and mix. Allow to cool and filter. Make adjustments if necessary.

2,4-Dinitrofluorobenzene reagent Prepare a stock solution of *2,4-dinitrofluorobenzene* in *ethanol* containing 10 mg per ml. This solution may be used for 5 days if refrigerated when not in use.

Tris(hydroxymethyl)aminomethane reagent Prepare a stock solution of *tris(hydroxymethyl)-aminomethane* in *water* containing 15 mg per ml. This stock solution may be used for 1 month if refrigerated when not in use. Transfer 40 ml of this stock solution to a 200-ml volumetric flask, add sufficient *dimethyl sulfoxide* with mixing, dilute with the same solvent to volume, and mix. Use this reagent within 4 hours. (**Note** If kept immersed in an ice-water bath below 10°, the reagent may be used for up to 8 hours.)

Standard preparation Transfer about 55 mg of Tobramycin RS, accurately weighed, to a 50-ml volumetric flask, add 1 ml of 0.5 M *sulfuric acid* and sufficient *water* to dissolve it, dilute with *water* to volume and mix. Transfer 10.0 ml of this solution to a second 50-ml volumetric flask, dilute with *water* to volume and mix. This solution contains about 220 µg of tobramycin per ml.

Assay preparation Transfer about 55 mg of Tobramycin, accurately weighed, to a 50-ml volumetric flask, add 1 ml of 0.5 M *sulfuric acid* and sufficient *water* to dissolve it, dilute with *water* to volume and mix. Transfer 10.0 ml of this solution to a second 50-ml volumetric flask, dilute with *water* to volume and mix.

Derivatization procedure (**Note** Heat all solutions at the same temperature and for the same duration of time as indicated. Move all flasks to and from the 60°

constant temperature bath at the same time.) To separate 50-ml volumetric flasks transfer 4.0 ml of *Standard preparation*, 4.0 ml of *Assay preparation*, and 4.0 ml of *water*. To each flask add 10 ml of *2,4-Dinitrofluorobenzene reagent* and 10 ml of *Tris(hydroxymethyl)aminomethane reagent*, shake, and insert the stopper. Place the flasks in a constant temperature bath at 60°±2° and heat for 50±5 minutes. Remove the flasks from the bath, and allow to stand for 10 minutes. Add *acetonitrile* to about 2 ml below the 50-ml mark, allow to cool to room temperature, dilute with *acetonitrile* to volume, and mix. The solutions thus obtained are Derivatized standard preparation, Derivatized assay preparation, and Blank preparation, respectively.

Resolution solution Prepare a fresh solution of p-*naphtholbenzein* in *acetonitrile* containing about 240 µg per ml. Transfer 2 ml of this solution to a 10-ml volumetric flask, dilute with *Derivatized standard preparation* to volume, and use promptly.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (30 cm × 3.9 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles (5 to 10 µm), (b) *Mobile phase* at a flow rate of about 1.2 ml per minute and (c) an ultraviolet photometer set at 365 nm.

To determine the suitability of the chromatographic system, chromatograph *Blank preparation* and record the peak responses as directed under *Procedure*. Identify the solvent and reagent peaks. Chromatograph *Resolution solution*, and record the peak responses as directed under *Procedure*: the relative retention times are about 1.0 for *p*-naphtholbenzein and 1.7 for tobramycin and the resolution factor between the two peaks is not less than 4.0. Chromatograph *Derivatized standard preparation*, and record the peak responses as directed under *Procedure*: the relative standard deviation for replicate injections is not more than 2.0 per cent.

Procedure Separately inject equal volumes (about 20 µl) of *Derivatized standard preparation* and *Derivatized assay preparation* into the chromatograph, record the chromatograms and measure the responses for the major peaks.

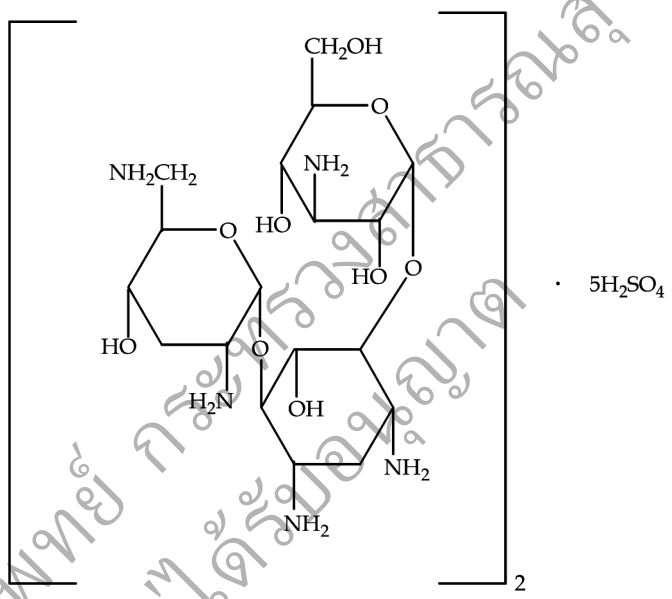
Calculation Calculate the content of $C_{18}H_{37}N_5O_9$ in the Tobramycin taken, using the declared content of $C_{18}H_{37}N_5O_9$ in Tobramycin RS.

Other requirements Tobramycin intended for parenteral administration complies with the following additional requirements.

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains not more than 2.00 Endotoxin Units per mg of tobramycin.

Sterilitys Complies with the "Sterility Test" (Method I, Appendix 10.1).

TOBRAMYCIN SULFATE



$(C_{18}H_{37}N_5O_9)_2.5H_2SO_4$ 1425.45 *79645-27-5*

D-Streptamine, *O*-3-amino-3-deoxy-α-D-glucopyranosyl-(1→6)-*O*-[2,6-diamino-2,3,6-trideoxy-α-D-*ribo*-hexopyranosyl-(1→4)]-2-deoxy-, sulfate (2:5) (salt).

Category Antibacterial.

Tobramycin Sulfate contains the equivalent of not less than 634 µg and not more than 739 µg of $C_{18}H_{37}N_5O_9$ per mg.

Description White to off-white powder; odourless.

Solubility Soluble in *water*.

Contra-indication; Warning; Precaution; Additional information See under *Gentamicin Sulfate*, p. 111.

Packaging and storage Tobramycin Sulfate shall be kept in tightly closed containers.

Labelling The label on the container states (1) the number of µg of activity per mg; (2) storage condition; (3) parenteral grade.

Identification

A. Complies with the tests for Identification A, B and C described under *Tobramycin*, p. 160.

B. It yields the *reactions* characteristic of sulfates (Appendix 5.1).

pH 6.0 to 8.0, in a 4.0 per cent w/v solution (Appendix 4.11).

Water Not more than 2.0 per cent w/w (Karl Fischer Method, Appendix 4.12).

Heavy metals Not more than 30 ppm (Method II, Appendix 5.2). Use 0.67 g; for the Standard Preparation, use *lead standard solution (1 ppm Pb)*.

Sulfated ash Not more than 1.0 per cent w/w, the charred residue being moistened with 2 ml of *nitric acid* and 5 drops of *sulfuric acid* (Appendix 5.3).

Chromatographic purity Not more than 1.0 per cent. Complies with the test described under *Tobramycin*, p. 160.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase, 2,4-Dinitrofluorobenzene reagent, Tris(hydroxymethyl)aminomethane reagent, Standard preparation, Derivatization procedure, Resolution solution, Chromatographic system, and **Procedure** Proceed as directed in the Assay under *Tobramycin*, p. 161.

Assay preparation Transfer an accurately weighed quantity of Tobramycin Sulfate, containing about 50 mg of tobramycin, to a 250-ml volumetric flask, dissolve in and dilute with *water* to volume, and mix.

Calculation Calculate the content of $C_{18}H_{37}N_5O_9$ in the Tobramycin Sulfate taken, using the declared content of $C_{18}H_{37}N_5O_9$ in Tobramycin RS.

Other requirements Tobramycin Sulfate intended for parenteral administration complies with the following additional requirements.

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains not more than 2.00 Endotoxin Units per mg of tobramycin.

Sterility Complies with the "Sterility Test" (Method I, Appendix 10.1).

TOBRAMYCIN SULFATE INJECTION

Category Antibacterial.

Tobramycin Sulfate Injection is a sterile solution of Tobramycin Sulfate in Water for Injection, or of Tobramycin in Water for Injection prepared with the aid of Sulfuric Acid. It contains not less than 90.0 per cent and not more than 120.0 per cent of the labelled amount of $C_{18}H_{37}N_5O_9$.

Description Clear, colourless solution.

Strengths available 10, 20, 40, 60, and 80 mg (base) per ml.

Dose Adults: *Intramuscular* or slow *intravenous infusion* (over 30 to 60 minutes), 0.75 to 1.25 mg per kg of body weight every 6 hours or 1 to 1.7 mg per kg of body weight every 8 hours for 7 to 10 days or more or up to 8 mg per kg of body weight daily.

Children and infants: *Intramuscular* or *slow intravenous infusion* (over 30 to 60 minutes), 1.5 to 1.9 mg per kg of body weight every 6 hours or 2 to 2.5 mg per kg of body weight every 8 to 16 hours.

Premature or full-term neonates: *Intramuscular* or slow *intravenous infusion* (over 30 to 60 minutes), up to 2 mg per kg of body weight every 12 to 24 hours. The maximum total dose should not exceed 4 mg per kg of body weight daily.

Contra-indication; Warning; Precaution See under *Gentamicin Sulfate*, p. 111.

Additional information Tobramycin Sulfate Injection may also be administered as an aerosol nebulization.

See also under *Gentamicin Sulfate*, p. 111.

Packaging and storage Tobramycin Sulfate Injection shall be kept in single-dose or in multiple-dose containers, preferably of Type I glass.

Labelling The label on the container states the quantity equivalent to the amount of tobramycin.

Identification

A. Complies with the test for Identification B described under *Tobramycin*, p. 160. For solution (A), dilute the injection with *water* to obtain a solution containing 6 mg of tobramycin per ml.

B. The retention time of the major peak in the chromatogram of the Derivatized assay preparation corresponds to that in the chromatogram of the Derivatized standard preparation, as obtained in the *Assay*.

C. It yields the *reactions* characteristic of sulfate (Appendix 5.1).

pH 3.0 to 6.5 (Appendix 4.11).

Particulate matter Complies with the requirments described under "Particulate Matter in Injections (Small-volume Injections, Appendix 4.27).

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains not more than 2.00 Endotoxin Units per mg of tobramycin.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

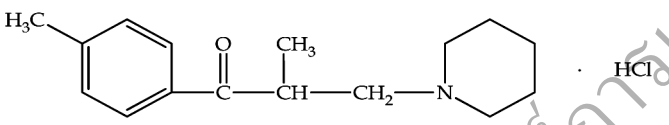
Mobile phase, 2,4-Dinitrofluorobenzene reagent, Tris(hydroxymethyl)aminomethane reagent, Standard preparation, Derivatization procedure, Resolution solution, Chromatographic system, and **Procedure** Proceed as directed in the Assay under *Tobramycin*, p 161.

Assay preparation Dilute an accurately measured volume of Tobramycin Sulfate Injection quantitatively, and stepwise if necessary, with *water* to obtain a solution containing the equivalent of about 200 µg of tobramycin per ml.

Calculation Calculate the content of $C_{18}H_{37}N_5O_9$ in each ml of the Injection taken, using the declared content of $C_{18}H_{37}N_5O_9$ in Tobramycin RS.

Other requirements Complies with the requirements described under "Parenteral Preparations" (Appendix 1.16).

TOLPERISONE HYDROCHLORIDE



$C_{16}H_{23}NO.HCl$ 281.83 *3644-61-9*

1-Propanone, 2-methyl-1-(4-methylphenyl)-3-(1 piperidinyl)-, hydrochloride.

Category Muscle relaxant.

Tolperisone Hydrochloride contains not less than 98.5 per cent of $C_{16}H_{23}NO.HCl$, calculated on the dried basis.

Description White, crystalline powder; odour, slight, characteristic; hygroscopic.

Solubility Freely soluble in *water* and in *ethanol*; very soluble in *acetic acid*; soluble in *acetic anhydride*; slightly soluble in *acetone*; practically insoluble in *ether*.

Stability It is hygroscopic.

Contra-indication It is contra-indicated in myasthenia gravis.

Warning

1. It may cause muscular weakness, headache, hypotension, nausea, vomiting, abdominal discomfort and anaphylactic reactions.

2. Risk-benefit should be considered if it is to be used in pregnant women.

Packaging and storage Tolperisone Hydrochloride shall be kept in well-closed containers.

Identification

A. Dissolve 200 mg in 2 ml of *ethanol*, add 2 ml of *1,3-dinitrobenzene TS* and 2 ml of *sodium hydroxide TS*, and heat: a red colour develops.

B. To 5 ml of a 5 per cent w/v solution of the test substance add 2 to 3 drops of *iodine TS*: a red-brown precipitate is produced.

C. It yields the *reactions* characteristic of chlorides (Appendix 5.1).

Melting range 167° to 174° (Appendix 4.3).

Clarity and colour of solution A 10.0 per cent w/v solution is clear (Appendix 4.11) and colourless.

pH 4.5 to 5.5, in a 5.0 per cent w/v solution (Appendix 4.11).

Absorbance Dissolve 5.0 mg (after drying) in *ethanol* and dilute to 500.0 ml with the same solvent. The light absorption spectrum of the diluted solution, when observed between 220 nm and 350 nm, exhibits a maximum at about 257 nm; the specific absorbance at the maximum is 555 to 585 (Appendix 2.2).

Loss on drying Not more than 0.5 per cent w/w after drying over *self-indicating silica gel* in vacuum for 3 hours (Appendix 4.15).

Sulfated ash Not more than 0.1 per cent w/w (Appendix 5.3).

Sulfate Not more than 0.005 per cent w/w (Appendix 5.2). A 4.0-g sample shows no more sulfate than that corresponds to 0.20 ml of 0.010 M *sulfuric acid*.

Heavy metals Not more than 20 ppm (Method II, Appendix 5.2). Use 1.0 g; for the Standard Preparation, use 2 ml of *lead standard solution (10 ppm Pb)*.

Piperidine hydrochloride Not more than 0.1 per cent w/w. Carry out the test as described in the "Ultraviolet and Visible Spectrophotometry" (Appendix 2.2). Transfer 200.0 mg of the test substance in a 10-ml volumetric flask. Add *water* to dissolve and dilute to volume. Use this solution as the test solution. Separately, dissolve 20.0 mg of *piperidine hydrochloride* in *water* to make 1000.0 ml and use this solution as the standard solution. Transfer 5.0 ml each of the test solution and the standard solution to different separators. Add 0.1 ml each of a 5 per cent w/v solution of *copper(II) sulfate*, then add 0.1 ml each of *strong ammonia solution* and 10.0 ml each of a mixture of 3 volumes of *2,2,4-trimethylpentane* and 1 volume of *carbon disulfide*, and shake vigorously for

30 minutes. Immediately after allowing to stand, separate the pentane-carbon disulfide mixture layer, and dehydrate with *anhydrous sodium sulfate*: the absorbance of the test solution at 438 nm is not more than that of the standard solution.

Assay Dissolve about 500 mg of Tolperisone Hydrochloride, accurately weighed, in 100 ml of *anhydrous glacial acetic acid*. Titrate with 0.1 M *perchloric acid VS*, determining the end-point potentiometrically (Appendix 6.1). Perform a blank determination, and make any necessary correction. Each ml of 0.1 M *perchloric acid* is equivalent to 28.18 mg of $C_{16}H_{23}NO.HCl$.

TOLPERISONE HYDROCHLORIDE TABLETS

Category Muscle relaxant.

Tolperisone Hydrochloride Tablets contain not less than 90.0 per cent and not more than 110.0 per cent of the labelled amount of $C_{16}H_{23}NO.HCl$.

Strengths available 50 and 150 mg.

Dose Adults: 150 to 450 mg daily in three equally divided doses.

Children 6 years of age and over: 2 to 4 mg per kg of body weight daily in three equally divided doses.

Children younger than 6 years of age: 5 mg per kg of body weight daily in three equally divided doses.

Contra-indication; Warning See under *Tolperisone Hydrochloride*, p. 164.

Identification

A. The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

B. A filtered 5 per cent w/v solution of the tablets yields *reaction A* characteristic of chlorides (Appendix 5.1).

Piperidine Hydrochloride Not more than 1.0 per cent w/w. Carry out the test as described in the "Ultraviolet and Visible Spectrophotometry" (Appendix 2.2). Transfer an accurately weighed portion of the powdered tablets equivalent to 20.0 mg of tolperisone hydrochloride to a 10-ml volumetric flask. Add sufficient 0.1 M *hydrochloric acid* and shake for 20 minutes to dissolve. Dilute with 0.1 M *hydrochloric acid* to volume, mix and filter. Use this solution as the test solution. Proceed as directed in the Piperidine Hydrochloride described under *Tolperisone Hydrochloride*, p. 164, beginning with " Separately, dissolve 20.0 mg of...".

Dissolution Carry out the test as described in the "Dissolution Test" (Appendix 4.24).

Dissolution medium: *water*; 900 ml.

Apparatus 2: 75 rpm.

Time: 45 minutes.

Procedure Determine the amount of $C_{16}H_{23}NO.HCl$ dissolved from absorbances at the maximum at about 260 nm of filtered portions of the test solution, suitably diluted with *Dissolution medium*, if necessary, in comparison with a standard solution having a known concentration of about 11 µg per ml of Tolperisone Hydrochloride RS in the same medium (Appendix 2.2).

Tolerances Not less than 75 per cent (*Q*) of the labelled amount of $C_{16}H_{23}NO.HCl$ is dissolved in 45 minutes.

Assay Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

pH 4.0 Buffer solution Mix 5.0 ml of *phosphoric acid* and 5.0 ml of *triethylamine* in 800 ml of *water* and adjust with *sodium hydroxide* to a pH of 4.0. Dilute with *water* to make 1000.0 ml.

Mobile phase Prepare a mixture of 650 volumes of *pH 4.0 Buffer solution* and 350 volumes of *acetonitrile*. Make adjustments if necessary.

Standard preparation Dissolve an accurately weighed quantity of Tolperisone Hydrochloride RS in 0.1 M *hydrochloric acid* to obtain a solution having a known concentration of about 1 mg per ml. Transfer 5.0 ml of this solution to a 50-ml volumetric flask, dilute with *Mobile phase* to volume and mix.

Assay preparation Weigh and finely powder not less than 20 Tolperisone Hydrochloride Tablets. Transfer an accurately weighed portion of the powder containing about 50 mg of tolperisone hydrochloride to a 50-ml volumetric flask. Add sufficient 0.1 M *hydrochloric acid* and shake for 20 minutes to dissolve. Dilute with 0.1 M *hydrochloric acid* to volume, mix and filter. Transfer 5.0 ml of the filtrate to a 50-ml volumetric flask, dilute with *Mobile phase* to volume and mix.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (25 cm × 4.6 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles (5 µm), (b) *Mobile phase* at a flow rate of about 1 ml per minute, and (c) an ultraviolet photometer set at 258 nm.

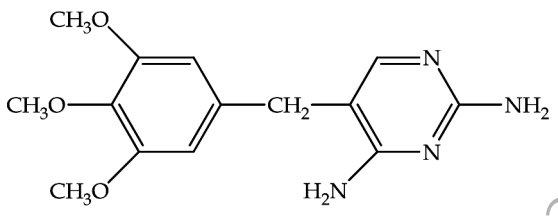
To determine the suitability of the chromatographic system, chromatograph *Standard preparation* and record the peak responses as directed under *Procedure*: the symmetry factor for the tolperisone peak is not more than 2.0 and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Procedure Separately inject equal volumes (about 10 µl) of *Standard preparation* and *Assay preparation* into the chromatograph, record the chromatograms, and measure the responses for the major peaks.

Calculation Calculate the content of $C_{16}H_{23}NO.HCl$ in the portion of the Tablets taken, using the declared content of $C_{16}H_{23}NO.HCl$ in Tolperisone Hydrochloride RS.

Other requirements Comply with the requirements described under "Tablets" (Appendix 1.16).

TRIMETHOPRIM



$C_{14}H_{18}N_4O_3$ 290.32 *738-70-5*

2,4-Pyrimidinediamine, 5-[(3,4,5-trimethoxyphenyl)-methyl]-.

Category Antibacterial.

Trimethoprim contains not less than 98.5 per cent and not more than 101.0 per cent of $C_{14}H_{18}N_4O_3$, calculated on the dried basis.

Description White to cream-coloured crystals or crystalline powder.

Solubility Very slightly soluble in *water*; soluble in *benzyl alcohol*; sparingly soluble in *chloroform* and in *methanol*; slightly soluble in *ethanol* and in *acetone*; practically insoluble in *ether* and in *carbon tetrachloride*.

Contra-indication It is contra-indicated in patients with megaloblastic anemia secondary to folate deficiency.

Warning

1. It should be used with caution in patients with impaired renal or hepatic function or with possible folate deficiency and in children or pregnant women who are at risk of having a child with the fragile X chromosomes associated with mental retardation.

2. It may cause rash, pruritus, photosensitivity, exfoliative dermatitis, toxic epidermal necrolysis, erythema multiforme, Stevens-Johnson Syndrome, nausea, vomiting, glossitis, abnormal taste sensation, elevation in serum aspartate transaminase (AST), serum alanine transaminase (ALT) and bilirubin levels, cholestatic jaundice and blood dyscrasias.

3. Risk-benefit should be considered if it is to be used in children, pregnant or nursing women.

4. Caution should be exercised if it is to be used concomitantly with other folate antagonists.

Precaution Monitoring and early signs and symptoms of a serious hematologic disorder, including fever, sore throat, pallor, or purpura are recommended. Periodic blood counts including platelet counts are recommended during therapy with the drug. If signs of folic or folinic acid deficiency develop, reduce the dosage or discontinue the drug according to the response of the patient. Folinic acid at a dose of 3 to 10 mg per day intramuscularly should be given until the blood picture returns to safe levels.

Additional information Safety and efficacy of trimethoprim in infants younger than 2 months of age and efficacy of the drug when used as a single agent in children younger than 12 years of age have not been established.

Packaging and storage Trimethoprim shall be kept in well-closed containers.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Trimethoprim RS (Appendix 2.1) or with the reference spectrum of Trimethoprim.

B. The ultraviolet absorption spectrum of a 0.002 per cent w/v solution in 0.1 M *sodium hydroxide*, when observed between 230 and 350 nm, exhibits a maximum only at 287 nm; the absorbance of a 1-cm layer at this wavelength is about 0.49 (Appendix 2.2).

C. Dissolve, with heating if necessary, 25 mg in 5 ml of 0.005 M *sulfuric acid* and add 2 ml of a 1.6 per cent w/v solution of *potassium permanganate* in 0.1 M *sodium hydroxide*. Heat to boiling and add to the hot solution 8 drops of *formaldehyde solution*. Mix, add 1 ml of 0.5 M *sulfuric acid*, mix and again heat to boiling. Cool to room temperature and filter. Add to the filtrate 2 ml of *chloroform* and shake vigorously: the chloroform layer exhibits a green fluorescence when examined under ultraviolet light (366 nm).

Melting range 199° to 203° (Appendix 4.3).

Loss on drying Not more than 1.0 per cent w/w after drying at 105° to constant weight (Appendix 4.15).

Sulfated ash Not more than 0.1 per cent w/w (Appendix 5.3).

Chromatographic purity Not more than 0.1 per cent w/w of any individual impurity; and not more than 0.2 per cent w/w of total impurities. Carry out the determination as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Buffer solution Prepare a 0.01 M *sodium perchlorate* solution in *water*, adjust with *phosphoric acid* to a pH of 3.6, and mix.

Mobile phase Prepare a mixture of 7 volumes of *Buffer solution* and 3 volumes of *methanol*. Make adjustments if necessary.

Resolution solution Dissolve accurately weighed quantities of Trimethoprim RS and *diaveridine*, and dilute quantitatively, and stepwise if necessary, with *Mobile phase* to obtain a solution having known concentrations of about 10 µg per ml and 5 µg per ml, respectively.

Test solution Transfer about 25.0 mg of the test substance, accurately weighed, to a 25-ml volumetric flask, dissolve in and dilute with *Mobile phase* to volume, and mix.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (25 cm × 4.6 mm) packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles (3 to 10 µm in diameter), (b) *Mobile phase* at a flow rate of 1.3 ml per minute, and (c) an ultraviolet photometer set at 280 nm.

To determine the suitability of the chromatographic system, chromatograph *Resolution solution*, and record the peak responses as directed under *Procedure*: the resolution factor between the peaks for trimethoprim and diaveridine is not less than 2.5; and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Procedure Inject a volume (about 20 µl) of *Test solution* into the chromatograph, record the chromatogram for not less than 11 times the retention time of the trimethoprim peak, and measure all of the peak responses.

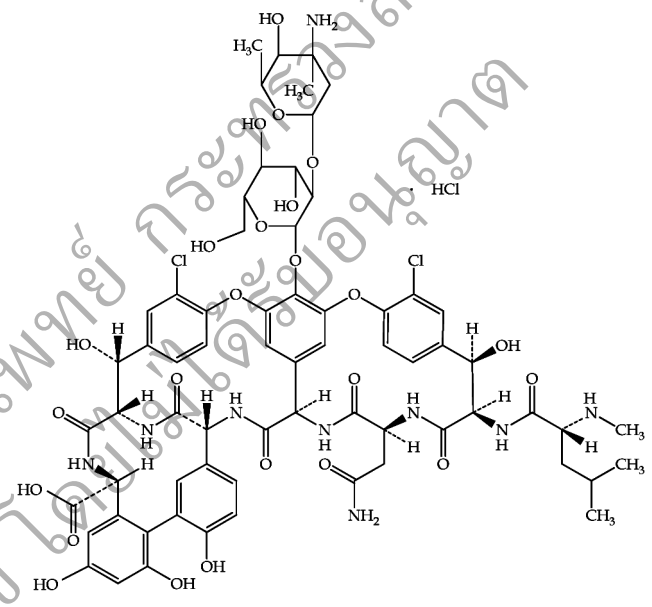
Calculation Calculate the percentage of each impurity in the portion of Trimethoprim taken by the expression:

$$100[Fri/[\sum(Fr_i) + Fr_t]] ,$$

in which F is a relative response factor, and is equal to 0.5 for any peak having a relative retention time of 0.9, 2.3, 2.7, or 10.3, and is equal to 1.0 for all other peaks; r_i is the peak response for each impurity; and r_t is the peak response for trimethoprim obtained from *Test solution*.

Assay Dissolve about 300 mg of Trimethoprim, accurately weighed, in 60 ml of *anhydrous glacial acetic acid* and titrate with 0.1 M *perchloric acid VS*, using *crystal violet TS* as indicator, to a blue-green end-point, or determining the end-point potentiometrically (Appendix 6.1). Perform a blank determination, and make any necessary correction. Each ml of 0.1 M *perchloric acid* is equivalent to 29.03 mg of $C_{14}H_{18}N_4O_3$.

VANCOMYCIN HYDROCHLORIDE



$C_{66}H_{75}Cl_2N_9O_{24}$·HCl 1485.71 *1404-93-9*

(*Sa*)-(3*S*,6*R*,7*R*,22*R*,23*S*,26*S*,36*R*,38a*R*)-44-[[2-*O*-(3-Amino 2,3,6-trideoxy-3-*C*-methyl-α-L-*lyxo*-hexopyranosyl)-β-D-glucopyranosyl]oxy]-3-(carbamoylmethyl)-10,19-dichloro-2,3,4,5,6,7,23,24,25,26,36,37,38,38a tetradecahydro-7,22,28,30,32-pentahydroxy-6-[(2*R*)-4-methyl-2-(methylamino)]valeramido]-2,5,24,38,39-pentaoxo-22*H*-8,11 : 18,21-dietheno-23,36-(iminomethano)-13,16 : 31,35-dimetheno-1*H*,16*H*-[1,6,9]oxadiazacyclohexadecino[4,5-*m*][10,2,16]benzoxadiazacyclotetracosine-26-carboxylic acid, monohydrochloride.

Category Antibacterial.

Vancomycin Hydrochloride is the hydrochloride salt of a kind of vancomycin, a substance produced by the growth of *Streptomyces orientalis* (Family Streptomycetaceae), or a mixture of two or more such salts. It has a potency equivalent to not less than 900 µg of vancomycin per mg, calculated on the anhydrous basis.

Description Tan to brown, free-flowing powder; odourless.

Solubility Freely soluble in *water*; insoluble in *ether* and in *chloroform*.

Warning

1. It may cause ototoxicity, nephrotoxicity, pseudomembranous colitis, neutropenia, red man syndrome (glycopeptide-induced anaphylactoid reaction), chemical peritonitis, or thrombocytopenia.

2. It should be used with caution in patients with renal function impairment.

3. Concomitant use with parenteral amphotericin B, aspirin or other salicylates, bacitracin, bumetanide, capreomycin, carmustine, cisplatin, cyclosporine, ethacrynic acid, furosemide, paromomycin, polymyxins or streptozocin or aminoglycosides should be avoided.

4. Risk-benefit should be considered if it is to be used in pregnant or nursing women.

Precaution

1. Audiograms, renal function determinations, white blood cell counts, urinalyses, or serum vancomycin concentration determination should be performed periodically.

2. Close monitoring of serum vancomycin concentration is recommended in premature neonates and young infants.

Packaging and storage Vancomycin Hydrochloride shall be kept in tightly closed containers, protected from light. It shall also be kept under sterile condition.

Labelling The label on the container states parenteral grade.

Identification

A. The infrared absorption spectrum is concordant with the spectrum obtained from Vancomycin Hydrochloride RS (Appendix 2.1) or with the reference spectrum of Vancomycin Hydrochloride.

B. The retention time of the major peak in the chromatogram of the Test solution A corresponds to that in the chromatogram of the Resolution solution, as obtained in the *Chromatographic purity*.

C. It yields the *reactions* characteristic of chlorides (Appendix 5.1).

pH 2.5 to 4.5, in a 5.0 per cent w/v solution (Appendix 4.11).

Water Not more than 5.0 per cent w/w (Karl Fischer Method, Appendix 4.12).

Chromatographic purity Carry out the test as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Triethylamine buffer Mix 4 ml of *triethylamine* and 2000 ml of *water*, and adjust with *phosphoric acid* to a pH of 3.2.

Mobile phase A Prepare a mixture of 92 volumes of *Triethylamine buffer*, 7 volumes of *acetonitrile* and 1 volume of *tetrahydrofuran*.

Mobile phase B Prepare a suitable mixture of 70 volumes of *Triethylamine buffer*, 29 volumes of *acetonitrile* and 1 volume of *tetrahydrofuran*.

Mobile phase Use variable mixtures of *Mobile phase A* and *Mobile phase B* as directed for *Chromatographic system*. Make adjustments if necessary, changing the acetonitrile proportion in *Mobile phase A* to obtain a retention time of 7.5 to 10.5 minutes for the main vancomycin peak.

Resolution solution Prepare a solution of Vancomycin Hydrochloride RS in *water* containing 0.5 mg per ml, heat at 65° for 48 hours, and allow to cool.

Test solution A Prepare a solution of the test substance in *Mobile phase A* containing 10 mg per ml.

Test solution B Transfer 2.0 ml of *Test solution A* to a 50-ml volumetric flask, dilute with *Mobile phase A* to volume, and mix.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (25 cm × 4.6 mm) packed with octadecylsilane chemical bonded to porous silica or ceramic microparticle (5 µm), (b) *Mobile phase* at a flow rate of about 2 ml per minute, and (c) an ultraviolet photometer set at 280 nm. The chromatograph is programmed as follows.

Time (min)	*Mobile Phase A* (Per Cent)	*Mobile Phase B* (Per Cent)	Elution
0 to 12	100	0	isocratic
12 to 20	100→0	0→100	linear gradient
20 to 22	0	100	isocratic
22 to 23	0→100	100→0	linear gradient
23 to 30	100	0	isocratic

To determine the suitability of the chromatographic system, chromatograph the *Resolution solution*, and record the peak responses as directed under *Procedure*: the elution order is resolution compound 1, vancomycin B, and resolution compound 2. The resolution, R, between resolution compound 1 and vancomycin B is not less than 3.0; and the column efficiency, calculated from the vancomycin B peak, is not less than 1500 theoretical plates. Resolution compound 2 is eluted at between 3 and 6 minutes after the start of the period

when the percentage of *Mobile phase B* is increasing from 0 to 100 per cent.

Procedure (**Note** Where baseline separation is not achieved, peak areas are defined by vertical lines extended from the valleys between peaks to the baseline. The main component peak may include a fronting shoulder, which is attributed to monodechlorovancomycin. This shoulder should not be integrated separately.) Inject separately equal volumes (about 20 µl) of *Test solution A* and *Test solution B* into the chromatograph, record the chromatograms, and measure the area responses for all of the peaks. (**Note** Correct any peak observed in the chromatograms obtained from *Test solution A* and *Test solution B* by subtracting the area response of any peak observed in the chromatogram of *Mobile phase A* at the corresponding elution time.)

Calculation Calculate the percentage of vancomycin B in the test substance by the expression:

$$2500r_B/(25r_B + r_A),$$

in which r_B is the corrected area response of the main peak obtained in the chromatogram of *Test solution B*; and r_A is the sum of the corrected area responses of all the peaks, other than the main peak, in the chromatogram obtained from *Test solution A*: not less than 80.0 per cent of vancomycin B is found.

Calculate the percentage of each other peak taken by the expression:

$$100r_{Ai}/(25r_B + r_A),$$

in which r_{Ai} is the corrected area response of any individual peak, other than the main peak, obtained in the chromatogram of *Test solution A*: not more than 9.0 per cent of any peak other than the main peak is found.

Assay Carry out the microbiological assay of vancomycin hydrochloride according to the "Microbiological Assay of Antibiotics" (Appendix 6.10).

Other requirements Vancomycin Hydrochloride intended for parenteral administration complies with the following additional requirements.

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains not more than 0.33 Endotoxin Unit per mg of vancomycin.

Sterility Complies with the "Sterility Test" (Method I, Appendix 10.1).

VANCOMYCIN HYDROCHLORIDE FOR INJECTION

Category Antibacterial.

Vancomycin Hydrochloride for Injection is a sterile dry mixture of Vancomycin Hydrochloride and a suitable stabilizing agent. It contains not less than 90.0 per cent and not more than 115.0 per cent of the labelled amount of vancomycin.

Strengths available 0.5, 1, 5 and 10 g (base).

Dose Adults: *Intravenous infusion*, 7.5 mg per kg of body weight or 500 mg every 6 hours; or 15 mg per kg of body weight or 1 g every 12 hours.
(**Note** After an initial loading dose of 750 mg to 1 g, but not less than 15 mg per kg of body weight, adults with impaired renal function may require a reduction in dose as indicated in the table. However, the preferred method is to adjust dosage based on serum vancomycin concentrations.)

Creatinine (ml/min)	Clearance (ml/sec)	Intravenous Dose
>80	1.33	See adult dose
50 to 80	0.83 to 1.33	1 g every 1 to 3 days
10 to 50	0.17 to 0.83	1 g every 3 to 7 days
<10	0.17	1 g every 7 to 14 days

Children and infants: *Intravenous infusion*, 10 mg per kg of body weight every 6 hours; or 20 mg per kg of body weight every 12 hours.

Neonates 1 week to 1 month of age: *Intravenous infusion*, 15 mg per kg of body weight initially, followed by 10 mg per kg of body weight every 8 hours.

Neonates up to 1 week of age: *Intravenous infusion*, 15 mg per kg of body weight initially, followed by 10 mg per kg of body weight every 12 hours.
(**Note** Doses up to 60 mg per kg of body weight per day have been used in some infections (e.g., staphylococcal infections of the central nervous system.))

Warning; **Precaution** See under *Vancomycin Hydrochloride*, p. 168.

Packaging and storage Vancomycin Hydrochloride for Injection shall be kept in Containers for Sterile Solids as described under "Parenteral Preparations" (Appendix 1.16), protected from light.

Labelling The label on the container states (1) the quantity equivalent to the amount of vancomycin; (2) the total number of µg of activity contained in it; (3) the number of µg of activity per mg.

Identification The retention time of the major peak in the chromatogram of the Test solution A corresponds to that in the chromatogram of the Resolution solution, as obtained in the *Chromatographic purity*.

pH 2.5 to 4.5, in a 5.0 per cent w/v solution (Appendix 4.11).

Particulate matter Complies with the requirements described under "Particulate Matter in Injections" (Small-volume Injections, Appendix 4.27).

Water Not more than 5.0 per cent w/w (Karl Fischer Method, Appendix 4.12); use 500 mg.

Heavy metals Not more than 30 ppm. (Method I, Appendix 5.2) Use 667 mg; for the Standard Preparation, use *lead standard solution (1 ppm Pb.)*.

Chromatographic purity Carry out the test as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Triethylamine buffer, Mobile phase, Resolution solution, **Chromatographic system**, and **Procedure** Proceed as directed in the test for Chromatographic purity under *Vancomycin Hydrochloride*, p. 168.

Test solution A Prepare a solution of the test substance in *Mobile phase A* containing 10 mg of vancomycin per ml.

Test solution B Transfer 2.0 ml of *Test solution A* to a 50-ml volumetric flask, dilute with *Mobile phase A* to volume, and mix.

Calculation Calculate the percentage of vancomycin B in the test substance by the expression:

$$2500r_B/(25r_B + r_A),$$

in which r_B is the corrected area response of the main peak obtained in the chromatogram of *Test solution B*; and r_A is the sum of the corrected area responses of all the peaks, other than the main peak, in the chromatogram obtained from *Test solution A*: not less than 88.0 per cent of vancomycin B is found.

Calculate the percentage of each other peak taken by the expression:

$$100r_{Ai}/(25r_B + r_A),$$

in which r_{Ai} is the corrected area response of any individual peak, other than the main peak, obtained in the chromatogram of *Test solution A*: not more than 4.0 per cent of any peak other than the main peak is found.

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendsix 8.5), it contains not more than 0.33 Endotoxin Unit per mg of vancomycin.

Assay Constitute a container of Vancomycin Hydrochloride for Injection in an accurately measured volume of *water* corresponding to the volume of diluent specified in the labelling. Withdraw all of the withdrawable contents, using a suitable hypodermic needle and syringe, and dilute quantitatively with *water* to obtain a solution containing about 1 mg of vancomycin per ml. Proceed as directed in the microbiological assay of vancomycin hydrochloride according to the "Microbiological Assay of Antibiotics" (Appendix 6.10) using an accurately measured volume of Vancomycin Hydrochloride for Injection, diluted quantitatively with Buffer No.3 to yield a solution having a concentration assumed to be equal to the median dose level of the Standard.

Other requirements Complies with the requirements described under "Parenteral Preparations" (Appendix 1.16).

WATER FOR INJECTION

Category Pharmaceutic aid (solvent).

Water for Injection is water purified by distillation or a purification process that is equivalent or superior to distillation in the removal of chemicals and micro-organisms. It is prepared from water that complies with the regulations on water intended for human consumption laid down by the competent authority. It contains no added substance.

(**Note** Water for Injection is intended for use as a solvent for the preparation of parenteral solutions subject to final sterilization. Use suitable means to minimize growth, or first render the Water for Injection sterile and thereafter protect it from microbial contamination. For parenteral solutions that are prepared under aseptic conditions and are not sterilized by appropriate filtration or in the final container, first render the Water for Injection sterile and, thereafter, protect it from microbial contamination.)

Description Clear, colourless liquid; odourless.

Packaging and storages Water for Injection shall be kept in unreactive storage containers that are designed to prevent microbial entry.

Labelling The label on the container states (1) that it contains no antimicrobial or other substance; (2) that it is not intended for direct parenteral administration.

Total organic carbon Complies with the "Total Organic Carbon" (Appendix 4.36). Alternatively the test method for Oxidizable substances may be performed. To

100 ml, add 10 ml of 1 M *sulfuric acid*, and heat to boiling. Add 0.20 ml of 0.020 M *potassium permanganate*, and boil for 5 minutes. The pink colour does not completely disappear.

Water conductivity Complies with the "Water Conductivity" (For Bulk Water, Appendix 4.37).

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains less than 0.25 Endotoxin Unit per ml.

WATER FOR INJECTION, BACTERIOSTATIC

Category Pharmaceutic aid (sterile vehicle).

Bacteriostatic Water for Injection is prepared from Water for Injection that is sterilized and suitably packaged, containing one or more suitable antimicrobial agents.

(**Note** Bacteriostatic Water for Injection is intended to be used as a diluent in the preparation of parenteral products, most typically for multi-dose products that require repeated content withdrawals. Use Bacteriostatic Water for Injection with due regard for the compatibility of the antimicrobial agent(s) it contains with the particular medicinal substance that is to be dissolved or diluted.)

Description Clear, colourless liquid; odourless or having the odour of the antimicrobial substance.

Packaging and storage Bacteriostatic Water for Injection shall be kept in single-dose or multiple-dose glass containers, preferably of Type I or Type II glass, or plastic containers, of not larger than 30-ml size.

Labelling The label on the container states the designation "**NOT FOR USE IN NEWBORNS**" in boldface capital letters on the label immediately under the official name, printed in a contrasting colour, preferably red. Alternatively, the statement may be placed prominently elsewhere on the label if the statement is enclosed within a box.

pH 4.5 to 7.0, determined potentiometrically in a solution prepared by the addition of 0.30 ml of a saturated solution of *potassium chloride* to 100 ml of the test substance (Appendix 4.11).

Calcium To 100 ml add 2 ml of *ammonium oxalate TS*: no turbidity is produced.

Carbon dioxide To 25 ml add 25 ml of *calcium hydroxide TS*: the mixture remains clear.

Sulfate To 100 ml add 1 ml of *barium chloride TS*: no turbidity is produced.

Particulate matter Complies with the requirements described under "Particulate Matter in Injections" (Small-volume Injection, Appendix 4.27).

Antimicrobial agent(s) Complies with the "Efficacy of Antimicrobial Preservation" (Appendix 10.6) and the "Content of Antimicrobial Agents" (Appendix 6.22).

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains less than 0.5 Endotoxin Unit per ml.

Sterility Complies with the "Sterility Test" (Appendix 10.1).

WATER FOR INJECTION, STERILE

Category Pharmaceutic aid (solvent).

Sterile Water for Injection is prepared from Water for Injection that is sterilized and suitably packaged. It contains no antimicrobial agent(s) or other added substance.

Description Clear, colourless liquid; odourless.

Packaging and storage Sterile Water for Injection shall be kept in glass containers, preferably of Type I or Type II glass, or plastic containers, of not larger than 1-litre size.

Labelling The label on the container states (1) that no antimicrobial or other substance has been added; (2) that it is not suitable for intravascular injection without first having been made approximately isotonic by the addition of a suitable solute.

Oxidizable substances To 100 ml add 10 ml of 1 M *sulfuric acid*, and heat to boiling. For Sterile Water for Injection in containers having a fill volume of less than 50 ml, add 0.40 ml of 0.020 M *potassium permanganate*, and boil for 5 minutes; where the fill volume is 50 ml or more, add 0.20 ml of 0.020 M *potassium permanganate*, and boil for 5 minutes. If a precipitate forms, cool in an ice-bath to room temperature, and pass through a sintered-glass filter: the pink colour does not completely disappear.

Water conductivity Complies with the "Water Conductivity" (For Sterile Water, Appendix 4.37).

Particulate matter Complies with the requirements described under "Particulate Matter in Injections" (Small-volume Injection, Appendix 4.27).

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains less than 0.25 Endotoxin Unit per ml.

Sterility Complies with the "Sterility Test" (Appendix 10.1).

WATER FOR INHALATION, STERILE

Category Pharmaceutic aid (solvent).

Sterile Water for Inhalation is prepared from Water for Injection that is sterilized and suitably packaged. It contains no antimicrobial agents. (**Note** Sterile Water for Inhalation is intended for use in inhalators and in the preparation of inhalation solutions. Do not use Sterile Water for Inhalation for parenteral administration or for other sterile compendial dosage forms.)

Description Clear, colourless liquid; odourless.

Packaging and storage Sterile Water for Inhalation shall be kept in glass containers, preferably of Type I or Type II glass, or plastic containers.

Labelling The label on the container states (1) that it is for inhalation therapy only; (2) that it is not for parenteral administration.

Oxidizable substances To 100 ml add 10 ml of 1 M *sulfuric acid*, and heat to boiling. For Sterile Water for Inhalation in containers having a fill volume of less than 50 ml, add 0.40 ml of 0.020 M *potassium permanganate*, and boil for 5 minutes; where the fill volume is 50 ml or more, add 0.20 ml of 0.020 M *potassium permanganate*, and boil for 5 minutes. If a precipitate forms, cool in an ice-bath to room temperature, and pass through a sintered-glass filter: the pink colour does not completely disappear.

Water conductivity Complies with the "Water Conductivity" (For Sterile Water, Appendix 4.37).

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains less than 0.5 Endotoxin Unit per ml.

Sterility Complies with the "Sterility Test" (Appendix 10.1).

WATER FOR IRRIGATION, STERILE

Category Irrigation solution

Sterile Water for Irrigation is prepared from Water for Injection that is sterilized and suitably packaged. It contains no antimicrobial agent or other added substance.

Description Clear, colourless liquid; odourless.

Packaging and storage Sterile Water for Irrigation shall be kept in single-dose glass containers, preferably of Type I or Type II glass, or plastic containers. The container may contain a volume of more than 1 litre, and may be designed to empty rapidly.

Labelling The label on the container states (1) that no antimicrobial or other substance has been added; (2) the prominent designations "For irrigation only" and "Not for injection".

Oxidizable substances To 100 ml add 10 ml of 1 M *sulfuric acid*, and heat to boiling. For Sterile Water for Irrigation in containers having a fill volume of less than 50 ml, add 0.40 ml of 0.020 M *potassium permanganate*, and boil for 5 minutes; where the fill volume is 50 ml or more, add 0.20 ml of 0.020 M *potassium permanganate*, and boil for 5 minutes. If a precipitate forms, cool in an ice-bath to room temperature, and pass through a sintered-glass filter: the pink colour does not completely disappear.

Water conductivity Complies with the "Water Conductivity" (For Sterile Water, Appendix 4.37).

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains less than 0.25 Endotoxin Unit per ml.

Sterility Complies with the "Sterility Test" (Appendix 10.1).

WATER FOR HEMODIALYSIS

Category Hemodialysis solution

Water for Hemodialysis is water that complies with the regulation on water intended for human consumption laid down by the competent authority and that has been subjected to further treatment, using a suitable process, to reduce chemical and microbiological components. It is produced and used onsite under the direction of qualified personnel. It contains no added antimicrobials and is not intended for injection.

Description Clear, colourless liquid; odourless.

Packaging and storage Water for Hemodialysis shall be kept in unreactive storage containers that are designed to prevent bacterial entry and stored at room temperature.

Oxidizable substances To 100 ml, add 10 ml of 1 M *sulfuric acid*, and heat to boiling. Add 0.20 ml of 0.020 M *potassium permanganate*, and boil for 5 minutes. The pink colour does not completely disappear. Alternatively the test method for Total Organic Carbon (Appendix 4.36) may be performed.

Water conductivity Complies with the "Water Conductivity" (For Bulk Water, Appendix 4.37).

Microbial limit Total aerobic microbial count does not exceed 2×10^2 CFU per ml, determined by membrane filtration (Appendix 10.2).

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains less than 2.0 Endotoxin Units per ml.

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ห้ามทำซ้ำ ดัดแปลง แก้ไข โดยไม่ได้รับอนุญาต

ลิขสิทธิ์ กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข
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MONOGRAPHS

BIOLOGICAL PRODUCTS

ลิขสิทธิ์ กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข
ห้ามทำซ้ำ ดัดแปลง แก้ไข โดยไม่ได้รับอนุญาต

ลิขสิทธิ์ กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข
ห้ามทำซ้ำ ดัดแปลง แก้ไข โดยไม่ได้รับอนุญาต

GENERAL INFORMATION

The biological products referred to in this Pharmacopoeia are products of biological origin whose potency and safety cannot be evaluated by chemical or physical tests alone. The testing of such products for potency and safety is therefore carried out by biological procedures requiring specialized knowledge and competence. These products may be of animal or microbial or vegetable origin (vaccines, diagnostic preparations) or of animal or human origin (antisera, blood products). The biological products are distributed under aseptic conditions in sterile containers that are inert towards the contents and sealed so as to exclude contamination. Moreover special care must be taken to the handling and storage of these products. Due to the above reasons, the biological products have been separately grouped.

Classification of Biological Products Biological Products to be included in this Pharmacopoeia, are classified as follows:

Human blood and blood products
Antisera
Vaccines
Diagnostic preparation

Labelling Statements required to appear on the "label on the container" may appear on a label fastened to the immediate container, or in indelible writing in or on the body of the container itself. They should also be demonstrated on the label on the package.

The label on the container or the label on the package states at least (1) the name of the product; (2) the name and address of the manufacturer; (3) the number of the final lot; (4) the nature and amount of the preservative added; (5) the storage condition; (6) the expiration date; (7) that it is to be well shaken before use; (8) for dried preparation, the liquid to be used for reconstitution and its volume.

Packaging and storage Biological products shall be stored at all times at controlled temperatures within a range which ensures optimal stability.

During distribution, short periods at ambient temperatures may have to be permitted.

Preservative A biological product contains a suitable preservative in a concentration without deleterious effects on its preparation and to cause no untoward reactions in man. If phenol or thiomersal is used, the content should be conformed to the limit stated in General Testing Methods for Biological Products, p. 178.

Glossary of Terms

IU International Unit is the specific activity of a stated amount of the International Standard as defined by the WHO Expert Committee on Biological Standardization.

The potency of the biological product is expressed in terms of International Unit when the assay is determined in parallel with the International Standard.

International Biological Standards and International Biological Reference Preparations

International Biological Standards and International Biological Reference Preparations are certain preparations authorized by the World Health Organization.

An International Biological Standard is a preparation of a biological substance to which an international unit has been assigned by the World Health Organization on the basis of data obtained in an international study.

An International Biological Reference Preparation is a preparation of a biological substance which may be used for a purpose similar to that of a standard but which has been established without a full collaborative study or after such a study has shown that it is not appropriate to establish it as an international standard.

National Biological Standards and National Biological Reference Preparations

National Biological Standards and National Biological Reference Preparations are specified products authorized by Ministry of Public Health, calibrated against the International Standards and International Reference Preparations to be used as measures for potency or toxicity of a product.

PFU Plaque-forming unit, the smallest quantity of virus suspension that will produce a plaque in monolayer cell cultures.

ED_{50} Effective Dose 50, the dose of a vaccine which protects 50 per cent of the immunized animals against a challenge dose of virulent micro-organism or toxin.

ID_{50} Infective Dose 50, the dose of the micro-organism that infects 50 per cent of the animals inoculated.

Human Dose A human dose is the dose stated on the label of the preparation being exam-

ined or in the accompanying information leaflet.

L+dose — The smallest quantity of a toxin that, in the conditions of the test, when mixed with 1 IU of antitoxin and administered by the specified route, causes the death of the test animals within the given period.

L+/10 dose — The smallest quantity of a toxin that, in the conditions of the test, when mixed with 0.1 IU of antitoxin and administered by the specified route, causes the death of the test animals within the given period.

LD_{50} — Lethal Dose 50, the amount of toxin or micro-organism that kills 50 per cent of a group of animals inoculated within specific time (the LD_{50} differs for different animal species and for different routes of administration).

Lf — Limits of Flocculation, the amount of toxin or toxoid which when mixed with one International Unit (1 IU) of antitoxin gives a flocculation reaction in the shortest time.

Lo/10 dose — The largest quantity of a toxin that, in the conditions of the test, when mixed with 0.1 IU of antitoxin and administered by the specified route, does not cause symptoms of toxicity in the test animals within a given speriod.

Lp/10 dose — The smallest quantity of a toxin that, in the conditions of the test, when mixed with 0.1 IU of antitoxin and administered by the specified route, causes paralysis in the test animals within a given period.

Lr — The minimum amount of toxin which when combined with a fixed amount of antitoxin (usually 0.002 IU of antitoxin) in a volume of 0.2 ml causes a local skin reaction that is just visible.

lr/100 dose — The smallest quantity of a toxin that, in the conditions of the test, when mixed with 0.01 IU of antitoxin and injected intracutaneously causes a characteristic reaction at the site of injection within a given period.

$CCID_{50}$ — Cell Culture Infective Dose 50, the quantity of a virus suspension that will infect 50 per cent of cell cultures inoculated.

GENERAL TESTING METHODS FOR BIOLOGICAL PRODUCTS

Determination of Aluminium

For adsorbed vaccines containing aluminium, not more than 1.25 mg per dose, unless otherwise stated in the monograph, determined by the following method.

Homogenize the preparation being examined and transfer a quantity expected to contain between 5 and 6 mg of aluminium to a 50-ml Kjeldahl flask. Add 1 ml of *sulfuric acid*, 0.3 ml of *nitric acid* and some glass beads. Heat the solution until dense, white fumes are evolved. If charring occurs, add a few more drops of *nitric acid* and continue boiling until the colour disappears. Allow to cool for a few minutes, carefully add 10 ml of *water* and boil until a clear solution is obtained. Allow to cool, add 0.1 ml of *methyl orange TS* and neutralize with 10 M *sodium hydroxide* (about 6.5 to 7.0 ml). If a precipitate forms, dissolve it by adding, dropwise, sufficient 1 M *sulfuric acid*. Transfer the solution to a flask, rinsing the Kjeldahl flask with 25 ml of *water*. Add 25.0 ml of 0.02 M *disodium edetate VS*, 10 ml of *acetate buffer pH 4.4* and a few glass beads and boil gently for 3 minutes. Add 0.25 ml of *pyridylazonaphthol TS* and titrate the excess of disodium edetate in the hot solution with 0.02 M *copper(II) sulfate VS* until the colour changes to purplish brown. Carry out a blank titration omitting the vaccine (Appendix 6.17). The difference between the titrations represents the volume of 0.02 M *disodium edetate VS* equivalent to the aluminium present. Each ml of 0.02 M *disodium edetate* is equivalent to 0.5396 mg of Al.

Determination of Calcium

For adsorbed vaccines containing calcium, not more than 1.3 mg per dose, unless otherwise stated in the monograph, determined by the following method.

Homogenize the preparation being examined. Add 0.2 ml of *hydrochloric acid* to 1.0 ml and dilute to 3.0 ml with *water*. Determine the content of calcium by the "Atomic Spectrometry: Emission and Absorption" (Appendix 2.3), measuring at 620 nm and using *calcium solution ASp*, diluted if necessary with *water*, for the preparation of the standard solutions.

Determination of Formaldehyde

For vaccines containing formaldehyde, not more than 0.02 per cent w/v of free formaldehyde (CH_2O).

Use test A unless otherwise prescribed.

Test B is suitable for vaccines where sodium metabisulfite has been used to neutralize excess formaldehyde.

Test A

To 1 ml of a tenfold dilution of the vaccine in *water* add 4 ml of *water* and 5 ml of *acetylacetone reagent*. Warm in a water-bath at 40° and allow to stand for 40 minutes. The solution is not more intensely coloured than a reference solution prepared at the same time and in the same manner using 1 ml of a solution containing 0.002 per cent w/v of *formaldehyde*, CH_2O, in place of the dilution of the vaccine. The comparison should be made by examining the tubes down their vertical axes.

Test B

Test solution

Prepare a 1 in 200 dilution of the vaccine being examined with *water*. If the vaccine is an emulsion, prepare an equivalent dilution using the aqueous phase separated by a suitable procedure. If one of the methods described below is used for separation of the aqueous phase, a 1 in 20 dilution of the latter is used.

Reference solutions

Prepare solutions containing 0.25 g/l, 0.50 g/l, 1.00 g/l and 2.00 g/l of CH_2O by dilution of *formaldehyde solution* with *water*.

Procedure

To 0.5 ml of the test solution and of each of the reference solutions in test-tubes, add 5.0 ml of a freshly prepared 0.05 per cent w/v solution of *3-methyl-2-benzothiazolinone hydrazone hydrochloride hydrate*. Close the tubes, shake and allow to stand for 60 minutes. Add 1 ml of *iron(III) chloride-sulfamic acid TS* and allow to stand for 15 minutes. Measure the absorbance (Appendix 2.2) of the solutions at 628 nm. Calculate the content of formaldehyde in the vaccine being examined from the calibration curve established using the reference solutions. The test is invalid if the correlation coefficient (r) of the calibration curve is less than 0.97.

Emulsions

If the vaccine being examined is an emulsion, the aqueous phase is separated using a suitable procedure and used for preparation of the test solution. The following procedures have been found suitable.

(a) Add 1.0 ml of the vaccine being examined to 1.0 ml of *isopropyl myristate* and mix. Add 1.3 ml of 1 M *hydrochloric acid*, 2.0 ml of *chloroform* and 2.7 ml of *saline TS*. Mix thoroughly. Centrifuge at 15,000 × *g* for 60 minutes. Transfer the aqueous phase to a 10-ml volumetric flask and dilute to volume with *water*. If this procedure fails to separate the aqueous phase, repeat the procedure but use a 10 per cent w/v solution of *polysorbate 20* in *saline TS* instead of *saline TS* and centrifuge at 22,500 × *g*.

(b) Add 1.0 ml of the vaccine being examined to 1.0 ml of a 10 per cent w/v solution of *sodium chloride* and mix. Centrifuge at 1000 × *g* for 15 minutes. Transfer the aqueous phase to a 10-ml volumetric flask and dilute to volume with *water*.

(c) Add 1.0 ml of the vaccine being examined to 2.0 ml of a 10 per cent w/v solution of *sodium chloride* and 3.0 ml of *chloroform* and mix. Centrifuge at 1000 × *g* for 15 minutes. Transfer the aqueous phase to a 10-ml volumetric flask and dilute to volume with *water*.

Determination of Phenol

For biological products containing phenol as preservative, not more than 0.25 per cent w/v, unless otherwise stated in the monograph, determined by the following method.

Homogenize the preparation being examined. Dilute an appropriate volume with *water* to give a solution expected to contain 15 µg of phenol per ml. To 5.0 ml of the resulting solution add 5 ml each of *borate buffer pH 9.0*, *aminophenazone TS* and a 5 per cent w/v solution of *potassium hexacyanoferrate(III)*. Allow to stand for 10 minutes and measure the absorbance at 546 nm (Appendix 2.2). Calculate the phenol content from the absorbance obtained, using a calibration curve prepared by repeating the operation using 5.0 ml of each of a series of reference solutions containing 5, 10, 15, 20, and 30 µg of *phenol* per ml, respectively.

Determination of Thiomersal[1]

For biological products containing thiomersal as preservative, not less than 0.005 per cent w/v and not more than 0.02 per cent w/v of thiomersal, determined by the following method.

Wash separators with *nitric acid* and rinse with tap water and *water*. Transfer two portions of the preparation being examined each of 1.0 ml to individual separators. Add a 1 per cent w/v solution of *ammonium acetate* to make 10 ml. To each separator, add 10.0 ml of *dithizone standard solution*. Shake vigorously for 45 seconds. Carefully separate the resulting chloroform layer, and measure the absorbance at 520 nm (Appendix 2.2), using *dithizone standard solution* as a blank. Calculate the thiomersal content from the absorbance obtained using a calibration curve prepared from Thiomersal RS by repeating the operation using 1.0 ml of each of a series of reference solutions containing 50, 75 and 100 µg of *thiomersal* per ml, respectively.

[1]Biological products available in some countries may contain thiomersal. Risk-benefit should be considered when medical problem exists.

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HUMAN BLOOD AND BLOOD PRODUCTS

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ห้ามทำซ้ำ ดัดแปลง แก้ไข โดยไม่ได้รับอนุญาต

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ANTICOAGULANT AND PRESERVATIVE SOLUTIONS FOR HUMAN BLOOD

Category Anticoagulant for storage of whole blood.

Anticoagulant and Preservative Solutions for Human Blood (Anticoagulant Citrate Dextrose Solution (ACD) and Anticoagulant Citrate Phosphate Dextrose Solution (CPD) are sterile and pyrogen-free solutions prepared with Water for Injection, filtered, distributed in the final containers and sterilized. The contents of sodium citrate dihydrate ($C_6H_5Na_3O_7.2H_2O$), dextrose monohydrate ($C_6H_{12}O_6.H_2O$) or anhydrous dextrose ($C_6H_{12}O_6$) and sodium dihydrogenphosphate dihydrate ($NaH_2PO_4.2H_2O$) are not less than 95.0 per cent and not more than 105.0 per cent of that stated in the formulae below. The contents of citric acid monohydrate ($C_6H_8O_7.H_2O$) or anhydrous citric acid ($C_6H_8O_7$) is not less than 90.0 per cent and not more than 110.0 per cent of that stated in the formulae below. Other substances, such as red-cell preservatives, may be included in the formula provided that their names and concentrations are stated on the label.

Requirements and advice concerning the containers are given in Appendix 11.3.

ANTICOAGULANT CITRATE DEXTROSE SOLUTION

Acid Citrate Dextrose Solution (ACD)

	Formula	
	A	B
Sodium Citrate Dihydrate	22.0 g	13.2 g
Citric Acid Monohydrate	8.0 g	4.8 g
or Anhydrous Citric Acid	7.3 g	4.4 g
Dextrose Monohydrate	24.5 g	14.7 g
or Anhydrous Dextrose	22.3 g	13.4 g
Water for Injection sufficient to produce	1000.0 ml	1000.0 ml
Volume of the solution for the collection of 100 ml of blood	15.0 ml	25.0 ml

Description Colourless or faintly yellow, clear liquid, free from particles.

Packaging and storage Anticoagulant Citrate Dextrose Solution shall be preserved in tightly closed, tamper-evident containers of plastic or other suitable materials.

Labelling Comply with the "General Information for Biological Products", p. 177. In addition the label on the container states (1) the composition and volume; (2) where applicable, the maximum amount of blood to be collected in the container.

Identification

A. Carry out the test as described in the "Thin-layer chromatography" (Appendix 3.1), using *silica gel G* as the coating substance and a mixture of 2 volumes of *water*, 3 volumes of *methanol*, 5 volumes of *anhydrous glacial acetic acid* and 10 volumes of *1,2-dichloroethane* as the mobile phase. The volumes of solvents have to be measured accurately since a slight excess of water produces cloudiness. Apply separately to the plate 2 µl of each of the following solutions. For solution (A) dilute 2 ml of the solution being examined (for formula A) or 3 ml (for formula B) to 100 ml with a mixture of 2 volumes of *water* and 3 volumes of *methanol*. For solution (B_1) dissolve 10 mg of Dextrose RS in a mixture of 2 volumes of *water* and 3 volumes of *methanol* and dilute to 20 ml with the same mixture of solvent. For solution (B_2) dissolve 10 mg each of Dextrose RS, Lactose RS, Fructose RS, and Sucrose RS in a mixture of 2 volumes of *water* and 3 volumes of *methanol* and dilute to 20 ml with the same mixture of solvents. After removal of the plate, allow it to dry in warm air, repeat the development immediately, after renewing the mobile phase. Dry the plate in a current of warm air and spray evenly with a solution of 0.5 g of *thymol* in a mixture of 5 ml of *sulfuric acid* and 95 ml of *ethanol*, heat at 130° for 10 minutes and allow it to cool. The principal spot in the chromatogram obtained from solution (A) is similar in position, colour and size to the principal spot in the chromatogram obtained from solution (B_1). The test is not valid unless the chromatogram obtained from solution (B_2) shows four clearly separated spots.

B. To 2 ml add 5 ml of *copper-citric TS* and heat to boiling: an orange precipitate is produced and the solution becomes yellow.

C. To 2 ml (for formula A) add 3 ml of *water* or to 4 ml (for formula B) add 1 ml of *water*. It yields the *reactions* characteristic of citrates (Appendix 5.1).

D. It yields *reaction B* characteristic of sodium salts (Appendix 5.1).

pH 4.7 to 5.3 (Appendix 4.11).

Hydroxymethylfurfural To 2.0 ml add 5.0 ml of a 10 per cent w/v solution of p-*toluidine* in *2-propanol* containing 10 per cent v/v of *glacial acetic acid* and 1.0 ml of a 0.5 per cent w/v solution of *barbituric acid*. The absorbance, determined at 550 nm after allowing the mixture to stand for 2 to 3 minutes, is not more than that of a standard prepared at the same time in the same

manner using 2.0 ml of a solution containing 5 ppm of *hydroxymethylfurfural* for formula A or 3 ppm of *hydroxymethylfurfural* for formula B.

Bacterial endotoxins and Pyrogens Perform one of the following tests.

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains not more than 5.56 Endotoxin Units per ml.

Pyrogens Complies with the "Pyrogen Test" (Appendix 8.2), using per kg of the rabbit's weight 10 ml of a dilution in *pyrogen-free saline TS* containing 0.5 per cent w/v of *sodium citrate dihydrate*.

Sterility Comply with the "Sterility Test" (Appendix 10.1).

Assay

FOR CITRIC ACID To 10.0 ml of Anticoagulant Citrate Dextrose Solution (for formula A) or to 20.0 ml (for formula B) add 0.1 ml of *phenolphthalein TS*. Titrate with 0.2 M *sodium hydroxide VS* until a pink colour is obtained. Perform a blank determination, and make any necessary correction. Each ml of 0.2 M *sodium hydroxide* is equivalent to 14.01 mg of $C_6H_8O_7.H_2O$ or to 12.81 mg of $C_6H_8O_7$.

FOR SODIUM CITRATE Prepare a chromatographic column (10 cm × 10 mm) and filled with *strongly acidic ion-exchange resin* (300 to 840 µm). Maintain a 1-cm layer of liquid above the resin at all times. Wash the column with 50 ml of de-ionized *water* at a flow rate of 12 to 14 ml per minute.

Dilute 10.0 ml of the solution being examined (for formula A) or 15.0 ml (for formula B) to about 40 ml with de-ionized *water* in a beaker and transfer to the column reservoir, washing the beaker three times with a few ml of de-ionized *water*. Allow the solution to run through the column at a flow rate of 12 to 14 ml per minute and collect the eluate. Wash the column with two 30-ml portions and with one 50-ml portion of de-ionized *water*. The column can be used for three successive determinations before regeneration with three times its volume of *dilute hydrochloric acid*. Titrate the combined eluate and washings (about 150 ml) with 0.20 M *sodium hydroxide*, using 0.1 ml of *phenolphthalein TS* as indicator.

Calculate the content of sodium citrate in g per litre from the following expressions:

For formula A: $1.961n - 1.40C$
or $1.961n - 1.53C'$
For formula B: $1.307n - 1.40C$
or $1.307n - 1.53C'$

where n = number of ml of 0.20 M *sodium hydroxide* used in the titration,
C = content of citric acid monohydrate in g per litre determined as prescribed above,
C' = content of anhydrous citric acid in g per litre determined as prescribed above.

FOR REDUCING SUGARS Dilute 5.0 ml (for formula A) or 10.0 ml (for formula B) to 100.0 ml with *water*. Introduce 25.0 ml of the solution into a 250-ml conical flask with ground-glass neck and add 25.0 ml of *copper-citric TS*. Add a few pieces of porous material, attach a reflux condenser, heat so that boiling begins within 2 minutes, and boil for exactly 10 minutes. Cool and add 3 g of *potassium iodide* dissolved in 3 ml of *water*. Add 25 ml of a 25 per cent w/w solution of *sulfuric acid* with caution and in small quantities. Titrate with 0.10 M *sodium thiosulfate* using 0.5 ml of *starch TS*, added towards the end of the titration, as indicator (n_1 ml). Carry out a blank titration using 25.0 ml of *water* (n_2 ml).

Calculate the content of reducing sugars as anhydrous dextrose or as dextrose monohydrate, as appropriate, from Table 1.

Table 1

Volume of 0.10 M *Sodium Thiosulfate* ($n_2 - n_1$ ml)	Anhydrous Dextrose in mg	Dextrose Monohydrate in mg
8	19.8	21.6
9	22.4	24.5
10	25.0	27.2
11	27.6	30.2
12	30.3	33.1
13	33.0	36.1
14	35.7	39.0
15	38.3	42.1
16	41.3	45.2

ANTICOAGULANT CITRATE PHOSPHATE DEXTROSE SOLUTION

Citrate Phosphate Dextrose Solution (CPD)

Sodium Citrate Dihydrate	26.3	g
Citric Acid Monohydrate	3.27	g
or Anhydrous Citric Acid	2.99	g
Dextrose Monohydrate	25.5	g
or Anhydrous Dextrose	23.2	g
Sodium Dihydrogenphosphate Dihydrate	2.51	g
Water for Injection sufficient to produce	1000.0	ml
Volume of the solution for the collection of 100 ml of blood	14.0	ml

Description; Packaging and storage; Labelling See under *Anticoagulant Citrate Dextrose Solution*, p. 183.

Identification

A. Complies with the tests for Identification described under *Anticoagulant Citrate Dextrose Solution*, p. 183.

B. It yields *reaction B* characteristic of phosphates (Appendix 5.1).

pH 5.3 to 5.9 (Appendix 4.11).

Hydroxymethylfurfural Complies with the tests for formula A described under *Anticoagulant Citrate Dextrose Solution*, p. 183.

Bacterial endotoxins and Pyrogens; Sterility Complies with the tests described under *Anticoagulant Citrate Dextrose Solution*, p. 183.

Assay

FOR SODIUM DIHYDROGENPHOSPHATE DIHYDRATE Dilute 10.0 ml of Anticoagulant Citrate Phosphate Dextrose Solution to 100.0 ml with *water*. To 10.0 ml of this solution add 10.0 ml of *nitro-vanado-molybdic TS*. Mix and allow to stand at 20° to 25° for 30 minutes. At the same time and in the same manner, prepare a reference solution using 10.0 ml of a standard solution containing 0.219 g of *potassium dihydrogenphosphate* per litre. Measure the absorbance of each of the two solutions at 450 nm (Appendix 2.2), using as the blank a solution prepared in the same manner using 10 ml of *water*. Calculate the content of $NaH_2PO_4.2H_2O$ (*P*) in g per litre from the expression:

$$\frac{11.46 \times C \times A_1}{A_2},$$

where C = concentration of *potassium dihydrogenphosphate* in the standard solution in g per litre,
A_1 = absorbance of the test solution, and
A_2 = absorbance of the reference solution.

FOR CITRIC ACID To 20.0 ml add 0.1 ml of *phenolphthalein TS* and titrate with 0.20 M *sodium hydroxide*. Calculate the content of citric acid monohydrate (*C*), or anhydrous citric acid (*C′*), in g per litre from the equations:

$$C = 0.7005n - 0.4490P$$
$$C' = 0.6404n - 0.4105P$$

where n = number of ml of 0.20 M *sodium hydroxide* used in the titration, and
P = content of sodium dihydrogenphosphate dihydrate in g per litre determined as prescribed above.

FOR SODIUM CITRATE Prepare a chromatographic column 0.10 m long and 10 mm in internal diameter and filled with *strongly acidic ion-exchange resin* (300 μm to 840 μm). Maintain a 1-cm layer of liquid above the resin at all times. Wash the column with 50 ml of de-ionized *water* at a flow rate of 12 to 14 ml per minute.

Dilute 10.0 ml of the solution being examined to about 40 ml with de-ionized *water* in a beaker and transfer to the column reservoir, washing the beaker three times with a few ml of de-ionized *water*. Allow the solution to run through the column at a flow rate of 12 to 14 ml per minute and collect the eluate. Wash the column with two 30-ml portions and with one 50-ml portion of de-ionized *water*. The column can be used for three successive determinations before regeneration with three times its volume of *dilute hydrochloric acid*. Titrate the combined eluate and washings (about 150 ml) with 0.20 M *sodium hydroxide*, using 0.1 ml of *phenolphthalein TS* as indicator. Calculate the content of $C_6H_5Na_3O_7.2H_2O$ in g per litre from the following expressions:

$$1.961n - 1.257P - 1.40C$$
$$1.961n - 1.257P - 1.53C'$$

where n = number of ml of 0.20 M *sodium hydroxide* used in the titration,
P = content of sodium dihydrogenphosphate dihydrate in g per litre determined as prescribed above,
C = content of citric acid monohydrate in g per litre determined as prescribed above, and
C' = content of anhydrous citric acid in g per litre determined as prescribed above.

FOR REDUCING SUGARS Carry out the tests for reducing sugar (for formula A) described under *Anticoagulant Citrate Dextrose Solution*, p. 183.

ANTICOAGULANT CITRATE PHOSPHATE DEXTROSE ADENINE SOLUTION

Category Anticoagulant for storage of whole blood.

Anticoagulant Citrate Phosphate Dextrose Adenine Solution is a sterile solution of Citric Acid, Sodium Citrate, Sodium Dihydrogenphosphate Monohydrate, Dextrose, and Adenine in Water for Injection. It contains, in each 1000 ml, not less than 2.11 g and not more than 2.33 g of sodium dihydrogenphosphate monohydrate ($NaH_2PO_4.H_2O$); not less than 30.30 g and not more than 33.50 g of dextrose ($C_6H_{12}O_6.H_5O$); not less than 19.16 g and not more than 21.18 g of total citrate, expressed as citric acid, anhydrous ($C_6H_8O_7$); not less than 6.21 g and not more than 6.86 g of sodium (Na); and not less than 0.247 g and not more than 0.303 g of adenine ($C_5H_5N_5$). It contains no antimicrobial agent.

Citrate Phosphate Dextrose Adenine Solution (CPDA-1)

Sodium Citrate Dihydrate	26.3	g
Citric Acid Monohydrate	3.27	g
or Anhydrous Citric Acid	2.99	g
Dextrose Monohydrate	31.9	g
or Anhydrous Dextrose	29.0	g
Sodium Dihydrogenphosphate Monohydrate	2.22	g
Adenine	0.275	g
Water for Injection sufficient to produce	1000.0	ml
Volume of the solution for the collection of 100 ml of blood	14.0	ml

Description; Packaging and storage; Labelling See under *Anticoagulant Citrate Dextrose Solution*, p. 183.

Identification The retention time of the adenine peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of the Standard preparation, as obtained in the *Assay*.

See also under *Anticoagulant Citrate Phosphate Dextrose Solution*, p. 183.

pH 5.0 to 6.0 (Appendix 4.11).

Chloride Not more than 0.0035 per cent w/v (Appendix 5.2), a 10-ml portion shows no more chloride than that corresponds to 0.50 ml of 0.020 M *hydrochloric acid*.

Bacterial endotoxins and Pyrogens; Sterility Complies with the tests described under *Anticoagulant Citrate Dextrose Solution*, p. 183.

Assay

FOR TOTAL CITRATE AND TOTAL PHOSPHATE

Mobile phase Transfer an appropriate volume of *water* (resistivity not less than 18 megohm-cm) to a suitable container, and degas with *helium* for not less than 20 minutes. Add an appropriate volume of a 50 per cent w/w carbonate-free *sodium hydroxide* or *potassium hydroxide* to obtain a 20 mM. Alternatively, a 20 mM sodium hydroxide or potassium hydroxide eluent can be generated electrolytically using an automatic eluent generator. (**Note** Protect *Mobile phase* from atmospheric carbon dioxide.)

Standard preparation Dissolve Citric Acid RS and *sodium dihydrogenphosphate* in freshly prepared 1 mM *sodium hydroxide* to obtain a solution having known concentrations of about 20 µg per ml and 12 µg per ml of citrate and phosphate (PO_4), respectively.

Assay preparation for total citrate assay Pipette 10 ml of Anticoagulant Citrate Phosphate Dextrose Adenine Solution into a suitable volumetric flask and add freshly prepared 1 mM *sodium hydroxide* to obtain a solution containing about 20 µg per ml of citrate in 1 mM *sodium hydroxide*.

Assay preparation for total phosphate assay Pipette 5 ml of Anticoagulant Citrate Phosphate Dextrose Adenine Solution into a suitable volumetric flask and add freshly prepared 1 mM *sodium hydroxide* to obtain a solution containing about 12 µg per ml of phosphate in 1 mM *sodium hydroxide*.

Chromatographic systems The chromatographic procedure may be carried out using (a) a guard column (5 cm × 4 mm) and a stainless steel analytical column (25 cm × 4 mm), both packed with a hydroxide selective strong anion-exchange resin consisting of a highly cross-linked core of 13-µm microporous particles having a pore size less than 10 Å and consisting of ethylvinylbenzene cross-linked with 55 per cent divinylbenzene with a latex coating composed of 85-nm diameter microbeads bonded with alkanol quaternary ammonium ions (6 per cent), (b) *Mobile phase* at a flow rate of about 2 ml per minute, (c) an electrochemical detector with suppressed conductivity detection using either a micromembrane anion autosuppressor or a suitable chemical suppression system. All columns are maintained at a temperature of 30°. (**Note** An anion trap column designed to remove trace anion contaminants in the *Mobile phase* should be added to the column assembly before the injector.)

To determine the suitability of the chromatographic system, chromatograph *Standard preparation*, and record the peak area responses as directed for *Procedure*: the symmetry factor is not more than 2.0, and the relative standard deviation of the peak areas for citrate and phosphate, for six replicate injections of *Standard Preparation*, is not more than 1.5 per cent.

Procedure Separately inject equal volumes (about 10 µl) of *Standard preparation* and *Assay preparation* into the chromatograph, record the chromatograms, and measure the peak areas for citrate and phosphate, as appropriate.

Calculation Calculate the quantity, in mg, of anhydrous citric acid ($C_6H_8O_7$) in the volume of the Solution taken by the expression:

$$0.001(192.12/189.10)\, C_S D(r_U/r_S),$$

in which 192.12 is the molecular weight of anhydrous citric acid; 189.10 is the molecular weight of citrate ($C_6H_5O_7$); C_S is the concentration, in µg per ml, of citrate in *Standard preparation*; D is the dilution factor; and r_U and r_S are the citrate peak areas obtained from *Assay preparation for total citrate assay* and *Standard preparation*, respectively.

Calculate the quantity, in mg, of phosphate, expressed as sodium dihydrogenphosphate monohydrate ($NaH_2PO_4.H_2O$), in the volume of the Solution taken by the expression:

$$0.001(137.99/94.97)C_S D(r_U/r_S),$$

in which 137.99 is the molecular weight of sodium dihydrogenphosphate monohydrate; 94.97 is the molecular weight of phosphate (PO_4); C_S is the concentration, in µg per ml, of phosphate in *Standard preparation; D* is the dilution factor; and r_U and r_S are the phosphate peak areas obtained from *Assay preparation for total phosphate assay* and *Standard preparation*, respectively.

FOR SODIUM

Lithium diluent solution Transfer 1.04 g of *lithium nitrate* to a 1000-ml volumetric flask, add a suitable nonionic surfactant, then add *water* to volume, and mix. This solution contains 15 mEq of lithium per litre.

Standard preparation Transfer 8.18 g of *sodium chloride*, previously dried at 105° for 2 hours and accurately weighed, to a 1000-ml volumetric flask, dilute with *water* to volume, and mix. This solution contains 140 mEq of sodium per litre. Transfer 50 µl of this solution to a 10-ml volumetric flask, dilute with *Lithium diluent solution* to volume, and mix.

Assay preparation Pipette 25 ml of Anticoagulant Citrate Phosphate Dextrose Adenine Solution into a 50-ml volumetric flask, dilute with *water* to volume, and mix. Transfer 50 µl of this solution to a 10-ml volumetric flask, dilute with *Lithium diluent solution* to volume, and mix.

Procedure Concomitantly measure the absorbances of *Standard preparation* and *Assay preparation* at the maximum at about 589 nm, using *Lithium diluent solution* as the blank (Appendix 2.2).

Calculation Calculate the quantity, in g, of Na in 1000 ml of the Solution taken by the expression:

$$2(8.18)(22.99/58.44)(r_U/r_S),$$

in which 8.18 is the weight, in g, of sodium chloride taken to make *Standard preparation*; 22.99 is the atomic weight of sodium; 58.44 is the molecular weight of sodium chloride; and r_U and r_S are the sodium emission readings obtained from *Assay preparation* and *Standard preparation*, respectively.

FOR DEXTROSE Tare a clean, medium-porosity filtering crucible containing several carborundum boiling chips or glass beads. Pipette 50 ml of freshly mixed *alkaline cupric tartrate TS* into a 400-ml beaker. Add the boiling chips or glass beads from the tared crucible, 45 ml of *water*, and 5.0 ml of Anticoagulant Citrate Phosphate Dextrose Adenine Solution to the beaker. Heat the beaker and contents over a burner that has been adjusted to cause boiling of the solution to start in 3.5 to 4 minutes. Boil the solution for 2 minutes, accurately timed, and filter immediately through the tared crucible, taking care to transfer all of the boiling chips or glass beads to the crucible. Wash the precipitate with hot *water* and 10 ml of *ethanol*. Dry the crucible and contents at 110° to constant weight. Perform a blank determination, and make any necessary correction. Each mg of cuprous oxide precipitate obtained is equivalent to 0.496 mg of $C_6H_{12}O_6.H_2O$.

FOR ADENINE

Mobile phase Dissolve 3.45 g of *ammonium dihydrogenphosphate* in 950 ml of *water* in a 1000-ml volumetric flask, add 10 ml of *glacial acetic acid,* dilute with *water* to volume, mix, pass through a membrane filter having a 1-µm or finer porosity, and degas.

Standard preparation Dissolve accurately weighed quantities of Adenine RS in diluted *hydrochloric acid* (1 in 120) in three separate volumetric flasks, dilute with the diluted hydrochloric acid solution to volume, and mix to obtain Standard preparations having known concen-

trations of about 250, 275, and 300 µg of adenine per ml, respectively. Protect from light.

Resolution solution Prepare a solution containing Adenine RS and *purine*, each at about 275 µg per ml, in diluted *hydrochloric acid* (1 in 120).

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (30 cm × 4 mm) packed with irregular or spherical, totally porous silica gel having a chemically bonded, strongly acidic cation-exchange coating, (3 to 10 µm), (b) *Mobile phase* at a flow rate of about 2.0 ml per minute, and (c) an ultraviolet photometer set at 254 nm.

To determine the suitability of the chromatographic system, chromatograph not less than four injections (about 20 µl) of *Resolution solution*: the relative standard deviation of the peak response of adenine is not more than 2.5 per cent, the relative standard deviation of the retention time of adenine is not more than 2.0 per cent, and the resolution factor of adenine and purine is not less than 3.0.

Procedure Separately inject equal volumes (about 20 µl) of Anticoagulant Citrate Phosphate Dextrose Adenine Solution and *Standard preparation*, record the chromatograms, and measure the responses for the major peaks. Plot the responses against the concentrations, in mg, of Adenine RS per ml of *Standard preparations*.

Calculation Calculate the quantity, in mg, of $C_5H_5N_5$ in each ml of the Solution taken as the value read directly from the Standard curve corresponding to the response obtained from the portion of the Solution chromatographed.

BLOOD, WHOLE

ACD Whole Blood; CPD Whole Blood; CPDA-1 Whole Blood; Heparin Whole Blood.

> ***Caution*** Whole blood shall be administered only through a 170 to 200 µm filter as transfusion equipment for medical use.

Category Human blood and blood products.

Whole Blood is blood that has been mixed with a suitable anticoagulant. The blood is obtained from a counselled healthy donor who must, as far as can be ascertained after medical examination, laboratory tests and a study of the donor's medical history, be free from detectable agents of infection transmissible by transfusion of blood or blood components. The examinations and tests to be carried out are decided by the National Blood Centre and Ministry of Public Health. Tests for syphilis, for hepatitis B surface antigen (HBsAg), for hepatitis C antibodies (anti-HCV) and for HIV antigen and antibodies (anti-HIV-1 and anti-HIV-2) and nucleic acid test (for HBV, HCV, HIV) are carried out by suitably sensitive methods and must give non-reactive results in all cases. The blood has a hemoglobin value, in terms of the National or International Standard for Hemiglobincyanide, of not less than 12.5 per cent w/v.

The blood is withdrawn aseptically through a closed sterile system consisting of a tube connecting the needle inserted in a donor's vein into a suitable, sterile plastic container holding one of the Anticoagulant and Preservative Solutions for Blood in a specific amount not exceeding 22 per cent v/v of the final volume of the mixture. No antimicrobial preservative is added. Requirements and advice concerning the container are given in Appendix 11.3. When withdrawal is complete, the container is sealed immediately so as to exclude micro-organisms and cooled at 2° to 6°. Firmly attached to each container is a smaller container with a small quantity of the blood for compatibility and other tests. The final mixture of blood and anticoagulant solution has a hemoglobin value in terms of the National or International Standard for Hemiglobincyanide of not less than 9.7 per cent v/v (calculated from the hemoglobin value of the donor's blood and the dilution due to the anticoagulant solution).

Description Deep red fluid which, on standing, separates into a lower layer of sedimented red cells and a yellow, upper layer of plasma free from signs of hemolysis. A layer may be visible between the two, appearing as a whitish film of white cells and platelets.

Dose *Intravenous infusion*, as needed to replenish blood volume; usually 1 Unit[1], repeated as necessary. The dose is based on the estimated blood loss, laboratory determination of need or central venous pressure. Units must be administered through a 170 to 200 µm filter.

Warning

1. The rapid transfusion of large volumes of whole blood may overload the circulation and cause pulmonary edema.
2. Repeated transfusion of blood, as in thalassemia, may lead to iron overload.
3. Disseminated intravascular coagulation may also occur in patients receiving large-volume transfusion.

[1]One unit is 450±45 ml of whole blood to which 63 ml of ACD, CPD or CPDA-1 solution has been added.

4. Group O blood samples whose sera show the presence of hemolysins should not be transfused to recipients of other groups.

Expiration date The expiration date depends on the anticoagulant used as follows:

ACD Whole Blood and CPD Whole Blood not later than 21 days.

CPDA-1 Whole Blood not later than 35 days.

Packaging and storage Whole Blood shall be stored at a temperature of 2° to 6°.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition, the label on the container states (1) the ABO group; (2) the Rh group and the nature of specific antisera used in testing; (3) the total volume of fluid, the proportion of blood and the composition and volume of anticoagulant solution; (4) a means whereby the individual source of the blood may be identified; (5) the date on which the blood was withdrawn; (6) that the contents shall not be used if there is any visible evidence of deterioration; (7) that the tests for HBV, HCV and HIV were non-reactive in all tests; (8) that a serologic test for syphilis was non-reactive; (9) for blood of group O, that the blood is for transfusion only to recipients belonging to group O unless the absence of hemolysins has been demonstrated.

Identification

A. Blood group Determine the blood group, using a separate sample of the donor's blood, as described in the "Determination of ABO Group of Donors" (Appendix 15.1.1) by examination of both cells and serum.

B. Rh type Determine the Rh type, using a separate sample of the donor's blood, as described in the "Determination of Rh Group of Donors" (Appendix 15.1.2) by examinations of the cells.

Sterility Complies with the "Sterility Test" (Appendix 10.1).

Visual inspection Inspect visually during storage and immediately prior to use. If the colour or physical appearance is abnormal or there is any indication or suspicion of microbial contamination, the unit is unsuitable for transfusion.

Leukocyte count (for units labelled as Whole Blood, Leukocytes Reduced) Proceed as directed under *Red Cell Concentrate,* p. 191, except to use 100 µl of Whole Blood.

Hemolysins Add 1 volume of fresh donor serum to 1 volume of a 10 per cent v/v suspension of A_1 cells in *saline TS* and add 1 volume to 1 volume of a similar suspension of B cells; a similar test using O cells is carried out as a negative control. If the serum is more than 24 hours old, add 1 volume of fresh group O serum free of lysins to each tube as a source of complement. Mix the contents of each tube, incubate at 37° for 1 hour and examine the supernatant liquid for hemolysis.

A serum giving a positive result in this test should be further examined as follows.

Dilute 1 volume of the serum with 3 volumes of *saline TS* and mix 1 volume of the diluted serum with 1 volume of fresh group O serum free of lysins and 1 volume of a 10 per cent v/v suspension, in *saline TS*, of A_1 or B cells (whichever were lysed in the first test). At the same time, in two further tubes, mix 1 volume of *saline TS* with 1 volume of the fresh group O serum free of lysins. To one of these tubes add 1 volume of a 10 per cent v/v suspension of A_1 cells in *saline TS* and to the other 1 volume of a similar suspension of B cells. Incubate the tubes for 1 hour at 37°, mix the contents of each tube and examine the supernatant liquid for hemolysis. Group O blood samples whose sera show the presence of hemolysins should be regarded as unsafe for transfusion to recipients of other groups and must be labelled accordingly.

Hemoglobin Complies with the "Determination of Hemoglobin Concentration by Hemiglobincyanide Method" (Appendix 14.2.3).

RED CELL CONCENTRATE

Red Blood Cells; Concentrated Red Blood Cells; Packed Red Cells (PRC).

> ***Caution*** Red Cell Concentrate shall be administered only through a 170 to 200 µm filter as transfusion equipment for medical use.

Category Human blood and blood products.

Red Cell Concentrate is the portion of blood that contains hemoglobin and is derived from human whole blood, from which plasma and platelets are removed by centrifugation, sedimentation, or by apheresis. In the case of apheresis, the plasma is automatically removed and returned directly to the donor. Red Cell Concentrate derived from whole blood may be prepared at any time during the dating period of the whole blood from which it is derived.

Red Cell Concentrate may be further processed by addition of red cell preservatives, irradiation to inactivate lymphocytes, filtration for removal of leukocytes,

washing to remove proteins, freezing and thawing, or rejuvenation using validated and approved procedures. The source blood for Red Cell Concentrate must comply with the requirements as described under *Blood, Whole,* p. 188. A unit (dose) of Red Cell Concentrate contains a minimum of 50 g of hemoglobin in a total volume of approximately 180 to 325 ml. A unit (dose) of Red Cell Concentrate, Leukocytes Reduced contains a minimum of 42.5 g of hemoglobin in a total volume of approximately 150 to 275 ml, and has a residual leukocyte count of less than 5×10^6. A unit (dose) of Red Cell Concentrate, Deglycerolized contains a minimum of 40 g of hemoglobin in a total volume of approximately 180 to 325 ml. A unit (dose) of Red Cell Concentrate, Leukocytes Reduced and Deglycerolized contains a minimum of 34 g of hemoglobin in a total volume of approximately 180 to 325 ml and has a residual leukocyte count of less than 5×10^6. A unit (dose) of Red Cell Concentrate, Pheresis contains a mean hemoglobin content of 60 g of hemoglobin. A unit (dose) of Red Cell Concentrate, Pheresis, Leucocytes Reduced contains a mean hemoglobin content of 51 g (or 153-ml packed red cell volume, and has a residual leukocyte count of less than 5×10^6.

Description Dark red fluid. After standing the red cells may sediment leaving a supernatant layer of yellow plasma.

Strength available The red cell equivalent of 1 Unit (450±45 ml) of human whole blood.

Dose *Intravenous infusion*, as needed to replenish red cells, prime extracorporeal circuits, etc, the equivalent of 1 Unit of Whole Blood, repeated as necessary.

Warning The high rate of alloimmunization among patients with sickle-cell anemia could be due to racial differences between donors and recipients.

See also under *Blood, Whole*, p. 188.

Additional information

1. Before transfusion of Red Cell Concentrate compatibility tests shall be carried out with the blood of the intended recipient and the identity of that recipient shall be indicated on the container.

2. One unit in a 70-kg recipient increases the hematocrit about 3 per cent.

Expiration date Red Cell Concentrate in ACD and CPD may be stored for up to 21 days at 2° to 6° after the blood has been drawn. Red Cell Concentrate in CPDA-1 may be stored for up to 35 days at 2° to 6°. Red Cell Concentrate may be stored in an approved additive solution (AS), for up to 42 days at 2° to 6°. If the hermetic seal of the container is broken during collection, preparation, or further processing, the expiration date is not later than 24 hours after the seal is broken (when blood is stored at 2° to 6°). The expiration date for frozen Red Cell Concentrate prepared with low glycerol content (20 per cent glycerol) is not later than 10 years from the date of collection when stored at –120° or colder, except when Red Cell Concentrate is prepared for freezing with high glycerol content (40 per cent glycerol), in which case it may be stored at –65° or colder for no later than 10 years from date of collection. If the frozen Red Cell Concentrate is processed for freezing or for thawing, in an open system, the expiration date for the thawed Red Cell Concentrate is 24 hours after removal from –65° storage, provided it is then stored at the temperature of unfrozen Red Cell Concentrate.

Packaging and storage Collect into an approved container containing a sterile, pyrogen-free approved anticoagulant. An approved additive solution may be added after removal of the plasma. Store Red Cell Concentrate in the original container, or transfer to an equivalent container using a technique that does not compromise sterility. Liquid Red Cell Concentrate is stored at a temperature between 2° and 6°. Frozen Red Cell Concentrate is stored at –65° or colder.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition, the label on the container states (1) the reference number of the donation of the Whole Blood from which the preparation was made; (2) the ABO group and the Rh type of the Whole Blood from which the preparation was made and, where appropriate, the specificities of the antisera used in testing; (3) the nature of the anticoagulant and any additive solutions added subsequent to collection; (4) that the preparation shall not be used if there is any visible evidence of deterioration; (5) the product name as indicated in Table 1; (6) the names of the adventitious agents tested and the results of the tests.

Table 1 Red Cell Concentrate Preparations

Product Name	Method of Preparation
Red Cell Concentrate	Prepared from whole human blood.
Red Cell Concentrate, Pheresis	Prepared using automated apheresis systems.
Red Cell Concentrate, Leukocyte Reduced	Prepared from Red Cell Concentrate or Red Cell concentrate, Pheresis (Total leukocytes count $<5\times10^6$).
Red Cell Concentrate, Frozen	Prepared from Red Cell Concentrate or Red Cells Concentrate, Pheresis suspended in cryoprotective solution (glycerol) and frozen at an appropriate temperature.
Red Cell Concentrate, Deglycerolyzed	Prepared from Red Cell Concentrate, Frozen from which glycerol is removed by washing by an approved procedure.

Identification

A. Blood group Determine the blood group, using a separate sample of the donor's blood, as described in the "Determination of ABO Group of Donors" (Appendix 15.1.1) by examination of both cells and serum.

B. Rh type Determine the Rh type, using a separate sample of the donor's blood, as described in the "Determination of Rh Group of Donors" (Appendix 15.1.2) by examinations of the cells.

Sterility Complies with the "Sterility Test" (Appendix 10.1).

Visual inspection Inspect visually during storage and immediately prior to use. If the colour or physical appearance is abnormal or there is any indication or suspicion of microbial contamination, the unit is unsuitable for transfusion.

Leukocyte count Pipette 40 µl of a suitable red cell-lysing agent into a clean test-tube, add 100 µl of Red Cell Concentrate diluted with *saline TS*, if necessary, such that the hematocrit of Red Cell Concentrate is not more than 60 per cent. Mix by pipetting up and down several times. Add 360 µl of a 0.01 per cent w/v solution of *crystal violet* in a 15 per cent v/v solution of *acetic acid* into the mixture, and mix thoroughly. Fit a hemocytometer with a 50-µl counting volume and a bright background, with a cover slip, and load the counting chamber with the mixture until the counting area is completely covered, but not overfull. Cover the counting chamber with a suitable moist lid to prevent evaporation, and allow to settle undisturbed for 10 to 15 minutes. Remove the lid, place the chamber on the stage of a light microscope fitted with 10× ocular lens and 20× objective. Count the leukocytes in the entire 50-µl counting volume. Calculate the leukocyte count in Red Cell Concentrate, expressed in leukocytes per µl, by dividing the observed leukocyte count by 10. Calculate the total number of leukocytes in the Red Cell Concentrate unit by using the following formula:

$$\text{Total leukocytes} = \text{leukocytes}/\mu l \times 10^3 \times \text{the volume of the Red Cell Concentrate unit in ml.}$$

Adequacy of deglycerolization (for Red Cell Concentrate, Deglycerolized) Interrupt the last wash cycle of the deglycerolization process at a point where the wash fluid is visible in the clear tubing segment leading to the waste receptacle. Hold the tubing against a well-lighted, white background. Note the coloration of the fluid in the tubing, and compare it to a suitable hemolysis colour comparator standard. The colour of the fluid should be no stronger than the block indicating 3 per cent hemolysis. (**Note** If the level of hemolysis is more than 3 per cent, continue the wash process, and repeat the test until the colour is within acceptable limits.)

Hemoglobin Complies with the "Determination of Hemoglobin Concentration by Hemiglobincyanide Method" (Appendix 14.2.3).

PLATELET CONCENTRATE

Caution A filter is to be used in the administration set.

Category Human blood and blood products.

Platelet Concentrate contains the platelets taken from plasma obtained by whole blood collection, by plasmapheresis, or by plateletpheresis, from a single suitable human donor of Whole Blood, except where a licensed physician has determined that the recipient is to be transfused with the platelets from a specific donor (in which case the plateletpheresis procedure is performed under the supervision of a licensed physician who is aware of the health status of the donor and has certified that the donor's health permits such procedure).

In all cases, the collection of source material is made by a single, uninterrupted venipuncture with minimal damage to and manipulation of the donor's tissue.

Concentrate consists of such platelets suspended in a specified volume of the original plasma, the separation of plasma and resuspension of the platelets being done in a closed system, within 6 to 8 hours of collection of the whole blood or plasma. The separation of platelets is by a procedure shown to yield an unclumped product without visible hemolysis, with a content of not less than 5.5×10^{10} platelets per unit in not less than 90 per cent of the units tested, and the volume of original plasma used for resuspension of the separated platelets is such that the product has a pH of not less than 6.2 during the storage period when kept at the selected storage temperature, the selected storage temperature and corresponding volume of resuspension plasma being 30 ml to 50 ml of plasma for storage at 20° to 24°. It meets the aforementioned requirements for platelet count, pH, and actual plasma volume, when tested 5 days after preparation.

Expiration date The expiration date of Platelet Concentrate processed in a closed system is not more than 5 days after the collection of the original whole blood.

Platelet Concentrate prepared in an open system shall be used within 4 hours of preparation.

Packaging and storage Platelet Concentrate shall be stored in tightly closed, sterile and pyrogen-free, colourless, transparent plastic containers, at the temperature relevant to the volume of resuspension plasma, either between 20° and 24° (a continuous gentle agitation shall be maintained) or between of 2° and 6°, the latter except during shipment, when the temperature may be between 1° and 10°.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition, the label on the container states (1) the volume of original plasma present; (2) the kind and volume of anticoagulant solution present in the original plasma; (3) the blood group designation of the source blood; (4) the hour of expiration on the stated expiration date; (5) the names of the adventitious agents tested and the results of the tests (6) where labelled for storage at 20° to 24°, that a continuous gentle agitation shall be maintained that such agitation is optional; (7) that it is to be used as soon as possible and not more than 4 hours after entering the container; (8) that a filter is to be used in the administration set.

Sterility Complies with the "Sterility Test" (Appendix 10.1).

FRESH FROZEN PLASMA

Fresh Frozen Plasma for Infusion; FFP.

Caution Fresh Frozen Plasma, after thawing, shall be administered only with transfusion equipment for medical use.

Category Human blood and blood products.

Fresh Frozen Plasma is human plasma that complies with the requirements stated on *Individual Plasma Units* under *Plasma for Fractionation*, p. 193, and with the following modifications.

Description When thawed, the preparation may vary in colour from light yellow to green liquid and may be turbid due to the presence of fat.

Warning Fresh Frozen Plasma derived from blood of group O whose sera show the presence of hemolysins should not be transfused to recipients of other groups.

Expiration date When stored under the prescribed condition, the expiration date is not later than 12 months from the date of collection.

Packaging and storage Fresh Frozen Plasma shall be kept in a sterile container, sealed so as to exclude micro-organisms and stored at a temperature not exceeding –18° until required for use, except during any period necessary for transportation providing this period does not exceed 30 minutes.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition, the label on the container states (1) the reference number of the donation of the Whole Blood from which the preparation was made; (2) the ABO group and the Rh group of the Whole Blood from which the preparation was made and the specificities of the antisera used in testing; (3) the weight in g of plasma in the container and the nature of the anticoagulant present; (4) that the contents shall not be used if there is any visible evidence of deterioration; (5) that, once thawed, the preparation should be used as soon as possible and, in any case, within 3 hours and that it should not be refrozen; (6) that, whenever possible, Fresh Frozen Plasma derived from ABO compatible blood should be used for transfusion; (7) for preparation obtained from blood of group O, that a test for hemolysins has been carried out and, if a positive result has been obtained, that the blood is safe for transfusion only to recipients belonging to group O; (8) that the transmission of infectious agents cannot be totally excluded when medicinal products prepared from human blood or plasma are administered.

Identification Thaw the preparation at a temperature not exceeding 37°. To 1 ml immediately add 0.2 ml of a 2.5 per cent w/v solution of *calcium chloride*. Coagulation occurs, which can be accelerated by incubation at 37°.

Hemolysins For Fresh Frozen Plasma derived from blood of group O, carry out the test as described under *Blood, Whole*, p. 189. Preparations that show the presence of hemolysins should be regarded as safe for transfusion only to recipients belonging to group O.

Sterility Complies with the "Sterility Test" (Appendix 10.1).

PLASMA FOR FRACTIONATION

Human Plasma for Fractionation is the liquid part of human blood remaining after separation of the cellular elements from blood collected in a receptacle containing an anticoagulant, or separated by continuous filtration or centrifugation of anticoagulated blood in an apheresis procedure; it is intended for the manufacture of plasma-derived products.

The plasma or serum obtained from counselled healthy donors who must, as far as can be ascertained after medical examination, laboratory blood tests and a study of their medical history, be free from detectable agents of infection transmissible by plasma-derived products, may be used in the collection of plasma for fractionation. The examinations and tests to be carried out are decided by the National Blood Centre and the Ministry of Public Health; in particular, tests for hepatitis B surface antigen (HBsAg), for hepatitis C antibodies (anti-HCV) and for HIV antibodies such as anti-HIV-1 and anti-HIV-2 are carried out by suitable sensitive methods. The nucleic acid test for HBV, HCV and HIV is also recommended and must give non-reactive results in all three cases.

***For individual plasma units*, the plasma is prepared by a method that removes cells and cell debris as completely as possible. Whether prepared from whole blood or by plasmapheresis, the plasma is separated from the cells by a method designed to prevent the introduction of micro-organisms. No antimicrobial agent is added to the plasma. Requirements and advice concerning the containers are given in Appendix 11.3. The containers are closed so as to prevent contamination.**

If two or more units are pooled prior to freezing, the operations are carried out using sterile connecting devices or under aseptic conditions and using containers that have not previously been used.

Plasma intended for the manufacture of coagulation factors and other labile derivatives is either processed shortly after separation or collection or frozen by cooling rapidly at –18° or below. Plasma obtained from whole blood and intended for the manufacture of coagulation factors and other labile derivatives is separated from cellular elements and frozen as soon as possible and at the latest within 24 hours of donation. Plasma intended for the manufacture of non-labile derivatives is separated within 5 days of the expiration date of the whole blood.

It is not intended that the determination of total protein and factor VIII shown below be carried out on each unit of plasma. They are rather given as guidelines for good manufacturing practice, the test for factor VIII being relevant for plasma intended for use in the preparation of concentrates of labile components.

The total protein content of a unit of plasma depends on the serum protein content of the donor and the degree of dilution inherent in the donation procedure. When plasma is obtained from a suitable donor and using the intended proportion of anticoagulant solution, a total protein content complying with the limit of 50 g per litre is obtained. If a volume of blood or plasma smaller than intended is collected into the anticoagulant solution, the resulting plasma is

not necessarily unsuitable for pooling for fractionation. The aim of good manufacturing practice must be to achieve the prescribed limit for all normal donations.

Preservation of factor VIII in the donation depends on the collection procedure and the subsequent handling of the blood and plasma. With good practice, 0.7 IU per ml can usually be achieved, but units of plasma with a lower content may still be suitable for use in the production of blood coagulation factor concentrates. The aim of good manufacturing practice is to conserve labile components as much as possible.

For pooled plasma, **during the manufacture of plasma products, the first homogeneous pool of plasma (for example, after removal of cryoprecipitate) is tested for hepatitis B surface antigen and for HIV antibodies such as anti-HIV-1 and anti-HIV-2 using test methods of suitable sensitivity and specificity; the pool must give non-reactive results in these tests.**

The pool is also tested for hepatitis C virus RNA using a validated nucleic acid amplification technique (Appendix 14.6). A positive control with 100 IU per ml of hepatitis C virus RNA and, to test for inhibitors, an internal control prepared by addition of a suitable marker to a sample of the plasma pool are included in the test. The test is invalid if the positive control is non-reactive or if the result obtained with the internal control indicates the presence of inhibitors. The plasma pool complies with the test if it is found non-reactive for hepatitis C virus RNA.

Description Before freezing, a clear or slightly turbid liquid without visible signs of hemolysis; it may vary in colour from light yellow to green.

Packaging and storage Store frozen plasma at or below –18°; the plasma may still be used for fractionation if a temperature of –18° is exceeded on at most one occasion for not more than 72 hours and if the plasma is at all times maintained at a temperature of –5° or lower.

Labelling The label enables each individual unit to be traced to a specific donor.

Total protein Not less than 5 per cent w/v of protein. Carry out the test using a pool of not less than 10 individual plasma units. Dilute the pool with *saline TS* to obtain a solution containing about 15 mg of protein in 2 ml. To 2.0 ml of this solution in a round-bottomed centrifuge tube add 2 ml of a 7.5 per cent w/v solution of *sodium molybdate*, 2 ml of a mixture of 1 volume of *nitrogen-free sulfuric acid* and 30 volumes of *water*. Shake, centrifuge for 5 minutes, decant the supernatant liquid, and allow the inverted tube to drain on filter paper. Using the residue thus obtained, carry out the "Determination of Nitrogen" (Method II, Appendix 6.7) and calculate the content of protein by multiplying by 6.25.

Factor VIII Thaw the samples of a pool of not less than 10 individual plasma units, if necessary, at a temperature not exceeding 37°. Carry out the "Biological Assay of Human Coagulation Factor VIII"(Appendix 15.1.5), using a reference plasma calibrated against the International Standard for blood coagulation factor VIII in plasma. The activity is not less than 0.7 IU per ml.

ALBUMIN SOLUTION

Albumin; Albumin, Human

Category Human blood and blood products (blood volume expander; antihyperbilirubinemic).

Albumin Solution is an aqueous solution of protein. It is a sterile, nonpyrogenic preparation of albumin obtained by fractionating human plasma that complies with the requirements stated under *Plasma for fractionation*, p. 193.

Separation of the albumin solution is carried out under controlled conditions, particularly of pH, ionic strength and temperature, so that in the final product not less than 95 per cent of the total protein is albumin.

Description Clear, slightly viscous liquid. It is almost colourless, yellow, amber or green, depending on protein concentration.

Strengths available 5, 20 and 25 per cent w/v (total protein).

Dose *Intravenous infusion*, as prescribed by the physician.

Contra-indication It is contra-indicated in patients with severe anemia or cardiac failure.

Warning

1. Adverse reactions (which may be caused by allergy or protein overload resulting from high dosage or repeated administrations) include fever, chills, nausea, vomiting, increased salivation, and urticaria. Variable effects on blood pressure, heart rate, and respiration may also occur.

2. It should be used with caution in patients with hypertension or low cardiac reserve.

3. The possibility that human albumin solutions may contain aluminium as a contaminant, which could accumulate in patients with impaired renal function, should be considered.

4. Sterile water for injection must not be used as a diluent for 20 and 25 per cent w/v solutions of albumin, because of the risk of potentially life-threatening hemolysis.

Additional information

1. It contains no blood group isoagglutinins and, therefore, may be given without regard to the blood group of the patient.

2. Transfusions of whole blood or packed red cell concentrate may be necessary following administration of large volumes of albumin to restore hemoglobin concentration and to prevent anemia.

3. It may be administered with plasma, packed red cells, or whole blood; however, albumin should not be added directly to any of these three components, except when used as a red blood cell resuspension medium.

Expiration date When stated to be stored at a temperature not higher than 25°, the expiration date is not more than 3 years; or at 2° to 8°, it is not more than 5 years.

Packaging and storage Albumin Solution shall be kept in tightly closed containers, and stored at the temperature recommended by the manufacturer or indicated on the label, protected from light; avoid freezing.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition, the label on the container states (1) the volume in the container; (2) the total amount of protein in the container expressed in g per litre or as a percentage; (3) the concentration of sodium expressed in mmol per litre; (4) the names and concentrations of stabilizing agents and of any other added substances present in the final solution; (5) that the solution must not be used if it is cloudy or contains a deposit; (6) that, once the container has been penetrated, the contents should be used within 4 hours and any unused preparation discarded; (7) that a filter is to be used in the administration set; (8) either that the preparation is suitable for administration to patients undergoing analysis and to premature infants or that it is not intended for such use.

Identification

A. Using an antiserum specific to human plasma proteins and a range of antisera specific to the plasma proteins of each species of domestic animal commonly used in the preparation of materials of biological origin, carry out precipitation tests on the preparation being examined. The preparation contains proteins of human origin and gives negative results with antisera specific to plasma proteins of other species.

B. Examine by a suitable immunoelectrophoresis technique. Using antiserum to normal human serum, compare normal human serum and the preparation being examined, both diluted to contain 1 per cent w/v of protein. The main component of the preparation being examined corresponds to the main component of the normal human serum. The solution may show the presence of small quantities of other plasma proteins.

pH 6.4 to 7.4, when diluted with *saline TS* to produce a solution containing 1 per cent w/v of protein (Appendix 4.11).

Aluminium Not more than 200 µg per litre when determined as described in the "Atomic Spectrometry: Emission and Absorption" (Appendix 2.3). Measure at 309.3 nm and use *aluminium standard solution (10 ppm Al)*, suitably diluted with *water*, as the standard solution.

Potassium Not more than 0.05 mmol of K per g of protein when determined as described in the "Atomic Spectrometry: Emission and Absorption" (Appendix 2.3). Measure at 766.5 nm and use *potassium solution Asp*, suitably diluted with *water*, as the standard solution.

Sodium 95 to 105 per cent of the content of Na stated on the label and, in any case, not more than 160 mmol of Na per litre when determined as described in the "Atomic Spectrometry: Emission and Absorption" (Appendix 2.3). Measure at 589 nm and use *sodium solution Asp*, suitably diluted with *water*, as the standard solution.

Haem Dilute with sufficient *saline TS* to produce a solution containing 1.0 per cent w/v of protein. The absorbance of the resulting solution at 403 nm is not more than 0.15 (Appendix 2.2). Use *water* in the reference cell.

Prekallikrein activator Not more than 35 IU per ml (Appendix 14.2.2).

Distribution of molecular size Carry out the test as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Dissolve and dilute 4.873 g of *disodium hydrogenphosphate dihydrate*, 1.741 g of *sodium dihydrogenphosphate monohydrate*, 11.688 g of *sodium chloride* and 50 mg of *sodium azide* with *water* to 1000.0 ml.

Test preparation Dilute the preparation being examined with *saline TS* to a concentration suitable for the chromatographic system used. A concentration in

the range of 0.4 to 1.2 per cent w/v of protein and the injection of 50 to 600 µg of protein are usually suitable.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (60 cm × 7.5 mm) packed with hydrophilic silica gel (3 to 10 µm), (b) *Mobile phase* at a flow rate of about 0.5 ml per minute and (c) an ultraviolet photometer set at 280 nm.

The peak corresponding to polymers and aggregates is located in the part of the chromatogram representing the void volume. The area of this peak divided by 2 is not more than 5 per cent of the total area of the chromatogram.

Protein composition Carry out the determination as described in the "Cellulose Acetate Electrophoresis" (Method II, Appendix 3.7), using one strip of cellulose acetate for each solution. For solution (A) dilute the preparation being examined with *saline TS* to produce a solution containing 2 per cent w/v of protein. For solution (B) dilute Albumin Solution (Human Albumin) for Electrophoresis RS with *saline TS* to produce a solution containing 2 per cent w/v of protein. In the strips prepared with solution (A) not more than 5 per cent of the protein is contained in bands other than the principal band. The test is not valid unless the proportion of protein in the principal band in the strip prepared with solution (B) is within the limits stated in the leaflet supplied with Human Albumin for Electrophoresis RS.

Total protein Not less than 95 per cent and not more than 105 per cent of the quantity of protein stated on the label. Dilute the preparation being examined with *saline TS* to obtain a solution containing about 15 mg of protein in 2 ml. To 2.0 ml of the resulting solution in a round-bottomed centrifuge tube add 2 ml of a 7.5 per cent w/v solution of *sodium molybdate* and 2 ml of a mixture of 30 volumes of *water* and 1 volume of *nitrogen-free sulfuric acid*. Shake, centrifuge for 5 minutes, decant the supernatant liquid, and allow the inverted tube to drain on filter paper. Using the residue thus obtained, carry out the "Determination of Nitrogen" (Method II, Appendix 6.7), and calculate the content of protein by multiplying the result by 6.25.

Pyrogens or Bacterial endotoxins Complies with the "Pyrogen Test" (Appendix 8.2) or, preferably and where justified and authorized, with a validated *in vitro* test such as the "Test for Bacterial Endotoxins" (Appendix 8.5).

For the pyrogen test, for a solution containing 3.5 to 5.0 per cent w/v of protein, use 10 ml of the preparation per kg of the rabbit's weight. For a solution containing 15.0 to 25.0 per cent w/v of protein, use 5 ml of the preparation per kg of the rabbit's weight.

Where the bacterial endotoxin test is used, it contains less than 0.5 Endotoxin Unit per ml for solutions with a protein content more than 5.0 per cent w/v solution, less than 1.3 Endotoxin Units per ml for solutions with a protein content more than 5.0 per cent w/v solution but not more than 20.0 per cent w/v solution, and less than 1.7 Endotoxin Units per ml for solutions with a protein content more than 20.0 per cent w/v solution but not more than 25.0 per cent w/v solution.

Sterility Complies with the "Sterility Test" (Appendix 10.1).

ANTITHROMBIN III CONCENTRATE

Category Anticoagulant; antithrombotic.

Antithrombin III Concentrate is a freeze-dried preparation of a glycoprotein fraction obtained from human plasma that inactivates thrombin in the presence of an excess of heparin. It is obtained from human plasma that complies with the requirements stated under *Plasma for Fractionation*, p. 193.

The antithrombin III is purified and concentrated, and a suitable stabilizer may be added. No antimicrobial preservative is added at any stage of production. The specific activity is not less than 3 IU of antithrombin III per mg of total protein, excluding albumin.

When reconstituted in the volume of solvent stated of the label, the potency is not less than 25 IU[1] of antithrombin III per ml.

Description White, or almost white, friable solid or a powder. Hygroscopic.

Strengths available 500 IU and 1000 IU.

Dose *Intravenous*, administered at a rate of 50 to 100 IU (not to exceed 100 IU) per minute:

Initial—A sufficient quantity to increase the antithrombin III activity, determined 30 minutes after administration, to 120 per cent of the normal activity.

Maintenance—A sufficient quantity to increase the antithrombin III activity to 80 per cent or more of the normal activity. Maintenance doses are generally

[1]The potency of Antithrombin III concentrate is expressed in International Unit (IU), defined as the amount of antithrombin III in 1 ml of pooled normal human plasma and is measured against a standard calibrated with a reference preparation from the World Health Organization.

administered at 24-hour intervals.

Warning It may cause nausea, flushing, and headache. Allergic reaction and fever can occur, but rarely.

Additional information

1. Concomitant use of very large doses of antithrombin III Concentrate and heparin may increase the risk of bleeding. Therefore, the dose of heparin should be monitored carefully when the two drugs are given concurrently.

2. In pregnant women receiving antithrombin III concentrate and heparin or oral anticoagulants near term, heparin should be discontinued at least 12 hours before delivery and the oral anticoagulant should be terminated several days before delivery or before a therapeutic or elective abortion.

Expiration date The expiration date is not later than 2 years from the date of manufacturing, as indicated on the label.

Packaging and storage Antithrombin III Concentrate shall be kept in tightly closed containers and stored between 2° to 8°, protected from light.

Labelling Complies with the "General Information for Biological Products" p. 177. In addition, the label on the container states (1) the content of antithrombin III expressed in International Units per container; (2) the name and volume of solvent to be used to reconstitute the preparation; (3) the amount of albumin present as a stabilizer; (4) that the transmission of infectious agents cannot be totally excluded when medicinal products prepared from human blood or plasma are administered.

Before carrying out the identification and the tests (except those for constituted solution and water) and the assay, immediately reconstitute the preparation to be examined as stated on the label.

Identification

A. Using a suitable range of species-specific antisera, carry out precipitation tests on the preparation to be examined and stain the gels with *acid blue 92*. It is recommended that the tests be carried out using antisera specific to the plasma proteins of each species of domestic animal commonly used in the preparation of materials of biological origin in the country concerned. The preparation is shown to contain proteins of human origin and gives negative results with antisera specific to plasma proteins of other species.

B. The assay for antithrombin III activity contributes to the identification of the preparation.

pH 6.0 to 7.5 (Appendix 4.11).

Osmolality Not less than 240 mOsmol/kg (Appendix 4.35).

Heparin Not more than 0.1 IU of heparin activity per IU of antithrombin III activity. Carry out the "Biological Assay of Heparin in Coagulation Factors" (Appendix 14.2.1). It is necessary to validate the method for assay of heparin for each specific preparation to be examined to allow for interference by antithrombin III.

Solubility test Reconstitute the preparation as stated on the label. It dissolves completely under gentle swirling within 10 minutes, giving a clear or slightly turbid, colourless solution.

Water Not more than 3.0 per cent w/w (Karl Fischer Method, Appendix 4.12).

Pyrogens Complies with the "Pyrogen Test" (Appendix 8.2), using a volume of the solution containing 50 IU of antithrombin III per kg of the rabbit's weight.

Sterility Complies with the "Sterility Test" (Appendix 10.1).

Total protein If necessary, dilute an accurately measured volume of the preparation to be examined with *water* to obtain a solution containing about 15 mg of protein in 2 ml. To 2.0 ml of the solution in a round-bottomed centrifuge tube add 2 ml of a 7.5 per cent w/v solution of *sodium molybdate* and 2 ml of a mixture of 1 volume of *nitrogen-free sulfuric acid* and 30 volumes of *water*. Shake, centrifuge for 5 minutes, decant the supernatant liquid and allow the inverted tube to drain on filter paper. Using residue thus obtained, carry out the "Determination of Nitrogen" (Method II, Appendix 6.7), and calculate the content of protein by multiplying the result by 6.25.

Assay

The antithrombin III content of the preparation to be examined is determined by comparing its ability to inactivate thrombin in the presence of an excess of heparin with the same ability of a reference preparation of human antithrombin III concentrate calibrated in International Units. Varying quantities of the preparation to be examined are mixed with a given quantity of thrombin and the remaining thrombin activity is determined using a suitable chromogenic substrate.

Prepare two independent series of three or four dilutions in the range of 1/75 to 1/200 from 1 IU per ml,

for both the preparation to be examined and the reference preparation, using *tris-EDTA BSA buffer solution pH 8.4* containing 15 IU of heparin per ml.

Warm 200 µl of each dilution at 37° for 1 to 2 minutes. Add to each dilution 200 µl of a solution of *bovine thrombin* containing 2 IU per ml in *tris-EDTA BSA buffer solution pH 8.4*. Mix and maintain at 37° for exactly 1 minute. Add 500 µl of a suitable chromogenic substrate (for example, D-phenylalanyl-L-pipecolyl-L-arginine-4-nitroanilide, reconstituted in *water* to give a solution containing 4 mmol per litre and further diluted for the assay using *tris-EDTA BSA buffer solution pH 8.4* without albumin). Immediately start measurement of the change in absorbance at 405 nm, continuing the measurement for at least 30 seconds. Calculate the rate of change of absorbance (ΔA/minute). (Alternatively, an end-point assay may be used by stopping the reaction with *acetic acid* and measuring the absorbance at 405 nm.)

The rate of change of absorbance is (ΔA/minute) is inversely proportional to antithrombin III activity. Plot the regression of absorbance of (ΔA/minute) against concentration on a linear scale and determine the potency by comparing the slopes for the reference preparation and the preparation to be examined.

Check the validity of the assay and calculate the potency of the test preparation by the usual statistical methods for a slope-ratio assay in the "Statistical Analysis of Results of Biological Assays and Tests" (Appendix 9).

The estimated potency is not less than 80 per cent and not more than 120 per cent of the potency stated on the label. The confidence limits (P = 0.95) are not less than 90 per cent and not more than 110 per cent of the estimated potency.

DRIED FACTOR VII FRACTION

Human Coagulation Factor VII

Category Human blood and blood products (coagulant; antihemorrhagic agent).

Dried Factor VII Fraction is a freeze-dried plasma protein fraction that contains the single-chain glycoprotein factor VII and may also contain small amounts of the activated form, the two-chain derivative factor VIIa, as well as coagulation factors II, IX and X and protein C and protein. It is obtained from human plasma that complies with the requirements stated under *Plasma for Fractionation*, p. 193. Heparin, antithrombin and other auxiliary substances such as a stabilizer may be added. No antimicrobial preservative is added.

The specific activity of the preparation before the addition of any protein stabilizer, is not less than 2 IU of factor VII per mg of protein. The potency of the preparation, reconstituted as stated on the label, is not less than 15 IU of factor VII per ml.

Description White or almost white, pale yellow, green or blue powder or friable solid; hygroscopic.

Strengths available 25 and 60 IU per ml.

Dose *Intravenous infusion*, as prescribed by the physician.

Additional information Factor VII may be used as replacement therapy in patients with rare genetic deficiencies of factor VII. It has a half-life of only 3 to 5 hours. As a result, it needs to be administered more frequently than other factor concentrates for other factor deficiencies. As an alternative, a new technique is to use continuous infusion of factor VIIa concentrate. Factor VIIa is used to treat serious bleeding episodes and to prevent bleeding associated with surgery in patients with hemophilia A or hemophilia B.

Expiration date When stored under the prescribed condition, the expiration date is not later than 3 years from the date of the last satisfactory test for potency.

Packaging and storage Dried Factor VII Fraction shall be kept in tightly closed containers, protected from light and stored at a temperature of 2° to 8°.

Labelling Complies with the "General Information for Biological Products" p. 177. In addition, the label on the container states (1) the content of factor VII expressed in International Units per container; (2) the maximum content of factor II, factor IX and factor X expressed in International Units per container; (3) the name and volume of solvent to be used to reconstitute the preparation; (4) the name and quantity of any added substances, including where applicable, heparin; (5) the amount of protein per container; (6) that the transmission of infectious agents cannot be totally excluded when medicinal products prepared from human blood or plasma are administered.

Before carrying out the identification, the tests (except those for constituted solution and water) and the assay, immediately reconstitute the preparation to be examined as stated on the label.

Identification

A. Using a suitable range of species-specific antisera, carry out precipitation tests on the preparation to be examined. It is recommended that the tests be carried out using antisera specific to the plasma proteins of each species of domestic animal commonly used in the preparation of materials of biological origin in the country concerned. The preparation is shown to contain proteins of human origin and gives negative results with antisera specific to the plasma proteins of other species.

B. The assay for factor VII activity contributes to the identification of the preparation.

pH 6.5 to 7.5 (Appendix 4.11).

Osmolality Not less than 240 mOsmol/kg (Appendix 4.35).

Activated coagulation factors If the preparation to be examined contains heparin, determine the amount present as described in the test for heparin and neutralize the heparin by addition of *protamine sulfate* (10 µg of protamine sulfate neutralizes 1 IU of heparin). Prepare 1 in 10 and 1 in 100 dilutions of the reconstituted preparation to be examined using *tris-chloride buffer pH 7.5*. Place a series of polystyrene tubes in a water-bath at 37° and add to each tube 0.1 ml of *platelet-poor plasma* and 0.1 ml of a suitable dilution of *cephalin TS* or *platelet substitute*. Allow to stand for 60 seconds. Add to each tube either 0.1 ml of one of the dilutions or 0.1 ml of the buffer solution (control tube). To each tube, add immediately 0.1 ml of a 0.37 per cent w/v solution of *calcium chloride*, previously heated to 37°, and measure within 30 minutes of the original dilution the time that elapses between addition of the calcium chloride solution and formation of a clot. For each of the dilutions, the coagulation time is not less than 150 seconds. The test is not valid unless the coagulation time measured for the control tube is 200 to 350 seconds.

Heparin If heparin has been added, the preparation being examined contains not more than the amount of heparin stated on the label and in any case not more than 0.5 IU of heparin per IU of factor VII (Appendix 14.2.1).

Thrombin If the preparation to be examined contains heparin, determine the amount present as described in the test for heparin and neutralize the heparin by addition of *protamine sulfate* (each 10 µg of protamine sulfate neutralizes 1 IU of heparin). In each of two test-tubes, mix equal volumes of the reconstituted preparation and a 0.3 per cent w/v solution of *fibrinogen*. Keep one of the tubes at 37° for 6 hours and the other at room temperature for 24 hours. In a third tube, mix a volume of the fibrinogen solution with an equal volume of a solution of *human thrombin* (1 IU per ml) and place the tube in a water-bath at 37°. No coagulation occurs in the tubes containing the preparation to be examined. Coagulation occurs within 30 seconds in the tube containing thrombin.

Factor II Carry out the "Biological Assay of Human Coagulation Factor II" (Appendix 15.1.7). The estimated content is not more than 125 per cent of the stated content. The confidence limits ($P = 0.95$) are not less than 90 per cent and not more than 111 per cent of the estimated potency.

Factor IX Carry out the "Biological Assay of Human Coagulation Factor IX" (Appendix 15.1.4). The estimated content is not more than 125 per cent of the stated content. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

Factor X Carry out the "Biological Assay of Human Coagulation Factor X" (Appendix 15.1.5). The estimated content is not more than 125 per cent of the stated content. The confidence limits ($P = 0.95$) are not less than 90 per cent and not more than 111 per cent of the estimated potency.

Solubility test Reconstitute the preparation as stated on the label or on the leaflet. It dissolves completely under gentle swirling within 10 minutes, giving a clear or slightly opalescent solution that may be coloured.

Water Not more than 3.0 per cent w/w (Karl Fischer Method, Appendix 4.12), add a suitable volume of *anhydrous methanol* to the container of the preparation to be examined, shake, allow to stand and carry out the determination on a known volume of the supernatant liquid.

Pyrogens Complies with the "Pyrogen Test" (Appendix 8.2), using a volume of the solution containing not less than 30 IU of Factor VII per kg of the rabbit's weight.

Sterility Complies with the "Sterility Test" (Appendix 10.1).

Total protein If necessary, dilute an accurately measured volume of the reconstituted preparation with *saline TS* to obtain a solution expected to contain about 15 mg of protein in 2 ml. To 2.0 ml of the solution in a round-bottomed centrifuge tube add 2 ml of a 7.5 per cent w/v solution of *sodium molybdate* and 2 ml of a mixture of 1 volume of *nitrogen-free sulfuric acid* and

30 volumes of *water*. Shake, centrifuge for 5 minutes, decant the supernatant liquid and allow the inverted tube to drain on filter paper. Using residue thus obtained, carry out the "Determination of Nitrogen (Method II, Appendix 6.7), and calculate the content of protein by multiplying the result by 6.25.

Assay Carry out the "Biological Assay of Human Coagulation Factor VII" (Appendix 15.1.8). The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

DRIED FACTOR VIII FRACTION

Factor VIII Concentrate; Antihemophilic Factor; Dried Human Antihemophilic Fraction; Human Coagulation Factor VIII.

Category Human blood and blood products (coagulant; antihemorrhagic agent).

Dried Factor VIII Fraction is a freeze-dried plasma protein fraction that contains the glycoprotein coagulation factor VIII together with varying amount of von Willebrand factor, depending on the method of preparation. It is obtained from human plasma that complies with the requirements stated under *Plasma for Fractionation*, p. 193. Auxiliary substances such as a stabilizer may be added. It contains no antimicrobial preservative.

The specific activity is not less than 1 IU of factor VIII:C per mg of total protein before the addition of any protein stabilizer. The potency of the preparation, reconstituted as stated on the label, is not less than 20 IU[1] of factor VIII:C per ml.

Description White or pale yellow powder or friable solid.

Strengths available 250, 500, 1000, and 1500 IU.

Dose *Intravenous infusion*, as prescribed by the physician. The dosage of factor VIII should be determined for each patient and will vary with the circumstances involving bleeding or type of surgery.

[1]The potency of Antihemophilic Factor (Factor VIII) is expressed in terms of antihemophilic factor units (AFU) or International Units (IU) of antihemophilic activity. One AFU is equivalent to one IU as defined by the World Health Organization Standard for Blood Coagulation Factor VIII, human, and is approximately equal to that quantity of antihemophilic factor present in 1 ml of fresh pooled human plasma. Since the activity standard is not a specifically defined value, the actual factor concentration per unit may vary.

Warning

1. Fever, chills, urticaria, stinging at the infusion site, and mild allergic reactions may occur.

2. Hypersensitivity, allergic reactions, severe anaphylaxis (including shock) may occur.

Expiration date When stored under the prescribed condition, the expiration date is not later than 2 years from the date of the last satisfactory test for potency.

Packaging and storage Dried Factor VIII Fraction shall be kept in tightly closed containers, protected from light and stored at a temperature of 2° to 8°.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition, the label on the container states (1) the number of IU of factor VIII:C in the container; (2) the concentration of protein in g per litre of the solution constituted as directed; (3) the maximum fibrinogen content; (4) the name and amount of any added substance; (5) the name and volume of solvent to be used for reconstitution; (6) that if solution is not complete or if a gel forms on reconstitution, the preparation shall not be used; (7) that the solution should be used as soon as possible and in any case within a stated time, not exceeding 3 hours, of constitution and any unused solution discarded; (8) whether the preparation is suitable for the treatment of von Willebrand's disease; (9) that a filter is to be used in the administration set.

Before carrying out the identification, the tests (except those for constituted solution and water) and the assay, immediately reconstitute the preparation to be examined as stated on the label.

Identification

A. Using an antiserum specific to human plasma proteins and a range of antisera specific to the plasma proteins of each species of domestic animal commonly used in the preparation of materials of biological origin, carry out precipitation tests on the preparation being examined. The preparation contains proteins of human origin and gives negative results with antisera specific to plasma proteins of the other species.

B. The assay for factor VIII:C contributes to the identification of the preparation.

pH 6.5 to 7.5 (Appendix 4.11).

Osmolality Not less than 240 mOsmol/kg (Appendix 4.35).

Solubility test Reconstitute the solution as stated on the label. The preparation dissolves completely under

gentle swirling within 10 minutes, giving a clear or slightly opalescent, colourless or slightly yellow solution.

Water Not more than 3.0 per cent w/w (Karl Fischer Method, Appendix 4.12). Add a suitable volume of *anhydrous methanol* to the container of the preparation to be examined, shake, allow to stand and carry out the determination on a known volume of the supernatant liquid.

Total protein (**Note** For some products, especially those without a protein stabilizer such as albumin, this method may not be applicable and another validated method for protein determination must therefore be performed.) If necessary, dilute the solution with *saline TS* to produce a solution containing about 15 mg of protein in 2 ml. To 2.0 ml of the resulting solution in a round-bottomed centrifuge tube add 2 ml of a 7.5 per cent w/v solution of *sodium molybdate* and 2 ml of a solution containing 1 volume of *nitrogen-free sulfuric acid* in 30 volumes of *water*. Shake, centrifuge for 5 minutes, decant the supernatant liquid, and allow the inverted tube to drain on filter paper. Using the residue thus obtained carry out the determination as described in the "Determination of Nitrogen" (Method II, Appendix 6.7), calculate the content of protein by multiplying the result by 6.25.

Hemagglutinins, anti-A and anti-B Dilute the constituted solution with *saline TS* to produce a solution containing 3 IU of factor VIII:C per ml. Carry out the test for hemagglutinins, anti-A and anti-B using a suitable indirect method such as that described below. The 1 in 64 dilutions do not show agglutination.

Prepare in duplicates serial dilutions of the preparation being examined in *saline TS*. To each dilution of one series add an equal volume of a 5 per cent v/v suspension of group A_1 red blood cells previously washed three times with *saline TS*. To each dilution of the other series add an equal volume of a 5 per cent v/v suspension of group B red blood cells previously washed three times with *saline TS*. Incubate the suspensions at 37° for 30 minutes and then wash the cells three times with *saline TS*. Leave the cells in contact with a polyvalent anti-human globulin reagent for 30 minutes. Without centrifuging, examine each suspension for agglutination under a microscope.

Pyrogens or Bacterial endotoxins Complies with the "Pyrogen Test" (Appendix 8.2) or, preferably and where justified and authorized, with a validated *in vitro* test such as the "Test for Bacterial Endotoxins" (Appendix 8.5).

For the pyrogen test, use a volume of the solution containing not less than 50 IU of factor VIII:C per kg of the rabbit's weight.

Where the bacterial endotoxin test is used, it contains less than 0.035 Endotoxin Unit per IU of factor VIII:C.

Sterility Complies with the "Sterility Test" (Appendix 10.1).

Assay

FACTOR VIII Carry out the "Biological Assay of Human Coagulation Factor VIII" (Appendix 15.1.3). The estimated potency is not less than 80 per cent and not more than 120 per cent of the stated potency. The confidence limits (P = 0.95) are not less than 80 per cent and not more than 120 per cent of the estimated potency.

VON WILLEBRAND FACTOR For preparation intended for the treatment of von Willebrand's disease, carry out the "Biological Assay of Human von Willebrand Factor" (Appendix 15.1.9). The estimated potency is not less than 60 per cent and not more than 140 per cent of the stated potency.

(**Note** Pending the availability of an International Standard for von Willebrand factor concentrate calibrated for use in the collagen-binding assay, only the ristocetin cofactor assay may be used.)

DRIED FACTOR VIII (rDNA)

Human Coagulation Factor VIII (rDNA)

Category Human blood and blood products (coagulant, antihemorrhagic agent).

Dried Factor VIII (rDNA) is a freeze-dried preparation of glycoproteins having the same activity as coagulation factor VIII in human plasma.

Human coagulation factor VIII circulates in plasma mainly as a two-chain glycosylated protein with one heavy chain (relative molecular weight of about 200,000) and one light chain (relative molecular weight 80,000) held together by divalent metal ions. Dried Factor VIII (rDNA) is prepared as full-length factor VIII (octocog alfa), or as a shortened two-chain structure (relative molecular weights 90,000 and 80,000), in which the B-domain has been deleted from the heavy chain (moroctocog alfa).

Full-length human rDNA Factor VIII contains 25 potential *N*-glycosylation sites, 19 in the B domain of the heavy chain, three in the remaining part of the heavy chain (relative molecular weight 90,000) and

three in the light chain (relative molecular weight 80,000). The different products are characterized by their molecular size and post-translational modification and/or other modifications.

The specific activity is not less than 2000 IU of factor VIII:C per milligram of total protein before the addition of any protein stabilizer, and varies depending on purity and the type of modification of molecular structure of factor VIII.

Description White or slightly yellow powder or friable mass.

Strengths available 50, 62.5, 100, 125, 200, 250, 300, 400, 500 and 600 IU per ml.

Dose *Intravenous infusion*, as prescribed by the physician.

Contra-indication It is contra-indicated in patients with hypersensitivity to mouse, hamster, or bovine protein, or to porcine or murine factor.

Warning

1. It is not indicated in the treatment of von Willebrand's disease.
2. Adverse local effects, including burning, pruritus, rash, and erythema may occur.
3. Systemic effects, including dizziness, lightheadedness, nausea, facial flushing, rash, pruritus, urticaria, rhinitis, depersonalization, chest discomfort, chest tightness with dyspnea, mouth dryness, cold feet, unpleasant, unusual, or metallic taste in the mouth, and a slight increase or decrease in blood pressure may occur.

Expiration date When stored under the prescribed condition, the expiration date is not later than 2 years from the date of the last satisfactory test for potency.

Packaging and storage Dried Factor VIII (rDNA) shall be kept in tightly closed containers, protected from light and stored at a temperature of 2° to 8°.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition, the label on the container states (1) the content of factor VIII expressed in International Units per container; (2) the name and amount of any added substance; (3) the composition and volume of the liquid to be used for reconstitution.

Before carrying out the identification, the tests (except those for constituted solution and water) and the assay, immediately reconstitute the preparation to be examined as stated on the label.

Identification

A. The assay for factor VIII contributes to the identification of the preparation.

B. The distribution of characteristic peptide bands corresponds with that of the manufacturer's reference preparation (SDS-PAGE or Western blot, Appendix 14.5).

pH 6.5 to 7.5 (Appendix 4.11).

Osmolality Not less than 240 mOsmol/kg (Appendix 4.35).

Solubility test Reconstitute the solution as stated on the label. The preparation dissolves completely under gentle swirling within 5 minutes at 20° to 25°, giving a clear or slightly opalescent solution.

Water Not more than 3.0 per cent w/w (Karl Fischer Method, Appendix 4.12). Add a suitable volume of *anhydrous methanol* to the container of the preparation to be examined, shake, allow to stand and carry out the determination on a known volume of the supernatant liquid.

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains less than 3 Endotoxin Units per 100 IU of factor VIII activity.

Sterility Complies with the "Sterility Test" (Appendix 10.1).

Assay Carry out the "Biological Assay of Human Coagulation Factor VIII" (Appendix 15.1.3). The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 120 per cent of the estimated potency.

FACTOR VIII, CRYOPRECIPITATED

Cryoprecipitated Antihemophilic Factor

Category Human blood and blood products (coagulant; antihemorrhagic agent).

Cryoprecipitated Factor VIII is a sterile, frozen concentrate of human antihemophilic factor[1] prepared from the factor VIII-rich cryoprotein fraction of human plasma obtained from suitable whole blood donors from a single unit of plasma derived from whole blood or by plasmapheresis, collected and processed in a closed system. It contains no antimicrobial preservative.

It meets the requirements of the test for potency by comparison with the Standard Antihemophilic Factor (Factor VIII) or with a working reference that has been calibrated with it, in having an average potency of not less than 70 IU per container, made at intervals of not more than 1 month during the dating period.

Description Yellowish frozen solid. On thawing becomes a very viscous, yellow, gummy liquid.

Strength available Not less than 70 IU of factor VIII and not less than 140 mg of fibrinogen per unit.

Dose *Intravenous infusion* after diluted with sterile saline solution, as prescribed by the physician.

Expiration date When stored under the prescribed condition, the expiration date is not later than 1 year from the date of collection of source materials.

Packaging and storage Cryoprecipitated Factor VIII shall be kept in tightly closed containers at a temperature of –18° or below.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition the label on the container states (1) the identification number of the donor from whom the source material was obtained; (2) a warning not to use if there is evidence of breakage or thawing; (3) instructions to thaw it before use to a temperature between 20° and 37°, and use as soon as possible; (4) that it is to be used within 4 hours after the container is entered; (5) that it is for intravenous administration; (6) that a filter is to be used in the administration equipment.

Fibrinogen Not less than 140 mg per container. Dilute 1 ml of the solution to 10 ml with *saline TS*. To a volume of the dilution containing 15 mg of fibrinogen add sufficient *thrombin* to coagulate the protein and allow to stand for 2 hours. Collect the clot, wash with *saline TS* and carry out the determination as described in the "Determination of Nitrogen" (Method II, Appendix 6.7).

Assay Compare the potency of cryoprecipitated factor VIII with that of an appropriate plasma or intermediate-purity standard, by measuring its ability to correct the prolonged activated partial thromboplastin time of hemophilia A plasma or by another suitable method.

[1]When cryoprecipitated factor VIII is produced from fresh-frozen plasma (frozen within 8 hours of donation), the yield should be more than 400 IU per litre of starting plasma. The average yield of factor VIII as freeze-dried cryoprecipitate is then at least 300 IU per litre of starting plasma. The acceptable yield shall be controlled by the Ministry of Public Health or by the National Blood Centre, Thai Red Cross Society.

DRIED FACTOR IX FRACTION

Human Coagulation Factor IX; Factor IX Concentrate

Category Human blood and blood products (coagulant; antihemorrhagic agent).

Dried Factor IX Fraction is a freeze-dried plasma protein fraction containing coagulation Factor IX, prepared by a method that effectively separates Factor IX from other prothrombin complex factors (factors II, VII and X). The activities of factor II, VII and X shall be shown to be not more than 5 per cent of the activity of factor IX. It is obtained from human plasma that complies with the requirements stated under *Plasma for Fractionation*, p. 193. Heparin, antithrombin and other auxiliary substances such as a stabilizer may be added. It contains no antimicrobial preservative.

The specific activity is not less than 50 IU of factor IX[1] per mg of total protein, before the addition of any protein stabilizer. The potency of the preparation, reconstituted as stated on the label, is not less than 20 IU of factor IX per ml.

Description White or pale yellow powder or friable solid.

Strengths available 250, 500, 1000, 1250, and 1500 IU.

Dose Slow *intravenous infusion*, as prescribed by the physician. The rate of administration of factor IX should be individualized according to the specific product and the response and comfort of the patient.

[1]The potency of factor IX is expressed in terms of the International Units (IU) as defined by the World Health Organization Standard. One unit of factor IX is defined as the average factor IX activity present in 1 ml of normal fresh pooled plasma less than 1 hour old.

Warning

1. Fever, chills and urticaria may occur.

2. Rapid administration may cause nausea, vomiting, headache, or flushing.

3. Caution should be exercised if large or frequently repeated doses are given to individuals with blood groups A, B, and AB since it may cause hemolysis due to the trace amounts of blood groups A and B isohemagglutinins.

4. It should be used with caution in neonates since there is a risk of hepatitis.

Expiration date When stored under the prescribed condition, the expiration date is not later than 2 years from the date of the last satisfactory test for potency.

Packaging and storage Dried Factor IX Fraction shall be kept in tightly closed containers, protected from light and stored at a temperature of 2° to 8°.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition, the label on the container states (1) the number of IU of coagulation factor IX in the container; (2) the concentration of protein in g per litre of the solution constituted as directed; (3) where appropriate, the number of IU of heparin in the container; (4) the name and amount of any other added substance; (5) the volume of Sterile Water for Injection necessary for reconstitution; (6) that if solution is not complete or if a gel forms on reconstitution, the preparation shall not be used; (7) that the solution should be used within 3 hours after reconstitution and any unused solution discarded; (8) that a filter is to be used in the administration set.

Before carrying out the identification, the tests (except those for constituted solution and water) and the assay, immediately reconstitute the preparation to be examined as stated on the label.

Identification

A. Using a suitable range of species-specific antisera, carry out precipitation tests on the preparation to be examined. It is recommended that the tests be carried out using antisera specific to the plasma proteins of each species of domestic animal commonly used in the preparation of materials of biological origin in the country concerned. The preparation is shown to contain proteins of human origin and gives negative results with antisera specific to plasma proteins of other species.

B. The assay for coagulation factor IX contributes to the identification of the preparation.

pH 6.5 to 7.5 (Appendix 4.11).

Osmolality Not less than 240 mOsmol/kg (Appendix 4.35).

Solubility test Reconstitute the solution as stated on the label or on the leaflet. The preparation dissolves completely under gentle swirling within 10 minutes, giving a clear or slightly opalescent, colourless solution.

Water Not more than 3.0 per cent w/w (Karl Fischer Method, Appendix 4.12). Add a suitable volume of *anhydrous methanol* to the container of the preparation being examined, shake, allow to stand and carry out the determination on a known volume of the supernatant liquid.

Total protein (**Note** For some products, especially those without a protein stabilizer such as albumin, this method may not be applicable. Another validated method for protein determination must therefore be performed.) If necessary, dilute the solution with *saline TS* to produce a solution containing about 15 mg of protein in 2 ml. To 2.0 ml of the resulting solution in a round-bottomed centrifuge tube, add 2 ml of a 7.5 per cent w/v solution of *sodium molybdate* and 2 ml of a solution containing 1 volume of *nitrogen-free sulfuric acid* in 30 volumes of *water*. Shake, centrifuge for 5 minutes, decant the supernatant liquid, and allow the inverted tube to drain on filter paper. Using the residue thus obtained, carry out the determination as described in the "Determination of Nitrogen" (Method II, Appendix 6.7). Calculate the content of protein by multiplying the result by 6.25.

Heparin If heparin has been added, the preparation being examined contains not more than the amount of heparin stated on the label and in any case not more than 0.5 IU of heparin per IU of factor IX (Appendix 14.2.1).

Activated coagulation factor For each of the dilutions, the coagulation time is not less than 150 seconds. If necessary, dilute the preparation to be examined to contain 20 IU of factor IX per ml.

Where applicable, determine the amount of heparin present and neutralize the heparin by addition of *protamine sulfate* (10 μg of protamine sulfate neutralizes 1 IU of heparin). Prepare 1 to 10 and 1 to 100 dilutions of the preparation being examined using *tris-(hydroxymethyl)aminomethane buffer solution pH 7.5*. Place a series of polystyrene tubes in a water-bath at 37° and add to each tube 0.1 ml of *platelet-poor plasma* and 0.1 ml of a suitable dilution of *cephalin TS* or *platelet substitute*. Allow to stand for 60 seconds. Add to each tube either

0.1 ml of one of the dilutions or 0.1 ml of the buffer solution (control tube). To each tube add immediately 0.1 ml of 0.025 M *calcium chloride* (previously warmed to 37°) and measure, within 30 minutes of the original dilution, the time that elapses between addition of the calcium chloride solution and the formation of a clot. The test is not valid unless the coagulation time measured for the control tube is 200 to 350 seconds.

Pyrogens Complies with the "Pyrogen Test" (Appendix 8.2), using a volume of the solution containing equivalent to not less than 50 IU of factor IX per kg of the rabbit's weight.

Sterility Complies with the "Sterility Test" (Appendix 10.1).

Assay Carry out the "Biological Assay of Human Coagulation Factor IX" (Appendix 15.1.4). The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

DRIED PROTHROMBIN COMPLEX

Prothrombin Complex Concentrate (PCC); Factor IX Complex Concentrate

Category Human blood and blood products (coagulant; antihemorrhagic agent).

Dried Prothrombin Complex is a sterile, freeze-dried powder consisting of partially purified Factor IX fraction as well as concentrated factors II, VII and X fractions. It is obtained from human plasma that complies with the requirements stated under *Plasma for Fractionation*, p. 193. Heparin, antithrombin and other auxiliary substances such as a stabilizer may be added. It contains no antimicrobial preservative.

The specific activity is not less than 0.6 IU of factor IX[1] per mg of total protein, before the addition of any protein stabilizer. The potency of the preparation, reconstituted as stated on the label, is not less than 20 IU of factor IX per ml.

Description White or slightly coloured powder or friable solid. Very hygroscopic.

Strengths available 400 to 1500 IU.

[1]The potency of factor IX is expressed in terms of the factor IX component. One unit of factor IX is defined as the average factor IX activity present in 1 ml of normal fresh pooled plasma less than 1 hour old.

Dose Slow *intravenous infusion*, as prescribed by the physician. The rate of administration of factor IX should be individualized according to the specific product and the response and comfort of the patient.

Warning It should be used with caution in neonates since there is a risk of hepatitis.

See also under *Dried Factor IX Fraction*, p. 204.

Expiration date When stored under the prescribed conditions, the expiration date is not later than 2 years from the date of the last satisfactory assay for potency.

Packaging and storage Dried Prothrombin Complex shall be kept in tightly closed containers, protected from light and stored at a temperature of 2° to 8°.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition, the label on the container states (1) the number of IU of coagulation factor IX, factor II, and factor X per container; (2) where applicable, the number of IU of factor VII per container; (3) the concentration of protein in g per litre of the solution constituted as directed; (4) where appropriate, the number of IU of heparin per container; (5) the name and amount of any other added substance; (6) the name and the volume of the liquid to be used for reconstitution; (7) that if solution is not complete or if a gel forms on reconstitution the preparation shall not be used; (8) that the solution should be used within 3 hours after reconstitution and any unused solution discarded; (9) that a filter is to be used in the administration set.

Before carrying out the identification, the tests (except those for constituted solution and water) and the assay, immediately reconstitute the preparation to be examined as stated on the label.

Identification

A. Using a suitable range of species-specific antisera, carry out precipitation tests on the preparation to be examined. It is recommended that the tests be carried out using antisera specific to the plasma proteins of each species of domestic animal commonly used in the preparation of materials of biological origin in the country concerned. The preparation is shown to contain proteins of human origin and gives negative results with antisera specific to plasma proteins of other species.

B. The assay for coagulation factor IX activity and, where applicable, those for factors II, VII, and X contribute to the identification of the preparation.

pH 6.5 to 7.5 (Appendix 4.11).

Osmolality Not less than 240 mOsmol/kg (Appendix 4.35).

Solubility test Reconstitute the solution as stated on the label or on the leaflet. The preparation dissolves completely under gentle swirling within 10 minutes, giving a clear solution that may be coloured.

Water Not more than 3.0 per cent w/w (Karl Fischer Method, Appendix 4.12). Add a suitable volume of *anhydrous methanol* to the container of the preparation to be examined, shake, allow to stand and carry out the determination on a known volume of the supernatant liquid.

Total protein (**Note** For some products, especially those without a protein stabilizer such as albumin, this method may not be applicable and another validated method for protein determination must therefore be performed.) If necessary, dilute the solution with *saline TS* to produce a solution containing about 15 mg of protein in 2 ml. To 2.0 ml of the resulting solution in a round-bottomed centrifuge tube add 2 ml of a 7.5 per cent w/v solution of *sodium molybdate* and 2 ml of a solution containing 1 volume of *nitrogen-free sulfuric acid* in 30 volumes of *water*. Shake, centrifuge for 5 minutes, decant the supernatant liquid, and allow the inverted tube to drain on filter paper. Using the residue thus obtained, carry out the determination as described in the "Determination of Nitrogen" (Method II, Appendix 6.7). Calculate the content of protein by multiplying the result by 6.25.

Heparin If heparin has been added, the preparation being examined contains not more than the amount of heparin stated on the label and in any case not more than 0.5 IU of heparin per IU of factor IX (Appendix 14.2.1).

Activated coagulation factor For each of the dilutions the coagulation time is not less than 150 seconds. If necessary, dilute the preparation to be examined to contain 20 IU of factor IX per ml.

Where applicable, determine the amount of heparin present and neutralize the heparin by addition of *protamine sulfate* (10 µg of protamine sulfate neutralizes 1 IU of heparin). Prepare 1 to 10 and 1 to 100 dilutions of the preparation to be examined using *tris-(hydroxymethyl)aminomethane buffer solution pH 7.5*. Place a series of polystyrene tubes in a water-bath at 37° and add to each tube 0.1 ml of *platelet-poor plasma* and 0.1 ml of a suitable dilution of *cephalin TS* or *platelet substitute*. Allow to stand for 60 seconds. Add to each tube either 0.1 ml of one of the dilutions or 0.1 ml of the buffer solution (control tube). To each tube add immediately 0.1 ml of 0.025 M *calcium chloride* (previously warmed to 37°) and measure, within 30 minutes of the original dilution, the time that elapses between addition of the calcium chloride solution and the formation of a clot. The test is not valid unless the coagulation time measured for the control tube is 200 to 350 seconds.

Thrombin No coagulation occurs in the tubes containing the preparation to be examined. Coagulation occurs within 30 seconds in the tube containing thrombin.

Where applicable, determine the amount of heparin present and neutralize the heparin by addition of *protamine sulfate* (10 g of protamine sulfate neutralizes 1 IU of heparin). In each of two test-tubes, mix equal volumes of the reconstituted preparation and a 0.3 per cent solution of *fibrinogen*. Keep one of the tubes at 37° for 6 hours and the other at room temperature for 24 hours. In the third tube, mix a volume of the fibrinogen solution with an equal volume of a solution containing 1 IU per ml of *human thrombin* and place the tube in a water-bath at 37°.

Pyrogens Complies with the "Pyrogen Test" (Appendix 8.2), using a volume of the solution containing equivalent to not less than 30 IU of coagulation factor IX per kg of the rabbit's weight.

Sterility Complies with the "Sterility Test" (Appendix 10.1).

Assay

FOR FACTOR IX Carry out the "Biological Assay of Human Coagulation Factor IX" (Appendix 15.1.4). The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

FOR FACTOR II Carry out the "Biological Assay of Human Coagulation Factor II" (Appendix 15.1.7). The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P = 0.95$) are not less than 90 per cent and not more than 111 per cent of the estimated potency.

FOR FACTOR VII If the label states that the preparation contains factor VII, carry out the "Biological Assay of Human Coagulation Factor VII" (Appendix 15.1.8). The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

FOR FACTOR X Carry out the "Biological Assay of Human Coagulation Factor X" (Appendix 15.1.5). The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits (P = 0.95) are not less than 90 per cent and not more than 111 per cent of the estimated potency.

FIBRIN SEALANT KIT

Category Human blood and blood products (antihemorrhagic agent, local).

Fibrin Sealant Kit is essentially composed of two components, namely fibrinogen concentrate (component 1), a protein fraction containing human fibrinogen and a preparation containing human thrombin (component 2). A fibrin clot is rapidly formed when the two thawed or reconstituted components are mixed. Other ingredients (for example, human coagulation factor XIII, a fibrinolysis inhibitor or calcium ions) and stabilizers (for example, *Albumin solution*) may be added. No antimicrobial preservative is added.

Human constituents are obtained from human plasma that complies with the requirements stated under *Plasma for fractionation*, p. 193. No antibiotic is added to the plasma used.

When thawed or reconstituted as stated on the label, component 1 contains not less than 40 mg per ml of clottable protein; the thrombin activity of component 2 varies over a wide range (approximately 4 to 1000 IU per ml).

Description

Freeze-dried constituents are hygroscopic, white or pale yellow powders or friable solids.

Frozen constituents are colourless or pale yellow, opaque solids.

Liquid constituents are colourless or pale yellow.

Strengths available 40 to 90 mg of fibrinogen per ml and 400 to 1000 IU of thrombin per ml.

Dose Adults: *Topical*, to the involved area, as prescribed by the physicians.

Contra-indication

1. It is contra-indicated in patients with arterial and strong venous bleeding or in individual known to have anaphylactic or severe systemic reaction to human blood and to any other components of the product.

2. It is contra-indicated for intravascular injection because life-threatening thromboembolic event may occur.

3. It is contra-indicated for application into the eyeball.

Warning

1. A fibrin sealant kit containing tranexamic acid may cause fatal neurotoxic reaction in patients during surgical operation where contact with cerebrospinal fluid or dura-mater can occur.

2. Caution should be exercised if it is to be used for tissue fixation or incisional closure in ocular surgery.

Expiration date When stored under the prescribed conditions, the expiration date is not later than 2 years from the date of manufacture, or as indicated on the label.

Packaging and storage Frozen Fibrin Sealant Kit shall be stored at a temperature of –20° or below, protected from light.

Freeze-dried Fibrin Sealant Kit shall be stored at a temperature of 2° to 8°, protected from light; avoid freezing.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition, the label on the container states (1) the amount of fibrinogen (mg of clottable protein), thrombin (IU), per container, and coagulation factor XIII, if this is more than 10 IU per ml; (2) the name and volume of solvent to be used to reconstitute the components, where applicable.

Before carrying out the identification and the tests (except those for solubility and water), immediately reconstitute or thaw the preparation to be examined as stated on the label.

FOR COMPONENT 1 (FIBRINOGEN CONCENTRATE)

Identification

A. The assay for fibrinogen contributes to the identification of the preparation.

B. The assay for factor XIII contributes to the identification of the preparation (where applicable).

pH 6.5 to 8.0 (Appendix 4.11).

Solubility test Freeze-dried concentrates dissolve within 20 minutes in the volume of solvent for reconstitution and at the temperature stated on the label, forming an almost colourless, clear or slightly turbid solution.

Stability of solution No gel formation appears within 120 minutes of reconstitution or thawing.

Water For freeze-dried contituents, not more than 3.0 per cent w/w (Karl Fischer Method, Appendix 4.12).

Sterility Complies with the "Sterility Test" (Appendix 10.1).

Assay

FIBRINOGEN (CLOTTABLE PROTEIN) The estimated content in mg of clottable protein is not less than 70 per cent and not more than 130 per cent of the content stated on the label.

Mix 0.2 ml of the reconstituted preparation with 2 ml of a suitable buffer solution (pH 6.6 to 7.4) containing sufficient *human thrombin* (approximately 3 IU per ml) and *calcium* (0.05 mol per litre). Maintain at 37° for 20 minutes, separate the precipitate by centrifugation (5000 × *g*, 20 minutes), and wash thoroughly with *saline TS*. Carry out the "Determination of Nitrogen" (Method II, Appendix 6.7). Calculate the protein content by multiplying the result by 6.0. If for a particular preparation this method cannot be applied, use another validated method for determination of fibrinogen.

FACTOR XIII Where the label indicates that the human coagulation factor XIII activity is more than 10 IU per ml, the estimated activity is not less than 80 per cent and not more than 120 per cent of the activity stated on the label.

Make at least three suitable dilutions of thawed or reconstituted component 1 and of normal human plasma (reference preparation) using as a diluent coagulation factor XIII deficient plasma or another suitable diluent. Add to each dilution suitable amounts of the following reagents:

activator reagent, containing bovine or human thrombin, a suitable buffer, calcium chloride and a suitable inhibitor such as Gly-Pro-Arg-Pro-Ala-NH_2 which inhibits clotting of the sample but does not prevent coagulation factor XIII activation by thrombin,

detection reagent, containing a suitable factor XIIIa-specific peptide substrate such as Leu-Gly-Pro-Gly-Glu-Ser-Lys-Val-Ile-Gly-NH_2 and glycine ethyl ester as second substrate in a suitable buffer solution,

NADH[1] reagent, containing glutamate dehydrogenase, α-ketoglutarate and NADH in a suitable buffer solution.

After mixing, the absorbance changes (Δ*A*/minute) are measured at a wavelength of 340 nm (Appendix 2.2), after the linear phase of the reaction is reached.

One IU of factor XIII is equal to the activity of 1 ml of human normal plasma.

Calculate the activity of the test preparation as described in the "Statistical Analysis of Results of Biological Assays and Tests" (Appendix 9).

The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 125 per cent of the estimated activity.

FOR COMPONENT 2 (THROMBIN PREPARATION)

Identification The assay for thrombin contributes to the identification of the preparation.

pH 5.0 to 8.0 (Appendix 4.11).

Solubility test Freeze-dried preparations dissolve within 5 minutes in the volume of solvent for reconstitution stated on the label, forming a colourless, clear to slightly turbid solution.

Water For freeze-dried constituents, not more than 3.0 per cent w/w (Karl Fischer Method, Appendix 4.12).

Sterility Complies with the "Sterility Test" (Appendix 10.1).

Assay

THROMBIN If necessary, dilute the reconstituted preparation to be examined to approximately 2 to 20 IU of thrombin per ml using as diluent a suitable buffer pH 7.3 to 7.5, such as *imidazole buffer solution pH 7.3* containing 1 per cent w/v of *human albumin* or *bovine serum albumin*. To a suitable volume of the dilution add a suitable volume of fibrinogen solution (a 0.1 per cent w/v solution of clottable protein) warmed to 37° and start measurement of the clotting time immediately. Repeat the procedure with each of at least three dilutions, in the range stated above, of a reference preparation of thrombin, calibrated in International Units. Draw a calibration curve on log-log graph paper using the measured clotting times for the dilutions of the reference preparation and the content in International Units of thrombin; use the curve obtained to determine the content in International Units of thrombin in the preparation to be examined.

Calculate the activity of the test preparation as described in the "Statistical Analysis of Results of Biological Assays and Tests" (Appendix 9).

The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 125 per cent of the estimated activity.

[1]β-Nicotinamide Adenine Dinucleotide, Reduced Form.

IMMUNOGLOBULIN

Human Normal Immunoglobulin for Intramuscular Administration; IGIM

Category Passive immunizing agent.

Immunoglobulin is a liquid or freeze-dried preparation containing immunoglobulins from normal human subjects, mainly immunoglobulin G (IgG). Other proteins may be present.

Immunoglobulin is obtained from plasma complies with the requirements stated under *Plasma for Fractionation*, p. 193. No antibiotic is added to the plasma used.

Description The liquid Immunoglobulin is clear and pale-yellow to light-brown; during storage it may show formation of slight turbidity or a small amount of particulate matter.

The freeze-dried Immunoglobulin is a white or slightly yellow powder or solid, friable mass. It is hygroscopic.

Stability Liquid Immunoglobulin should be discarded if it has been frozen. The reconstituted solution of freeze-dried Immunoglobulin should be used immediately or as stated on the label.

Strengths available Liquid or reconstituted preparation, 150 to 180 mg of protein per ml.

Dose *Intramuscular*, preferably in the gluteal region, as directed by the physician.

Contra-indication

1. It is not for intravenous administration.
2. It is contra-indicated in patients with immunoglobulin A deficiencies since anaphylaxis may occur.

Warning

1. It may cause pain, tenderness and muscle stiffness at the site of injection.
2. It should be used with extreme caution in individuals with severe thrombocytopenia or any bleeding disorder.
3. Repeated injections of immunoglobulin, especially in allergic individuals, may result in sensitization which is usually manifested as fever, chills and sweating.
4. Risk-benefit should be considered if it is to be used in pregnant women.

Additional information The antibodies in immunoglobulin preparations may interfere with the immune response to certain live virus vaccines such as measles, mumps, rubella including MMR and varicella. These vaccines should be administered at least 2 to 3 weeks before or 3 months after treatment with immunoglobulin. However, there appears to be no interference between immunoglobulin and oral poliomyelitis vaccine (OPV), yellow fever vaccine, oral typhoid (strain Ty 21a) vaccine, or adsorbed diphtheria, pertussis (whole cell) and tetanus vaccine (DPT).

Expiration date When stored under the prescribed conditions, the expiration date is not later than 2 years from the date of manufacturing, or as indicated on the label.

Packaging and storage Liquid Immunoglobulin shall be kept in a sealed and colourless glass container, protected from light, and stored at a temperature of 2° to 8°; avoid freezing.

Freeze-dried Immunoglobulin shall be kept in a tightly closed colourless glass container, protected from light, and stored at a temperature not exceeding 25°, unless otherwise specified by manufacturers.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition, the label on the container states (1) the volume and the total amount of the protein expressed in mg per ml or, for freeze-dried immunoglobulin, the total amount of protein in the container; (2) the route of administration; (3) where applicable, that the preparation is suitable for use in the prophylaxis of hepatitis A infection; (4) where applicable, the anti-hepatitis A virus activity in IU per ml.

Before carrying out the identification and the tests (except those for constituted solution and water), immediately reconstitute the preparation to be examined as stated on the label.

Identification

A. Carry out the precipitation tests on the preparation being examined, using an antiserum specific to human plasma proteins and a range of antisera specific to the plasma proteins of each species of domestic animal commonly used in the preparation of materials of biological origin. The preparation is shown to contain proteins of human origin and gives negative reactions with antisera specific to plasma proteins of other species.

B. Examine by a suitable immunoelectrophoresis technique. Using antiserum to normal human serum, compare normal human serum and the preparation being examined, both diluted to contain 1 per cent w/v of protein. The main component of the preparation being examined corresponds to the IgG component of

normal human serum. The solution may show the presence of small quantities of other plasma proteins.

pH 5.0 to 7.2, when diluted with *saline TS* to produce a solution containing 1 per cent w/v of protein (Appendix 4.11).

Total protein Not less than 10 per cent w/v and not more than 18 per cent w/v of protein and not less than 90 per cent and not more than 110 per cent of the labelled quantity of protein stated on the label. Dilute the preparation to be examined with *saline TS* to obtain a solution containing about 15 mg of protein in 2 ml. To 2.0 ml of this solution in a round-bottomed centrifuge tube add 2 ml of a 7.5 per cent w/v solution of *sodium molybdate* and 2 ml of a mixture of 1 volume of *nitrogen-free sulfuric acid* and 30 volumes of *water*. Shake, centrifuge for 5 minutes, decant the supernatant liquid, and allow the inverted tube to drain on filter paper. Using the residue thus obtained, carry out the "Determination of Nitrogen" (Method II, Appendix 6.7) and calculate the content of protein by multiplying by 6.25.

Protein composition Carry out the test as described in the "Cellulose Acetate Electrophoresis" (Method II, Appendix 3.7), but applying an electric field such that the albumin band of normal human serum applied in a control strip migrates at least 30 mm. Prepare the following solutions. For solution (A), dilute the preparation being examined with *saline TS* to produce a solution containing 5 per cent w/v of protein. For solution (B), dilute Human Immunoglobulin for Electrophoresis RS with *saline TS* to produce a solution containing 5 per cent w/v of protein. In the strips prepared from solution (A) not more than 10 per cent of the protein is contained in bands other than the principal band. The test is not valid unless the proportion of protein in the principal band in the strips prepared from solution (B) is within the limits stated in the leaflet supplied with Human Immunoglobulin for Electrophoresis RS.

Distribution of molecular size Carry out the test as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase Dissolve and dilute 4.873 g of *disodium hydrogenphosphate dihydrate*, 1.741 g of *sodium dihydrogenphosphate monohydrate*, 11.688 g of *sodium chloride* and 50 mg of *sodium azide* with *water* to 1000.0 ml.

Standard preparation Dilute Human Immunoglobulin RS with *saline TS* to the same protein concentration as *Test preparation*.

Test preparation Dilute the preparation being examined with *saline TS* to a concentration suitable for the chromatographic system used. A concentration in the range 0.4 to 1.2 per cent w/v of protein and the injection of 50 µg to 600 µg of protein are usuallysuitable.

Chromatographic system The chromatographic procedure may be carried out using (a) a stainless steel column (60 cm × 7.5 mm) packed with *hydrophilic silica gel* (3 to 10 µm), (b) *Mobile phase* at a flow rate of about 0.5 ml per minute and (c) an ultraviolet photometer set at 280 nm.

In the chromatogram obtained from *Standard preparation*, the principal peak corresponds to IgG monomer and there is a peak corresponding to dimer with a retention time relative to monomer of about 0.85. Identify the peaks in the chromatogram obtained from *Test preparation* by comparison with the chromatogram obtained from *Standard preparation*; any peak with retention time shorter than that of dimer corresponds to polymers and aggregates. The preparation to be examined complies with the test if, in the chromatogram obtained from *Test preparation*: for monomer and dimer, retention time relative to the corresponding peak in the chromatogram obtained from *Standard preparation* is 1±0.02; the sum of monomer and dimer represents not less than 85 per cent of the total area of the chromatogram and polymers and aggregates represent not more than 10 per cent of the total area of the chromatogram. This requirement does not apply to products where albumin has been added as a stabilizer; for products stabilized with albumin, a test for distribution of molecular size is carried out during manufacture before addition of the stabilizer.

Hemagglutinins, anti-A and anti-B Dilute the constituted solution with *saline TS* to produce a solution containing 3 per cent w/v of immunoglobulin. Carry out the test for hemagglutinins, anti-A and anti-B using a suitable indirect method such as that described below. The 1 in 64 dilutions do not show agglutination.

Prepare in duplicates serial dilutions of the preparation being examined in *saline TS*. To each dilution of one series add an equal volume of a 5 per cent v/v suspension of group A_1 red blood cells previously washed three times with *saline TS*. To each dilution of the other series add an equal volume of a 5 per cent v/v suspension of group B red blood cells previously washed three times with *saline TS*. Incubate the suspensions at 37° for 30 minutes and then wash the cells three times with *saline TS*. Leave the cells in contact with a

polyvalent anti-human globulin reagent for 30 minutes. Without centrifuging, examine each suspension for agglutination under a microscope.

Anti-D antibodies If Immunoglobulin is intended for subcutaneous administration, it complies with the "Test for Anti-D Antibodies in Human Normal Immunoglobulin for Intravenous Administration" (Appendix 15.1.10).

Antibody to hepatitis B surface antigen Not less than 0.5 IU per g of immunoglobulin, determined by a suitable immunochemical method (Appendix 14.5).

Antibody to hepatitis A virus If intended for use in the prophylaxis of hepatitis A, it complies with the following additional requirement.

Carry out the "Immunochemical Method" (Appendix 14.5). Determine the antibody content by comparison with a reference preparation calibrated in International Units, using an immunoassay of suitable sensitivity and specificity.

The stated potency is not less than 100 IU per ml. The estimated potency is not less than the stated potency. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

Sterility Complies with the "Sterility Test" (Method II, Appendix 10.1).

Pyrogens or Bacterial endotoxins Complies with the "Pyrogen Test" (Appendix 8.2) or, preferably and where justified and authorized, with a validated *in vitro* test such as the "Test for Bacterial Endotoxins" (Appendix 8.5).

For the pyrogen test, use 1 ml of the preparation being examined per kg of the rabbit's weight.

Where the bacterial endotoxin test is used, it contains less than 5 Endotoxin Units per ml.

Other requirements Freeze-dried IGIM complies with the following additional requirements.

Solubility test Add the volume of the liquid stated on the label. The preparation dissolves completely within 20 minutes at 20° to 25°.

Water Not more than 3.0 per cent w/w (Karl Fischer Method, Appendix 4.12).

HUMAN NORMAL IMMUNOGLOBULIN FOR INTRAVENOUS ADMINISTRATION

Category Passive immunizing agent.

Human Normal Immunoglobulin for Intravenous Administration (IGIV) is a liquid or freeze-dried preparation containing immunoglobulins from normal human subjects, mainly immunoglobulin G (IgG). Other proteins may be present. This monograph does not apply to products intentionally prepared to contain fragments or chemically modified IgG.

IGIV is obtained from plasma complies with the requirements stated under *Plasma for Fractionation*, p. 193. No antibiotic is added to the plasma used.

Description The liquid IGIV is clear or slightly opalescent and colourless or pale yellow.

The freeze-dried IGIV is a white or slightly yellow powder or solid friable mass. It is hygroscopic.

Stability Liquid IGIV should be discarded if it has been frozen. The reconstituted solution of freeze-dried IGIV should be used IGIV immediately or as stated on the label.

Strengths available Liquid or reconstituted preparation, 30 to 120 mg of protein per ml.

Dose Immunodeficiency: *Intravenous infusion*, 200 to 800 mg per kg of body weight once a month.

Idiopathic thrombocytopenic purpura: *Intravenous infusion*, 400 mg per kg of body weight per day for 2 to 5 consecutive days or 1 g per kg of body weight per day 1 to 2 days. If the patient's response is inadequate, up to 2 g per kg of body weight may be administered as a single maintenance dose every 2 weeks.

Bacterial infection secondary to B-cell chronic lymphocytic leukemia: *Intravenous infusion*, 400 mg per kg of body weight per day for 4 days or 2 g per kg of body weight as a single dose.

Prophylaxis of infection after bone marrow transplantation: *Intravenous infusion*, 500 mg per kg of body weight weekly.

Kawasaki diaease: *Intravenous infusion*, 400 mg per kg of body weight per day for 4 days or 1 to 2 g per kg of body weight as a single dose.

Bone marrow transplantation: *Intravenous infusion*, 500 mg per kg of body weight beginning on day 7 and day 2 pretransplantation (or at the time conditioning therapy for transplantation is begun), then weekly through 3 months posttransplantation.

Contra-indication It is contra-indicated in patients with immunoglobulin A deficiencies since anaphylaxis may occur.

Warning

1. Backache, chills, flushing, headache, hypotension, myalgia, nausea or pyrexia usually begins within 1 hour of the start of the infusion of IGIV. Symptoms usually subside within 30 minutes.

2. It may cause dyspnea, tachycardia, burning sensation in head, cyanosis, faintness, fatigue, wheezing, and renal dysfunction.

3. Risk-benefit should be considered if it is to be used in cardiac function impairment patients and pregnant women.

4. Caution should be exercised if it is to be used in patients with agammaglobulinemia or severe hypogammaglobulinemia.

Additional information Antibodies contained in IGIV may interfere with the body's immune response to certain live virus vaccines, such as measles, mumps, and rubella including MMR which should be administered at least 14 days before or 3 months after treatment with IGIV. However, there appears to be no interference between IGIV and oral poliomyelitis vaccine (OPV), yellow fever vaccine, oral typhoid (strain Ty 21a) vaccine, or adsorbed diphtheria, pertussis (whole cell) and tetanus vaccine (DPT).

Expiration date When stored under the prescribed conditions, the expiration date is not later than 2 years from the date of manufacturing, or as indicated on the label.

Packaging and storage Liquid IGIV shall be kept in a sealed and colourless glass container, protected from light, and stored at a temperature of 2° to 8°; avoid freezing.

Freeze-dried IGIV shall be kept in a tightly closed colourless glass container, protected from light, and stored at a temperature not exceeding 25°, unless otherwise specified by manufacturers.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition, the label on the container states (1) for the liquid IGIV, the volume of the preparation in container and the protein content expressed in grams per litre or for freeze-dried IGIV, the total amount of protein in the container; (2) the amount of immunoglobulin in the container; (3) the route of administration; (4) the distribution of subclasses of immunoglobulin G present in the preparation; (5) where applicable, the amount of albumin added as a stabilizer; (6) the maximum content of immunoglobulin A.

Before carrying out the identification and the tests (except those for constituted solution and water), immediately reconstitute the preparation to be examined as stated on the label.

Identification Complies with the Identification described under *Immunoglobulin*, p. 209.

pH 4.0 to 7.4, when diluted with sufficient *saline TS* to produce a solution containing 1 per cent w/v of protein (Appendix 4.11).

Osmolality Not less than 240 mOsmol/kg (Appendix 4.35).

Total protein Not less than 3 per cent w/v of protein and not less than 90 per cent and not more than 110 per cent of the labelled quantity of protein stated on the label. Dilute the preparation to be examined with *saline TS* to obtain a solution containing about 15 mg of protein in 2 ml. To 2.0 ml of this solution in a round-bottomed centrifuge tube add 2 ml of a 7.5 per cent w/v solution of *sodium molybdate* and 2 ml of a mixture of 1 volume of *nitrogen-free sulfuric acid* and 30 volumes of *water*. Shake, centrifuge for 5 minutes, decant the supernatant liquid, and allow the inverted tube to drain on filter paper. Using the residue thus obtained, carry out the "Determination of Nitrogen" (Method II, Appendix 6.7).

Protein composition Carry out the test as described in the "Cellulose Acetate Electrophoresis" (Method II, Appendix 3.7) or other suitable electrophoresis methods, but applying an electric field such that the albumin band of normal human serum applied in a control strip migrates at least 30 mm. Prepare the following solutions. For solution (A), dilute the preparation being examined with *saline TS* to produce a solution containing 3 per cent w/v of protein. For solution (B), dilute Human Immunoglobulin for Electrophoresis RS with *saline TS* to produce a solution containing 3 per cent w/v of protein. In the strips prepared with solution (A) not more than 5 per cent of the protein is contained in bands other than the principal band. This limit is not applicable if albumin has been added to the preparation as a stabilizer; for such preparations, a test for protein composition is carried out during manufacture before addition of the stabilizer. The test is not valid unless the proportion of protein in the principal band in the strips prepared with solution (B) is within the limits stated in

the leaflet supplied with Human Immunoglobulin for Electrophoresis RS.

Distribution of molecular size Carry out the test as described in the "High-pressure Liquid Chromatography" (Appendix 3.5).

Mobile phase, Standard preparation, Test preparation, and **Chromatographic system** Proceed as directed in the test for distribution of molecular size described under *Immunoglobulin*, p. 210.

Anticomplementary activity (ACA) The consumption of complement is not more than 50 per cent (1 CH_{50} per mg of immunoglobulin) (Appendix 15.1.11).

Prekallikrein activator (PKA) Not more than 35 IU per ml, calculated with reference to a solution containing 3 per cent w/v of immunoglobulin (Appendix 14.2.2).

Hemagglutinins, anti-A and anti-B Dilute the solution with *saline TS* to produce a solution containing about 3 per cent w/v of immunoglobulin. Carry out the test for hemagglutinins, anti-A and anti-B, using a suitable indirect method such as that described below. The 1 in 64 dilutions do not show agglutination.

Prepare in duplicates serial dilutions of the preparation being examined in *saline TS*. To each dilution of one series add an equal volume of a 5 per cent v/v suspension of group A_1 red blood cells previously washed three times with *saline TS*. To each dilution of the other series, add an equal volume of a 5 per cent v/v suspension of group B red blood cells previously washed three times with *saline TS*. Incubate the suspension at 37° for 30 minutes and then wash the cells three times with *saline TS*. Leave the cells in contact with a polyvalent anti-human globulin reagent for 30 minutes. Without centrifuging, examine each suspension for agglutination under a microscope.

Anti-D antibodies It complies with the "Test for Anti-D antibodies in Human Normal Immunoglobulin for Intravenous Administration" (Appendix 15.1.10).

Antibody to hepatitis B surface antigen Not less than 0.5 IU per g of immunoglobulin, determined by a suitable immunochemical method (Appendix 14.5).

Immunoglobulin A Not more than the maximum content stated on the label, determined by a suitable immunochemical method (Appendix 14.5).

Sterility Complies with the "Sterility Test" (Method II, Appendix 10.1).

Pyrogens or Bacterial endotoxins Complies with the "Pyrogen Test" (Appendix 8.2) or, preferably and where justified and authorized, with a validated *in vitro* test such as the "Test for Bacterial Endotoxins" (Appendix 8.5).

For the pyrogen test, inject a volume equivalent to 0.5 g of immunoglobulin per kg of the rabbit's weight, but not more than 10 ml per kg of the rabbit's weight.

Where the bacterial endotoxin test is used, it contains less than 0.5 Endotoxin Unit per ml for solutions with a protein content not more than 5 per cent, and less than 1.0 Endotoxin Unit per ml for solutions with a protein content more than 5 per cent but not more than 10 per cent.

Other requirements Freeze-dried IGIV complies with the following additional requirements.

Solubility test Add the volume of the liquid stated on the label. The preparation dissolves completely within 30 minutes at 20° to 25°.

Water Not more than 3.0 per cent w/w (Karl Fischer Method, Appendix 4.12).

ANTI-Rh_0 (D) IMMUNOGLOBULIN

Anti-D (Rh_0) Immunoglobulin; Anti-D Immunoglobulin; Rh_0 (D) Immune Human Globulin; Rh Immune Globulin; Rh_0 (D) IGIM

Category Passive immunizing agent (Immunosuppressive).

Anti-Rh_0 (D) Immunoglobulin is a liquid or freeze-dried preparation containing human immunoglobulins, mainly immunoglobulin G (IgG). The preparation is intended for intramuscular administration. It is obtained from plasma from D-negative donors immunized against D-antigen. It contains specific antibodies against erythrocyte D-antigen and may also contain small quantities of other blood-group antibodies. *Immunoglobulin* and/or *Albumin solution* may be added.

Anti-Rh_0 (D) Immunoglobulin complies with the requirements stated under *Immunoglobulin*, p.=, except for the minimum total protein content.

The test for anti-D antibodies (Appendix 15.1.10) is not carried out, since it is replaced by the assay of human anti-D immunoglobulin (Appendix 15.1.9) as prescribed below under *Assay*.

Description The Anti-Rh_0 (D) Immunoglobulin is clear or slightly opalescent, colourless to pale yellow or light brown solution.

The freeze-dried Anti-Rh_0 (D) Immunoglobulin is white to off-white powder.

Stability Liquid Anti-Rh_0 (D) Immunoglobulin should be discarded if it has been frozen. The reconstituted solution of freeze-dried Anti-Rh_0 (D) Immunoglobulin may be stored at room temperature for up to 4 hours. It should be discarded if it is not used within 4 hours.

Both solutions should not be used if they are discoloured or contain particulate matter.

Strengths available 250 and 1500 IU (50 and 300 μg) per single-dose container.

Dose *Intramuscular*, 250 or 1500 IU (50 or 300 μg), preferably in the anterolateral aspects of the upper thigh or the deltoid muscle of the upper arm, as prescribed by the physician.

Contra-indication

1. It is not for intravenous administration.
2. It is contra-indicated in patients who have had anaphylactic or severe systemic reactions to preparations containing human immunoglobulin.

Warning

1. It should be administered cautiously to individuals with a specific IgA deficiency since anaphylaxis may occur.
2. It should be used with caution in individuals with thrombocytopenia or bleeding disorders, since bleeding may occur following intramuscular administration of the drug.
3. It should be used with caution in individuals with a history of prior systemic allergic reactions to preparations containing human immunoglobulin.

Additional information The antibodies contained in anti-Rh_0 (D) immunoglobulin may interfere with the immune response to some live virus vaccines such as measles, mumps and rubella virus vaccine live, poliovirus vaccine live (oral). These vaccines should be administered at least 3 months after treatment with anti-Rh_0 (D) immunoglobulin. However, postpartum administration of a vaccine containing rubella virus vaccine live to women who are susceptible to rubella should not be delayed because they received antepartum or postpartum anti-Rh_0 (D) immunoglobulin. These women should receive rubella virus vaccines live or measles, mumps and rubella virus vaccine live (MMR) immediately after delivery and serologic tests should be performed 3 months after vaccination to determine if seroconversion occurred.

Expiration date When stored under the prescribed conditions, the expiration date is not later than 1 year from the date of manufacturing, or as indicated on the label.

Packaging and storage Liquid Anti-Rh_0 (D) Immunoglobulin shall be kept in a sealed and colourless glass container, protected from light, and stored at a temperature of 2° to 8°; avoid freezing.

Freeze-dried Anti-Rh_0 (D) Immunoglobulin shall be kept in a tightly closed colourless glass container, protected from light, and stored at a temperature not exceeding 25°, unless otherwise specified by manufacturers.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition the label on the container states the total number of IU of Anti-Rh_0 (D) Immunoglobulin in the container.

See also under *Immunoglobulin*, p. 209.

Assay Carry out the "Biological Assay of Anti-Rh_0 (D) Immunoglobulin" (Method A, Appendix 15.1.6). The estimated potency is not less than 90 per cent of the stated potency. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 120 per cent of the estimated potency.

Method B or C (Appendix 15.1.6) may be used for potency determination if a satisfactory correlation with the results obtained by Method A has been established for the particular product.

ANTI-Rh_0 (D) IMMUNOGLOBULIN FOR INTRAVENOUS ADMINISTRATION

Rh_0 (D) Immune Globulin IV; Rh_0 (D) IGIV

Category Passive immunizing agent (immunosuppressive).

Anti-Rh_0 (D) Immunoglobulin for Intravenous Administration is a liquid or freeze-dried preparation containing human immunoglobulins, mainly immunoglobulin G (IgG). It is obtained from plasma from D-negative donors immunized against D-antigen. It contains specific antibodies against erythrocyte D-antigen and may also contain small quantities of other blood-group antibodies. ***Human Normal Immunoglobulin for Intravenous Administration*** **and/or** ***Albumin solution*** **may be added.**

Anti-Rh_0 (D) Immunoglobulin for Intravenous Administration complies with the requirements stated under *Human Normal Immunoglobulin for Intravenous Administration*, p. 211, except for the minimum total protein content, the limit for osmolality and the limit for prekallikrein activator.

The test for anti-D antibodies (Appendix 15.1.10) is not carried out, since it is replaced by the assay of

anti-Rh_0 (D) immunoglobulin (Appendix 15.1.6) as prescribed below under *Assay*.

Description The Anti-Rh_0 (D) Immunoglobulin for Intravenous Administration is clear or slightly opalescent, colourless to pale yellow solution.

The freeze-dried Anti-Rh_0 (D) Immunoglobulin for Intravenous Administration is white to off-white lyophilized powder.

Stability Liquid Anti-Rh_0 (D) Immunoglobulin for Intravenous Administration should be discarded if it has been frozen. The reconstituted solution of freeze-dried Anti-Rh_0 (D) Immunoglobulin for Intravenous Administration may be stored at room temperature for up to 4 hours. It should be discarded if it is not used within 4 hours.

Both solutions should not be used if they are discoloured or contain particulate matter.

Strengths available 600, 1500, 2500, 5000 and 15,000 IU (120, 300, 500, 1000, and 3000 μg) per single-dose container.

Dose *Intravenous*, should be given over 3 to 5 minutes, or *intramuscular*, preferably in the anterolateral aspects of the upper thigh or the deltoid muscle of the upper arm, as prescribed by the physician.

Contra-indication It is contra-indicated in patients who have had anaphylactic or severe systemic reactions to preparations containing human immunoglobulin.

Warning

1. It should be administered cautiously to individuals with a specific IgA deficiency since anaphylaxis may occur.

2. It should be used with caution in individuals with a history of prior systemic allergic reactions to preparations containing human immunoglobulin.

Additional information The antibodies contained in anti-Rh_0 (D) immunoglobulin for intravenous administration may interfere with the immune response to some live virus vaccines such as measles, mumps and rubella virus vaccine live, poliovirus vaccine live (oral). These vaccines should be administered at least 3 months after treatment with anti-Rh_0 (D) immunoglobulin for intravenous administration. However, postpartum administration of a vaccine containing rubella virus vaccine live to women who are susceptible to rubella should not be delayed because they received antepartum or postpartum anti-Rh_0 (D) immunoglobulin for intravenous administration. These women should receive rubella virus vaccines live or measles, mumps and rubella virus vaccine live (MMR) immediately after delivery and serologic tests should be performed 3 months after vaccination to determine if seroconversion occurred.

Expiration date When stored under the prescribed conditions, the expiration date is not later than 3 years from the date of manufacturing, or as indicated on the label.

Packaging and storage Liquid Anti-Rh_0 (D) Immunoglobulin for Intravenous Administration shall be kept in a sealed and colourless glass container, protected from light, and stored at a temperature of 2° to 8°; avoid freezing.

Freeze-dried Anti-Rh_0 (D) Immunoglobulin for Intravenous Administration shall be kept in a tightly closed colourless glass container, protected from light, and stored at a temperature not exceeding 25°, unless otherwise specified by manufacturers.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition the label on the container states the total number of IU of Anti-Rh_0 (D) Immunoglobulin in the container.

See also under *Human Normal Immunoglobulin for Intravenous Administration*, p. 212.

Assay Carry out the "Biological Assay of Anti-Rh_0 (D) Immunoglobulin" (Method A, Appendix 15.1.6). The estimated potency is not less than 90 per cent of the stated potency. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 120 per cent of the estimated potency.

Method B or C (Appendix 15.1.6) may be used for potency determination if a satisfactory correlation with the results obtained by Method A has been established for the particular product.

HEPATITIS B IMMUNOGLOBULIN

Hepatitis B Immune Globulin; Human Hepatitis B Immunoglobulin; HBIG

Category Passive immunizing agent.

Hepatitis B Immunoglobulin is a liquid or freeze-dried preparation containing immunoglobulins, mainly immunoglobulin G (IgG). The preparation is intended for intramuscular administration. It is obtained from plasma from selected and/or immunized donors having antibodies against hepatitis B surface antigen. *Immunoglobulin* may be added.

Hepatitis B Immunoglobulin complies with the requirements stated under *Immunoglobulin*, p. 209, except for the minimum total protein content, and with those under this monograph.

Description The liquid Hepatitis B Immunoglobulin is clear or slightly opalescent and colourless or pale yellow to light brown.

The freeze-dried Hepatitis B Immunoglobulin is a white or slightly yellow powder or solid friable mass.

Stability Liquid Hepatitis B Immunoglobulin should be discarded if it has been frozen. The reconstituted solution of freeze-dried Hepatitis B Immunoglobulin should be used immediately or as stated on the label.

Strengths available 100, 180, 200, and 220 IU per ml.

Dose Adults and children over 10 years of age: *Intramuscular*, 500 IU as a single dose.

Children 5 to 10 years of age: *Intramuscular*, 300 IU as a single dose.

Children under 5 years of age and infants: *Intramuscular*, 200 IU as a single dose.

Contra-indication It is not for intravenous administration.

Warning

1. It should be administered cautiously to individuals with a specific IgA deficiency since anaphylaxis may occur.

2. It should be used with caution in individuals with thrombocytopenia or bleeding disorders, since bleeding may occur following intramuscular administration of the drug.

3. Local pain, tenderness, swelling, and erythema may occur at the injection site.

4. Risk-benefit should be considered if it is to be used in nursing women.

5. Urticaria, rash, pruritus, angioedema, nausea, faintness, fever, body and joint pains, dizziness, leg cramps, or malaise and lassitude may occur.

Additional information

1. It should be administered as soon as possible within 48 hours and not more than 1 week after exposure to the virus for adults and children over 10 years of age or within 48 hours of birth of hepatitis B infected mothers.

2. It should be administered at different sites when administering concomitantly with hepatitis B vaccine.

3. The antibodies in hepatitis B immunoglobulin preparations may interfere with the immune responses to certain live virus vaccines such as measles, mumps and rubella including MMR. These vaccines should be administered at least 14 days before or 3 months after treatment with hepatitis B immunoglobulin. However, there appears to be no interference between hepatitis B immunoglobulin and oral poliomyelitis vaccine (OPV), yellow fever vaccine or oral typhoid (strain Ty 21a) vaccine.

Expiration date When stored under the prescribed conditions, the expiration date is not later than 2 years from the date of manufacturing, or as indicated on the label.

Packaging and storage Liquid Hepatitis B Immunoglobulin shall be kept in a sealed and colourless glass container, protected from light, and stored at a temperature of 2° to 8°; avoid freezing.

Freeze-dried Hepatitis B Immunoglobulin shall be kept in a sealed and colourless glass container, protected from light, and stored at a temperature not exceeding 25°, unless otherwise specified by manufacturers.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition the label on the container states the number of IU per container.

See also under *Immunoglobulin*, p. 205.

Assay Carry out the "Immunochemical Methods (Appendix 14.5). The potency is determined by comparing the antibody titre of the immunoglobulin to be examined with that of a standard preparation calibrated in International Units, using an immunoassay of suitable sensitivity and specificity.

The stated potency is not less than 100 IU per ml. The estimated potency is not less than the stated potency. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

RABIES IMMUNOGLOBULIN

Human Rabies Immune Globulin; Antirabies Immunoglobulin Injection; Human Antirabies Immunoglobulin Injection; HRIG

Category Passive immunizing agent.

Rabies immunoglobulin is a liquid or freeze-dried preparation containing human immunoglobulins, mainly immunoglobulin G (IgG). The preparation is intended for intramuscular administration. It is obtained from plasma from donors immunized against rabies. It contains specific antibodies neutralizing the rabies virus. *Immunoglobulin* may be added.

Rabies Immunoglobulin complies with the requirements stated under *Immunoglobulin*, p. 209, except for the minimum total protein content, and with those under this monograph.

Description The liquid Rabies Immunoglobulin is clear or slightly opalesscent and colourless or pale yellow to light brown.

The freeze-dried Rabies Immunoglobulin is a white or slightly yellow powder or solid friable mass.

Stability Liquid Rabies Immunoglobulin should be discarded if it has been frozen. The reconstituted solution of freeze-dried Rabies Immunoglobulin should be used immediately or as stated on the label.

Strength available 150 IU per ml.

Dose *Intramuscular*, 20 IU per kg of body weight with as much as possible of the volume infiltrated at the site(s) of the bite(s) and the remainder should be administered at a distant site, deltoid preferred.

Contra-indication It is not for intravenous administration.

Warning

1. Rabies immunoglobulin should be administered cautiously to individuals with a specific IgA deficiency since anaphylaxis may occur.
2. Rabies immunoglobulin should be used with caution in individuals with thrombocytopenia or bleeding disorders, since bleeding may occur following intramuscular administration of the drug.
3. Rabies immunoglobulin should not be administered to an individual who has been previously immunized with rabies vaccine and has a known adequate rabies antibody titer.
4. Local tenderness, pain, soreness or stiffness of the muscles may occur at the injection site.
5. Low-grade fever, urticaria, or angioedema may occur.

Additional information The antibodies in rabies immunoglobulin preparations may interfere with the immune response to certain live virus vaccines such as measles, mumps and rubella including MMR. These vaccines should be administered at least 14 days before or 4 months after treatment of rabies. However, there appears to be no interference between rabies immunoglobulin and oral poliomyelitis vaccine (OPV), yellow fever vaccine or oral typhoid (strain Ty 21a) vaccine.

Expiration date When stored under the prescribed conditions, the expiration date is not later than 2 years from the date of manufacture, or as indicated on the label.

Packaging and storage Liquid Rabies Immunoglobulin shall be kept in a sealed colourless glass container, protected from light, and stored at a temperature of 2° to 8°; avoid freezing.

Freeze-dried Rabies Immunoglobulin shall be stored in a sealed and colourless glass containers, protected from light at a temperature not exceeding 25°, unless otherwise specified by manufacturers.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition the label on the container states the number of IU per container.

See also under *Immunoglobulin*, p. 209.

Assay The potency is determined by comparing the dose of immunoglobulin required to neutralize the infectivity of a rabies virus suspension with the dose of a reference preparation, calibrated in International Units, required to produce the same degree of neutralization.

Carry out the determination as described in the "Immunochemical Method" (Appendix 14.5). The test is performed in sensitive cell cultures and the presence of unneutralized virus is revealed by immunofluorescence. Rapid Fluorescent Focus Inhibition Test (RFFIT) is described as the reference immunofluorescent technique for rabies immunoglobulin. Carry out the test in suitable sensitive cells. It is usual to use the BHK 21 cell line, grown in the medium described below, between the 18th and 30th passage levels counted from the ATCC seed lot. Harvest the cells after 2 to 4 days of growth, treat with trypsin and prepare a suspension containing 500,000 cells per ml (cell suspension). Ten minutes before using the cell suspension, add 10 µg of *diethylamino-ethyldextran* per ml, if necessary, to increase the sensitivity of the cells.

Use a fixed virus strain grown in sensitive cells, such as the CVS strain of rabies virus adapted to growth in the BHK 21 cell line (seed virus suspension). Estimate the titre of the seed virus suspension as follows.

Prepare a series of dilutions of the viral suspension. In the chambers of cell-culture slides (8 chambers per slide), place 0.1 ml of each dilution and 0.1 ml of medium and add 0.2 ml of the cell suspension. Incubate in an atmosphere of *carbon dioxide* at 37° for 24 hours.

Carry out fixation, immunofluorescence staining and evaluation as described below. Determine the endpoint titre of the seed virus suspension and prepare the working virus dilution corresponding to 100 $CCID_{50}$ per 0.1 ml.

For each assay, check the amount of virus used by performing a control titration: from the dilution corresponding to 100 $CCID_{50}$ per 0.1 ml, make three tenfold dilutions. Add 0.1 ml of each dilution to four chambers

containing 0.1 ml of medium and add 0.2 ml of the cell suspension. The test is not valid unless the titre lies between 30 $CCID_{50}$ and 300 $CCID_{50}$.

Dilute the reference preparation to a concentration of 2 IU per ml using non-supplemented culture medium (stock reference dilution, stored below –80°). Prepare two suitable predilutions (1:8 and 1:10) of the stock reference dilution so that the dilution of the reference preparation that reduces the number of fluorescent fields by 50 per cent lies within the four dilutions of the cell-culture slide. Add 0.1 ml of the medium to each chamber, except the first in each of two rows, to which add respectively 0.2 ml of the two predilutions of the stock reference dilution transferring successively 0.1 ml to the other chambers.

Dilute the preparation to be examined 1 in 100 using non-supplemented medium (stock immunoglobulin dilution) to reduce to a minimum errors due to viscosity of the undiluted preparation and make three suitable predilutions so that the dilution of the preparation to be examined that reduces the number of fluorescent fields by 50 per cent lies within the four dilutions of the cell-culture slide. Add 0.1 ml of the medium to all the chambers except the first in each of three rows, to which add respectively 0.2 ml of the three predilutions of the stock immunoglobulin dilution. Prepare a series of two-fold dilutions transferring successively 0.1 ml to the other chambers.

To all the chambers containing the dilutions of the reference preparation and the dilutions of the preparation to be examined, add 0.1 ml of the virus suspension corresponding to 100 $CCID_{50}$ per 0.1 ml (working virus dilution). Shake manually and allow to stand in an atmosphere of carbon dioxide at 37° for 90 minutes. Add 0.2 ml of the cell suspension, shake manually and allow to stand in an atmosphere of carbon dioxide at 37° for 24 hours.

After 24 hours, discard the medium and remove the plastic walls. Wash the cell monolayer with *phosphate buffered saline pH 7.4* and then with a mixture of 20 volumes of *water* and 80 volumes of *acetone* and fix in a mixture of 20 volumes of *water* and 80 volumes of *acetone* at –20° for 3 minutes. Spread on the slides *fluorescein-conjugated rabies antiserum* ready for use. Allow to stand in an atmosphere with a high level of moisture at 37° for 30 minutes. Wash with *phosphate buffered saline pH 7.4* and dry. Examine twenty fields in each chamber at a magnification of 250×, using a microscope equipped for fluorescence readings. Note the number of fields with at least one fluorescent cell. Check the test dose used in the virus titration slide and determine the dilution of the reference preparation and the dilution of the preparation to be examined that reduce the number of fluorescent fields by 50 per cent, calculating the two or three dilutions together using probit analysis. The test is not valid unless the statistical analysis shows a significant slope of the dose response curve and no evidence of deviation from linearity or parallelism.

The stated potency is not less than 150 IU per ml. The estimated potency is not less than the stated potency and is not more than two times the stated potency. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

CULTURE MEDIUM FOR GROWTH OF BHK 21 CELLS

Commercially available media that have a slightly different composition from that shown below may also be used.

Sodium chloride	6.4	g
Potassium chloride	0.40	g
Calcium chloride, anhydrous	0.20	g
Magnesium sulfate	0.20	g
Sodium dihydrogenphosphate, monohydrate	0.124	g
Dextrose monohydrate	4.5	g
Iron(III) nitrate nonahydrate	0.10	mg
L-Arginine hydrochloride	42.0	mg
L-Cystine	24.0	mg
L-Histidine	16.0	mg
L-Isoleucine	52.0	mg
L-Leucine	52.0	mg
L-Lysine hydrochloride	74.0	mg
L-Phenylalanine	33.0	mg
L-Threonine	48.0	mg
L-Tryptophan	8.0	mg
L-Tyrosine	36.0	mg
L-Valine	47.0	mg
L-Methionine	15.0	mg
L-Glutamine	0.292	g
i-Inositol	3.60	mg
Choline chloride	2.0	mg
Folic acid	2.0	mg
Nicotinamide	2.0	mg
Calcium pantothenate	2.0	mg
Pyridoxine hydrochloride	2.0	mg
Thiamine hydrochloride	2.0	mg
Riboflavine	0.2	mg
Phenol red	15.0	mg
Sodium hydrogencarbonate	2.75	g
Water to	1000	ml

The medium is supplemented with:

Fetal calf serum (heated at 56° for 30 minutes)	10	per cent
Tryptose phosphate broth	10	per cent
Benzylpenicillin sodium	60	mg/l
Streptomycin	0.1	g/l

TETANUS IMMUNOGLOBULIN

Tetanus Immune Globulin; Antitetanus Immunoglobulin Injection; Human Antitetanus Immunoglobulin Injection; TIG

Category Passive immunizing agent.

Tetanus Immunoglobulin is a liquid or freeze-dried preparation containing immunoglobulins, mainly immunoglobulin G (IgG). It is obtained from plasma containing specific antibodies against the toxin of *Clostridium tetani*. *Immunoglobulin* may be added.

Tetanus Immunoglobulin complies with the requirements stated under *Immunoglobulin*, p. 209, except for the minimum total protein content, and with those under this monograph.

Description The liquid Tetanus Immunoglobulin is clear or slightly opalescent and colourless or pale yellow to light brown.

The freeze-dried Tetanus Immunoglobulin is a white or slightly yellow powder or solid friable mass.

Stability Liquid Tetanus Immunoglobulin should be discarded if it has been frozen. The reconstituted solution of freeze-dried Tetanus Immunoglobulin should be used immediately or as stated on the label.

Strength available 250 IU per single-dose container.

Dose For prophylaxis of tetanus—Adults and adolescents: *Intramuscular*, 250 to 500 IU.

For treatment of tetanus: *Intramuscular*, 150 IU per kg of body weight into multiple sites, usual dose of 3000 to 6000 IU may be administered.

Contra-indication It is not for intravenous administration.

Warning

1. Local pain, tenderness, and erythema may occur at the injection site.

2. It should be used with caution in individuals with thrombocytopenia or bleeding disorders, since bleeding may occur following intramuscular administration of the drug.

3. It should be administered cautiously to individuals with a specific IgA deficiency since anaphylaxis may occur.

4. Low-grade fever, urticaria, angioedema and local inflammation may occur.

Additional information

1. It should be administered at different sites when administering concomitantly with tetanus toxoid.

2. The antibodies in tetanus immunoglobulin preparations may interfere with the immune responses to certain live virus vaccines such as measles, mumps, rubella including MMR, and oral polimyelitis vaccine (OPV). These vaccines should be administered at least 14 days before or 3 months after treatment with tetanus immunoglobulin. However, there appears to be no interference between tetanus immunoglobulin and yellow fever vaccine or oral typhoid (strain Ty 21a) vaccine.

Expiration date When stored under the prescribed conditions, the expiration date is not later than 2 years from the date of manufacturing, or as indicated on the label.

Packaging and storage Liquid Tetanus Immunoglobulin shall be kept in a sealed colourless glass container, protected from light, and stored at a temperature of 2° to 8°; avoid freezing.

Freeze-dried Tetanus Immunoglobulin shall be kept in a seal and colourless glass container, protected from light, and stored at a temperature not exceeding 25°, unless otherwise specified by manufacturers.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition, the label on the container states the number of IU per container.

See also under *Immunoglobulin*, p. 209.

Assay Carry out the "Immunochemical Methods (Appendix 14.5). The potency is determined by comparing the antibody titre of the immunoglobulin to be examined with that of a reference preparation calibrated in International Units, using an immunoassay of suitable sensitivity and specificity.

The stated potency is not less than 100 IU per ml. The estimated potency is not less than the stated potency. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

VARICELLA IMMUNOGLOBULIN

Varicella-Zoster Immune Globulin (VZIG)

Category Passive immunizing agent.

Varicella Immunoglobulin (VIG) is a liquid or freeze-dried preparation containing immunoglobulins, mainly immunoglobulin G (IgG). The preparation is intended for intramuscular administration. It is obtained from plasma from selected donors having antibodies against *Herpesvirus varicellae*. *Immunoglobulin* may be added.

Varicella Immunoglobulin complies with the requirements stated under *Immunoglobulin*, p. 209, except for the minimum number of donors, the minimum total protein content and, where authorized, the test for antibody to hepatitis B surface antigen and with those under this monograph.

Description The liquid varicella Immunoglobulin is clear or slighty opalescent and colourless or pale yellow.

The freeze-dried Varicella Immunoglobulin is a white or slightly yellow powder or solid friable mass.

Stability Liquid Varicella Immunoglobulin should be discarded if it has been frozen. The reconstituted solution of freeze-dried Varicella Immunoglobulin should be used immediately or as stated on the label.

Strengths available 125 and 625 IU per single-dose container.

Dose *Intramuscular*, as prescribed by the physician or as follows:

Body weight (kg)	Dose (IU)
Up to 10	125
>10 to 20	250
>20 to 30	375
>30 to 40	500
>40	625

Contra-indication

1. It is not for intravenous administration.
2. It is contra-indicated in patients who have exhibited previous systemic allergic reactions to immunoglobulin, and in those with immunoglobulin A deficiencies and severe thrombocytopenia.

Warning

1. It is recommended that no more than 125 IU be given in a single injection site.
2. Swelling, tenderness and pain at the site of intramuscular injection may follow the administration of Varicella Immunoglobulin.
3. Rapid administration may lead to systemic reactions which include fever, chills, facial flushing, headache, gastro-intestinal and respiratory symptoms.
4. Severe reactions such as angioedema and anaphylactic shock may occur.
5. Risk-benefit should be considered if it is to be used in pregnant women.
6. It is not recommended for use in patients with varicella or herpers zoster infection.

Additional information

1. It should be administered as soon as possible within 48 hours but not later than 96 hours after exposure to the disease or virus.
2. Fractional doses of less than 125 IU of Varicella Immunoglobulin are not recommended.
3. Postexposure prophylaxis with Varicella Immunoglobulin could be repeated at three-week intervals in persons with continued or repeated exposure to varicella until such risk is no longer present.
4. Antibodies contained in Varicella Immunoglobulin could interfere with the immune response to varicella live virus vaccine, and other certain live virus vaccines such as measles, mumps and rubella including MMR. These vaccines should be administered 5 months or longer after the treatment of Varicella Immunoglobulin. However, there appears to be no interference between Varicella Immunoglobulin and oral poliomyelitis vaccine (OPV), yellow fever vaccine or oral typhoid (strain Ty 21a) vaccine.

Expiration date When stored under the prescribed conditions, the expiration date is not later than 2 years from the date of manufacturing, or as indicated on the label.

Packaging and storage Liquid Varicella Immunoglobulin shall be kept in a sealed colourless glass container, protected from light, and stored at a temperature of 2° to 8°; avoid freezing.

Freeze-dried Varicella Immunoglobulin shall be kept in a sealed colourless glass container, protected from light, and stored at a temperature not exceeding 25°, unless otherwise specified by manufacturers.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition the label on the

container states the total number of IU of Varicella Immunoglobulin in the container.

See also under *Immunoglobulin*, p. 209.

Assay Carry out the "Immunochemical Method" (Appendix 14.5). The potency is determined by comparing the antibody titer of the immunoglobulin to be examined with that of a standard preparation calibrated in International Units, using an immunoassay of suitable sensitivity and specificity.

The stated potency is not less than 100 IU per ml. The estimated potency is not less than the stated potency. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

ลิขสิทธิ์ กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข
ห้ามทำซ้ำ ดัดแปลง แก้ไข โดยไม่ได้รับอนุญาต

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ANTISERA

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ANTISERA

Antisera are preparations containing the specific immunoglobulins obtained from serum of animals by purification. Antisera have the specific power of combining with venins or with the toxins formed by bacteria, or of combining with the bacterium, virus or other antigen used for their preparation.

Antisera are obtained from healthy animals immunized by injections of the appropriate toxins or toxoids, venins or suspensions of micro-organisms or other antigens. The animals are not treated with penicillin antibiotics. If the animals are treated with other antibiotics, a suitable withdrawal period is allowed before collection of blood or plasma. The specific immunoglobulins may be obtained from the immune serum by fractional precipitation and enzyme treatment or by other chemical or physical methods.

A suitable antimicrobial preservative may be added and is invariably added if the preparations are issued in multidose containers. The final sterile product is distributed aseptically in sterile containers that are then sealed so as to exclude contamination. Alternatively, the final product, after distribution in the sterile containers, may be freeze-dried by a procedure that reduces the water content of the finished product to not more than 3.0 per cent w/w. The containers may then be sealed under vacuum or they may be filled with oxygen-free nitrogen or another suitable inert gas before being sealed; in either case they are sealed so as to exclude contamination. The antiserum is reconstituted immediately before use.

Antisera prepared by enzyme treatment and fractional precipitation are most stable at about pH 6. The method of preparation of antisera is such that the product loses not more than 5 per cent of its activity per year at this pH when stored at 20° and not more than 20 per cent per year when stored at 37°.

Antisera are almost colourless or very faintly yellow liquids free from turbidity and almost odourless except for the odour of any added antimicrobial preservative. Freeze-dried antisera consist of white or pale yellow crusts or powders, freely soluble in water to form colourless or pale yellow liquids having the same characteristics as the corresponding liquid preparations.

The antiserum, reconstituted where necessary as stated on the label, complies with the following requirements.

Packaging and storage Liquid Antisera shall be stored at a temperature of 2° to 8°, protected from light; avoid freezing. Freeze-dried Antisera shall be stored at a temperature not exceeding 25°, protected from light.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition, the label on the container states (1) the number of IU or mg per ml, where applicable; (2) the amount of protein per container; (3) the route of administration; (4) the major precautions to be employed in administering animal serum or plasma; (5) the animal source of the preparation.

pH 6.0 to 7.0 (Appendix 4.11), when diluted with *saline TS* to give a solution containing 1 per cent w/v of protein.

Foreign proteins When examined by precipitation tests with specific antisera, consists exclusively of protein of the declared animal species.

Phenol For an antiserum containing phenol as a preservative, not more than 0.25 per cent w/v when determined by the method described under the "Determination of Phenol", p. 179.

Protein content Not more than 17 per cent w/v. Carry out the determination as described in the "Determination of Nitrogen" (Method II, Appendix 6.7).

Assay Determine the potency by comparison with an established reference preparation, using the method described in the individual monograph, and express the result in IU or mg per ml, where applicable.

Pyrogens Complies with the "Pyrogen Test" (Appendix 8.2), using not less than 1 ml of Antiserum per kg of the rabbit's weight.

Sterility Complies with the "Sterility Test" (Appendix 10.1).

Abnormal toxicity Complies with the "Abnormal Toxicity Test" (Appendix 8.1).

Water For freeze-dried antisera, not more than 3.0 per cent w/w. Use Karl Fischer Method (Appendix 4.12), unless otherwise specified in the individual monograph.

The provisions of this monograph apply to the following antisera:

Botulinum Antitoxin
Diphtheria Antitoxin
Tetanus Antitoxin
Rabies Antiserum
Cobra Antivenin
King Cobra Antivenin
Banded Krait Antivenin
Malayan Krait Antivenin
Green Pit Viper Antivenin
Malayan Pit Viper Antivenin
Russell's Viper Antivenin

Hematotoxic Polyvalent Antivenin (Green Pit Viper, Malayan Pit Viper, and Russell's Viper Antivenin)

Neurotoxic Polyvalent Antivenin (Cobra, King Cobra, Banded Krait, and Malayan Krait Antivenin).

BOTULINUM ANTITOXIN

Botulism Antitoxin

Category Passive immunizing agent.

Botulinum Antitoxin is a sterile liquid or freeze-dried preparation containing antitoxic globulins that have the power of specifically neutralizing the toxins formed by *Clostridium botulinum* type A, type B or type E, or any mixture of these types. It is obtained by fractionation from serum or plasma of horses, or other mammals, that have been immunized against *Clostridium botulinum* type A, type B and type E toxins.

The antitoxin complies with the requirements stated under Antisera, with the following modifications.

Description Transparent or slightly opalescent liquid, practically colourless, and practically odourless or having an odour because of the antimicrobial agent.

Freeze-dried antitoxin, when reconstituted, becomes colourless or slightly yellowish brown, clear or slightly whitish turbid.

Strengths available One ml contains 500 to 750 IU of type A, 500 to 550 IU of type B, and/or 50 to 850 IU of type E antibodies.

Dose *Intramuscular* or *intravenous infusion* as directed by the physician.

Contra-indication Botulinum antitoxin prepared from horse serum is contra-indicated in individuals with history of hypersensitivity or anaphylaxis to equine-derived serums.

Warning Tachycardia, cardiovascular collapse, flushing, cyanosis, sinus tachycardia, serum sickness, photophobia, bronchospasm, apnea, and anaphylaxis may occur.

Additional information

1. For intravenous infusion, dilute 1 volume of antitoxin with 10 volumes of sodium chloride injection (0.9 per cent) and give slowly over at least 30 minutes.

2. Patients should be kept under medical observation after the administration of full dose.

3. The relative proportions of types A and B present in Botulinum Antitoxin Bivalent (Equine) Types A and B are believed to be adequate to establish a level of neutralizing capacity in the bloodstream, against the homologous toxins, of several times the level shown to be clinically efficacious for type E antitoxin.

4. The type E Botulinum Antitoxin is administered consecutively with Botulinum Antitoxin Bivalent, types A and B, only when type E botulinum (usually associated with consumption of contaminated fish products) is suspected.

Expiration date The expiration date for Antitoxin containing a 20 per cent excess of potency is not later than 5 years after the date of the last satisfactory test for potency.

Labelling Complies with the "General Information for Antisera", p. 225. In addition the label states the types of *Clostridium botulinum* toxin neutralized by the preparation as stated in the accompanying leaflets.

Identification It specifically neutralizes the types of *Clostridium botulinum* toxin stated on the label, rendering them harmless to susceptible animals.

Assay Not less than the value stated on the label for each of types A, B and E.

The potency of botulinum antitoxin is determined by comparing the dose necessary to protect mice against the lethal effects of a fixed dose of botulinum toxin with the quantity or the standard preparation of botulinum antitoxin necessary to give the same protection. For this comparison a reference preparation of each type of botulinum antitoxin, calibrated in International Units, and suitable preparations of botulinum toxins, for use as test toxins, are required. The potency of each test toxin is determined in relation to the specific reference preparation; the potency of the botulinum antitoxin to be examined is determined in relation to the potency of the test toxins by the same method.

International Units of the antitoxin are the specific neutralizing activity for botulinum toxin type A, type B and type E contained in stated amounts of the International Standards which consist of dried immune horse sera of types A, B and E. The equivalence in International Units of the International Standard is stated from time to time by the World Health Organization.

Selection of animals Use mice having body masses such that the difference between the lightest and the heaviest does not exceed 5 g.

Preparation of test toxins

> ***Caution*** Botulinum toxin is extremely toxic. Exceptional care must be taken in any procedure in which it is employed.

Prepare type A, B and E toxins from sterile filtrates of approximately 7-day cultures in liquid medium of *Clostridium botulinum* types A, B and E. To the filtrate, add 2 volumes of *glycerol*, concentrate, if necessary, by dialysis against glycerol and store at or slightly below 0°.

Selection of test toxins Select toxins of each type for use as test toxins by determining for mice the L+/10 dose and the LD_{50}, the observation period being 96 hours. The test toxins contain at least 1000 LD_{50} in an L+/10 dose.

Determination of test doses of the toxins (L+/10 dose) Prepare solutions of the reference preparations in a suitable liquid such that each contains 0.25 IU of antitoxin per ml. Using each solution in turn, determine the test dose of the corresponding test toxin.

Prepare mixtures of the solution of the reference preparation and the test toxin such that each contains 2.0 ml of the solution of the reference preparation, one of a graded series of volumes of the test toxin and sufficient of a suitable liquid to bring the total volume to 5.0 ml. Allow the mixtures to stand at room temperature, protected from light, for 60 minutes. Using four mice for each mixture, inject a dose of 1.0 ml intraperitoneally into each mouse. Observe the mice for 96 hours.

The test dose of toxin is the quantity in 1.0 ml of the mixture made with the smallest amount of toxin capable of causing, despite partial neutralization by the reference preparation, the death of all four mice injected with the mixture within the observation period.

Determination of potency of the antitoxin Prepare solutions of each reference preparation in a suitable liquid such that each contains 0.25 IU of antitoxin per ml.

Prepare solutions of each test toxin in a suitable liquid such that each contains 2.5 test doses per ml.

Using each toxin solution and the corresponding reference preparation in turn, determine the potency of the antitoxin. Prepare mixtures of the solution of the test toxin and the antitoxin to be examined such that each contains 2.0 ml of the solution of the test toxin, one of a graded series of volumes of the antitoxin to be examined, and sufficient of a suitable liquid to bring the total volume to 5.0 ml. Also prepare mixtures of the solution of the test toxin and the solution of the reference preparation such that each contains 2.0 ml of the solution of the test toxin, one of a graded series of volumes of the solution of the reference preparation centred on that volume (2.0 ml) that contains 0.5 IU, and sufficient of a suitable liquid to bring the total volume to 5.0 ml. Allow the mixtures to stand at room temperature, protected from light, for 60 minutes. Using four mice for each mixture, inject a dose of 1.0 ml intraperitoneally into each mouse. Observe the mice for 96 hours.

The mixture that contains the largest volume of antitoxin that fails to protect the mice from death contains 0.5 IU. This quantity is used to calculate the potency of the antitoxin in International Units per ml.

The test is not valid unless all the mice injected with mixtures containing 2.0 ml or less of the solution of the reference preparation die and all those injected with mixtures containing more survive.

DIPHTHERIA ANTITOXIN

Category Passive immunizing agent.

Diphtheria Antitoxin is a sterile non-pyrogenic solution of the refined and concentrated proteins, chiefly globulins, containing antitoxic antibodies obtained from serum or plasma of horses, or other mammals, that have been immunized against diphtheria toxin or toxoid.

The antitoxin complies with the requirements stated under Antisera, with the following modifications.

Description Transparent or slightly opalescent, practically colourless liquid; odour, practically odourless or of the antimicrobial agent.

Strength available 10,000 IU per container.

Dose Prophylaxis: *Intramuscular*, 5000 to 10,000 IU.

Therapeutic—

Mild or moderate: *Intramuscular*, 10,000 to 30,000 IU.

Severe: *Intramuscular*, 40,000 to 100,000 IU.

The dosage may be administered as prescribed by the physicians.

Warning

1. It is to be administered only by or under the supervision of the physician or other health care professionals.

2. It should be used with extreme caution in individuals with a history of allergic disorders and/or who have exhibited previous sensitivity to equine serum. Epinephrine and other supportive measures should be available for immediate management of an anaphylactic reaction if it occurs.

3. A few days after the injection of a heterologous serum, serum sickness may also occur.

Expiration date The expiration date for Diphtheria Antitoxin containing a 20 per cent excess of potency is not later than 5 years from the date of the last satisfactory test for potency.

Labelling Complies with the "General Information for Antisera", p. 225. In addition the label on the container states the recommended human dose for the various prophylactic and therapeutic uses.

Identification It specifically neutralizes the toxin formed by *Corynebacterium diphtheriae* rendering it harmless to susceptible animals. The potency test may serve as an identification test.

Assay Not less than 500 IU of antitoxin per ml. The potency of diphtheria antitoxin is determined by comparing the dose necessary to protect guinea-pigs or rabbits against the erythrogenic effects of a fixed dose of diphtheria toxin with the quantity of the standard preparation of diphtheria antitoxin necessary to give the same protection. For this comparison a reference preparation of diphtheria antitoxin, calibrated in International Units, and a suitable preparation of diphtheria toxin, for use as a test toxin, are required.

The potency of the test toxin is determined in relation to the reference preparation; the potency of the diphtheria antitoxin being examined is determined in relation to the potency of the test toxin by the same method.

The International Unit of antitoxin is the specific neutralizing activity for diphtheria toxin contained in a stated amount of the International Standard, which consists of a quantity of dried immune horse serum.

Preparation of test toxin Prepare diphtheria toxin from cultures of *Corynebacterium diphtheriae* in a liquid medium. Filter the culture to obtain a sterile toxic filtrate and store at 4°.

Selection of test toxin Select a toxin for use as a test toxin by determining for guinea-pigs or rabbits the lr/100 dose and the minimal reacting dose, the observation period being 48 hours. The test toxin has at least 200 minimal reacting doses in the lr/100 dose.

Minimal reacting dose This is the smallest quantity of toxin which, when injected intracutaneously into guinea-pigs or rabbits, causes a small, characteristic reaction at the site of injection within 48 hours.

The test toxin is allowed to stand for some months before being used for the assay of antitoxin. During this time its toxicity declines and the lr/100 dose may be increased. Determine the minimal reacting dose and the lr/100 dose at frequent intervals. When the experiment shows that the lr/100 dose is constant, the test toxin is ready for use and may be used for a long period. Store the test toxin in the dark at 0° to 5°. Maintain its sterility by the addition of toluene or other antimicrobial preservatives that do not cause rapid decline in specific toxicity.

Determination of test dose of toxin (lr/100 dose). Prepare a solution of the reference preparation in a suitable liquid such that it contains 0.1 IU of antitoxin per ml.

Prepare mixtures of the solution of the reference preparation and of the test toxin such that each contains 1.0 ml of the solution of the reference preparation, one of a graded series of volumes of the test toxin and sufficient of a suitable liquid to bring the total volume to 2.0 ml. Allow the mixtures to stand at room temperature, protected from light, for 15 to 60 minutes. Using two animals for each mixture, inject a dose of 0.2 ml intracutaneously into the shaven or depilated flanks of each animal. Observe the animals for 48 hours.

The test dose of toxin is the quantity in 0.2 ml of the mixture made with the smallest amount of toxin capable of causing, despite partial neutralization by the reference preparation, a small but characteristic erythematous lesion at the site of injection.

Determination of potency of the antitoxin Prepare a solution of the reference preparation in a suitable liquid such that it contains 0.125 IU of antitoxin per ml.

Prepare a solution of the test toxin in a suitable liquid such that it contains 12.5 test doses per ml.

Prepare mixtures of the solution of the test toxin and of the antitoxin being examined such that each contains 0.8 ml of the solution of the test toxin, one of a graded series of volumes of the antitoxin being examined and sufficient of a suitable liquid to bring the total volume to 2.0 ml. Also prepare mixtures of the solution of the test toxin and the solution of the reference preparation such that each contains 0.8 ml of the solution of the test toxin, one of a graded series of volumes of the solution of the reference preparation centred on that volume (0.8 ml) that contains 0.1 IU and sufficient of a suitable liquid to bring the total volume to 2.0 ml. Allow the mixtures to stand at room temperature, protected from light, for 15 to 60 minutes. Using two animals for each mixture, inject a dose of 0.2 ml intracutaneously into the shaven or depilated flanks of each animal. Observe the animals for 48 hours.

The mixture that contains the largest volume of antitoxin that fails to protect the guinea-pigs from the erythematous effects of the toxin contains 0.1 IU. This quantity is used to calculate the potency of the antitoxin in International Units per ml.

The test is not valid unless all the sites injected with mixtures containing 0.8 ml or less of the solution of the reference preparation show erythematous lesions and at all those injected with mixtures containing more there are no lesions.

TETANUS ANTITOXIN

Category Passive immunizing agent.

Tetanus Antitoxin for human use is a sterile, non-pyrogenic solution containing the specific antitoxic globulins obtained from the serum or plasma of horses, or other mammals, that have been immunized against tetanus toxin or toxoid. It has the power of neutralizing the toxin formed by *Clostridium tetani*.

The antitoxin complies with the requirements stated under Antisera, with the following modification.

Description Transparent or slightly opalescent, faint brownish or yellowish or greenish liquid; odour, practically odourless or of the antimicrobial agent.

Strengths available 1500 IU and 20,000 IU per container.

Dose Prophylaxis: *Intramuscular* or *subcutaneous*, 1500 to 5000 IU.

Therapeutic: *Intravenous* or *intramuscular*, 50,000 to 100,000 IU; part of this dose is administered by intravenous injection with the remainder being given intramuscularly.

The dosage may be administered as prescribed by the physician.

Warning

1. Reactions to Tetanus Antitoxin are rather common and often severe. In sensitized persons the administration of a heterologous serum may trigger an anaphylactic reaction. Therefore, it is recommended to take a precise anamnesis and to perform an intradermal test in every case (eventually to begin with a high serum dilution). Before the application of a heterologous serum all the preparations for the treatment of cardiovascular and respiratory complications which may accompany such anaphylactic reactions have to be made. A few days after the injection of a heterologous serum, serum sickness may also occur.

2. Difficulty in breathing and swallowing; feeling of discomfort; fever; hives; itching; muscle aches; rash; reddening of skin, especially around ears; swelling of eyes, face, or inside of nose; swelling of joints; swollen lymph glands; unusual tiredness or weakness, sudden and severe, may occur.

Expiration date The expiration date for Tetanus Antitoxin containing a 20 per cent excess of potency is not later than 5 years from the date of the last satisfactory test for potency.

Packaging and storage Tetanus Antitoxin shall be stored at a temperature of 2° to 8°; avoid freezing.

Labelling See under *Diphtheria Antitoxin*, p. 228.

Identification It specifically neutralizes the toxin formed by *Clostridium tetani*, rendering it harmless to susceptible animals. The potency test may serve as an identification test.

Assay Not less than 400 IU of antitoxin per ml. The potency of tetanus antitoxin is determined by comparing the dose necessary to protect guinea-pigs or mice against the paralytic effects of a fixed dose of tetanus toxin with the quantity of the standard preparation of tetanus antitoxin necessary to give the same protection. If the lethal method is used, the number of animals and the procedure are identical with those described for the paralysis method but the end-point is the death of the animal rather than the onset of paralysis and the L+/10 dose is used instead of the Lp/10 dose. For this comparison a reference preparation of tetanus antitoxin, calibrated in International Units, and a suitable preparation of tetanus toxin, for use as a test toxin, are required. The potency of the test toxin is determined in relation to the reference preparation; the potency of the tetanus antitoxin being examined is determined in relation to the potency of the test toxin by the same method.

The International Unit of antitoxin is the specific neutralizing activity for tetanus toxin contained in a stated amount of the International Standard, which consists of a quantity of dried immune horse serum.

Selection of animals If mice are used, the body masses should be such that the difference between the lightest and the heaviest does not exceed 5 g.

Preparation of test toxin Prepare the test toxin from a sterile filtrate of an approximately 9-day culture in liquid medium of *Clostridium tetani*. To the filtrate add 1 to 2 volumes of *glycerol* and store slightly below 0°. Alternatively, treat the filtrate with *ammonium sulfate*, collect the precipitate, which contains the toxin, dry *in vacuum* over *phosphorus pentoxide desiccant* and store dry, either in sealed ampoules or *in vacuum* over *phosphorus pentoxide desiccant*.

Determination of test dose of toxin (Lp/10 dose) Prepare a solution of the reference preparation in a suitable liquid such that it contains 0.5 IU of antitoxin per ml.

If the test toxin is stored dry, reconstitute it using a suitable liquid.

Prepare mixtures of the solution of the reference preparation and the test toxin such that each contains 2.0 ml of the solution of the reference preparation, one of a graded series of volumes of the test toxin and sufficient of a suitable liquid to bring the volume to 5.0 ml. Allow the mixtures to stand at room tempera-

ture, protected from light, for 60 minutes. Using six mice for each mixture, inject a dose of 0.5 ml subcutaneously into each mouse. Observe the mice for 96 hours. Mice that become paralyzed may be killed.

The test dose of toxin is the quantity in 0.5 ml of the mixture made with the smallest amount of toxin capable of causing, despite partial neutralization by the reference preparation, paralysis in all six mice injected with the mixture within the observation period.

Determination of potency of the antitoxin Prepare a solution of the reference preparation in a suitable liquid such that it contains 0.5 IU of antitoxin per ml.

Prepare a solution of the test toxin in a suitable liquid such that it contains five test doses per ml.

Prepare mixtures of the solution of the test toxin and the antitoxin being examined such that each contains 2.0 ml of the solution of the test toxin, one of a graded series of volumes of the antitoxin being examined and sufficient of a suitable liquid to bring the total volume to 5.0 ml. Also prepare mixtures of the solution of the test toxin and the solution of the reference preparation such that each contains 2.0 ml of the solution of the test toxin, one of a graded series of volumes of the solution of the reference preparation centred on that volume (2.0 ml) that contains 1 IU and sufficient of a suitable liquid to bring the total volume to 5.0 ml. Allow the mixtures to stand at room temperature, protected from light, for 60 minutes. Using six mice for each mixture, inject into each mouse subcutaneously a dose of 0.5 ml. Observe the mice for 96 hours. Mice that become paralyzed may be killed.

The mixture that contains the largest volume of antitoxin that fails to protect the mice from paralysis contains 1 IU. This quantity is used to calculate the potency of the antitoxin in International Units per ml.

The test is not valid unless all the mice injected with mixtures containing 2.0 ml or less of the solution of the reference preparation show paralysis and all those injected with mixtures containing more do not.

RABIES ANTISERUM

Equine Rabies Immunoglobulin

Category Passive immunizing agent.

Rabies Antiserum is a sterile preparation containing the specific antirabies globulins from immunized horses or other mammals.

The antiserum complies with the requirements stated under Antisera, with the following modifications.

Description Transparent or slightly opalescent faint brownish, yellowish or greenish liquid; odour, practically odourless or of the antimicrobial agent.

Warning See under *Diphtheria Antitoxin*, p. 227.

Expiration date The expiration date for Rabies Antiserum is not later than 3 years from the date of the last satisfactory test for potency.

Labelling Complies with the "General Information for Antisera", p. 225. In addition the label on the container states (1) the recommended human dose; (2) the nature of the preparation, e.g. natural serum or purified immunoglobulin or treated immunoglobulin and, if purified or treated, the nature of the process.

Identification Specifically neutralizes the rabies virus rendering it harmless to susceptible animals. The potency test may serve as an identification test.

Assay The potency is determined by comparing the dose of Rabies Antiserum required to neutralize the infectivity of a rabies virus suspension with the dose of a reference preparation, calibrated in International Units, required to produce the same degree of neutralization.

Carry out the determination as described in the "Immunochemical Method" (Appendix 14.5). The test is performed in sensitive cell cultures and the presence of unneutralized virus is revealed by immunofluorescence. Rapid Fluorescent-Focus Inhibition Test (RFFIT) is described as the reference immunofluorescent technique for rabies immunoglobulin. Carry out the test in suitable sensitive cells. It is usual to use the BHK 21 cell line, grown in the medium described below, between the 18^{th} and 30^{th} passage levels counted from the ATCC seed lot. Harvest the cells after 2 to 4 days of growth, treat with trypsin and prepare a suspension containing 500,000 cells per ml (cell suspension). Ten minutes before using the cell suspension, add 10 µg of *diethylamino-ethyldextran* per ml, if necessary, to increase the sensitivity of the cells.

Use a fixed virus strain grown in sensitive cells, such as the CVS strain of rabies virus adapted to growth in the BHK 21 cell line (seed virus suspension). Estimate the titre of the seed virus suspension as follows.

Prepare a series of dilutions of the viral suspension. In the chambers of cell-culture slides (8 chambers per slide), place 0.1 ml of each dilution and 0.1 ml of medium and add 0.2 ml of the cell suspension. Incubate in an atmosphere of *carbon dioxide* at 37° for 24 hours.

Carry out fixation, immunofluorescence staining and evaluation as described below. Determine the end-

point titre of the seed virus suspension and prepare the working virus dilution corresponding to 100 $CCID_{50}$ per 0.1 ml.

For each assay, check the amount of virus used by performing a control titration: from the dilution corresponding to 100 $CCID_{50}$ per 0.1 ml, make three tenfold dilutions. Add 0.1 ml of each dilution to four chambers containing 0.1 ml of medium and add 0.2 ml of the cell suspension. The test is not valid unless the titre lies between 30 $CCID_{50}$ and 300 $CCID_{50}$.

Dilute the reference preparation to a concentration of 2 IU per ml using non-supplemented culture medium (stock reference dilution, stored below –80°). Prepare two suitable predilutions (1:8 and 1:10) of the stock reference dilution so that the dilution of the reference preparation that reduces the number of fluorescent fields by 50 per cent lies within the four dilutions of the cell-culture slide. Add 0.1 ml of the medium to each chamber, except the first in each of two rows, to which add respectively 0.2 ml of the two predilutions of the stock reference dilution transferring successively 0.1 ml to the other chambers.

Dilute the preparation to be examined 1 in 100 using non-supplemented medium (stock immunoglobulin dilution) to reduce to a minimum errors due to viscosity of the undiluted preparation and make three suitable predilutions so that the dilution of the preparation to be examined that reduces the number of fluorescent fields by 50 per cent lies within the four dilutions of the cell-culture slide. Add 0.1 ml of the medium to all the chambers except the first in each of three rows, to which add respectively 0.2 ml of the three predilutions of the stock immunoglobulin dilution. Prepare a series of two fold dilutions transferring successively 0.1 ml to the other chambers.

To all the chambers containing the dilutions of the reference preparation and the dilutions of the preparation to be examined, add 0.1 ml of the virus suspension corresponding to 100 $CCID_{50}$ per 0.1 ml (working virus dilution). Shake manually and allow to stand in an atmosphere of carbon dioxide at 37° for 90 minutes. Add 0.2 ml of the cell suspension, shake manually and allow to stand in an atmosphere of carbon dioxide at 37° for 24 hours.

After 24 hours, discard the medium and remove the plastic walls. Wash the cell monolayer with *phosphate buffered saline pH 7.4* and then with a mixture of 20 volumes of *water* and 80 volumes of *acetone* and fix in a mixture of 20 volumes of *water* and 80 volumes of *acetone* at –20° for 3 minutes. Spread on the slides *fluorescein-conjugated rabies antiserum* ready for use. Allow to stand in an atmosphere with a high level of moisture at 37° for 30 minutes. Wash with *phosphate buffered saline pH 7.4* and dry. Examine twenty fields in each chamber at a magnification of 250×, using a microscope equipped for fluorescence readings. Note the number of fields with at least one fluorescent cell. Check the test dose used in the virus titration slide and determine the dilution of the reference preparation and the dilution of the preparation to be examined that reduce the number of fluorescent fields by 50 per cent, calculating the two or three dilutions together using probit analysis. The test is not valid unless the statistical analysis shows a significant slope of the dose response curve and no evidence of deviation from linearity or parallelism.

The stated potency is not less than 150 IU per ml. The estimated potency is not less than the stated potency and is not more than two times the stated potency. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

CULTURE MEDIUM FOR GROWTH OF BHK 21 CELLS

Commercially available media that have a slightly different composition from that shown below may also be used.

Sodium chloride	6.4	g
Potassium chloride	0.40	g
Calcium chloride, anhydrous	0.20	g
Magnesium sulfate	0.20	g
Sodium dihydrogenphosphate, monohydrate	0.124	g
Dextrose monohydrate	4.5	g
Iron(III) nitrate nonahydrate	0.10	mg
L-Arginine hydrochloride	42.0	mg
L-Cystine	24.0	mg
L-Histidine	16.0	mg
L-Isoleucine	52.0	mg
L-Leucine	52.0	mg
L-Lysine hydrochloride	74.0	mg
L-Phenylalanine	33.0	mg
L-Threonine	48.0	mg
L-Tryptophan	8.0	mg
L-Tyrosine	36.0	mg
L-Valine	47.0	mg
L-Methionine	15.0	mg
L-Glutamine	0.292	g
i-Inositol	3.60	mg
Choline chloride	2.0	mg
Folic acid	2.0	mg
Nicotinamide	2.0	mg

Calcium pantothenate	2.0	mg
Pyridoxine hydrochloride	2.0	mg
Thiamine hydrochloride	2.0	mg
Riboflavin	0.2	mg
Phenol red	15.0	mg
Sodium hydrogencarbonate	2.75	g
Water to	1000	ml
The medium is supplemented with:		
Fetal calf serum (heated at 56° for 30 minutes)	10	per cent
Tryptose phosphate broth	10	per cent
Benzylpenicillin sodium	60	mg/l
Streptomycin	0.1	g/l

Usual dose Rabies Antiserum should be given as soon as possible after exposure, preferably within 24 hours. The dose of 40 IU of rabies antiserum per kg of body weight should be infiltrated around the wound(s) as much as possible, the remainder being injected intramuscularly. Active immunization should be started immediately after rabies antiserum has been given.

COBRA ANTIVENIN

Naja Antivenin

Thai name เซรุ่มแก้พิษงูเห่า (SERUM KAE PHIT NGU HAO)

Category Passive immunizing agent.

Cobra Antivenin is a sterile preparation containing the specific antitoxic globulins from immunized animals. It has the power of neutralizing the cobra *(Naja kaouthia)* venom.

The antivenin, reconstituted if necessary as stated on the label, complies with the requirements stated under Antisera, with the following modifications.

Description Colourless or slightly yellowish brown transparent liquid. Freeze-dried antivenin consists of solid exhibiting the characteristic structure of a freeze-dried solid, light cream in colour.

Expiration date When stored under the prescribed conditions, the expiration date is not later than 3 years for liquid antivenin.

For freeze-dried antivenin, the expiration date have been allowed of up to 5 years.

Warning Reactions to Cobra Antivenin are unfortunately common and often severe. In sensitized persons the administration of a heterologous serum may trigger an anaphylactic reaction. Therefore, it is recommended to take a precise anamnesis and to perform an intradermal test in every case (eventually to begin with a high serum dilution). Before the application of a heterologous serum all the preparations for the treatment of cardiovascular and respiratory complications which may accompany such anaphylactic reactions have to be made. A few days after the injection of a heterologous serum, serum sickness may also occur.

Labelling Complies with the "General Information for Antisera", p. 225. In addition the label on the container states (1) the number in mg of reference cobra venom neutralized by a specified quantity of the antivenin; (2) the recommended human dose; (3) the nature of the preparation, e.g. natural serum or purified immunoglobulin or treated immunoglobulin and, if purified or treated, the nature of the process.

Identification Specifically neutralizes the cobra venom, rendering it harmless to susceptible animals. The assay may serve as an identification test.

Assay Each ml contains sufficient antitoxic globulins to neutralize the amount of the cobra venom stated on the label when determined by the "Biological Assay of Cobra Antivenin" (Appendix 15.2.7).

Usual dose Initial dose of 100 ml of liquid or freeze-dried Cobra Antivenin (after reconstitution by accompanying diluent) should be given by slow intravenous infusion (approximately 2 ml per minute). During the observation period, subsequent doses can be given every 12 hours according to the clinical symptoms.

KING COBRA ANTIVENIN

Thai name เซรุ่มแก้พิษงูจงอาง (SERUM KAE PHIT NGU CHONG ANG)

Category Passive immunizing agent.

King Cobra Antivenin is a sterile preparation containing the specific antitoxic globulins from immunized animals. It has the power of neutralizing the king cobra (*Ophiophagus hannah*) venom.

The antivenin, reconstituted if necessary as stated on the label, complies with the requirements stated under Antisera, with the following modifications.

Description Colourless or slightly yellowish brown transparent liquid. Freeze-dried antivenin consists of solid exhibiting the characteristic structure of a freeze-dried solid, light cream in colour.

Expiration date; Warning See under *Cobra Antivenin*, p. 232.

Labelling Complies with the "General Information for Antisera", p. 225. In addition the label on the container

states (1) the number in mg of reference king cobra venom neutralized by a specified quantity of the antivenin; (2) the recommended human dose; (3) the nature of the preparation, e.g. natural serum or purified immunoglobulin or treated immunoglobulin and, if purified or treated, the nature of the process.

Identification Specifically neutralizes the king cobra venom, rendering it harmless to susceptible animals. The assay may serve as an identification test.

Assay Each ml contains sufficient antitoxic globulins to neutralize the amount of the king cobra venom stated on the label when determined by the "Biological Assay of Cobra Antivenin" (Appendix 15.2.7); use the king cobra test venom for determining its capacity to neutralize the lethal effect.

Usual dose Initial dose 50 ml of liquid or freeze-dried King Cobra Antivenin (after reconstitution by accompanying diluent) should be given by slow intravenous infusion (approximately 2 ml per minute). During the observation period, subsequent doses can be given every 12 hours according to the clinical symptoms.

BANDED KRAIT ANTIVENIN

Thai name เซรุ่มแก้พิษงูสามเหลี่ยม (SERUM KAE PHIT NGU SAMLIAM)

Category Passive immunizing agent.

Banded Krait Antivenin is a sterile preparation containing the specific antitoxic globulins from immunized animals. It has the power of neutralizing the banded krait (*Bungarus fasciatus*) venom.

The antivenin, reconstituted if necessary as stated on the label, complies with the requirements stated under Antisera, with the following modifications.

Description Colourless or slightly yellowish brown transparent liquid. Freeze-dried antivenin consists of solid exhibiting the characteristic structure of a freeze-dried solid, light cream in colour.

Expiration date; Warning See under *Cobra Antivenin*, p. 232.

Labelling Complies with the "General Information for Antisera", p. 225. In addition the label on the container states (1) the number in mg of reference banded krait venom neutralized by a specified quantity of the antivenin; (2) the recommended human dose; (3) the nature of the preparation, e.g. natural serum or purified immunoglobulin or treated immunoglobulin and, if purified or treated, the nature of the process.

Identification Specifically neutralizes the banded krait venom, rendering it harmless to susceptible animals. The assay may serve as an identification test.

Assay Each ml contains sufficient antitoxic globulins to neutralize the amount of the banded krait venom stated on the label when determined by the "Biological Assay of Cobra Antivenin" (Appendix 15.2.7); use the banded krait test venom for determining its capacity to neutralize the lethal effect.

Usual dose Initial dose 50 ml of liquid or freeze-dried Banded Krait Antivenin (after reconstitution by accompanying diluent) should be given by slow intravenous infusion (approximately 2 ml per minute). During the observation period, subsequent doses can be given every 12 hours according to the clinical symptoms.

MALAYAN KRAIT ANTIVENIN

Thai name เซรุ่มแก้พิษงูทับสมิงคลา (SERUM KAE PHIT NGU TAPSAMINGKHLA)

Category Passive immunizing agent.

Malayan Krait Antivenin is a sterile preparation containing the specific antitoxic globulins from immunized animals. It has the power of neutralizing the Malayan krait (*Bungarus candidus*) venom.

The antivenin, reconstituted if necessary as stated on the label, complies with the requirements stated under Antisera, with the following modifications.

Description Colourless or slightly yellowish brown transparent liquid. Freeze-dried antivenin consists of solid exhibiting the characteristic structure of a freeze-dried solid, light cream in colour.

Expiration date; Warning See under *Cobra Antivenin*, p. 232.

Labelling Complies with the "General Information for Antisera", p. 225. In addition the label on the container states (1) the number in mg of reference Malayan krait venom neutralized by a specified quantity of the antivenin; (2) the recommended human dose; (3) the nature of the preparation, e.g. natural serum or purified immunoglobulin or treated immunoglobulin and, if purified or treated, the nature of the process.

Identification Specifically neutralizes the Malayan krait venom, rendering it harmless to susceptible animals. The assay may serve as an identification test.

Assay Each ml contains sufficient antitoxic globulins to neutralize the amount of the Malayan krait venom stated on the label when determined by the "Biological

Assay of Cobra Antivenin" (Appendix 15.2.7); use the Malayan krait test venom for determining its capacity to neutralize the lethal effect.

Usual dose Initial dose 50 ml of liquid or freeze-dried Malayan Krait Antivenin (after reconstitution by accompanying diluent) should be given by slow intravenous infusion (approximately 2 ml per minute). During the observation period, subsequent doses can be given every 12 hours according to the clinical symptoms.

GREEN PIT VIPER ANTIVENIN

Thai name เซรุ่มแก้พิษงูเขียวหางไหม้ (SERUM KAE PHIT NGU KHIAW HANG MAI)

Category Passive immunizing agent.

Green Pit Viper Antivenin is a sterile preparation containing the specific antitoxic globulins from immunized animals. It has the power of neutralizing the green pit viper (*Trimeresurus albolabris*) venom.

The antivenin, reconstituted if necessary as stated on the label, complies with the requirements stated under Antisera, with the following modifications.

Description Colourless or slightly yellowish brown transparent liquid. Freeze-dried antivenin consists of solid exhibiting the characteristic structure of a freeze-dried solid, light cream in colour.

Expiration date; Warning See under *Cobra Antivenin*, p. 232.

Labelling Complies with the "General Information for Antisera", p. 225. In addition the label on the container states (1) the number in mg of reference green pit viper venom neutralized by a specified quantity of the antivenin; (2) the recommended human dose; (3) the nature of the preparation, e.g. natural serum or purified immunoglobulin or treated immunoglobulin and, if purified or treated, the nature of the process.

Identification Specifically neutralizes the green pit viper venom, rendering it harmless to susceptible animals. The assay may serve as an identification test.

Assay Each ml contains sufficient antitoxic globulins to neutralize the amount of the green pit viper venom stated on the label when determined by the "Biological Assay of Cobra Antivenin" (Appendix 15.2.7); use the green pit viper test venom for determining its capacity to neutralize the lethal effect.

Usual dose Initial dose 30 ml of liquid or freeze-dried Green Pit Viper Antivenin (after reconstitution by accompanying diluent) should be given by slow intravenous infusion (approximately 2 ml per minute). During the observation period, subsequent doses can be given every 6 hours according to the clinical symptoms.

MALAYAN PIT VIPER ANTIVENIN

Thai name เซรุ่มแก้พิษงูกะปะ (SERUM KAE PHIT NGU KAPA)

Category Passive immunizing agent.

Malayan Pit Viper Antivenin is a sterile preparation containing the specific antitoxic globulins from immunized animals. It has the power of neutralizing the Malayan pit viper (*Calloselasma rhodostoma*) venom.

The antivenin, reconstituted if necessary as stated on the label, complies with the requirements stated under Antisera, with the following modifications.

Description Colourless or slightly yellowish brown transparent liquid. Freeze-dried antivenin consists of solid exhibiting the characteristic structure of a freeze-dried solid, light cream in colour.

Expiration date; Warning See under *Cobra Antivenin*, p. 232.

Labelling Complies with the "General Information for Antisera", p. 225. In addition the label on the container states (1) the number in mg of reference Malayan pit viper venom neutralized by a specified quantity of the antivenin; (2) the recommended human dose; (3) the nature of the preparation, e.g. natural serum or purified immunoglobulin or treated immunoglobulin and, if purified or treated, the nature of the process.

Identification Specifically neutralizes the Malayan pit viper venom, rendering it harmless to susceptible animals. The assay may serve as an identification test.

Assay Each ml contains sufficient antitoxic globulins to neutralize the amount of the Malayan pit viper venom stated on the label when determined by the "Biological Assay of Cobra Antivenin" (Appendix 15.2.7); use the Malayan pit viper test venom for determining its capacity to neutralize the lethal effect.

Usual dose Initial dose 30 ml of liquid or freeze-dried Malayan Pit Viper Antivenin (after reconstitution by accompanying diluent) should be given by slow intravenous infusion (approximately 2 ml per minute). During the observation period, subsequent doses can be given every 6 hours according to the clinical symptoms.

RUSSELL'S VIPER ANTIVENIN

Thai name เซรุ่มแก้พิษงูแมวเซา (SERUM KAE PHIT NGU MAEW SAO)

Category Passive immunizing agent.

Russell's Viper Antivenin is a sterile preparation containing the specific antitoxic globulins from immunized animals. It has the power of neutralizing the Russell's viper (*Daboia russelii siamensis*) venom.

The antivenin, reconstituted if necessary as stated on the label, complies with the requirements stated under Antisera, with the following modifications.

Description Colourless or slightly yellowish brown transparent liquid. Freeze-dried antivenin consists of solid exhibiting the characteristic structure of a freeze-dried solid, light cream in colour.

Expiration date; Warning See under *Cobra Antivenin*, p. 232.

Labelling Complies with the "General Information for Antisera", p. 225. In addition the label on the container states (1) the number in mg of reference Russell's viper venom neutralized by a specified quantity of the antivenin; (2) the recommended human dose; (3) the nature of the preparation, e.g. natural serum or purified immunoglobulin or treated immunoglobulin and, if purified or treated, the nature of the process.

Identification Specifically neutralizes the Russell's viper venom, rendering it harmless to susceptible animals. The assay may serve as an identification test.

Assay Each ml contains sufficient antitoxic globulins to neutralize the amount of the Russell's viper venom stated on the label when determined by the "Biological Assay of Cobra Antivenin" (Appendix 15.2.7); use the Russell's viper test venom for determining its capacity to neutralize the lethal effect.

Usual dose Initial dose 30 ml of liquid or freeze-dried Russell's Viper Antivenin (after reconstitution by accompanying diluent) should be given by slow intravenous infusion (approximately 2 ml per minute). During the observation period, subsequent doses can be given every 6 hours according to the clinical symptoms.

HEMATOTOXIC POLYVALENT ANTIVENIN (GREEN PIT VIPER, MALAYAN PIT VIPER, AND RUSSELL'S VIPER ANTIVENIN)

Thai name เซรุ่มแก้พิษงูระบบโลหิตชนิดรวม (SERUM KAE PHIT NGU RABOP LOHIT CHANID RUAM)

Category Passive immunizing agent.

Hematotoxic Polyvalent Antivenin is a sterile preparation containing the specific antitoxic globulins from immunized animals. It has the power of neutralizing the green pit viper (*Trimeresurus albolabris*) venom, the Malayan pit viper (*Calloselasma rhodostoma*) venom, and the Russell's viper (*Daboia russelii siamensis*) venom.

The antivenin, reconstituted if necessary as stated on the label, complies with the requirements stated under Antisera, with the following modifications.

Description Colourless or slightly yellowish brown transparent liquid. Freeze-dried antivenin consists of solid exhibiting the characteristic structure of a freeze-dried solid, light cream in colour.

Expiration date; Warning See under *Cobra Antivenin*, p. 232.

Labelling Complies with the "General Information for Antisera", p. 225. In addition the label on the container states (1) the number in mg of reference green pit viper venom, reference Malayan pit viper venom, and reference Russell's viper venom, neutralized by a specified quantity of the antivenin; (2) the recommended human dose; (3) the nature of the preparation, e.g. natural serum or purified immunoglobulin or treated immunoglobulin and, if purified or treated, the nature of the process.

Identification Specifically neutralizes the green pit viper venom, the Malayan pit viper venom, and the Russell's viper venom, rendering it harmless to susceptible animals. The assay may serve as an identification test.

Assay Each ml contains sufficient antitoxic globulins to neutralize the amount of the green pit viper venom, the Malayan pit viper venom, and the Russell's viper venom stated on the label when determined by the "Biological Assay of Cobra Antivenin" (Appendix 15.2.7); use the green pit viper test venom, the Malayan pit viper test venom, and the Russell's viper test venom, for determining its capacity to neutralize the lethal effect.

Usual dose Initial dose 20 ml of liquid or freeze-dried Hematotoxic Polyvalent Antivenin (after reconstitution by accompanying diluent) should be given by slow intravenous infusion (not more than 1 ml per minute). If symptoms still persist, the second dose should be repeated 2 hours or even earlier after the initial dose. The further dose should be repeated every 6 hours according to the clinical symptoms.

NEUROTOXIC POLYVALENT ANTIVENIN (COBRA, KING COBRA, BANDED KRAIT AND MALAYAN KRAIT ANTIVENIN)

Thai name เซรุ่มแก้พิษงูระบบประสาทชนิดรวม (SERUM KAE PHIT NGU RABOP PRASAT CHANID RUAM)

Category Passive immunizing agent.

Neurotoxic Polyvalent Antivenin is a sterile preparation containing the specific antitoxic globulins from immunized animals. It has the power of neutralizing the cobra (*Naja kaouthia*) venom, the king cobra (*Ophiophagus hannah*) venom, the banded krait (*Bungarus fasciatus*) venom, and the Malayan krait (*Bungarus candidus*) venom.

The antivenin, reconstituted if necessary as stated on the label, complies with the requirements stated under Antisera, with the following modifications.

Description Colourless or slightly yellowish brown transparent liquid. Freeze-dried antivenin consists of solid exhibiting the characteristic structure of a freeze-dried solid, light cream in colour.

Expiration date; Warning See under *Cobra Antivenin*, p. 232.

Labelling Complies with the "General Information for Antisera", p. 225. In addition the label on the container states (1) the number in mg of reference cobra venom, reference banded krait venom, reference king cobra venom, and reference Malayan krait venom, neutralized by a specified quantity of the antivenin; (2) the recommended human dose; (3) the nature of the preparation, e.g. natural serum or purified immunoglobulin or treated immunoglobulin and, if purified or treated, the nature of the process.

Identification Specifically neutralizes the cobra venom, the banded krait venom, the king cobra venom, and the Malayan krait venom, rendering it harmless to susceptible animals. The assay may serve as an identification test.

Assay Each ml contains sufficient antitoxic globulins to neutralize the amount of the cobra venom, the banded krait venom, the king cobra venom, and Malayan krait venom stated on the label when determined by the "Biological Assay of Cobra Antivenin" (Appendix 15.2.7); use the cobra test venom, the banded krait test venom, the king cobra test venom, and the Malayan krait test venom, for determining its capacity to neutralize the lethal effect.

Usual dose Initial dose 20 ml of liquid or freeze-dried Neurotoxic Polyvalent Antivenin (after reconstitution by accompanying diluent) should be given by slow intravenous infusion (not more than 1 ml per minute). If symptoms still persist, the second dose should be repeated 2 hours or even earlier after the initial dose. The further dose should be repeated every 6 hours according to the clinical symptoms.

ลิขสิทธิ์ กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข ห้ามทำซ้ำ ดัดแปลง แก้ไข เผยแพร่ โดยไม่ได้รับอนุญาต

VACCINES

ลิขสิทธิ์ กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข
ห้ามทำซ้ำ ดัดแปลง แก้ไข โดยไม่ได้รับอนุญาต

ลิขสิทธิ์ กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข
ห้ามทำซ้ำ ดัดแปลง แก้ไข โดยไม่ได้รับอนุญาต

VACCINES

Vaccines for human use are preparations containing antigenic substances capable of inducing a specific and active immunity in man against an infecting agent or the toxin or the antigen elaborated by it or an allergen. They shall have been shown to have acceptable immunogenic activity in man with the intended vaccination schedule.

Vaccines for human use may contain: organisms inactivated by chemical or physical means that maintain adequate immunogenic properties; living organisms that are naturally avirulent or that have been treated to attenuate their virulence whilst retaining adequate immunogenic properties; antigens extracted from the organisms or secreted by them or produced by genetic engineering. The antigens may be used in their native state or may be detoxified by chemical or physical means and may be aggregated, polymerized or conjugated to a carrier to increase their immunogenicity.

Allergen vaccines Allergen vaccines are preparations of allergens obtained by extraction of the active constituents from micro-organism, animal or vegetable substances with a suitable menstruum.

Bacterial vaccines Bacterial vaccines are prepared from cultures of suitable strains grown on suitable solid media or in suitable liquid media and contain live or inactivated bacteria or their immunogenic components. They are suspensions of various degrees of opacity in colourless or almost colourless liquids or they may be freeze-dried.

Inactivated bacterial vaccines contain bacteria or their immunogenic components that have been inactivated in such a way that adequate immunogenicity is retained.

Living bacterial vaccines are prepared from strains of attenuated virulence that are capable of stimulating immunity against the pathogenic strains of the same or antigenically related species of bacteria.

The concentration of living or inactivated bacteria of each of the bacterial varieties or species present is measured in terms of International Units of opacity or, where appropriate, by direct cell count or, in the case of living bacteria, by viable count.

Bacterial toxoids Bacterial toxoids are prepared from toxins by diminishing their toxicity to a non-detectable level or by completely eliminating it, without destroying their immunogenicity, by methods that avoid the reversion of toxoid to toxin. The toxins are obtained from selected strains of specific micro-organisms grown in media free, as far as possible, from ingredients known to cause toxic, allergic or other undesirable reactions in man.

Toxoids may be liquid or freeze-dried. They may be purified and adsorbed. Adsorbed toxoids are suspensions of white or grey particles dispersed in colourless or pale yellow liquids and may form a sediment at the bottom of the container.

Viral vaccines Viral vaccines are prepared from viruses grown in animals, in embryonated eggs, in suitable cell cultures or in suitable tissues or by culture of genetically engineered cells. They are liquids that vary in opacity according to the type of preparation or may be freeze-dried. Liquid preparations and freeze-dried preparations after reconstitution may be coloured if a pH indicator such as phenol red has been used in the culture medium.

Living viral vaccines are usually prepared from strains of the specific virus that are of attenuated virulence.

Combined vaccines Combined vaccines are mixtures of two or more vaccines.

Vaccines, reconstituted where necessary, comply with the following requirements unless otherwise stated in the individual monographs.

Packaging and storage Unless otherwise stated in the monograph, vaccines shall be stored at a temperature between 2° and 8°, protected from light; avoid freezing.

Aluminium For adsorbed vaccines containing aluminium, not more than 1.25 mg of aluminium per single human dose, unless otherwise stated in the monograph when determined by the method described under the "Determination of Aluminium", p. 178.

Calcium For adsorbed vaccines containing calcium, not more than 1.3 mg of calcium per single human dose, unless otherwise stated in the monograph, when determined by the method described under the "Determination of Calcium", p. 178.

Phenol For vaccines containing phenol as a preservative, not more than 0.25 per cent w/v, unless otherwise stated in the monograph, when determined by the method described under the "Determination of Phenol", p. 179.

Thiomersal[1] For vaccines containing thiomersal as a preservative, not less than 0.005 per cent w/v and not more than 0.02 per cent w/v, when determined by the method described under the "Determination of Thiomersal", p. 179.

[1]Vaccines available in some countries may contain thiomersal. Risk-benefit should be considered when medical problem exists.

Formaldehyde For vaccines containing formaldehyde, not more than 0.02 per cent w/v of free formaldehyde, when determined by the method described under the "Determination of Formaldehyde", p. 178.

Sterility Unless otherwise stated, all vaccines comply with the "Sterility Test" (Appendix 10.1), except that for living bacterial vaccines, growth of the organism from which the vaccine was prepared is permitted.

Abnormal toxicity Complies with the "Abnormal Toxicity Test" (Appendix 8.1).

Water For freeze-dried vaccines, not more than 3.0 per cent w/w (Karl Fischer Method, Appendix 4.12).

The provisions of this monograph apply to the following vaccines:

Allergen Vaccines
- House Dust Mite Allergen Vaccine

Bacterial Vaccines
- Bacillus Calmette-Guérin Vaccine, Freeze-Dried
- Percutaneous Bacillus Calmette-Guérin Vaccine
- Cholera Vaccine
- Diphtheria Vaccine, Adsorbed
- Haemophilus Type b Conjugate Vaccine
- Meningococcal Polysaccharide Vaccine
- Pertussis Vaccine, Adsorbed
- Pertussis Vaccine, Adsorbed (Acellular Component)
- Pneumococcal Polysaccharide Vaccine
- Pneumococcal Polysaccharide Conjugate Vaccine, Adsorbed
- Tetanus Vaccine, Adsorbed
- Typhoid Polysaccharide Vaccine
- Typhoid Vaccine, Oral

Viral Vaccines
- Hepatitis A Vaccine, Inactivated
- Hepatitis B Vaccine, Recombinant
- Influenza Vaccine, Inactivated (Whole Virion)
- Influenza Vaccine, Inactivated (Split Virion)
- Influenza Vaccine, Inactivated (Surface Antigen)
- Influenza Vaccine, Inactivated (Surface Antigen, Virosome)
- Japanese Encephalitis Vaccine, Inactivated
- Measles Vaccine, Live
- Mumps Vaccine, Live
- Poliomyelitis Vaccine, Inactivated
- Poliomyelitis Vaccine, Oral
- Rabies Vaccine, Inactivated
- Rubella Vaccine, Live
- Varicella Vaccine, Live
- Yellow Fever Vaccine

Combined Vaccines
- Diphtheria and Tetanus Vaccine, Adsorbed
- Diphtheria, Tetanus and Pertussis Vaccine, Adsorbed
- Diphtheria, Tetanus and Pertussis (Acellular Component) Vaccine, Adsorbed
- Measles, Mumps and Rubella Vaccine, Live
- Tetanus and Diphtheria Vaccine for Adult Use, Adsorbed

BACILLUS CALMETTE-GUÉRIN VACCINE, FREEZE-DRIED

BCG Vaccine

Category Active immunizing agent.

Freeze-dried BCG Vaccine is a preparation of live bacteria derived from a culture of the bacillus Calmette and Guérin Strain of *Mycobacterium tuberculosis* var *bovis* whose capacity to protect against tuberculosis has been established.

The vaccine, reconstituted as stated on the label, complies with the requirements stated under Vaccines, with the following modifications.

Description Freeze-dried BCG Vaccine is a white to off-white dried mass having the characteristic texture of material dried in the frozen state. When reconstituted, it becomes a whitish or pale yellowish turbid liquid.

Strengths available 2×10^6 to 26×10^6 and 1×10^6 to 33×10^6 CFU of Bacillus Calmette-Guérin per ml.

Dose *Intracutaneous*, 0.1 or 0.05 ml as state on the label.

Contra-indication

1. It is contra-indicated in individuals with immunosuppression, including those with hypogammaglobulinemia, congenital immunodeficiency, sarcoidosis, leukemia, lymphoma, and generalized malignancy.
2. It is contra-indicated in HIV infected, severe immune deficiency syndrome or immunocompromised infants, children or adults.
3. It is contra-indicated in individuals in whom immunologic responses have been suppressed because of prolonged treatment with immunosuppressive therapy (e.g., corticosteroids, certain antineoplastic agents, alkylating agents, antimetabolites, radiation therapy).
4. It is contra-indicated in individuals with a positive tuberculin skin test, individuals with recent smallpox immunizations, and burn patients.
5. It is contra-indicated in individuals with hypersensitivity to the product.

Warning

1. It is not for intravenous, subcutaneous, or intramuscular administration.

2. BCG vaccine is strictly given intracutaneously. A local reaction at the vaccination site is normal after a BCG vaccination. It may take the form of a nodule, which in many cases will break down and suppurate. The reaction developing at the vaccination site usually subsides within 2 to 5 months and in practically all children leaves a superficial scar of 2 to 10 mm in diameter. The nodule may persist and ulcerate. Swelling of regional lymph nodes may also be seen, and this may be regarded as a normal reaction. Keloid and lupoid reactions may occur at the site of the vaccination. Children with such reactions should not be revaccinated. Inadvertent subcutaneous injections produce abscess formations and may lead to ugly retracted scars.

Among the major complications, suppurative lymphadenitis has been observed.

3. Risk-benefit should be considered if it is to be used in pregnant women.

Additional information

1. It has been administered concurrently with yellow fever vaccine without interference with the immune response to either vaccine.

2. It may not be effective if administered during therapy with some antituberculosis agents.

3. If acute overdosage of BCG vaccine occurs and there is reason to suspect that a generalized BCG infection may develop, isoniazid or other appropriated antituberculosis agent therapy should be initiated.

4. Healthy neonates and infants younger than 2 months of age who have not been exposed to tuberculosis, may receive freeze-dried BCG vaccine without tuberculin skin testing.

Expiration date The expiration date is not later than 3 years from the date of the last satisfactory test for the number of culturable particles, if stored at a temperature between 2° and 8° or as stated on the label.

Packaging and storage Freeze-Dried BCG Vaccine shall be kept in tightly closed containers, preferably of Type I glass, protected from light, and stored at a temperature between 2° and 8°; avoid freezing.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition, the label on the container states (1) the description "intracutaneous use"; (2) the number of culturable particles in each single human dose; (3) the volume and nature of the reconstituting fluid; (4) that it shall not be exposed to strong daylight either before or after reconstitution; (5) that after reconstitution it should be used immediately after preparation and any portion not used within 2 hours should be discarded.

Identification

1. When examined microscopically in stained-smears, the bacilli exhibit the characteristics of an authentic strain of the bacillus of Calmette and Guérin.

2. Colonies grown on a suitable solid culture medium have a characteristic appearance.

Virulent mycobacteria Inject subcutaneously or intramuscularly into each of six guinea-pigs, each weighing 250 to 400 g and having received no treatment likely to interfere with the test, a quantity of the vaccine equivalent to at least 50 human doses. Observe the animals for at least 6 weeks. At the end of this period, kill the guinea-pigs and examine by autopsy for signs of infection with tuberculosis, ignoring any minor reactions at the site of injection. Animals that die during the observation period are also examined for signs of tuberculosis. The vaccine complies with the test if none of the guinea-pigs shows signs of tuberculosis and if not more than one animal dies during the observation period. If two animals die during this period and autopsy does not reveal signs of tuberculosis, repeat the test on six other guinea-pigs. The vaccine complies with the test if not more than one animal dies during the 6 weeks following the injection and autopsy does not reveal any sign of tuberculosis.

Bacterial and fungal contamination The reconstituted vaccine complies with the "Sterility Test" (Appendix 10.1) except for the presence of mycobacteria.

Test of skin reaction (Excessive dermal reactivity) Use six healthy white or palecoloured guinea-pigs, each weighing not less than 250 g and having received no treatment likely to interfere with the test. Inject intracutaneously into each guinea-pig, according to a randomized plan, 0.1 ml of the reconstituted vaccine and of 2 tenfold serial dilutions of the vaccine and identical doses of the comparison vaccine. Observe the lesions formed at the site of the injection for 4 weeks. The vaccine complies with the test if the reaction it produces is not markedly different from that produced by the National or International Reference Preparation of Freeze-Dried BCG Vaccine.

Temperature stability Maintain samples of the freeze-dried vaccine at 37° for 4 weeks. Determine the number of viable units in the heated vaccine and in unheated vaccine as described below. The number of viable units in the heated vaccine is not less than 20 per cent that in unheated vaccine.

Number of culturable particles Determine the number of viable units in the reconstituted vaccine by using the method described below.

Reconstitute the vaccine being examined and further dilute in sterile water to a concentration of 0.5×10^{-4} mg of moist bacteria per ml. Inoculate each dilution to a suitable validated medium at a dose of 0.1 ml; incubate the cultures at 37° to 38° for 4 weeks, and count the number of appearing colonies per culture tube. Count the National or International Reference Preparation of Freeze-Dried BCG Vaccine in parallel for comparison.

Freeze-Dried BCG Vaccine contains not less than and not more than the number of viable units stated on the label.

PERCUTANEOUS BACILLUS CALMETTE-GUÉRIN VACCINE

Percut. BCG Vaccine

Category Active immunizing agent.

Percutaneous Bacillus Calmette-Guérin Vaccine is a suspension of living cells of an authentic strain of the bacillus of Calmette and Guérin with a higher viable bacterial count than Bacillus Calmette-Guérin Vaccine. It is prepared immediately before use by reconstitution from the dried vaccine with an appropriate volume of a suitable sterile liquid.

The vaccine, reconstituted as stated on the label, complies with the requirements stated under Vaccines, with the following modifications.

Strengths available 1×10^8 to 8×10^8 CFU of Bacillus Calmette-Guérin per ml.

Dose *Percutaneous*, 0.2 to 0.3 ml, using the multiple puncture device.

Neonates: the dosage should be decreased by 50 per cent by reconstituting the vaccine with 2 ml instead of 1 ml of sterile water for injection without preservatives.

Contra-indication; Warning See under *Bacillus Calmette-Guérin Vaccine, Freeze-Dried*, p. 240.

Additional information

1. It has been administered concurrently with yellow fever vaccine without interference with the immune response to either vaccine.

2. It may not be effective if administered during therapy with some antituberculosis agents.

3. If acute overdosage of BCG vaccine occurs and there is reason to suspect that a generalized BCG infection may develop, isoniazid or other appropriated antituberculosis agent therapy should be initiated.

4. Healthy neonates and infants younger than 2 months of age who have not been exposed to tuberculosis may receive BCG vaccine without prior tuberculin skin testing.

5. Individuals with chronic diseases of the skin (e.g., eczema) should receive immunization with BCG vaccine in a healthy area of skin.

Expiration date The expiration date is not later than 6 months from the date of the last satisfactory test for potency, or not later than 1 year after the date of the last satisfactory test for potency, if stored at a temperature less than 5°.

Packaging and storage Percutaneous Bacillus Calmette-Guérin Vaccine shall be kept in tightly closed containers, preferably of Type I glass, protected from light, and stored at a temperature between 2° and 8°; avoid freezing.

Labelling Complies with the "General Information for Biological Products", p 177. In addition, the label on the container stated (1) that the vaccine is a living culture of the bacillus of Calmette and Guérin; (2) that any portion of the reconstituted vaccine not used at once should be discarded; (3) that the vaccine is for percutaneous administration and must not be given by the intracutaneous (intradermal) route.

Identification

1. When examined microscopically in stained smears, the bacilli exhibit the characteristics of an authentic strain of the bacillus of Calmette and Guérin.

2. Colonies grown on a suitable solid culture medium have a characteristic appearance.

Virulent mycobacteria Prepare a fivefold dilution of the vaccine using an appropriate sterile liquid. Inject 1 ml intramuscularly into each of six guinea-pigs weighing 250 to 400 g. None of the animals dies within 6 weeks or if one dies, a post-mortem examination establishes that it is free from tuberculosis. If two of the animals die within this period and a post-mortem examination establishes that both are free from tuberculosis, repeat the test on six further guinea-pigs. None of the second group of animals dies within 6 weeks or, if one dies, a post mortem examination establishes that it is free from tuberculosis.

Test of skin reaction (Excessive dermal reactivity) Inject intracutaneously into each of two guinea-pigs a volume of 0.1 ml of 1, 0.1, 0.01 and 0.001 doses of the vaccine being examined and of the comparison vaccine. Use an appropriate sterile liquid as diluent. The vaccine

passes the test if the skin reactions produced within 3 weeks do not differ markedly from those produced by the comparison vaccine.

Skin-sensitizing potency Prepare a 25-fold dilution of the vaccine using an appropriate sterile liquid. Inject 0.5 ml subcutaneously or intramuscularly into each of two guinea-pigs. Within 4 weeks of injection, inject intracutaneously into each guinea-pig 10 IU of Old Tuberculin, or of Tuberculin Purified Protein Derivative, in a volume of 0.1 ml. An inflammatory area of induration and edema not less than 5 mm in diameter, irrespective of the area of erythema, is induced within 24 hours.

CHOLERA VACCINE

Category Active immunizing agent.

Cholera Vaccine is a sterile suspension, in isotonic sodium chloride solution or other suitable diluent, of the smooth strains of the two main serological types, *Inaba* and *Ogawa* of killed *Vibrio cholerae* classical biotype. Each 1.0 ml contains not less than 4000 million organisms of each serotype. Either a single strain or several strains of each type may be included. All strains must contain, in addition to their type *O* antigens, the heat-stable *O* antigen common to *Inaba* and *Ogawa*.

The vaccine complies with the requirements stated under Vaccines, with the following modifications.

Description Slightly turbid to milky suspension; odour, nearly odourless or of the antimicrobial agent.

Strength available 8000 million killed *V. cholerae* per ml.

Dose Adults and children 10 years of age and over: *Subcutaneous* or *intramuscular*, 0.5 ml, repeat at 1- to 4-week intervals, 0.5 ml.

Intradermal, 0.2 ml, repeat at 1- to 4-week intervals, 0.2 ml.

Children 5 to 10 years of age: *Subcutaneous* or *intramuscular*, 0.3 ml, repeat at 1- to 4-week intervals, 0.3 ml.

Intradermal, 0.2 ml, repeat at 1- to 4-week intervals, 0.2 ml.

Children under 5 years of age: *Subcutaneous* or *intramuscular*, 0.2 ml, repeat at 1- to 4-week intervals, 0.2 ml.

Infant 6 months to 1 year of age: *Subcutaneous* or *intramuscular*, 0.2 ml, repeat at 1- to 4-week intervals, 0.2 ml.

Booster doses are required every 6 months while an individual is at continual risk.

Contra-indication

1. It is contra-indicated in individuals with acute infections.
2. It is contra-indicated in individuals with immune deficiency conditions who are taking corticosteroids or other immunosuppressive agents.
3. It is contra-indicated in individuals with a history of severe systemic or allergic reactions to cholera vaccine.

Warning

1. It is not for intravenous administration.
2. Do not administer intramuscularly to persons with thrombocytopenia or any coagulation disorders.
3. Fever; headache; malaise; pain, redness or swelling at the injection site may occur.

Additional information

1. Immunization against cholera is not recommended in infants under 6 months of age.
2. Appropriate precautions should be taken prior to vaccine injection to prevent allergic or any other unwanted reactions. This should include review of the patient's history regarding possible sensitivity and the ready availability of epinephrine 1:1000 and other appropriate agents used for control of immediate allergic reactions.
3. Even after immunization with cholera vaccine, not all recipients of the vaccine will be fully protected against cholera. Travellers should take all necessary precautions to avoid contact with, or ingestion of, potentially contaminated food or water.
4. Cholera vaccine does not prevent *V. cholerae* excretion. Therefore, it should not be used to manage contacts of imported cholera cases or to control the spread of infection, even during epidemics.

Expiration date When stored under the prescribed conditions, the expiration date is not later than 18 months from the date of the last satisfactory potency test.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition the label on the container states (1) the method used to inactivate the bacteria; (2) the number of bacteria in each human dose; (3) that it is to be well shaken before use.

Identification Identify the organisms by specific agglutination.

pH 6.8 to 7.4 (Appendix 4.11).

Phenol If phenol has been used in the preparation, the concentration is not more than 0.50 per cent w/v. Carry out the test as described under the "Determination of Phenol", p. 179.

Assay The potency of a Cholera Vaccine is determined by comparing the dose necessary to protect mice against a lethal intraperitoneal dose of *Vibrio cholerae* with the dose of the National of International Reference Preparation of Cholera Vaccine necessary to give the same protection.

SUGGESTED METHOD

Selection of challenge strain Select a suitable strain of each serotype, Inaba and Ogawa of *V. cholerae* classical biotype. The strains, suspended in gastric mucin or other resistance-lowering substance, should have adequate virulence which is evaluated by determining the LD_{50} in mice, after 72-hour intraperitoneal injection. The LD_{50} should be 10,000 or fewer colony-forming units.

LD_{50} The number of *V. cholerae* organisms that kills 50 per cent of a group of mice within 72 hours when injected by the intraperitoneal route.

Preparation of challenge suspension Make one subculture from the strain and suspend the harvested *V. cholerae* in *saline TS*. Determine the opacity of the suspension. Prepare in gastric mucin or other resistance-lowering substance a series of dilutions of the suspension, and allocate each dilution to a group of 10 mice. Inject intraperitoneally into each mouse a dose (0.5 ml) of the dilution allocated to its group and count the number of mice surviving in each group after 72 hours. Calculate the titre of the challenge suspension in LD_{50} per challenge dose by the standard statistical methods. From the result calculate the opacity of a suspension containing not less than 100 and not more than 10,000 LD_{50} per challenge dose.

For the determination of the potency of the vaccine being examined make a fresh subculture from the same strain of *V. cholerae* and from the harvested organisms prepare a suspension of an opacity corresponding to about 1000 LD_{50} in each challenge dose. Prepare three dilutions of the challenge suspension.

Selection and distribution of test animals Use 3 to 4 weeks old mice of a suitable strain, drawn from a uniform stock, keeping the range of weight not more than 5 g. For the test of each serotype, distribute the mice into six groups of not less than 15 and four groups of 10; the mice should be of the same sex or the sexes should be equally distributed among the groups. Three of the groups of 15 receive the Reference Preparation and the other three the vaccine being tested; the four groups of 10 are used for the titration of the LD_{50} of the challenge suspension.

Determination of the potency of the vaccine For the test of each serotype, use three doses of the Reference Preparation and three of the vaccine being tested, diluted with *saline TS*. In each case, the three doses should be so arranged that the dose protecting 50 per cent of the mice is as near as possible to the middle of the dose range. Give each mouse one dose (0.5 ml) by intraperitoneal injection.

Twelve to fourteen days later, challenge the mice reserved for Inaba serotype test intraperitoneally with *V. cholerae* Inaba, and the mice reserved for Ogawa serotype test with *V. cholerae* Ogawa. The challenge dose should be chosen to contain approximately 1000 LD_{50}. This is confirmed by titrating at the same time the challenge suspension in the four groups of 10 nonimmunized control mice. Inject one group of control mice with the challenge dose and the other three, with graded tenfold dilutions of the challenge dose. Simultaneously, inoculate a suitable solid medium with measured amounts of appropriate dilutions of the challenge culture to determine the number of colony-forming units in 1 ml. Observe the mice for 72 hours and record the number of deaths and survivals. Calculate the results of the assay by standard statistical methods from the number of mice surviving. The vaccine passes the test if the potency of the vaccine under test is not less than that of the Reference Preparation of both serotypes.

The test is not valid unless, for both the test vaccine being examined and the Reference Preparation, the 50 per cent protective dose lies between the largest and smallest doses given to the mice, the slopes of the dose response curves are significant with no significant deviation from linearity or parallelism and the challenge dose is approximately 1000 LD_{50}.

The estimated potency of the test vaccine shall be not less than that of the National or International Reference Preparation of Cholera Vaccine upon statistical comparison of the results.

DIPHTHERIA VACCINE, ADSORBED

Adsorbed Diphtheria Toxoid; Adsorbed Diphtheria Prophylactic

Category Active immunizing agent.

Adsorbed Diphtheria Vaccine is a sterile suspension of suitable adjuvant(s), such as aluminium hydroxide, aluminium phosphate, onto which purified diphtheria toxoid is adsorbed. The diphtheria toxoid is prepared from diphtheria toxin produced by the growth in suitable media of *Corynebacterium diphtheriae* by treatment with formaldehyde. Each 0.5 ml contains not less than 30 IU of adsorbed diphtheria vaccine.

The vaccine complies with the requirements stated under Vaccines, with the following modifications.

Strength available 30 IU of adsorbed diphtheria toxoid per 0.5 ml.

Description Light colour suspension, free from evident clumps after shaking.

Dose Children under 6 years of age: *Intramuscular* or *deep subcutaneous*, 0.5 ml.

Contra-indication It is contra-indicated in individuals with serious diseases, fever, or with a history of severe systemic or allergic reactions to Adsorbed Diphtheria Vaccine.

Warning Low fever; fatigue; headache; erythema and swelling at the injection site may occur.

Additional information Adsorbed Diphtheria Vaccine should not be used in persons over 6 years of age.

Expiration date When stored under the prescribed conditions, the expiration date is not later than 2 years from the date of the last satisfactory test for potency.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition the label on the container states (1) the number of IU in each single human dose; (2) the name and the amount of adjuvant(s); (3) that it is not to be frozen.

Identification Dissolve in the vaccine to be examined sufficient *sodium citrate dihydrate* to give a 10 per cent w/v solution. Maintain at 37° for about 16 hours and centrifuge until a clear supernatant liquid is obtained. The clear supernatant liquid reacts with a suitable diphtheria antitoxin, producing a precipitate.

pH 6.0 to 7.0 (Appendix 4.11).

Phenol Phenol should not be added to Adsorbed Diphtheria Vaccine, since it has been shown to have deleterious effects on antigenic properties of the vaccine.

Specific toxicity Inject subcutaneously five times the single human dose stated on the label into each of five healthy guinea-pigs, each weighing 250 to 350 g, that have not previously been treated with any material that will interfere with the test. The vaccine complies with the test if within 6 weeks of the injection, none of the animals shows signs or dies from diphtheria toxaemia. If more than one animal dies from non-specific causes, repeat the test once. The vaccine complies with the test if not more than one animal dies in the second test.

Assay Carry out the "Biological Assay of Adsorbed Diphtheria Vaccine" (Appendix 15.3.1). The lower confidence limit ($P = 0.95$) of the estimated potency is not less than 30 IU per single human dose (0.5 ml).

HAEMOPHILUS TYPE B CONJUGATE VACCINE

Category Active immunizing agent.

Haemophilus Type b Conjugate Vaccine is a sterile liquid or freeze-dried preparation of a polysaccharide, derived from a suitable strain of *Haemophilus influenzae* type b (Hib), covalently bound to a carrier protein. The polysaccharide, polyribosylribitol phosphate (PRP), is a linear copolymer composed of repeated units of 3-β-D-ribofuranosyl-(1→1)-ribitol-5-phosphate [$(C_{10}H_{19}O_{12}P)_n$], with a defined molecular size. The carrier protein, when conjugated to PRP, is capable of inducing a T-cell-dependent B-cell immune response to the polysaccharide.

The vaccine reconstituted, if necessary as stated on the label, complies with the requirements stated under Vaccines, with the following modifications.

Description Liquid vaccine is clear and colourless.

Freeze-dried vaccine, when reconstituted, becomes a clear and colourless liquid.

Strengths available 7.5 to 10 μg of haemophilus b capsular polysaccharide bound to 125 μg of an outer membrane protein (OMP) complex of the *Neisseria meningitidis*, or bound to approximately 20 to 30 μg of tetanus toxoid, or bound to approximately 25 μg of diptheria CRM 197 protein, per 0.5 ml.

Dose Children under 6 years of age: *Intramuscular*, 0.5 ml, into the deltoid region; for small children and

infants, injection at anterolateral thigh is more preferable.

Contra-indication

1. It is not for intravenous administration.
2. It should not be administered to anyone who developed a reaction to a previous dose.

Warning

1. It may cause soreness, pain or tenderness, erythema, swelling and induration at injection site.
2. Fever, irritability, drowsiness, and anorexia may occur.
3. Risk-benefit should be considered if it is to be used in patients with a prior history of Guillain-Barré syndrome.

Additional information

1. It is not recommended for use in adults and adolescences except for patients with certain chronic conditions associated with an increased risk of Hib disease.
2. Safety and efficacy are not established for use in infants under 6 weeks of age.

Expiration date When stored under the prescribed conditions, the expiration date of liquid vaccine is not later than 2 years and of the freeze-dried vaccine is not later than 3 years, from the date of the last satisfactory test for potency, or shall be based on the stability data of the vaccine.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition the label on the container states (1) the number of µg of PRP per human dose; (2) the type and nominal amount of carrier protein per single human dose.

Identification The vaccine is identified by a suitable immunochemical method (Appendix 14.5) for PRP.

pH 5.0 to 7.5 (Appendix 4.11).

Antimicrobial preservative Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The content is not less than the minimum amount shown to be effective and not more than 115 per cent of the quantity stated on the label.

Pyrogens Complies with the "Pyrogen Test" (Appendix 8.2), using 1 µg of PRP for a vaccine with diphtheria toxoid or CRM 197 diphtheria protein as carrier; 0.1 µg of PRP for a vaccine with tetanus toxoid as carrier; 0.025 µg of PRP for a vaccine with OMP as carrier, per kg of the rabbit's weight.

PRP content Not less than 80 per cent of the amount of PRP stated on the label.

PRP is determined either by assay of ribose or phosphorus, by an immunochemical method (Appendix 14.5) or by anion-exchange liquid chromatography with pulsed amperometric detection (Appendix 3.5).

FOR RIBOSE

Test solution Use a volumetric flask with a suitable volume for preparation of a solution containing about 5 mg per ml of dry polysaccharide. Transfer the contents of a container quantitatively to the flask and dilute to volumes with *water*. Dilute the solution so that the volumes used in the test contain 2.5 to 25 µg of ribose. Introduce 0.20 and 0.40 ml of the diluted solution into tubes in triplicate.

Reference solution Dissolve 25 mg of *ribose* in *water* and dilute to 100.0 ml with the same solvent (stock solution containing 0.25 g per litre of ribose). Immediately before use, dilute 1 ml of the stock solution to 10.0 ml with *water* (working dilution containing 25 mg per litre of ribose). Introduce 0.10 ml, 0.20 ml, 0.40 ml, 0.60 ml, 0.80 ml, and 1.0 ml of the working dilution into six tubes.

Prepare a blank using 2 ml of *water*.

Make up the volume in each tube to 2 ml with *water*. Mix and add 2 ml of a 0.05 per cent w/v solution of *iron(III) chloride* in *hydrochloric acid* to each tube. Mix and add 0.2 ml of a 10 per cent w/v solution of *orcinol* in *ethanol*. Place the tubes in a water-bath for 20 minutes. Cool in iced water.

Meassure the absorbance (Appendix 2.2) of each solution at 670 nm, using the blank solution in the reference cell. Draw a calibration curve from the absorbance readings for the six reference solutions and the corresponding content of ribose and read from the curve the quantity of ribose in the test solution for each volume tested. Calculate the mean of the three values.

FOR PHOSPHORUS

Test solution Use a volumetric flask with a suitable volume for preparation of a solution containing about 5 mg per ml of dry polysaccharide. Transfer the contents of a container quantitatively to the flask and dilute to volume with *water*. Dilute the solution so that the volume used in the test (1 ml) contains about 6 µg of phosphorus. Transfer 1.0 ml of the solution to a 10-ml ignition tube.

Reference solutions Dissolve 0.2194 g of *potassium dihydrogenphosphate* in 500 ml of *water* to give a solution containing the equivalent of 0.1 mg of phosphorus per

ml. Dilute 5.0 ml of the solution to 100.0 ml with *water*. Introduce 0.5 ml, 1.0 ml and 2.0 ml of the dilute solution into three ignition tubes.

Prepare a blank solution using 2.0 ml of *water* in an ignition tube.

To all the tubes add 0.2 ml of *sulfuric acid* and heat in an oil-bath at 120° for 1 hour and then at 160° until white fumes appear (about 1 hour). Add 0.1 ml of *perchloric acid* and heat at 160° until the solution is decolourized (about 90 minutes). Cool and add to each tube 4 ml of *water* and 4 ml of *ammonium molybdate with ascorbic acid TS*. Heat in a water-bath at 37° for 90 minutes and cool. Adjust the volume to 10.0 ml with *water*. The blue colour is stable for several hours.

Measure the absorbance (Appendix 2.2) of each solution at 820 nm, using the blank solution in the reference cell. Draw a calibration curve with the absorbances of the three reference solutions as a function of the quantity of phosphorus in the solutions and read from the curve the quantity of phosphorus in the test solution.

HEPATITIS A VACCINE, INACTIVATED

Hepatitis A Vaccine (Inactivated, Adsorbed)

Category Active immunizing agent.

Inactivated Hepatitis A Vaccine is a suspension consisting of a suitable strain of hepatitis A virus (HAV) grown in cell cultures, inactivated by a validated method and adsorbed onto suitable adjuvant(s), such as aluminium hydroxide or hydrated aluminium phosphate.

The vaccine complies with the requirements stated under Vaccines, with the following modifications.

Description Whitish turbid suspension.

Strengths available 50 units of viral antigen per ml (0.5 ml or 1 ml per vial); 1440 enzyme linked immunosorbent assay (ELISA) units of viral antigen per ml (0.5 ml or 1 ml per vial).

Dose *Intramuscular*, at deltoid region.

Adults over 18 years: 50 units or 1440 ELISA units, the volume injected usually not exceeding 1 ml.

Adults 18 years of age and under: 25 units or 720 ELISA units, the volume injected usually not exceeding 0.5 ml.

Children 2 years of age and over: 25 units or 720 ELISA units, the volume injected usually not exceeding 0.5 ml.

Contra-indication

1. It is contra-indicated in individuals with hypersensitivity to any component of the vaccine.
2. It is not for intravenous administration.

Warning

1. It may cause soreness, tenderness or warmth at the injection site.
2. Anorexia, fever, headache or malaise may occur.
3. It should be used with caution in individuals with thrombocytopenia or a bleeding disorder (e.g., hemophilia) or individuals receiving anticoagulant therapy since bleeding may occur following intramuscular administration of the drug.
4. It should not be administered in the gluteal region since administration at this site may result in suboptimal response.
5. It may not prevent hepatitis A infection in individuals who have an unrecognized hepatitis A infection at the time of vaccination.

Additional information

1. It is well tolerated and highly immunogenic and effective in children 2 years of age and older.
2. Active immunization against hepatitis A may be less effective in immunocompromized individuals and in individuals receiving immunosuppressive therapy since the immune response to the vaccine may be decreased.
3. If it is administered concurrently with any routine childhood vaccines, it should be administered at a different site using a separate syringe.

Expiration date When stored under the prescribed conditions, the expiration date is not later than 3 years from the date of the last satisfactory test for potency.

Labelling Complies with the "General Information for Biological Products" p. 177. In addition the label on the container states (1) the biological origin of the cells used for the preparation of the vaccine; (2) the virus strain used for the production of the vaccine; (3) the method used for inactivating the virus; (4) the nature and amount of adjuvant(s) and preservative present.

Identification The vaccine is shown to contain hepatitis A virus antigen by a suitable immunochemical method (Appendix 14.5) using specific antibodies or by the *in vivo* assay.

Antimicrobial preservative Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than the minimum amount shown to

be effective and is not more than 115 per cent of that stated on the label.

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains not more than 2 Endotoxin Units per single human dose.

Assay

The assay of Inactivated Hepatitis A Vaccine is carried out either *in vivo*, by comparing in given conditions its capacity to induce specific antibodies in mice with the same capacity of a reference preparation, or *in vitro*, by an immunochemical determination of the antigen content.

IN VIVO ASSAY

The test in mice shown below is given as an example of a method that has been found suitable for a given vaccine; other validated methods may also be used.

Selection and distribution of the test animals Use in the test healthy mice from the same stock, about 5 weeks old and from a strain shown to be suitable. Use animals of the same sex. Distribute the animals in at least seven equal groups of a number suitable for the requirements of the assay.

Determination of potency of the vaccine to be examined Using *saline TS* containing the aluminium adjuvant used for the vaccine or another appropriate diluent, prepare at least three dilutions of the vaccine to be examined and matching dilutions of the reference preparation. Allocate the dilutions one to each of the groups of animals and inject subcutaneously not more than 1.0 ml of each dilution into each animal in the group to which that dilution is allocated. Maintain a group of unvaccinated controls, injected subcutaneously with the same volume of diluent. After 28 to 32 days, anaesthetize and bleed all animals, keeping the individual sera separate. Assay the individual sera for specific antibodies against hepatitis A virus by a suitable immunochemical method (Appendix 14.5).

Calculations Carry out the calculations by the "Statistical Analysis of Results of Biological Assay and Tests" (Quantal response, Appendix 9).

From the distribution of reaction levels measured on all the sera in the unvaccinated group, determine the maximum reaction level that can be expected to occur in an unvaccinated animal for that particular assay. Any response in vaccinated animals that exceeds this level is by definition a seroconversion.

Make a suitable transformation of the percentage of animals showing seroconversion in each group (for example, a probit transformation) and analyze the data according to a parallel-line log dose-response model. Determine the potency of the test preparation relative to the reference preparation.

Validity conditions The test is not valid unless:

– for both the test and the reference vaccine, the ED_{50} lies between the smallest and the largest doses given to the animals,

– the statistical analysis shows no significant deviation from linearity or parallelism,

– the confidence limits ($P = 0.95$) are not less than 33 per cent and not more than 300 per cent of the estimated potency.

Potency requirement The upper confidence limit ($P = 0.95$) of the estimated relative potency is not less than 1.0.

IN VITRO ASSAY

Carry out the "Immunochemical Method" (Appendix 14.5) for determination of antigen content with acceptance criteria validated against the *in vivo* test.

The acceptance criteria are approved for a given reference preparation by the competent authority in the light of the validation data.

HEPATITIS B VACCINE, RECOMBINANT

Hepatitis B Vaccine (rDNA)

Category Active immunizing agent.

Recombinant Hepatitis B Vaccine is a noninfectious subunit viral vaccine containing hepatitis B surface antigen (HBsAg) that stimulates active immunity to hepatitis B virus infection. The antigen is obtained by recombinant DNA technology and adsorbed onto suitable adjuvant(s) such as aluminium hydroxide or hydrated aluminium phosphate.

The vaccine complies with the requirements stated under Vaccines, with the following modifications.

Description Whitish turbid suspension, free from evident clumps after shaking.

Strengths available For pediatric, 5 and 10 μg of hepatitis B surface antigen per 0.5 ml; for adults, 10, 20 and 40 μg of hepatitis B surface antigen per ml.

Dose *Intramuscular*, at deltoid region (for newborn infants injection at anterolateral thigh is more preferable).

Adults: 10 to 20 μg, the volume injected usually not exceeding 1 ml.

Children under 10 years of age: 5 to 10 μg, the vol-

ume injected usually not exceeding 0.5 ml.

Dialysis patients and immunocompromized patients: as directed by the physician.

The dose may vary depending on the specific preparation used, the recipient's age, the HBsAg status of the mother (for neonates), and the presence of underlying disease.

Contra-indication

1. It is contra-indicated in individuals with hypersensitivity to yeast or any component of the vaccines.
2. It is not for intravenous administration.

Warning

1. It may cause soreness, pain, induration, tenderness, pruritus, erythema, ecchymosis, swelling, warmth, burning, and nodule formation at the injection site.
2. Fatigue, weakness, headache or fever may occur.
3. It should be used with caution in individuals with thrombocytopenia or a bleeding disorder (e.g., hemophilia) since bleeding may occur following intramuscular administration of the drug.
4. It should be administered with caution to individuals with severely compromized cardiopulmonary status or to other individuals in whom a febrile or adverse systemic reaction could pose a substantial risk.
5. Unrecognized hepatitis B virus infection may be present in some individuals at the time of vaccination since the infection has a long incubation period; recombinant hepatitis B vaccine may not prevent infection in these individuals.
6. For individuals receiving immunosuppressive therapy, deferral of vaccination for not less than 3 months after therapy may be considered.

Additional information Hemodialysis patients and immunocompromized patients (i.e., those with immunodeficiencies and those receiving immunosuppressive therapy) generally require higher doses of recombinant hepatitis B vaccine than other individuals to stimulate adequate circulating antibody titers.

Expiration date When stored under the prescribed conditions, the expiration date is not later than 3 years from the date of the last satisfactory test for potency.

Labelling Complies with the "General Information for Biological Products" p. 177. In addition the label on the container states (1) the amount of HBsAg per container; (2) the type of cells used for production of the vaccine; (3) the name and amount of the adjuvant(s) used.

Identification The assay or, where applicable, the electrophoretic profile, may serve as an identification test.

Antimicrobial preservative Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than the minimum amount shown to be effective and is not more than 115 per cent of that stated on the label.

Pyrogens Complies with the "Pyrogen Test" (Appendix 8.2), using one human dose of the vaccine per each rabbit.

Assay

The assay of Recombinant Hepatitis B Vaccine is carried out either *in vivo*, by comparing in given conditions its capacity to induce specific antibodies against hepatitis B surface antigen (HBsAg) in mice or guinea-pigs with the same capacity of a reference preparation, or *in vitro*, by an immunochemical determination of the antigen content.

IN VIVO ASSAY

Selection and distribution of the test animals Use in the test healthy mice from the same stock, about 5 weeks old. The strain of mice used for this test must give a significant slope for the dose-response curve to the antigen; mice with haplotype H-2^q or H-2^d are suitable. Healthy guinea-pigs weighing 300 to 350 g (about 7 weeks old) from the same stock are also suitable. Use animals of the same sex. Distribute the animals in at least seven equal groups of a number appropriate to the requirements of the *assay*.

Determination of potency of the vaccine to be examined Using *saline TS* containing the aluminium adjuvant used for the vaccine or another appropriate diluent, prepare at least three dilutions of the vaccine to be examined and matching dilutions of the reference preparation. Allocate the dilutions one to each of the groups of animals and inject intraperitoneally not more than 1.0 ml of each dilution into each animal in the group to which that dilution is allocated. One group of animals remains unvaccinated and is injected intraperitoneally with the same volume of diluent. After an appropriate time interval (for example, 4 to 6 weeks), anaesthetize and bleed the animals, keeping the individual sera separate. Assay the individual sera for specific antibodies against HBsAg by a suitable immunochemical method (Appendix 14.5).

Calculations Calculations are carried out by the "Statistical Analysis of Results of Biological Assay and Test" (Quantal response, Appendix 9).

From the distribution of reaction levels measured on all the sera in the unvaccinated group, the maximum reaction level that can be expected to occur in an unvac-

cinated animal for that particular assay is determined. Any response in vaccinated animals that exceeds this level is by definition a seroconversion.

Make a suitable transformation of the percentage of animals showing seroconversion in each group (for example, a probit transformation) and analyze the data according to a parallel-line log dose-response model. Determine the potency of the test preparation relative to the reference preparation.

Validity conditions The test is not valid unless:

– for both the test and the reference vaccine, the ED_{50} lies between the smallest and the largest doses given to the animals,

– the statistical analysis shows no significant deviation from linearity or parallelism,

– the confidence limits ($P = 0.95$) are not less than 33 per cent and not more than 300 per cent of the estimated potency.

Potency requirement The upper confidence limit ($P = 0.95$) of the estimated relative potency is not less than 1.0.

IN VITRO ASSAY

Carry out the "Immunochemical Method" (Appendix 14.5) for determination of antigen content with acceptance criteria validated against the *in vivo* test.

Enzyme-linked immunosorbent assay (ELISA) and radio-immunoassay (RIA) using monoclonal antibodies specific for protection-inducing of HBsAg have been shown to be suitable. Suitable numbers of dilutions of the vaccine to be examined and the reference preparation are used and a parallel-line model is used to analyze the data which may be suitably transformed. Kits for measuring HBsAg *in vitro* are commercially available and it is possible to adapt their test procedures for use as an *in vitro* potency assay.

The acceptance criteria are approved for a given reference preparation by the competent authority in the light of the validation data.

HOUSE DUST MITE ALLERGEN VACCINE

Domestic Dust Mite Allergen Vaccine; Pure Mite Bodies Allergen Vaccine; Dust Mite Allergen Vaccine.

Category Diagnostic agent; active immunizing agent.

House Dust Mite Allergen Vaccine is a sterile liquid or freeze-dried preparation of one or more species obtained from suitable strains of *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae* or other species of *Dermatophagoides*. It is intended for the diagnosis and treatment of dust mite allergy.

The vaccine, reconstituted as stated on the label, complies with the requirements stated under Vaccines, with the following modifications.

Description Colourless transparent liquid. Freeze-dried vaccine consists of solid exhibiting the characteristic structure of a freeze-dried solid. When reconstituted, it becomes a colourless transparent liquid.

Strength available 10,000 AU per ml.

Dose Diagnosis: *Skin prick test* or *intradermal*, as prescribed by the physician.

Treatment: *Subcutaneous*, initial and increasing dose as prescribed by the physician, using optimal maintenance dose for each patient, 5 to 20 μg of major allergen.

Contra-indication

1. It is not for intramuscular or intravenous administration.
2. It is contra-indicated in patients with febrile conditions, serious immunological illness, or acute asthma and during periods of exacerbation of symptoms.
3. It is contra-indicated in patients with high degree of hypersensitivity by skin testing or specific IgE measurements.

Warning

1. Risk-benefit should be considered if it is to be used in pregnant or nursing women.
2. Pain, redness and/or swelling at the injection site and/or fever may occur.
3. Concomitant use with β-blockers should be avoided.

Precaution

1. The dose should be decreased if previous injection causes severe skin and/or systemic reactions.
2. Decreasing dose should be considered when new vial is used.
3. Emergency equipment must be available when performing allergen immunotherapy.

Additional information

1. Patients should be kept under medical observations for at least 30 to 60 minutes after treatment.
2. If a patient is receiving two different allergen extracts concurrently, try to avoid giving injections of both on the same day. If this is unavoidable, give the injections in different arms and allow at least 30 minutes between injections.
3. If possible, avoid all other immunization procedures (e.g., for poliomyelitis) during treatment with allergen extracts. If they are essential, give immunization one week after allergen injection. The next allergen

injection should be 2 weeks after immunization and the dose reduced by 50 per cent.

Expiration date When stored under the prescribed conditions, the expiration date is not later than 1 year from the date of the last satisfactory test for potency.

Packaging and storage Liquid Vaccine shall be stored at a temperature not exceeding 4°, protected from light. Do not freeze.

Freeze-Dried Vaccine shall be stored at a temperature below 25°, protected from light, unless otherwise specified by manufacturers.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition the label on the container states (1) the species of *Dermatophagoides*; (2) the protein content and/or total allergenic activity and individual allergens; (3) the route of administration and the intended use; (4) for freeze-dried preparation, the period of time within which the preparation is to be used after reconstitution; (5) where applicable the name and amount of adsorbent.

Identification It is identified by a suitable immunochemical method (Appendix 14.5). The protein composition corresponds to that of the International Standard.

Water Not more than 5.0 per cent w/w for freeze-dried vaccine (Appendix 4.12).

Aluminium For the adsorbed Vaccine containing aluminium, not less than 80 per cent and not more than 120 per cent of the stated amount but in any case not more than 1.25 mg of aluminium per single human dose, when determined by the method described under the "Determination of Aluminium", p. 178.

Calcium For the adsorbed Vaccine containing calcium, not less than 80 per cent and not more than 120 per cent of the stated amount but in any case not more than 1.3 mg of calcium per single human dose, when determined by the method described under the "Determination of Calcium", p. 178.

Abnormal toxicity Complies with the "Abnormal Toxicity Test" (appendix 8.1).

Antigen profile The antigens are identified by means of a suitable immunochemical method using antigen-specific animal antibodies (Appendix 14.5).

Allergen profile Relevant allergenic components are identified by means of a suitable immunochemical method using allergen-specific human antibodies (Appendix 14.5).

Protein profile The protein composition determined by suitable methods corresponds to that of the In-House Reference Preparation (IHRP[1]) (Appendix 3.7).

Protein content Not less than 80 per cent and not more than 120 per cent of the stated content of a given batch (Appendix 6.7). If the biological potency can be determined, then the test for protein content may be omitted.

Individual allergens Not less than 50 per cent and not more than 200 per cent of the stated amount, determined by a suitable immunochemical method (Appendix 14.5).

Total allergenic activity The activity is not less than 50 per cent and not more than 200 per cent of the stated amount as assayed by inhibition of the binding capacity of specific immunoglobulin E antibodies (Appendix 14.5) or a suitable equivalent *in vitro* method.

[1]The preparation prepared from In-House reference serum pool which is collected from whole blood of the house dust mite allergic patients who have positive history and positive skin prick testing to house dust mite vaccine.

ลิขสิทธิ์ กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข ห้ามทำซ้ำ ดัดแปลง แก้ไข ใช้ในเชิงพาณิชย์ โดยไม่ได้รับอนุญาต

INFLUENZA VACCINE, INACTIVATED (WHOLE VIRION)

Category Active immunizing agent.

Inactivated Influenza Vaccine (Whole Virion) is a sterile, aqueous suspension of a suitable strain or strains of influenza virus, type A or B, or a mixture of strains of the two types grown individually in embryonated chicken eggs and inactivated in such a manner that their antigenic properties are retained.

The vaccine complies with the requirements stated under Vaccines, with the following modifications.

Description Slightly opalescent liquid.

Strength available Not less than 15 μg hemagglutinin antigen for each strain per dose (0.5 ml).

Dose Adults: *Intramuscular*, at deltoid region, 0.5 ml.

Contra-indication

1. It is contra-indicated in individuals with a history of hypersensitivity to egg proteins.
2. It is not for intravenous administration.

Warning

1. It may cause soreness, pain or tenderness, erythema, and induration or mass at the injection site.
2. Fever, malaise, myalgia, and other systemic manifestations may occur.
3. Immunization should be delayed in persons with an active neurological disorder characterized by changing neurological findings, but can be initiated when the disease process has been stabilized.
4. Risk-benefit should be considered if it is to be used in patients with a prior history of Guillain-Barré syndrome.
5. It is not recommended for children under 12 years of age.

Expiration date When stored under the prescribed conditions, the expiration date should not exceed 1 year from the date of issue stated by the manufacturer.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition the label on the container states (1) that the vaccine has been prepared in eggs; (2) the strain or strains of influenza virus used to prepare the vaccine; (3) the method of inactivation; (4) the hemagglutinin content in micrograms per virus strain per dose; (5) the maximum amount of ovalbumin; (6) the season during which the vaccine is intended to protect.

Identification The identity of the hemagglutinins in the vaccine should be determined by an immunological technique, such as immunodiffusion or hemagglutinin inhibition, using the appropriate specific immune serum.

Residual infectious virus Inoculate 0.2 ml of the vaccine into the allantoic cavity of each of 10 fertilized eggs and incubate at 35°±2° for 3 days. The test is not valid unless at least 8 of the 10 embryos survive. Harvest 0.5 ml of the allantoic fluid from each surviving embryo and pool the fluids. Inoculate 0.2 ml of the pooled fluid into a further 10 fertilized eggs and incubate at 35°±2° for 3 days. The test is not valid unless at least 8 of the 10 embryos survive. Harvest about 0.1 ml of the allantoic fluid from each surviving embryo and examine each individual harvest for live virus by a hemagglutination test. If hemagglutination is found for any of the fluids, carry out for that fluid a further passage in eggs and test for hemagglutination, hemagglutinin activity should not be detected in these new groups of eggs.

Antimicrobial preservative Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not more than 115 per cent of the quantity stated on the label.

Ovalbumin Not more than the quantity stated on the label and in any case not more than 1 μg per human dose, determined by a suitable immunochemical method (Appendix 14.5) using a suitable reference preparation of ovalbumin.

Total protein Not more than six times the total hemagglutinin content of the vaccine as determined in the assay, but in any case, not more than 100 μg of protein per virus strain per human dose and not more than a total of 300 μg of protein per human dose. Carry out the test as described in the "Determination of Nitrogen" (Method III, Appendix 6.7).

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains less than 100 Endotoxin Units per single human dose.

Assay Determine the content of hemagglutinin antigen by an immunodiffusion test (Appendix 14.5), by comparison with the National or International Reference Preparation of Influenza Hemagglutinin. Carry out the test at 20° to 25°. Each dose contains 15 μg of hemagglutinin of each strain of virus used in the preparation of the vaccine. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 125 per cent of the

estimated hemagglutinin antigen content. The lower confidence limit ($P = 0.95$) is not less than 80 per cent of the amount stated on the label for each strain.

INFLUENZA VACCINE, INACTIVATED (SPLIT VIRION)

Category Active immunizing agent.

Inactivated Influenza Vaccine (Split Virion) is a sterile, aqueous suspension of a strain or strains of influenza virus, type A or B, or a mixture of strains of the two types grown individually in embryonated chicken eggs, inactivated and treated so that the integrity of the virus particles has been disrupted without diminishing the antigenic properties of the hemagglutinin and neuraminidase antigens.

The vaccine complies with the requirements stated under Vaccines, with the following modifications.

Description Slightly opalescent liquid.

Strength available Each 0.5 ml dose contains not less than 15 μg hemagglutinin antigen for each strain.

Dose Adults and children 3 years of age and older: *Intramuscular*, at deltoid region, 0.5 ml.

Children under 3 years of age and infants 6 months of age and older: *Intramuscular*, at anterolateral aspect of the thigh, 0.25 ml.

(**Note** Dose should be repeated at least 4 weeks if the children younger than 8 years of age have not been previously vaccinated.)

Contra-indication

1. It is contra-indicated in individuals with a history of hypersensitivity to egg proteins.
2. It is not for intravenous administration.

Warning

1. It may cause soreness, pain or tenderness, erythema, and induration or mass at injection site.
2. Fever, malaise, myalgia, and other systemic manifestations may occur.
3. Immunization should be delayed in persons with an active neurological disorder characterized by changing neurological findings, but can be initiated when the disease process has been stabilized.
4. Risk-benefit should be considered if it is to be used in patients with a prior history of Guillain-Barré syndrome.
5. It is not recommended for infants under 6 months of age.

Expiration date When stored under the prescribed conditions, the expiration date should not exceed 1 year from the date of issue stated by the manufacturer.

Labelling Complies with the "General Information for Biological Products" p. 177. In addition the label on the container states (1) that the vaccine has been prepared in eggs; (2) the strain or strains of influenza virus used to prepare the vaccine; (3) the method of inactivation; (4) the hemagglutinin content in micrograms per virus strain per dose; (5) the maximum amount of ovalbumin; (6) the season during which the vaccine is intended to protect.

Identification The identity of the hemagglutinins in the vaccine should be determined by an immunological technique, such as immunodiffusion or hemagglutinin inhibition, using the appropriate specific immune serum.

Residual infectious virus Inoculate 0.2 ml of the vaccine into the allantoic cavity of each of 10 fertilized eggs and incubate at 35°±2° for 3 days. The test is not valid unless at least 8 of the 10 embryos survive. Harvest 0.5 ml of the allantoic fluid from each surviving embryo and pool the fluids. Inoculate 0.2 ml of the pooled fluid into a further 10 fertilized eggs and incubate at 35°±2° for 3 days. The test is not valid unless at least 8 of the 10 embryos survive. Harvest about 0.1 ml of the allantoic fluid from each surviving embryo and examine each individual harvest for live virus by a hemagglutination test. If hemagglutination is found for any of the fluids, carry out for that fluid a further passage in eggs and test for hemagglutination, hemagglutinin activity should not be detected in these new groups of eggs.

Antimicrobial preservative Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not more than 115 per cent of the quantity stated on the label.

Ovalbumin Not more than the quantity stated on the label and in any case not more than 1 μg per human dose, determined by a suitable immunochemical method (Appendix 14.5) using a suitable reference preparation of ovalbumin.

Total protein Not more than six times the total hemagglutinin content of the vaccine as determined in the assay, but in any case, not more than 100 μg of protein per virus strain per human dose and not more than a

total of 300 μg of protein per human dose. Carry out the test as described in the "Determination of Nitrogen" (Method III, Appendix 6.7).

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains less than 100 Endotoxin Units per single human dose.

Assay Determine the content of hemagglutinin antigen by an immunodiffusion test (Appendix 14.5), by comparison with the National or International Reference Preparation of Influenza Hemagglutinin. Carry out the test at 20° to 25°. Each dose contains 15 μg of hemagglutinin of each strain of virus used in the preparation of the vaccine. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 125 per cent of the estimated hemagglutinin antigen content. The lower confidence limit ($P = 0.95$) is not less than 80 per cent of the amount stated on the label for each strain.

For some vaccines, quantitative determination of hemagglutinin antigen with respect to available reference preparations is not possible. An immunological identification of the hemagglutinin antigen and a semi-quantitative determination are carried out instead by suitable methods.

INFLUENZA VACCINE, INACTIVATED (SURFACE ANTIGEN)

Influenza Vaccine, Inactivated (Subunit)

Category Active immunizing agent.

Inactivated Influenza Vaccine (Surface Antigen) is a sterile suspension of a strain or strains of influenza virus, type A or B, or a mixture of strains of the two types grown individually in embryonated chicken eggs, inactivated and treated so that the preparation consists predominantly of hemagglutinin and neuraminidase antigens, without diminishing the antigenic properties of these antigens. The vaccine may contain an adjuvant.

The vaccine complies with the requirements stated under Vaccines, with the following modifications.

Strength available Each 0.5 ml dose contains not less than 15 μg hemagglutinin antigen for each strain.

Dose Adults and children 3 years of age and older: *Intramuscular*, at deltoid region, 0.5 ml.

Children under 3 years of age and infants 6 months of age and older: *Intramuscular*, at anterolateral aspect of the thigh, 0.25 ml.

(**Note** Dose should be repeated at least 4 weeks if the children younger than 8 years of age has not been previously vaccinated.)

Contra-indication

1. It is contra-indicated in individuals with a history of hypersensitivity to egg proteins.
2. It is not for intravenous administration.

Warning

1. It may cause soreness, pain or tenderness, erythema, and induration or mass at injection site.
2. Fever, malaise, myalgia, and other systemic manifestations may occur.
3. Immunization should be delayed in persons with an active neurological disorder characterized by changing neurological findings, but can be initiated when the disease process has been stabilized.
4. Risk-benefit should be considered if it is to be used in patients with a prior history of Guillain-Barré syndrome.
5. It is not recommended for infants under 6 months of age.

Expiration date When stored under the prescribed conditions, the expiration date should not exceed 1 year from the date of issue stated by the manufacturer.

Labelling Complies with the "General Information for Biological Products" p. 177. In addition the label on the container states (1) that the vaccine has been prepared in eggs; (2) the strain or strains of influenza virus used to prepare the vaccine; (3) the method of inactivation; (4) the hemagglutinin content in micrograms per virus strain per dose; (5) the maximum amount of ovalbumin; (6) the season during which the vaccine is intended to protect; (7) where applicable, name and quantity of the adjuvant used.

Identification The identity of the hemagglutinins in the vaccine should be determined by an immunological technique, such as immunodiffusion or hemagglutinin inhibition, using the appropriate specific immune serum.

Residual infectious virus Inoculate 0.2 ml of the vaccine into the allantoic cavity of each of 10 fertilized eggs and incubate at 35°±2° for 3 days. The test is not valid unless at least 8 of the 10 embryos survive. Harvest 0.5 ml of the allantoic fluid from each surviving embryo and pool the fluids. Inoculate 0.2 ml of the pooled fluid into a further 10 fertilized eggs and incubate at 35°±2° for 3 days. The test is not valid unless at least 8 of the 10 embryos survive. Harvest about 0.1 ml

of the allantoic fluid from each surviving embryo and examine each individual harvest for live virus by a hemagglutination test. If hemagglutination is found for any of the fluids, carry out for that fluid a further passage in eggs and test for hemagglutination, hemagglutinin activity should not be detected in these new-groups of eggs.

Antimicrobial preservative Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not more than 115 per cent of the quantity stated on the label.

Ovalbumin Not more than the quantity stated on the label and in any case not more than 1 μg per human dose, determined by a suitable immunochemical method (Appendix 14.5) using a suitable reference preparation of ovalbumin.

Total protein Not more than 40 μg of protein other than hemagglutinin per virus strain per human dose and not more than a total of 120 μg of protein other than hemagglutinin per human dose. Carry out the test as described in the "Determination of Nitrogen" (Method III, Appendix 6.7).

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains less than 100 Endotoxin Units per single human dose.

Assay Determine the content of hemagglutinin antigen by an immunodiffusion test (Appendix 14.5), by comparison with the National or International Reference Preparation of Influenza Hemagglutinin. Carry out the test at 20° to 25°. Each dose contains 15 μg of hemagglutinin of each strain of virus used in the preparation of the vaccine. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 125 per cent of the estimated content. The lower confidence limit ($P = 0.95$) hemagglutinin antigen is not less than 80 per cent of the amount stated on the label for each strain.

INFLUENZA VACCINE, INACTIVATED (SURFACE ANTIGEN, VIROSOME)

Category Active immunizing agent.

Inactivated Influenza Vaccine (Surface Antigen, Virosome) is a sterile, aqueous suspension of a strain or strains of influenza virus, type A or B, or a mixture of strains of the two types grown individually in embryonated chicken eggs, inactivated and treated so that the preparation consists predominantly of hemagglutinin and neuraminidase antigens reconstituted to virosomes with phospholipids and without diminishing the antigenic properties of the antigens.

The vaccine complies with the requirements stated under Vaccines, with the following modifications.

Description Slightly opalescent liquid.

Strength available Each 0.5 ml dose contains not less than 15 μg hemagglutinin antigen for each strain.

Dose Adults and children 3 years of age and older: *Intramuscular*, at deltoid region, 0.5 ml.

Children under 3 years of age and infants 6 months of age and older: *Intramuscular*, at anterolateral aspect of the thigh, 0.25 ml.

(**Note** Dose should be repeated at least 4 weeks if the children younger than 8 years of age has not been previously vaccinated.)

Contra-indication

1. It is contra-indicated in individuals with a history of hypersensitivity to egg proteins.
2. It is not for intravenous administration.

Warning

1. It may cause soreness, pain or tenderness, erythema, and induration or mass at injection site.
2. Fever, malaise, myalgia, and other systemic manifestations may occur.
3. Immunization should be delayed in persons with an active neurological disorder characterized by changing neurological findings, but can be initiated when the disease process has been stabilized.
4. Risk-benefit should be considered if it is to be used in patients with a prior history of Guillain-Barré syndrome.
5. It is not recommended for infants under 6 months of age.

Expiration date When stored under the prescribed conditions, the expiration date should not exceed 1 year from the date of issue stated by the manufacturer.

Labelling Complies with the "General Information for Biological Products" p. 177. In addition the label on the

container states (1) that the vaccine has been prepared in eggs; (2) the strain or strains of influenza virus used to prepare the vaccine; (3) the method of inactivation; (4) the hemagglutinin content in micrograms per virus strain per dose; (5) the maximum amount of ovalbumin; (6) the season during which the vaccine is intended to protect.

Identification The identity of the hemagglutinins in the vaccine should be determined by an immunological technique, such as immunodiffusion or hemagglutinin inhibition, using the appropriate specific immune serum.

Residual infectious virus Inoculate 0.2 ml of the vaccine into the allantoic cavity of each of 10 fertilized eggs and incubate at 35°±2° for 3 days. The test is not valid unless at least 8 of the 10 embryos survive. Harvest 0.5 ml of the allantoic fluid from each surviving embryo and pool the fluids. Inoculate 0.2 ml of the pooled fluid into a further 10 fertilized eggs and incubate at 35°±2° for 3 days. The test is not valid unless at least 8 of the 10 embryos survive. Harvest about 0.1 ml of the allantoic fluid from each surviving embryo and examine each individual harvest for live virus by a hemagglutination test. If hemagglutination is found for any of the fluids, carry out for that fluid a further passage in eggs and test for hemagglutination, hemagglutinin activity should not be detected in these new groups of eggs.

pH 6.5 to 7.8 (Appendix 4.11).

Phospholipid The content and identity of the phospholipids is determined by a suitable immunochemical (Appendix 14.5) or physico-chemical method.

Ratio of hemagglutinin to phospholipid The ratio of hemagglutinin content to phospholipid content is within the limits approved for the particular product.

Antimicrobial preservative Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not more than 115 per cent of the quantity stated on the label.

Ovalbumin Not more than the quantity stated on the label and in any case not more than 1 µg per human dose, determined by a suitable immunochemical method (Appendix 14.5) using a suitable reference preparation of ovalbumin.

Total protein Not more than 40 µg of protein other than hemagglutinin per virus strain per human dose and not more than 120 µg of protein other than hemagglutinin per human dose. Carry out the test as described in the "Determination of Nitrogen" (Method III, Appendix 6.7).

Virosome size distribution The size of the virosomes, determined by a suitable method such as laser light scattering, is not less than 100 nm and not more than 500 nm.

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains less than 100 Endotoxin Units per single human dose.

Assay Determine the content of hemagglutinin antigen by an immunodiffusion test (Appendix 14.5), by comparison with the National or International Reference Preparation of Influenza Hemagglutinin. Carry out the test at 20° to 25°. Each dose contains 15 µg of hemagglutinin of each strain of virus used in the preparation of the vaccine. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 125 per cent of the estimated hemagglutinin antigen content. The lower confidence limit ($P = 0.95$) is not less than 80 per cent of the amount stated on the label for each strain.

JAPANESE ENCEPHALITIS VACCINE, INACTIVATED

Category Active immunizing agent.

Inactivated Japanese Encephalitis Vaccine is a sterile liquid or freeze-dried preparation of a suitable strain of Japanese encephalitis virus, either Nakayama or Beijing, grown in mouse brain or in cell cultures and inactivated by a suitable method.

The vaccine, reconstituted if necessary as stated on the label, complies with the requirements stated under Vaccines, with the following modifications.

Description Liquid vaccine is clear or slightly whitish turbid and colourless liquid.

Freeze-dried vaccine is a white amorphous pellet; when reconstituted, it becomes clear or slightly whitish turbid and colourless liquid.

Strengths available As specified by the manufacturers.

Dose According to the strength specified.

Warning

1. It may cause tenderness, redness and swelling at the injection site.

2. Headache, rash, edema and generalized urticaria or angioedema may occur shortly after vaccination or up to 17 days following vaccination.

3. It should not be administered to anyone who developed a reaction to a previous dose.

4. It is not recommended for infants and neonates.

Expiration date When stored under the prescribed conditions, the expiration date of liquid vaccine is not later than 1 year or shall be based on the stability data of the vaccine, and of the freeze-dried vaccine is not later than 5 years, from the date of the last satisfactory test for potency.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition the label on the container states (1) whether the vaccine was prepared by an *in vivo* or an *in vitro* method; (2) the strain included in the vaccine; (3) the method used to inactivate the virus; (4) if the vaccine is in dried form, a statement that, after its reconstitution, it shall be used as soon as possible or stored at 2° to 8° and discarded at the end of the day; (5) the name and the concentration of the preservative.

Identification The test for potency may serve as an identification test.

pH 6.8 to 7.4 (Appendix 4.11).

Protein content Not more than 80 μg per ml when neural tissue is used, and not more than 200 μg per ml when human albumin is added in cell culture. Carry out the test as described in the "Determination of Nitrogen" (Method III, Appendix 6.7).

Inactivation test Inject intracerebrally at a dose of 0.03 ml of vaccine into at least 10 mice of about 4 weeks of age. Observe the animals for 14 days. No animal shows any abnormal sign during the observation period.

Pyrogens Complies with the "Pyrogen Test" (Appendix 8.2), using 1.0 ml of the vaccine per kg of the rabbit's weight.

Assay

The assay of Japanese Encephalitis Vaccine is determined by assessing the ability of the vaccine to stimulate the production of antibody to Japanese encephalitis virus in mice to which the appropriate vaccine has been administered. The sera of the mice are titrated for antibody by comparing their ability to neutralize 50 per cent of a fixed dose of Japanese encephalitis virus with the ability of that of the National or International Reference Preparation of Japanese Encephalitis Vaccine to give the same effects.

Preparation of challenge virus suspension The challenge virus is prepared by inoculating intracerebrally the virus strain for challenge into suckling mice of about 2 days of age. The brains of mice showing typical signs of infection are harvested and triturated in a suitable diluent containing calf serum to make a 10 per cent w/v suspension. Centrifuge the brain suspension at about 2000 × *g* for 30 minutes. The supernatant is diluted to contain about 100 PFU of virus per 0.2 ml to serve as virus suspension for challenge.

Determination of the potency of the vaccine Dilute the test vaccine and the reference preparation with *saline TS*. Each dilution is injected intraperitoneally in two doses of 0.5 ml each at 7-day intervals into at least 10 mice of 4 weeks of age. Bleed the animals after 7 days of the second injection. The separated serum is pooled at each dilution of vaccine and then inactivated at 56° for 30 minutes and may be stored at –20° or below.

The serum is appropriately diluted and mixed with an equal volume of the challenge virus. The mixtures are kept at 37° for 90 minutes for neutralization and inoculated at a dose which contains challenge virus about 100 PFU, onto at least three wells of chick embryo cell or BHK-21 or other suitable cell.

The challenge virus suspension is diluted and inoculated onto chick embryo cell or BHK-21 cell to serve as the virus control. All the inoculated cell cultures are kept standing at 35° to 37° for 90 minutes in a CO_2-adjusted incubator, and then overlayed with *agar* or *methylcellulose*.

After incubation for 5 to 7 days, the inoculated cell cultures are stained and further incubated for one day. The number of plaques on cell cultures are counted to obtain the plaque reduction rates for the test vaccine and the Reference preparation. The neutralizing antibody titres are calculated for each group. The mean number of plaques of the virus control shall be 50 to 150 per well.

Validity conditions The test is not valid unless:

– for both the test vaccine and the reference preparation, the slopes of the plaque neutralizing reduction rates of the highest and the lowest serum dilutions are significant,

– the statistical analysis shows no significant deviation from linearity or parallelism. The test may be repeated but when more than one test is performed, the results of all valid tests must be combined in the estimated potency and its confidence limit.

MEASLES VACCINE, LIVE

Category Active immunizing agent.

Live Measles Vaccine is a freeze-dried preparation of a suitable attenuated strain of measles virus, propagated in human diploid cells or in cell cultures of chick-embryo derived from a chicken flock free from specified pathogens. The vaccine does not contain any added preservative.

The vaccine, reconstituted as stated on the label, complies with the requirements stated under Vaccines, with the following modifications.

Description Solid having the characteristic appearance of substances dried from frozen state. When reconstituted, it becomes a clear liquid that may be coloured owing to the presence of a pH indicator.

Stability The reconstituted solution of freeze-dried vaccine should be stored at a temperature between 2° and 8° protected from light. Discoloured or cloudy solutions should not be used. Discard unused reconstituted vaccines after 8 hours.

Strength available Not less than 1000 $CCID_{50}$ per 0.5 ml.

Dose *Subcutaneous*, preferably into the outer aspect of the upper arm, 0.5 ml.

Contra-indication

1. It is contra-indicated in pregnant women; patients with a history of hypersensitivity reactions to this vaccine or any of its components; patients receiving immunosuppressive therapy; patients with blood dyscrasia, leukemia, lymphoma of any type, or other malignant neoplasms affecting the bone marrow or lymphatic systems; patients with primary or acquired immunodeficiency, or active untreated tuberculosis; patients with a family history of congenital or hereditary immunodeficiency.
2. Do not administer this vaccine during febrile respiratory illness or other active febrile infections.
3. Do not vaccinate individuals who are immunosuppressed in association with AIDS or other clinical manifestations of infection with HIV, cellular immune deficiencies, and hypogammaglobulinemic and dysgammaglobulinemic states.
4. Defer immunization during the course of any acute illness.

Warning

1. The administration of the vaccine less than one month before or after administration of other live viral vaccines should be avoided unless given simultaneously.
2. It may cause burning or stinging of short duration at the injection site.
3. It should be used with caution in individuals with a history of febrile seizures, cerebral injury, or family histories of convulsions, or any other conditions in which fever-induced stress should be avoided.
4. It should be used with caution in individuals with a history of thrombocytopenic purpura or thrombocytopenia.
5. Delayed administration of the vaccine in an individual with a current or recent febrile illness depends largely on the severity and etiology of the illness.

Precaution

1. Avoid pregnancy for 3 months following vaccination.
2. Individuals with current thrombocytopenia may develop more severe thrombocytopenia following vaccination.
3. The immune status of patients about to undergo immunosuppressive therapy should be evaluated.

Additional information

1. Measles, mumps, and rubella vaccine are commercially available as a combination vaccine.
2. The primary immunization against measles, mumps and rubella can be integrated with primary immunization against diphtheria, tetanus, pertussis, *H. influenzae* type b (Hib), hepatitis B, pneumococcal disease, poliomyelitis, and varicella.
3. The recommended age for primary vaccination is 9 to 12 months of age, followed by revaccination prior to elementary school entry.
4. It can be administered to people with minor illnesses such diarrhea, mild upper respiratory infection with or without low-grade fever, or other low-grade febrile illness.
5. Children under treatment for tuberculosis may be immunized with the vaccine since no evidence of exacerbation of the disease has been reported.
6. Vaccine originated from cell cultures of chick embryo or vaccine containing neomycin should be used with caution in persons with severe hypersensitive reactions caused by such antigens.

Expiration date When stored under the prescribed conditions, the expiration date is not later than 2 years after the date of the last satisfactory test for virus titre.

Labelling Complies with the labelling described under the "General Information for Biological Products", p. 177. In addition the label on the container states (1) the strain of measles virus used for the preparation of

the vaccine; (2) the type and origin of the cells used for the preparation of the vaccine; (3) the minimum virus concentration contained in one recommended human dose; (4) that after reconstitution it should be used without delay or, if not used immediately, stored between 2° and 8° for a period not exceeding 8 hours; (5) that contact with disinfectants is to be avoided.

Identification When the reconstituted vaccine is mixed with an appropriate amount of specific measles antibodies, it is no longer able to infect susceptible cell cultures.

Bovine serum albumin Not more than 50 ng per single human dose. Carry out the "Immunochemical Methods" (Appendix 14.5).

Assay Titrate the vaccine for infective virus at least in triplicate, using at least five cell cultures for each 0.5 $\log_{10}$ dilution step or by a method of equal precision. Use an appropriate virus reference preparation to validate each assay. The estimated measles virus concentration is not less than that stated on the label; the minimum measles virus concentration stated on the label is not less than 1000 $CCID_{50}$ per single human dose (0.5 ml). The assay is not valid if the confidence interval ($P = 0.95$) of the logarithm of the virus concentration is more than ±0.3.

National or International Reference Preparation of Live Measles Vaccine is suitable for use as a reference preparation.

MENINGOCOCCAL POLYSACCHARIDE VACCINE

Category Active immunizing agent.

Meningococcal Polysaccharide Vaccine is a freeze-dried preparation of one or more purified capsular polysaccharides obtained from one or more suitable strains of *Neisseria meningitidis* group A, group C, group Y and group W 135 that are capable of consistently producing polysaccharides.

***N. meningitidis* group A polysaccharide consists of partly *O*-acetylated repeating units of *N*-acetyl-mannosamine, linked with 1α → 6 phosphodiester bonds.**

***N. meningitidis* group C polysaccharide consists of partly O-acetylated repeating units of sialic acid, linked with 2α → 9 glycosidic bonds.**

***N. meningitidis* group Y polysaccharide consists of partly *O*-acetylated alternating units of sialic acid and D-glucose, linked with 2α → 6 and 1α → 4 glycosidic bonds.**

***N. meningitidis* group W135 polysaccharide consists of partly *O*-acetylated alternating units of sialic acid and D-galactose, linked with 2α → 6 and 1α → 4 glycosidic bonds.**

The polysaccharide component or components stated on the label together with calcium ions and residual moisture account for over 90 per cent of the weight of the preparation.

The vaccine, reconstituted as stated on the label, complies with the requirements stated under Vaccines, with the following modifications.

Description The dried vaccine is a white or cream-coloured powder or pellet. When reconstituted, it becomes a clear colourless liquid.

Solubility Freely soluble in *water*.

Stability The reconstituted solution of freeze-dried vaccine should be stored at a temperature between 2° and 8° and used within 30 minutes, unless otherwise specified by the manufacturer. The solution should not be used if there is extraneous particulate matter and/or discoloration.

Strength available 50 µg of polysaccharide from each of the serogroup of meningococci represented in the vaccine per dose (0.5 ml).

Dose *Subcutaneous*, 0.5 ml.

Warning

1. It may cause erythema, pain or induration at the injection site.
2. Headache, malaise, fatigue, lethargy, fever, chills, rash, coryza, and gastro-intestinal symptoms may occur.
3. It should not be administered concomitantly with any vaccine containing whole-cell pertussis or whole-cell typhoid antigens because of concerns related to combined endotoxin content.
4. If the vaccine is used in people receiving immunosuppressive therapy, the expected immune response may not be obtained.

Additional information

1. It is indicated in persons travelling to countries where the risk of infection is high.
2. It is not indicated for infants and children under two years of age except as short-term protection of infants at least 3 months of age against Group A.
3. It may be administered concurrently with other vaccines, using separate body sites, separate syringes, and the precautions that apply to each immunizing agent.
4. Revaccination may be indicated for persons at high risk of infection, particularly children at high risk

who were first immunized before they were 4 years of age; such children should be considered for revaccination after 2 or 3 years if they remain at high risk.

Expiration date When stored under the prescribed conditions, the expiration date is not later than 2 years from the date of the last satisfactory assay.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition the label on the container states (1) the number of micrograms of polysaccharide in each dose; (2) the total quantity of polysaccharide in the container; (3) the group or groups of polysaccharides A, C, Y, or W135 and, for multispecific vaccines, the quantity of each polysaccharide per dose.

Identification Identify by a suitable immunochemical method (Appendix 14.5) such as immuno-precipitation, ELISA or radio-immunoassay.

Molecular size Carry out the test as described in the "Size-exclusion Chromatography" (Appendix 3.6), applying a quantity of the vaccine containing about 2.5 mg of each polysaccharide in a volume of about 1.5 ml.

The chromatographic procedure may be carried out using (a) a column (about 900 mm × 16 mm) packed with *cross-linked agarose for chromatography* for a divalent vaccine or *cross-linked agarose for chromatography 1* for a tetravalent vaccine and (b) a solvent having an ionic strength of 0.2 molal and a pH of 7.0 to 7.5 as the mobile phase with a flow rate of approximately 20 ml per hour.

Collect fractions of about 3 ml and determine the content of each polysaccharide by a suitable method.

FOR A DIVALENT VACCINE (GROUP A + GROUP C) The vaccine complies with the test if: 65 per cent of group A polysaccharide is eluted before distribution coefficient (K_D) of 0.50 is reached; 75 per cent of group C polysaccharide is eluted before K_D of 0.50 is reached.

FOR A TETRAVALENT VACCINE (GROUP A + GROUP C + GROUP Y + GROUP W135) Apply a suitable immunochemical method (Appendix 14.5) to establish the elution pattern of the different polysaccharides.

The vaccine complies with the test if K_D for the principal peak is:

– not more than 0.70 for group A and group C polysaccharide,

– not more than 0.57 for group Y polysaccharide,

– not more than 0.68 for group W135 polysaccharide.

Pyrogens Complies with the "Pyrogen Test" (Appendix 8.2), using a solution containing a quantity of the vaccine equivalent to 0.025 µg purified polysaccharide per ml for a monovalent vaccine; 0.050 µg of purified polysaccharide per ml for a divalent vaccine; 0.075 µg of purified polysaccharide per ml for a trivalent vaccine; 0.10 µg of purified polysaccharide per ml for a tetravalent vaccine and injecting 1.0 ml per kg of the rabbit's weight.

Assay Carry out an assay of each polysaccharide present in the vaccine.

For a divalent vaccine (group A + group C), use measurement of phosphorus to determine the content of polysaccharide A and measurement of sialic acid to determine the content of polysaccharide C.

To determine sialic acid, use as reference solution a 150 mg per litre solution of *N-acetylneuraminic acid*.

For a tetravalent vaccine (group A + group C + group Y + group W135) a suitable immunochemical method (Appendix 14.5) is used with a reference preparation of purified polysaccharide for each group.

The vaccine contains not less than 70 per cent and not more than 130 per cent of the quantity of each polysaccharide stated on the label.

FOR PHOSPHORUS

Test solution Use a volumetric flask with a suitable volume for preparation of a solution containing about 5 mg per ml of dry polysaccharide. Transfer the contents of a container quantitatively to the flask and dilute to volume with *water*. Dilute the solution so that the volume used in the test (1 ml) contains about 6 µg of phosphorus. Transfer 1.0 ml of the solution to a 10-ml ignition tube.

Reference solutions Dissolve 0.2194 g of *potassium dihydrogenphosphate* in 500 ml of *water* to give a solution containing the equivalent of 0.1 mg of phosphorus per millilitre. Dilute 5.0 ml of the solution to 100.0 ml with *water*. Introduce 0.5 ml, 1.0 ml and 2.0 ml of the dilute solution into three ignition tubes.

Prepare a blank solution using 2.0 ml of *water* in an ignition tube.

To all the tubes add 0.2 ml of *sulfuric acid* and heat in an oil-bath at 120° for 1 hour and then at 160° until white fumes appear (about 1 hour). Add 0.1 ml of *perchloric acid* and heat at 160° until the solution is decolourized (about 90 minutes). Cool and add to each tube 4 ml of *water* and 4 ml of *ammonium molybdate with ascorbic acid TS*. Heat in a water-bath at 37° for 90 minutes and cool. Adjust the volume to 10.0 ml with *water*. The blue colour is stable for several hours.

Measure the absorbance at 820 nm (Appendix 2.2), using in the reference cell 2.0 ml of *water* that has been treated in the same manner. Draw a calibration curve

with the absorbances of the three reference solutions as a function of the quantity of phosphorus in the solutions and read from the curve the quantity of phosphorus in the test solution.

FOR SIALIC ACID

Test solution Transfer quantitatively the contents of one or several containers to a volumetric flask of a suitable volume that will give a solution with a known concentration of about 250 µg per millilitre of polysaccharide and dilute to volume with *water*. Using a syringe, transfer 4.0 ml of this solution to a10-ml ultrafiltration cell suitable for the passage of molecules of relative molecular mass less than 50,000. Rinse the syringe twice with *water* and transfer the rinsings to the ultrafiltration cell. Carry out the ultrafiltration, with constant stirring, under *nitrogen* at a pressure of about 150 kPa (1125 Torr). Refill the cell with *water* each time the volume of liquid in it has decreased to 1 ml and continue until 200 ml has been filtered and the remaining volume in the cell is about 2 ml. Using a syringe, transfer this residual liquid to a 10-ml volumetric flask. Wash the cell with 3 quantities, each of 2 ml, of *water*, transfer the washings to the flask and dilute to 10.0 ml with *water* (test solution). In each of two test-tubes place 2.0 ml of the test solution.

Reference solution Use a 0.015 per cent w/v solution of N-*acetylneuraminic acid*.

Prepare two series of three test-tubes, place in the tubes of each series 0.5 ml, 1.0 ml and 1.5 ml, respectively, of the reference solution and adjust the volume in each tube to 2.0 ml with *water*.

Prepare blank solutions using 2.0 ml of *water* in each of two test-tubes.

To all the tubes add 5.0 ml of *resorcinol with copper(II) sulfate TS*. Heat at 105° for 15 minutes, cool in cold water and transfer the tubes to a bath of iced water. To each tube add 5 ml of *3-methyl-1-butanol* and mix thoroughly. Place in the bath of iced water for 15 minutes. Centrifuge the tubes and keep them in the bath of iced water until the examination by absorption spectrophotometry. Measure the absorbance of each supernatant solution at 580 nm and 450 nm (Appendix 2.2), using *3-methyl-1-butanol* as the blank. For each wavelength, calculate the absorbance as the mean of the values obtained with two identical solutions. Subtract the mean value for the blank solution from the mean values obtained for the other solutions.

Draw a graph showing the difference between the absorbances at 580 nm and 450 nm of the reference solutions as a function of the content of *N*-acetyl neruaminic acid and read from the graph the quantity of *N*-acetylneuraminic acid (sialic acid) in the test solution.

MUMPS VACCINE, LIVE

Category Active immunizing agent.

Live Mumps Vaccine is a freeze-dried preparation of a suitable attenuated strain of mumps virus, propagated in human diploid cells or in cell cultures of chick-embryo or in the amniotic cavity of chick embryonated eggs, derived from a chicken flock free from specified pathogens. The vaccine does not contain any added preservative.

The vaccine, reconstituted as stated on the label, complies with the requirements stated under Vaccines, with the following modifications.

Description Solid having the characteristic appearance of substances dried from frozen state. When reconstituted, it becomes a clear liquid that may be coloured owing to the presence of a pH indicator.

Stability The reconstituted solution of freeze-dried vaccine should be stored at a temperature between 2° and 8°, protected from light. Discoloured or cloudy solutions should not be used. Discard unused reconstituted vaccine after 8 hours.

Strengths available Not less than 5000 $CCID_{50}$ per 0.5 ml and not less than 20,000 $CCID_{50}$ per 0.5 ml, as applicable.

Dose *Subcutaneous*, preferably into the outer aspect of the upper arm, 0.5 ml.

Contra-indication, Warning, Precaution, Additional information See under *Measles Vaccine, Live*, p. 258.

Expiration date When stored under the prescribed conditions, the expiration date is not later than 2 years after the date of the last satisfactory test for virus titre.

Labelling Complies with the labelling described under the "General Information for Biological Products", p.=. In addition the label on the container states (1) the strain of mump virus used for the preparation of the vaccine; (2) the type and origin of the cells used for the preparation of the vaccine; (3) the minimum virus concentration contained in one recommended human dose; (4) that after reconstitution it should be used without delay or, if not used immediately, stored be tween 2° and 8° for a period not exceeding 8 hours; (5) that contact with disinfectants is to be avoided.

Identification When the reconstituted vaccine is mixed with an appropriate amount of specific mumps antibodies, it is no longer able to infect susceptible cell cultures.

Bovine serum albumin Not more than 50 ng per single human dose. Carry out the "Immunochemical Methods" (Appendix 14.5).

Ovalbumin If vaccine is produced in chick embryonated eggs, it contains not more than 1 μg of ovalbumin per single human dose. Carry out the "Immunochemical Methods" (Appendix 14.5).

Assay Titrate the vaccine for infective virus at least in triplicate, using at least five cell cultures for each 0.5 $\log_{10}$ dilution step or by a method of equal precision. Use an appropriate virus reference preparation to validate each assay. The estimated mumps virus concentration is not less than that stated on the label; the minimum mumps virus concentration stated on the label is not less than 5000 $CCID_{50}$ per single human dose (0.5 ml). The assay is not valid if the confidence interval ($P = 0.95$) of the logarithm of the virus concentration is more than ±0.3.

National or International Reference Preparation of Live Mumps Vaccine is suitable for use as a reference preparation.

PERTUSSIS VACCINE, ADSORBED

Whooping-cough Vaccine

Category Active immunizing agent.

Adsorbed Pertussis Vaccine is a sterile suspension, in saline solution or other appropriate solution isotonic with the blood, of killed *Bordetella pertussis* of one or more strains selected for high antigenic efficiency, which are adsorbed onto suitable adjuvant(s), such as aluminium hydroxide or aluminium phosphate. Each 0.5 ml contains not less than 4 IU of adsorbed pertussis vaccine.

The vaccine complies with the requirements stated under Vaccine, with the following modifications.

Description Markedly turbid, whitish liquid; odour, practically odourless or of the antimicrobial agent.

Dose *Intramuscular* or *deep subcutaneous*, 0.5 ml, usually as a component in Adsorbed Diptheria, Tetanus and Pertussis Vaccine.

Contra-indication

1. It is contra-indicated in children who have suffered a severe reaction to a previous dose.

2. It is contra-indicated in children with poorly controlled epilepsy or other neurological problems.

Warning

1. It may cause pain, redness, or local tenderness at the injection site.

2. Fever, drowsiness, irritable, loss of appetite or vomiting may occur.

Additional information

1. It is not recommended to use as a single vaccine.

2. Whooping-cough is most dangerous in early life. The first dose of Adsorbed Pertussis Vaccine should be injected when the infant is 2 months old. The vaccine should not be administered after the age of 6 years. It is best used in the form of adsorbed diphtheria, pertussis and tetanus vaccine.

3. Immunization can be carried out in children with a history of cerebral damage in the neonatal period. In children with a neurological problem that is still evolving it is recommended that immunization be deferred until the condition is stable.

4. If a patient is receiving Diphtheria, Tetanus, and Pertussis Vaccine and Inactivated Poliomyelitis Vaccine concurrently, try to avoid giving injections of both on the same site.

Expiration date When stored under the prescribed conditions, the expiration date is not later than 2 years from the date of the last satisfactory test for potency.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition the label on the container states (1) the number of IU in each single human dose; (2) the name and the amount of the adjuvant(s); (3) that it is not to be frozen; (4) that it must be well shaken before use; (5) the method used to inactivate the bacteria.

Identification

A. When injected into mice, the vaccine induces the production of pertussis antibodies. The test for potency may serve as an identification test.

B. Agglutination of the organisms with specific antipertussis serum is served as an identity test. Mix three loopfuls of vaccine with one loopful of monospecific antiserum on a microscope slide. After mixing, rock the slide gently for a few minutes. Rapid, heavy agglutination should be observed with all three specific antisera within about 3 minutes.

pH 6.0 to 7.0 (Appendix 4.11).

Specific toxicity Use not less than 5 healthy mice each weighing 14 to 16 g for the vaccine group and for the

saline control. Use mice of the same sex or distribute males and females equally between the groups. Allow the animals access to food and water for at least 2 hours before injection and during the test. Inject each mouse of the vaccine group intraperitoneally with 0.5 ml, containing a quantity of the vaccine equivalent to not less than half the single human dose. Inject each mouse of the control group with 0.5 ml of *saline TS*, preferably containing the same amount of antimicrobial preservative as that injected with the vaccine. Weigh the groups of mice immediately before the injection and 72 hours and 7 days after the injection. The vaccine complies with the test if: (a) at the end of 72 hours the total weight of the group of vaccinated mice is not less than that preceding the injection; (b) at the end of 7 days the average weight gain per vaccinated mouse is not less than 60 per cent of that per control mouse; and (c) not more than 5 per cent of the vaccinated mice die during the test. The test may be repeated and the results of the test combined.

Assay Carry out the "Biological Assay of Adsorbed Pertussis Vaccine" (Appendix 15.3.2). The estimated potency is not less than 4 IU per single human dose (0.5 ml) and the lower confidence limit ($P = 0.95$) of the estimated potency is not less than 2 IU per single human dose.

PERTUSSIS VACCINE (ACELLULAR COMPONENT), ADSORBED

Pertussis Vaccine (Acellular, Component, Adsorbed); Whooping-cough Vaccine (Acellular Component)

Category Active immunizing agent.

Adsorbed Pertussis Vaccine (Acellular Component) is a sterile suspension, in saline solution or other appropriate solution isotonic with the blood, of the individually prepared and purified antigenic components of *Bordetella pertussis*, which are adsorbed onto suitable adjuvant(s), such as aluminium hydroxide or aluminium phosphate.

The vaccine contains either pertussis toxoid or a pertussis-toxin-like protein free from toxic properties, produced by expression of a genetically modified form of the corresponding gene. Pertussis toxoid is prepared from pertussis toxin by a method that renders the latter harmless while maintaining adequate immunogenic properties and avoiding reversion to toxin. The vaccine may also contain filamentous haemagglutinin, pertactin (a 69 kDa outer-membrane protein) and other defined components of *B. pertussis*, such as fimbrial-2 and fimbrial-3 antigens. The latter 2 antigens may be copurified. The antigenic composition and characteristics are based on evidence of protection and freedom from unexpected reactions in the target group for which the vaccine is intended.

The vaccine complies with the requirements stated under Vaccine, with the following modifications.

Dose *Intramuscular* or *deep subcutaneous*, 0.5 ml, usually as a component in Adsorbed Diphteria, Tetanus and Pertussis (Acellular Component) Vaccine.

Contra-indication; Warning See under *Pertussis Vaccine, Adsorbed*, p. 262.

Additional information Adsorbed Pertussis Vaccine (Acellular Component) has been developed to reduce the frequency and severity of both local and systemic adverse reactions associated with Adsorbed Pertussis Vaccine.

See also under *Pertussis Vaccine, Adsorbed*, p. 262.

Expiration date When stored under the prescribed conditions, the expiration date is not later than 2 years from the date of the last satisfactory test for potency.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition the label on the container states (1) the names and amounts of the components present in the vaccine; (2) where applicable, that the vaccine contains a pertussis toxin-like protein produced by genetic modification; (3) the name and the amount of the adjuvant(s); (4) that it is not to be frozen; (5) that it must be well shaken before use.

Identification Subject the vaccine to a suitable desorption procedure such as the following: dissolve in the vaccine being examined sufficient *sodium citrate dihydrate* to give a 1 per cent w/v solution; maintain at 37° for about 16 hours and centrifuge until a clear supernatant liquid is obtained. Examined by a suitable immunochemical method (Appendix 14.5), the clear supernatant liquid reacts with specific antisera to the components stated on the label.

Absence of residual pertussis toxin and irreversibility of pertussis toxiod

(**Note** This test is not necessary for the product obtained by genetic modification.) Use three groups each of not less than five histamine-sensitive mice. Inject intraperitoneally into the first group twice the single human dose of the vaccine stored at 2° to 8°. Inject intraperitoneally into the second group twice the single human dose of the vaccine incubated at 37° for 4 weeks. Inject diluent into the third group of mice. After 5 days, inject into each mouse 2 mg of histamine

base intraperitoneally in a volume not exceeding 0.5 ml and observe for 24 hours. The test is invalid if one or more control mice die following histamine challenge. The vaccine complies with the test if no animal in the first or second group dies following histamine challenge. If one mouse dies in either or both of the first and second groups, the test may be repeated with the same number of mice or with a greater number and the results of valid tests combined; the vaccine complies with the test if, in both of the groups given the vaccine, not more than 5 per cent of the total number of mice die following histamine challenge.

The histamine sensitivity of the strain of mice used is verified at suitable intervals as follows: inject intravenously threefold dilutions of a reference pertussis toxin preparation in phosphate-buffered saline solution containing 0.2 per cent w/v of *gelatin* and challenge with histamine as above; the strain is suitable if more than 50 per cent of the animals are sensitized by 50 ng of pertussis toxin and none of the control animals injected with only diluent and challenged similarly with histamine show symptoms of sensitization.

Antimicrobial preservative Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physicochemical method. The amount is not less than the minimum amount shown to be effective and is not more than 115 per cent of the quantity stated on the label.

Assay

The capacity of the vaccine to induce the formation of specific antibodies is compared with the same capacity of a reference preparation examined in parallel; anitbodies are determined using suitable immunochemical methods (Appendix 14.5) such as enzyme-linked immunosorbent assay (ELISA). The test in mice shown below uses a three-point model but, after validation, for routine testing a single-dilution method may be used.

Reference vaccine A batch of vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The stability of the reference vaccine shall be documented.

Reference antiserum *Bordetella pertussis* mouse antiserum of National or International Standard is suitable for use as a reference antiserum.

Requirement The capacity to induce antibodies is not significantly ($P = 0.95$) less than that of the reference vaccine.

(**Note** The following test model is given as an example of a method that has been found to be satisfactory.)

Selection and distribution of test animals Use in the test healthy mice of the same stock 4 to 8 weeks old. Distribute the animals in 6 groups of a number appropriate to the requirements of the assay. Use 3 dilutions of the vaccine being examined and 3 dilutions of a reference preparation and attribute each dilution to a group of mice. Inject intraperitoneally or subcutaneously into each mouse 0.5 ml of the dilution attributed to its group.

Collection of serum samples After 4 to 5 weeks of vaccination, bleed the mice individually under anaesthesia. Store the sera at –20° until tested for antibody content.

Antibody determination Assay the individual sera for content of specific antibodies to each component using a validated method such as the ELISA test shown below.

ELISA test Coat microtitre plates (polyvinyl chloride or polystyrene as appropriate for the specific antigen) with the purified antigen at a concentration of 100 ng per well. After washing, block unreacted sites by incubating with a solution of bovine serum albumin and then washing. Make twofold dilutions of sera from mice immunized with test or reference vaccines on the plates. After incubation at 22° to 25° for 1 hour, wash the plates. Add a suitable solution of anti-mouse IgG enzyme conjugate to each well and incubate at 22° to 25° for 1 hour. After washing, add a substrate from which the bound enzyme conjugate liberates a chromophore which can be quantified by measurement of absorbance (Appendix 2.2). Design the test conditions to obtain a linear response for absorbance with respect to antibody content over the range of measurement used and absorbance values within the range 0.1 to 2.0.

Use a reference antiserum of assigned potency in the test as the basis for calculation of the antibody levels in test sera. Also include a standardized control serum in the test.

The test is not valid if: (a) the value found for the control serum differs by more than 2 standard deviations from the assigned value; (b) the confidence limits ($P = 0.95$) are less than 50 per cent or more than 200 per cent of the estimated potency.

Calculation Calculate the antibody titers in the sera of mice immunized with reference and test vaccines and from the values obtained calculate the potency of the test vaccine in relation to the reference vaccine by the "Statistical Analysis of Results of Biological Assay and Test" (Appendix 9).

PNEUMOCOCCAL POLYSACCHARIDE VACCINE

Category Active immunizing agent.

Pneumococcal Polysaccharide Vaccine consists of a mixture of equal parts of purified capsular polysaccharide antigens prepared from suitable pathogenic strains of *Streptococcus pneumoniae* whose capsules have been shown to be made up of polysaccharides that are capable of inducing satisfactory levels of specific antibodies in man. It contains 23 immunochemically different capsular polysaccharides of *S. pneumoniae* serotypes: 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17A or 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F.

The vaccine complies with the requirements stated under Vaccines, with the following modifications.

Description Clear, colourless liquid.

Strength available 25 μg of polysaccharide for each of the 23 serotypes per 0.5 ml.

Dose Adults and children 2 years of age and over: *Intramuscular* or *subcutaneous*, 0.5 ml.

Warning

1. It may cause pain, redness, induration and swelling at the injection site.
2. Headache, paresthesia, radiculoneuropathy, Guillain-Barré syndrome, rash, urticaria, nausea, and vomiting may occur.
3. It is not recommended for children younger than 2 years of age because they may not have a satisfactory antibody response.

Expiration date When stored under the prescribed conditions, the expiration date is not later than 2 years from the date of the last satisfactory tests.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition, the label on the container states (1) the number of micrograms of each polysaccharide per human dose; (2) the total amount of polysaccharide in the container.

Identification The assay may serve as an identification test.

pH 4.5 to 7.4 (Appendix 4.11).

Antimicrobial preservative Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not more than 115 per cent of the quantity stated on the label.

Abnormal toxicity Complies with the "Abnormal Toxicity Test" (Appendix 8.1), modified as follows for the test in guinea-pigs: inject intraperitoneally 10 human doses into each guinea-pig and observe for 12 days.

Pyrogens Complies with the "Pyrogen Test" (Appendix 8.2), using 2.5 μg per ml of each polysaccharide, per kg of the rabbit's weight.

Assay Determine the content of each polysaccharide by a suitable immunochemical method (Appendix 14.5), using antisera specific for each polysaccharide contained in the vaccine, including factor sera for types within groups, and purified polysaccharides of each type as standards.

The estimated amount of polysaccharide per dose is not less than 70 per cent and not more than 130 per cent of the content stated on the label. The confidence limits ($P = 0.95$) of the estimated amount of polysaccharide are not less than 80 per cent and not more than 120 per cent.

PNEUMOCOCCAL POLYSACCHARIDE CONJUGATE VACCINE, ADSORBED

Category Active immunizing agent.

Adsorbed Pneumococcal Polysaccharide Conjugate Vaccine is a sterile solution of purified capsular polysaccharide antigens obtained from suitable pathogenic strains of *Streptococcus pneumoniae* individually conjugated to a carrier protein. The vaccine may be adsorbed on a suitable adjuvant or adsorbant. The choice of polysaccharide depends on the frequency of the serotypes responsible for acute diseases and their geographical distribution. It contains immunochemically different capsular polysaccharides.

The vaccine complies with the requirements stated under Vaccines, with the following modifications.

Description White suspension.

Strengths available 2 μg of each polysaccharide for *S. pneumoniae* serotypes 4, 9V, 14, 18C, 19F, and 23F, 4 μg of polysaccharide for serotype 6B (16 μg total polysaccharide) and approximately 20 μg of CRM 197 protein per 0.5 ml.

Dose Children younger than 10 years of age and infants 6 weeks of age and over: *Intramuscular*, 0.5 ml.

Warning

1. It may cause pain, redness, induration and swelling at the injection site.

2. Fever, diarrhea, vomiting, decreased appetite, drowsiness, restless sleep, and irritability may occur.

Expiration date When stored under the prescribed conditions, the expiration date is not later than 3 years from the date of the last satisfactory tests.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition, the label on the container states (1) the number of micrograms of each polysaccharide per single human dose; (2) the number of micrograms of carrier protein per single human dose.

Identification Each polysaccharide present in the vaccine is identified by a suitable immunochemical method (Appendix 14.5).

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains less than 12.5 Endotoxin Units per single human dose, unless otherwise justified and authorized.

Assay

POLYSACCHARIDE CONTENT The polysaccharide content for each serotype is determined by a suitable immunochemical method (for example, nephelometry assay) (Appendix 14.5).

The estimated amount of polysaccharide per dose is not less than 70 per cent and not more than 130 per cent of the content stated on the label. The confidence limits ($P = 0.95$) of the estimated amount of polysaccharide are not less than 80 per cent and not more than 120 per cent.

POLIOMYELITIS VACCINE, ORAL

Poliomyelitis Vaccine, Live (Oral); Poliovirus Vaccine Live Oral; Sabin Vaccine; OPV

Category Active immunizing agent.

Oral Poliomyelitis Vaccine is a preparation of approved strains of live attenuated poliovirus type 1, 2 or 3 grown *in vitro* cultures of approved cells, containing any one type or any combination of the three types of Sabin strains, presented in a form suitable for oral administration.

The vaccine complies with the requirements stated under Vaccines, with the following modifications.

Description Clear liquid; may be coloured owing to the presence of a pH indicator. It is generally frozen.

Strength available $10^{6.0}$ $CCID_{50}$ of type 1, $10^{5.0}$ $CCID_{50}$ of type 2, and $10^{5.5}$ $CCID_{50}$ of type 3 per human dose.

Dose One human dose as stated.

Contra-indication

1. It is contra-indicated in individuals with primary immunodeficiencies or in individuals with suppressed immune response resulting from human immunodeficiency virus (HIV) infection, leukemia, lymphoma, other malignancies affecting the bone marrow or lymphatic system, certain blood dyscrasias, or therapy with immunosuppressive agents since replication of attenuated polioviruses may not be limited in such individuals.

2. Do not administer parenterally.

Warnning

1. Defer vaccination of persons with any acute, febrile illness until after recovery.

2. Postpone or avoid the vaccine in the presence of persistent vomiting or diarrhea and in patients with any advanced debilitated condition.

Additional information It is recommended for outbreak control if local epidemics of wild-type poliomyelitis occur.

Expiration date The expiration date is not later than 1 year from the date of the last satisfactory test for virus titre, when stored at a temperature below –5°, and not later than 2 years, when stored at a temperature below –20°.

Packaging and storage Oral Poliomyelitis Vaccine shall be stored, whenever possible in a frozen state, and protected from light. When thawed it shall be kept at a temperature of 2° to 8° and used within 30 days; such vaccine may be kept for longer periods of time if a suitable stabilizer has been added during manufacture. When exposed to higher temperature it shall be used within a few hours.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition, the label on the container states (1) the strain and type of poliomyelitis virus contained in the vaccine; (2) the cell substrate used for the preparation of the vaccine; (3) the nature and amount of any stabilizer present in the vaccine; (4) the nature and amount of any antibiotics used in the preparation of the vaccine; (5) the amount of virus of each type contained in one recommended dose; (6) the number of doses in the container; (7) that the vaccine is not for injection.

Identification When the vaccine is mixed with an appropriate amount of specific poliomyelitis antiserum, it is no longer able to infect susceptible cell cultures.

Thermal stability Maintain not less than three vials of the final lot at 37°±1° for 48 hours. Determine the total virus concentration as described under *Assay* in parallel for the heated vaccine and for vaccine maintained at the temperature recommended for storage. The estimated difference between the total virus concentration of the unheated and heated vaccines is not more than 0.5 $\log_{10}$ infectious virus units ($CCID_{50}$) per single human dose.

The test is not valid if:

– the confidence interval ($P = 0.95$) of the logarithm of the virus concentration of the reference preparation is more than ±0.3;

– the virus concentration of the reference preparation differs by more than 0.5 log $CCID_{50}$ from the assigned value;

– the range of virus concentrations found for the replicates of any sample is more than 0.8 log $CCID_{50}$.

Assay Titrate for infectious virus using not less than three separate vials of vaccine, following the method described below. Use one vial of an appropriate virus reference preparation to validate each assay. If the vaccine contains more than one poliovirus type, titrate each type separately, using appropriate type-specific antiserum (or preferably a monoclonal antibody) to neutralize each of the other types present.

For a trivalent vaccine, the estimated mean virus titres must be: not less than $10^{6.0}$ infectious virus units ($CCID_{50}$) per single human dose for type 1; not less than 105.0 infectious virus units ($CCID_{50}$) for type 2; and not less than $10^{5.5}$ infectious virus units ($CCID_{50}$) for type 3.

For a monovalent or divalent vaccine, the minimum virus titres are decided by the competent authority.

METHOD Inoculate groups of 8 to 12 flat-bottomed wells in a microtitre plate with 0.1 ml of each of the selected dilutions of virus followed by a suitable cell suspension of the Hep-2 (Cincinnati) line. Incubate the plates at a suitable temperature. Examine the cultures on days 7 to 9.

The assay is not valid if:

– the confidence interval ($P = 0.95$) of the logarithm of the virus concentration of the reference preparation is greater than ±0.3;

– the virus concentration of the reference preparation differs by more than 0.5 log $CCID_{50}$ from the assigned value;

– the range of virus concentrations found for the replicates for any sample is more than 0.8 log $CCID_{50}$.

POLIOMYELITIS VACCINE, INACTIVATED

Inactivated Poliomyelitis Vaccine; Poliovirus Vaccine Inactivated; Salk Vaccine; IPV

Category Active immunizing agent.

Inactivated Poliomyelitis Vaccine is a sterile liquid preparation of suitable strains of human poliovirus types 1, 2 and 3 grown in suitable cell cultures and inactivated by a validated method.

The vaccine complies with the requirements stated under Vaccines, with the following modifications.

Description Clear liquid; may be coloured owing to the presence of a pH indicator.

Strength available 40 D-antigen units of type 1, 8 D-antigen units of type 2, and 32 D-antigen units of type 3 per 0.5 ml.

Dose *Subcutaneous*, 0.5 ml.

Contra-indication

1. It is contra-indicated in individuals with hypersensitivity to any component of the vaccine, including neomycin, streptomycin and polymyxin B.
2. Do not administer intravenously.

Warnning Defer vaccination of person with any acute, febrile illness until after recovery.

Expiration date When stored under the prescribed conditions, the expiration date is not later than 18 months from the date of the last satisfactory test for potency.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition, the label on the container states (1) the nature of the cell cultures used; (2) the types of poliovirus contained in the vaccine; (3) the nominal amount of virus of each type (1, 2 and 3), expressed in units of D-antigen, per single human dose; (4) that it is not to be frozen.

Identification The vaccine is shown to contain human poliovirus types 1, 2 and 3 by a suitable immunochemical method (Appendix 14.5) such as the determination of D-antigen by enzyme-linked immunosorbent assay (ELISA).

Inactivation test

The vaccine shows the absence of any living extraneous virus when determined by one of the following methods.

A. Inoculate the vaccine to be examined into cell cultures sensitive to human and simian viruses and

incubate for a sufficient time, including sub-cultures, to detect any extraneous viruses as well as any living poliomyelitis virus.

B. Inoculate the vaccine into mice, guinea-pigs and rabbits.

Antimicrobial preservative Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physicochemical method. The amount is not less than the minimum amount shown to be effective and is not mores than 115 per cent of that stated on the label.

Protein nitrogen content Not more than 10 μg per single human dose. Carry out the determination as described in the "Determination of Nitrogen" (Method III, Appendix 6.7).

Bovine serum albumin Not more than 50 ng per single human dose, determined by a suitable immunochemical method (Appendix 14.5).

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains not more than 5 Endotoxin Units per single human dose.

Assay

IN VITRO TEST

D-antigen content Determine the D-antigen content for human poliovirus types 1, 2 and 3 by a suitable immunochemical method (Appendix 14.5) using a reference preparation calibrated in International Units of D-antigen. For each type, the content, expressed with reference to the amount of D-antigen stated on the label, is within the limits approved for the particular product.

IN VIVO TEST The capacity of the vaccine to induce the formation of neutralizing antibodies is determined *in vivo* by one of the following methods.

Test in chicks or guinea-pigs Prepare a suitable series of not less than three dilutions of the vaccine to be examined using a suitable buffered saline solution. Distribute either guinea-pigs weighing 250 to 350 g or 3-week-old chicks into groups of 10, and allocate a group to each dilution of the vaccine. Inject intramuscularly into each animal 0.5 ml of the dilution intended for its group. Bleed the animals after 5 to 6 days and separate the sera. Examine the sera for the presence of neutralizing antibodies, at a dilution of 1 in 4, to each of the human polioviruses 1, 2 and 3. Mix 100 $CCID_{50}$ of virus with the dilution of serum and incubate at 37° for 4.5 to 6 hours. Keep at a temperature between 2° and 8° for 12 to 18 hours where necessary for consistency of results. Inoculate the mixtures into cell cultures for the detection of unneutralized virus and read the results up to 7 days after inoculation. For each group of animals, note the number of sera which have neutralizing antibodies and calculate the dilution of the vaccine giving an antibody response in 50 per cent of the animals. Carry out in parallel a control test using a suitable reference preparation. The vaccine complies with the test if a dilution of 1 in 100 or more produces an antibody response for each of the three types of virus in 50 per cent of the animals.

Test in rats Prepare a suitable series of not less than three dilutions of the vaccine to be examined and a reference vaccine. Inject each dilution intramuscularly into the hind limb(s) of a group of 10 specific pathogen-free rats of a suitable strain. Use of four dilutions is often necessary to obtain valid results for all three serotypes. The number of animals per group must be sufficient to obtain results that meet the validity criteria; groups of 10 rats are usually sufficient although valid results may be obtained with fewer animals per group. If animals of different sex are used, males and females are evenly distributed between all groups. A weight range of 175 to 250 g has been found suitable. An inoculum of 0.5 ml per rat is used. The dose range is chosen such that a dose response to all three poliovirus type is obtained. Bleed the animals after 20 to 22 days. Neutralizing titres against all three poliovirus types are measured separately using 100 $CCID_{50}$ of the Sabin strains as challenge viruses, Vero of Hep2 as indicator cells, and neutralization conditions of 3 hours at 35° to 37° followed by 18 hours at 2° to 8° where necessary for consistency of results. Results are read following fixation and staining after 7 days of incubation at 35°. For a valid antibody assay, the titre of each challenge virus must be shown to be within the range of 10 $CCID_{50}$ to 1000 $CCID_{50}$ and the neutralizing antibody titre of a control serum must be within two twofold dilutions of the geometric mean titre of the serum. The potency is calculated by comparison of the proportion of responders for the vaccine to be examined and the reference vaccine by the probit method or, after validation, using a parallel-line model. For the probit method it is necessary to establish a cut-off neutralizing antibody titre for each poliovirus type to define a responder. Due to interlaboratory variation, it is not possible to define cut-off values that could be applied by all laboratories. Rather, the cut-off values are determined for each laboratory based on a minimum series of three tests with the reference vaccine. The mid-point on a $\log_2$ scale of the minimum and maximum geometric mean

titres of the series of three or more tests is used as the cut-off value. For each of the three poliovirus types, the potency of the vaccine is not significantly less than that of the reference preparation.

The test is not valid unless:

– for both the test and reference vaccines the ED_{50} lies between the smallest and the largest doses given to the animals;

– the statistical analysis shows no significant deviation from linearity or parallelism;

– the confidence limits ($P = 0.95$) are not less than 25 per cent and not more than 400 per cent of the estimated potency.

RABIES VACCINE, INACTIVATED

Category Active immunizing agent.

Inactivated Rabies Vaccine for human use is a sterile liquid or freeze-dried preparation of a suitable strain of fixed rabies virus grown in cell cultures or embryonated duck eggs, purified and inactivated by a suitable method.

The vaccine reconstituted, if necessary as stated on the label, complies with the requirements stated under Vaccines, with the following modifications.

Description Liquid vaccine is slightly whitish turbid that may be coloured owing to the presence of a pH indicator.

Freeze-dried vaccine, when reconstituted, becomes clear liquid that may be coloured owing to the presence of a pH indicator.

Strengths available Liquid or reconstituted preparation, not less than 2.5 IU rabies virus antigen per 0.5 or 1.0 ml.

Dose *Intramuscular*, one human dose, at deltoid region (for small children and infants injection at anterolateral thigh is more preferable).

Warning

1. It may cause transient pain, erythema, swelling or itching at the injection site.

2. Nausea, vomiting, abdominal pain, diarrhea, headache, fatigue, sore throat, low-grade fever (up to 38.3°), chills, muscle aches, arthralgia, myalgia, fainting, dizziness, and malaise may occur.

3. Patients should be closely observed during the period of immunization.

4. It should not be injected into the gluteal region or near blood vessels or nerve.

Additional information

1. Pregnancy is not a contra-indication to post-exposure therapy.

2. For postexposure therapy in previously unvaccinated individuals, passive immunization with a single dose of rabies immunoglobulin should always be administered in conjunction with the first dose of rabies vaccine.

Expiration date When stored under the prescribed conditions, the expiration date is not later than 2 years for the liquid vaccine and not later than 3 years for the freeze-dried vaccine, from the date of the last satisfactory test for potency.

Labelling Complies with the "General Information for Biological Products" p. 177. In addition the label on the container states (1) that it contains rabies virus antigen equivalent to not less than 2.5 IU per dose; (2) the cell culture in which the vaccine was prepared or embryonated duck eggs; (3) the method used for inactivating the virus; (4) that after reconstitution, it shall be used immediately unless data are provided to show that it may be stored for a limited time without loss of potency; (5) the name and amount of adjuvant(s) and preservative(s).

Identification The vaccine is shown to contain rabies virus antigen by a suitable immunochemical method (Appendix 14.5) using specific antibodies, preferably monoclonal; alternatively, the assay serves also to identify the vaccine.

Residual infectious virus Inoculate a quantity equivalent to not less than 25 human doses of vaccine into cell cultures of the same type as those used for production of the vaccine. A passage may be made after 7 days. Maintain the cultures for a total of 21 days and then examine the cell cultures for rabies virus using an immunofluorescence test. No rabies virus is detected.

Bovine serum albumin Not more than 50 ng per single human dose. Carry out the "Immunochemical Methods" (Appendix 14.5).

Ovalbumin If the vaccine is produced in a duck embryonated egg, it contains not more than 1 µg of ovalbumin per human single dose, determined by a suitable immunochemical method (Appendix 14.5).

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains less than 25 Endotoxin Units per single human dose.

Pyrogens Complies with the "Pyrogen Test" (Appendix 8.2), injecting into each rabbit a single human dose of the vaccine diluted to 10 times its volume.

Assay The potency of rabies vaccine is determined by comparing the dose necessary to protect mice against the effects of a lethal dose of rabies virus, administered intracerebrally, with the quantity of a reference preparation of rabies vaccine necessary to provide the same protection. For this comparison a reference preparation of rabies vaccine, calibrated in International Units, and a suitable preparation of rabies virus for use as the challenge preparation are necessary.

The International Unit is the activity contained in a stated quantity of the International Standard.

The test described below uses a parallel-line model with at least three points for the vaccine to be examined and the reference preparation.

Selection and distribution of the test animals Use healthy mice about 4 weeks old, each weighing 11 to 15 g, from the same stock. Distribute the mice into six groups of 16 for potency test and four groups of 10 for titrating the challenge virus. The mice must all be of the same sex, or, if of different sexes, the sexes must be distributed equally among the groups.

Preparation of the challenge virus suspension Inoculate mice intracerebrally with the Challenge Virus Standard (CVS) strain of rabies virus and when the mice show signs of rabies, but before they die, euthanize them, remove the brains and prepare a homogenate of the brain tissue in a suitable diluent. Centrifuge and use the supernatant liquid as the challenge suspension. Distribute the suspension in small volumes in ampoules, seal and store at a temperature below –60°. Determine the LD_{50} of challenge virus suspension by thawing one ampoule of the suspension and make serial dilutions in a suitable diluent. Allocate each dilution to a group of five mice and inject intracerebrally into each mouse 0.03 ml of the dilution allocated to its group. Observe the mice for 14 days. Calculate the LD_{50} of the undiluted suspension using the number in each group that, between the fifth and fourteenth days, die or develop signs of rabies.

Determination of potency of the vaccine to be examined Prepare at least three fivefold serial dilutions of the vaccine to be examined and at least three fivefold serial dilutions of the reference preparation of rabies vaccine. Prepare the dilutions such that the most concentrated suspensions may be expected to protect more than 50 per cent of the animals to which they are administered and the least concentrated suspensions may be expected to protect less than 50 per cent of the animals to which they are administered. Allocate the six dilutions, one to each of the six groups of 16 mice, and inject intraperitoneally into each mouse 0.5 ml of the dilution allocated to its group. After 7 days, prepare three identical dilutions of the vaccine to be examined and of the reference preparation and repeat the injections. Seven days after the second injection, prepare a suspension of the challenge virus such that, on the basis of the preliminary titration, 0.03 ml contains 12 to 50 LD_{50}. Inject intracerebrally into each vaccinated mouse 0.03 ml of this suspension.

Prepare three suitable serial dilutions of the challenge suspension. Allocate the challenge suspension and the three dilutions one to each of the four groups of 10 control mice and inject intracerebrally into each mouse 0.03 ml of the suspension or one of the dilutions allocated to its group. Observe the animals in each group for 14 days and record the number in each group that die or show signs of rabies for a period of 5 to 14 days after challenge.

Validity conditions The test is not valid unless:

– for both the vaccine to be examined and the reference preparation the 50 per cent protective dose lies between the largest and smallest doses given to the mice;

– the titration of the challenge suspension shows that 0.03 ml of the suspension contained 12 to 50 LD_{50};

– the statistical analysis shows a significant slope and no significant deviations from linearity or parallelism of the dose-response curves;

– the confidence limits ($P = 0.95$) are not less than 25 per cent and not more than 400 per cent of the estimated potency.

The vaccine complies with the test if the estimated potency is not less than 2.5 IU per human dose.

RUBELLA VACCINE, LIVE

Category Active immunizing agent.

Live Rubella Vaccine is a freeze-dried preparation of a suitable attenuated strain of rubella virus, propagated in human diploid cells. The vaccine does not contain any added preservative.

The vaccine, reconstituted as stated on the label, complies with the requirements stated under Vaccines, with the following modifications.

Description Solid having the characteristic appearance of substances dried from frozen state. When reconstituted, it becomes a clear liquid that may be coloured owing to the presence of a pH indicator.

Stability The reconstituted solution of freeze-dried vaccine should be stored at a temperature between 2° and 8°, protected from light. Discoloured or cloudy solutions should not be used. Discard unused reconstituted vaccine after 8 hours.

Strength available Not less than 1000 $CCID_{50}$ per 0.5 ml.

Dose *Subcutaneous*, preferably into the outer aspect of the upper arm, 0.5 ml.

Contra-indication; Precaution; Additional information See under *Measles Vaccine, Live*, p. 258.

Warning Rubella-like symptoms (mild regional lymphadenopathy, rash, urticaria, fever, malaise, sorethroat, dizziness, headache, nausea, vomiting, diarrhea, general aches, and polyneuritis) may occur 11 to 20 days after vaccination and are usually mild and transient, generally persisting 1 to 5 days.

See also under *Measles Vaccine, Live*, p. 258.

Expiration date When stored under the prescribed conditions, the expiration date is not later than 2 years after the date of the last satisfactory test for virus titre.

Labelling Complies with the labelling described under the "General Information for Biological Products", p. 177. In addition, the label on the container states (1) the strain of rubella virus used for the preparation of the vaccine; (2) the type and origin of the cells used for the preparation of the vaccine; (3) the minimum virus concentration contained in one recommended human dose; (4) that after reconstitution it should be used without delay or, if not used immediately, stored between 2° and 8° for a period not exceeding 8 hours; (5) that contact with disinfectants is to be avoided; (6) that the vaccine must not be given to a pregnant woman and that a woman must not become pregnant within 3 months after having the vaccine.

Identification When the reconstituted vaccine is mixed with an appropriate amount of specific rubella antibodies, it is no longer able to infect susceptible cell cultures.

Bovine serum albumin Not more than 50 ng per single human dose. Carry out the "Immunochemical Methods" (Appendix 14.5).

Assay Titrate the vaccine for infective virus at least in triplicate, using at least five cell cultures for each 0.5 $\log_{10}$ dilution step or by a method of equal precision. Use an appropriate virus reference preparation to validate each assay. The estimated rubella virus concentration is not less than that stated on the label; the minimum rubella virus concentration stated on the label is not less than 1000 $CCID_{50}$ per single human dose (0.5 ml). The assay is not valid if the confidence interval (P = 0.95) of the logarithm of the virus concentration is more than ±0.3.

National or International Reference Preparation of Live Rubella Vaccine is suitable for use as a reference preparation.

MEASLES, MUMPS, AND RUBELLA VACCINE, LIVE

MMR (Live)

Category Active immunizing agent.

Live Measles, Mumps, and Rubella Vaccine is a freeze-dried preparation of a combination of suitable attenuated strains of measles virus, mumps virus and rubella virus such that each component is prepared in conformity with and meets the requirements for Live Measles Vaccine, for Live Mumps Vaccine, and for Live Rubella Vaccine, whichever is applicable.

The vaccine, reconstituted as stated on the label, complies with the requirements stated under Vaccines, with the following modifications.

Description Solid having the characteristic appearance of substances dried from frozen state. When reconstituted, it becomes a clear liquid that may be coloured owing to the presence of a pH indicator.

Stability The reconstituted solution of freeze-dried vaccine should be stored at a temperature between 2° and 8°, protected from light. Discoloured or cloudy solutions should not be used. Discard unused reconstituted vaccine after 8 hours.

Strength available Not less than 1000 $CCID_{50}$ of measles virus, not less than 5000 or 20,000 $CCID_{50}$ of mumps virus and not less than 1000 $CCID_{50}$ of rubella virus per 0.5 ml.

Dose *Subcutaneous*, preferably into the outer aspect of the upper arm, 0.5 ml.

Contra-indication; Precaution; Additional information See under *Measles Vaccine, Live*, p. 258.

Warning Rubella-like symptoms (mild regional lymphadenopathy, rash, urticaria, fever, malaise, sore throat, dizziness, headache, nausea, vomiting, diarrhea, general aches, and polyneuritis) may occur 11 to 20 days after vaccination and are usually mild and transient, generally persisting 1 to 5 days.

See also under *Measles Vaccine, Live*, p. 258.

Expiration date When stored under the prescribed conditions, the expiration date is not later than 2 years after the date of the last satisfactory test for virus titre.

Labelling Complies with the labelling described under the "General Information for Biological Products", p. 177. In addition the label on the container states (1) the strain of measles, mumps and rubella virus used in the preparation of the vaccine; (2) where applicable, that chick embryos have been used for the preparation of the vaccine; (3) the type and origin of the cells used for the preparation of the vaccine; (4) the minimum virus concentration for each component of the vaccine; (5) that contact with disinfectants is to be avoided; (6) that after reconstitution it should be used without delay or, if not used immediately, stored between 2° and 8° for a period not exceeding 8 hours; (7) that both lyophilized and reconstituted vaccines should be protected from light; (8) that the vaccine must not be given to a pregnant woman and that a woman must not become pregnant within 3 months after having the vaccine.

Identification When the reconstituted vaccine is mixed with antibodies specific for measles virus, mumps virus and rubella virus, it is no longer able to infect cell culture susceptible to these viruses. When the reconstituted vaccine is mixed with appropriate amount of specific antibodies sufficient to neutralize any two viral components, the third viral component infects susceptible cell cultures. However, selection of cell cultures susceptible only to mumps and measles, but resisting to rubella can be used instead of rubella neutralization.

Bovine serum albumin Not more the 50 ng per single human dose. Carry out the "Immunochemical Methods" (Appendix 14.5).

Ovalbumin If the mumps component is produced in chick embryonated eggs, the vaccine contains not more than 1 μg of ovalbumin per single human dose. Carry out the "Immunochemical Methods" (Appendix 14.5).

Thermal stability Maintain samples of the freeze-dried vaccine in dry state at 37° for 7 days. Determine the virus concentration as described under *Assay* in parallel for the heated vaccine and for unheated vaccine stored at a temperature between 2° and 8°. For each component, the virus concentration of the heated vaccine is not more than 1.0 $\log_{10}$ lower than that of the unheated vaccine.

Assay

FOR MEASLES Mix the vaccine with a sufficient quantity of antibodies specific for mumps virus. Titrate the vaccine for infective measles virus at least in triplicate, using at least five cell cultures of susceptible cells such as vero cells for each 0.5 $\log_{10}$ dilution step or by a method of equal precision. Use an appropriate virus reference preparation to validate each assay. The estimated measles virus concentration is not less than that stated on the label; the minimum measles virus concentration stated on the label is not less than 1000 $CCID_{50}$ per single human dose. The assay is not valid if the confidence interval ($P = 0.95$) of the logarithm of the virus concentration is more than ±0.3.

National or International Reference Preparation of Live Measles Vaccine is suitable for use as reference preparation.

FOR MUMPS Mix the vaccine with a sufficient quantity of antibodies specific for measles virus. Titrate the vaccine for infective mumps virus at least in triplicate, using at least five cell cultures of susceptible cells such as vero cells for each 0.5 $\log_{10}$ dilution step or by amethod of equal precision. Use an appropriate virus reference preparation to validate each assay. The estimated mumps virus concentration is not less than that stated on the label; the minimum mumps virus concentration stated on the label is not less than 5000 or 20,000 $CCID_{50}$ per single human dose. The assay is not valid if the confidence interval ($P = 0.95$) of the logarithm of the virus concentration is more than ±0.3.

National or International Reference Preparation of Live Mump Vaccine is suitable for use as reference preparation.

FOR RUBELLA Mix the vaccine with a sufficient quantity of antibodies specific for mumps virus. Titrate the vaccine for infective rubella virus at least in triplicate, using at least five cell cultures of susceptible cells

such as RK-13 cells for each 0.5 $\log_{10}$ dilution step or by a method of equal precision. Use an appropriate virus reference preparation to validate each assay. The estimated rubella virus concentration is not less than that stated on the label; the minimum rubella virus concentration stated on the label is not less than 1000 $CCID_{50}$ per single human dose. The assay is not valid if the confidence interval ($P = 0.95$) of the logarithm of the virus concentration is more than ±0.3.

National or International Reference Preparation of Live Rubella Vaccine is suitable for use as reference preparation.

TETANUS VACCINE, ADSORBED

Adsorbed Tetanus Toxoid

Category Active immunizing agent.

Adsorbed Tetanus Vaccine is a sterile suspension of suitable adjuvant(s), such as aluminium hydroxide, aluminium phosphate, onto which purified tetanus toxoid is adsorbed. The tetanus toxoid is prepared from tetanus toxin produced by the growth in suitable media of *Clostridium tetani* by treatment with formaldehyde. Each 0.5 ml contains not less than 40 IU of adsorbed tetanus vaccine.

The vaccine complies with the requirements stated under Vaccines, with the following modifications.

Description Light colour suspension, free from evident clumps after shaking.

Strength available 40 IU of adsorbed tetanus toxoid per 0.5 ml.

Dose *Intramuscular*, 0.5 ml.

Contra-indication

1. It is contra-indicated in individuals who have a history of systemic hypersensitivity or neurologic reactions to the vaccine.

2. It is contra-indicated in individuals with thrombocytopenia or any coagulation disorder; in this situation, subcutaneous administration may be advisable.

Warning

1. It is not recommended for immunizing children under 7 years of age.

2. If a contra-indication to using a tetanus vaccine-containing preparation exists in an individual who has previously received less than 3 doses of tetanus vaccine or tetanus vaccine adsorbed, only passive immunization with tetanus immunoglobulin (TIG) should be considered when injury other than a clean, minor wound (not tetanus prone) is sustained.

3. Local reactions and mild systemic reactions may occur.

Expiration date When stored under the prescribed conditions, the expiration date is not later than 2 years from the date of the last satisfactory test for potency.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition the label on the container states (1) the number of IU in each single human dose; (2) the name and the amount of the adjuvant(s); (3) that it is not to be frozen; (4) it is to be well-shaken before use.

Identification Dissolve in the vaccine to be examined sufficient *sodium citrate dihydrate* to give a 10 per cent w/v solution. Maintain at 37° for about 16 hours and centrifuge until the clear supernatant liquid is obtained. The clear supernatant liquid reacts with a suitable tetanus antitoxin, producing a precipitate.

pH 6.0 to 7.0 (Appendix 4.11).

Phenol Phenol should not be added to Adsorbed Tetanus Vaccine, since it has been shown to have deleterious effects on antigenic properties of the toxoid.

Specific toxicity Inject subcutaneously five times the single human dose stated on the label into each of five healthy guinea-pigs, each weighing 250 to 350 g, that have not previously been treated with any material that will interfere with the test. The vaccine complies with the test if within 3 weeks of the injection, none of the animals shows signs or dies from tetanus. If more than one animal dies from non-specific causes, repeat the test once. The vaccine complies with the test if not more than one animal dies in the second test.

Assay Carry out the "Biological Assay of Adsorbed Tetanus Vaccine" (Appendix 15.3.3). The lower confidence limit ($P = 0.95$) of the estimated potency is not less than 40 IU per single human dose (0.5 ml).

DIPHTHERIA AND TETANUS VACCINE, ADSORBED

Adsorbed Diphtheria and Tetanus Toxoids; DT Vaccine

Category Active immunizing agent.

Adsorbed Diphtheria and Tetanus Vaccine is a sterile suspension of suitable adjuvant(s), such as aluminium hydroxide or aluminium phosphate, onto which purified diphtheria toxoid and tetanus toxoid are adsorbed. The diphtheria toxoid and tetanus toxoid are prepared from diphtheria toxin and tetanus toxin produced by the growth in suitable media of *Corynebacterium diphtheriae* and *Clostridium tetani*, respectively, by treatment with formaldehyde. Each 0.5 ml contains not less than 30 IU of adsorbed diphtheria toxoid and 40 IU of adsorbed tetanus toxoid.

The vaccine complies with the requirements stated under Vaccines, with the following modifications.

Description Light colour suspension, free from evident clumps after shaking.

Strengths available 30 IU of adsorbed diphtheria toxoid and 40 IU of adsorbed tetanus toxoid per 0.5 ml.

Dose *Intramuscular*, preferably into the deltoid or the midlateral muscles of the thigh, 0.5 ml.

Contra-indication

1. It is contra-indicated in individuals hypersensitive to any ingredient in the formulations.
2. It is contra-indicated in individuals who have had immediate anaphylactic reaction to a previous dose.
3. It is not for subcutaneous or intravenous administration.
4. It is not given for adults and children 7 years of age and older.

Warning

1. It should be used with caution to individuals with thrombocytopenia or any coagulation disorder that would contra-indicate intramuscular injection unless the potential benefits outweigh the risk of administration.
2. It may cause mild to moderate local reactions at the injection site, including tenderness, erythema, and induration, which may persist for several days.
3. Fever, chills, malaise, fatigue, arthralgias or generalized aches and pains, nausea and vomiting, erythema multiforme or other rash, flushing, generalized urticaria or pruritus, tachycardia, dizziness, and hypotension may occur.
4. Children receiving immunosuppresive therapy, including radiation, corticosteroids, antimetabolites, alkylating agents, and cytotoxic drugs, or with other immunodeficiencies may have diminished antibody response to active immunization.
5. It may be administered simultaneously at a separate site with *H. influenzae* type b (Hib) conjugate vaccines, hepatitis B vaccine, poliovirus vaccine inactivated (IPV), measles virus vaccine live, mumps virus vaccine live, and/or rubella virus vaccine live.

Additional information

1. Before additional dose of Adsorbed Diphtheria and Tetanus Vaccine, the health status of the patient should be assessed. In addition, information should be obtained regarding any symptom and/or sign of an adverse reaction that occurred after the previous dose.
2. Routine immunization of children should be deferred during an outbreak of poliomyelitis in the community, unless there is also an outbreak of diphtheria. In either case, emergency tetanus prophylaxis for wounds should be administered as usual.
3. Children with impaired immune response may be immunized, but may have reduced antibody response to Adsorbed Diphtheria and Tetanus Vaccine. Children infected with human immunodeficiency virus (HIV) including those who are immunosuppressed in association with acquired immunodeficiency syndrome (AIDS) or other clinical manifestations of HIV infection may receive Adsorbed Diphtheria and Tetanus Vaccine whether they have asymptomatic or symptomatic HIV infection.
4. Diphtheria infection may not (and tetanus infection does not) confer immunity; therefore, initiation or completion of active immunization with Adsorbed Diphtheria and Tetanus Vaccine is indicated at the time of recovery from either of these infections.
5. Interruption of the recommended schedule for the primary immunizing series of Adsorbed Diphtheria and Tetanus Vaccine by a delay between doses does not interfere with the final immunity achieved and does not necessitate starting the series over again, regardless of the length of time that elapsed between doses.

Expiration date When stored under the prescribed conditions, the expiration date is not later than 2 years from the date of the last satisfactory test for potency.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition the label on the container states (1) the number of IU in each single human dose; (2) where applicable, that the vaccine is intended for primary vaccination of children; (3) the name and the amount of the adjuvant(s); (4) that it is not to be frozen; (5) that it must be well shaken before use.

Identification Complies with the test for Identification of *Diphtheria Vaccine, Adsorbed*, p. 245, and of *Tetanus Vaccine, Adsorbed*, p. 273.

Antimicrobial preservative Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not more than 115 per cent of the quantity stated on the label.

Phenol Phenol should not be added to Adsorbed Diphtheria and Tetanus Vaccine, since it has been shown to have deleterious effects on antigenic properties of the toxoids.

Specific toxicity Complies with the test for Specific toxicity described under *Diphtheria Vaccine, Adsorbed*, p. 245, and under *Tetanus Vaccine, Adsorbed*, p. 273. For these two tests, the same animals are used and are observed for 6 weeks in order to cover the observation period specified for diphtheria.

Assay

A. Carry out the "Biological Assay of Adsorbed Diphtheria Vaccine" (Appendix 15.3.1). The lower confidence limit ($P = 0.95$) of the estimated potency is not less than 30 IU per single human dose (0.5 ml).

B. Carry out the "Biological Assay of Adsorbed Tetanus Vaccine" (Appendix 15.3.3). The lower confidence limit ($P = 0.95$) of the estimated potency is not less than 40 IU per single human dose (0.5 ml).

DIPHTHERIA, TETANUS AND PERTUSSIS VACCINE, ADSORBED

Diphtheria and Tetanus Toxoids and Pertussis Vaccine Adsorbed; Adsorbed Diphtheria, Tetanus and Whooping-cough Prophylactic; DTP Vaccine; DTwP Vaccine

Category Active immunizing agent.

Adsorbed Diphtheria, Tetanus and Pertussis Vaccine is a sterile suspension of purified and adsorbed diphtheria and tetanus toxoids and killed *Bordetella pertussis* of one or more strains selected for high antigenic efficiency. The diphtheria toxoid and tetanus toxoid are prepared from toxins produced by the growth in suitable media of *Corynebacterium diphtheriae* and *Clostridium tetani*, respectively, by treatment with formaldehyde. Each 0.5 ml contains not less than 30 IU of adsorbed diphtheria toxoid, 40 IU of adsorbed tetanus toxoid and 4 IU of adsorbed pertussis vaccine.

The vaccine complies with requirements stated under Vaccines, with the following modifications.

Description Whitish turbid suspension, free from evident clumps after shaking.

Strength available 30 IU of adsorbed diphtheria toxoid, 40 IU of adsorbed tetanus toxoid and 4 IU of adsorbed pertussis vaccine per 0.5 ml.

Dose *Intramuscular* or *deep subcutaneous*, 0.5 ml.

Warning

1. It should not be used in persons over 6 years of age.
2. It should not be used in children who had convulsions or brain disorders (encephalopathy) within three days of a previous dose of vaccine.
3. High fever, persistent crying, collapse, or convulsions may occur.
4. It may cause prolonged seizures.
5. Risk-benefit should be considered if it is to be used in infants and children with underlying neurologic disorders.

Additional information

1. Children with a personal or close family history of epilepsy may also be at increased risk of seizures after vaccination.
2. Children experiencing a seizure during the course of immunization should be carefully assessed before deciding whether to continue immunization with the vaccine.
3. The first dose of the vaccine should be administered at 2 months of age.
4. The decision about whether to give the vaccine to infants and children with underlying neurologic disorders can be difficult and must be made on an individual basis after careful and continuing consideration of the risks and benefits.

Expiration date When stored under the prescribed conditions, the expiration date is not later than 2 years from the date of the last satisfactory test for potency.

Labelling Complies with the labelling described under the "General Information for Biological Products", p. 177. In addition the label on the container states (1) the number of IU of each component per single human dose; (2) the name and the amount of the adjuvant(s); (3) that it is not to be frozen; (4) that it must be well shaken before use; (5) the vaccine is intended for vaccination of children under 6 years of age.

Identification Complies with the tests for Identification of *Diphtheria Vaccine, Adsorbed*, p. 245, of *Tetanus Vaccine,*

Adsorbed, p. 273, and of *Pertussis Vaccine, Adsorbed*, p. 262.

pH 6.0 to 7.0 (Appendix 4.11).

Antimicrobial preservative Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not more than 115 per cent of the quantity stated on the label.

Phenol Phenol should not be added to Adsorbed Diphtheria, Pertussis and Tetanus Vaccine, since it has been shown to have deleterious effects on antigenic properties of the vaccine.

Specific toxicity Complies with the test for Specific toxicity described under *Diphtheria Vaccine, Adsorbed*, p. 245, *Tetanus Vaccine, Adsorbed*, p. 273, and of *Pertussis Vaccine, Adsorbed*, p. 262. For Diphtheria Vaccine, Adsorbed and Tetanus Vaccine, Adsorbed, the same animals are used and are observed for 6 weeks in order to cover the observation period specified for diphtheria.

Assay

A. Carry out the "Biological Assay of Adsorbed Diphtheria Vaccine" (Appendix 15.3.1). The lower confidence limit ($P = 0.95$) of the estimated potency is not less than 30 IU per single human dose (0.5 ml).

B. Carry out one of the prescribed methods in the "Biological Assay of Adsorbed Tetanus Vaccine" (Appendix 15.3.3).

If the test is carried out in guinea-pigs (Method A), the lower confidence limit ($P = 0.95$) of the estimated potency is not less than 40 IU per single human dose (0.5 ml). If the tests is carried out in mice (Method B), the lower confidence limit ($P = 0.95$) of the estimated potency is not less than 60 IU per single human dose (0.5 ml) due to whole-cell pertussis component.

C. Carry out the "Biological Assay of Adsorbed Pertussis Vaccine" (Appendix 15.3.2). The estimated potency is not less than 4 IU per single human dose (0.5 ml) and the lower confidence limit ($P = 0.95$) of the estimated potency is not less than 2 IU per single human dose.

DIPHTHERIA, TETANUS AND PERTUSSIS (ACELLULAR COMPONENT) VACCINE, ADSORBED

Adsorbed Diphtheria, Tetanus and Pertussis (Acellular Component) Vaccine; DTaP Vaccine; Acellular DTP

Category Active immunizing agent.

Adsorbed Diphtheria, Tetanus and Pertussis (Acellular Component) Vaccine is a sterile suspension of purified and adsorbed diphtheria and tetanus toxoids and individually purified antigenic components of *Bordetella pertussis*, which are adsorbed onto suitable adjuvant(s), such as aluminium hydroxide or aluminium phosphate.

The diphtheria toxoid and tetanus toxoid are prepared from toxins produced by the growth in suitable media of *Corynebacterium diphtheriae* and *Clostridium tetani*, respectively, by treatment with formaldehyde.

The vaccine contains either pertussis toxoid or a pertussis-toxin-like protein free from toxic properties, produced by expression of a genetically modified form of the corresponding gene. Pertussis toxoid is prepared from pertussis toxin by a method that renders the latter harmless while maintaining adequate immunogenic properties and avoiding reversion to toxin. The vaccine may also contain filamentous haemagglutinin, pertactin (a 69 kDa outer-membrane protein) and other defined components of *B. pertussis*, such as fimbrial-2 and fimbrial-3 antigens. The latter 2 antigens may be copurified. The antigenic composition and characteristics are based on evidence of protection and freedom from unexpected reactions in the target group for which the vaccine is intended.

The vaccine complies with the requirements stated under Vaccine, with the following modifications.

Description Turbid liquid, white deposit and colourless or white opalescent liquid.

Strength available 30 IU of adsorbed diphtheria toxoid, 40 IU of adsorbed tetanus toxoid, 5 to 25 µg of pertussis toxin (PT), 2.5 to 25 µg of filamentous hemagglutinin (FHA), and may also contain 2.5 to 8 µg of pertactin, with or without 5 µg of fimbrial-2 and fimbrial-3 antigens per 0.5 ml.

Dose *Intramuscular*, at the anterolateral aspect of the thigh (for children under 1 year of age) or the deltoid muscle of the upper arm (for older children) is preferred, 0.5 ml.

Warning See under *Diphtheria, Tetanus and Pertussis Vaccine, Adsorbed*, p. 275.

Additional information Adsorbed Diphtheria, Tetanus and Pertussis (Acellular Component) Vaccine has been developed to reduce the frequency and severity of both local and systemic adverse reactions associated with Adsorbed Diphtheria, Tetanus and Pertussis Vaccine.

See also under *Diphtheria, Tetanus and Pertussis Vaccine, Adsorbed*, p. 275.

Expiration date When stored under the prescribed conditions, the expiration date is not later than 3 years from the date of the last satisfactory test for potency.

Labelling Complies with the labelling described under the "General Information for Biological Products", p.177. In addition the label on the container states (1) the minimum number of IU of diphtheria and tetanus toxoid per single human dose; (2) the names and amounts of the pertussis components per single human dose; (3) the name and the amount of the adjuvant(s); (4) that it is not to be frozen; (5) that it must be well shaken before use; (6) where applicable, that the vaccine is intended for primary vaccination of children and is not necessarily suitable for reinforcing doses or for administration to adults; (7) where applicable, that the vaccine contains a pertussis toxin-like protein produced by genetic modification.

Identification Complies with the tests for Identification of *Diphtheria Vaccine, Adsorbed*, p. 245, of *Tetanus Vaccine, Adsorbed*, p. 273, and of *Pertussis Vaccine (Acellular Component), Adsorbed*, p. 263.

pH 5.8 to 7.2 (Appendix 4.11).

Absence of residual pertussis toxin and irreversibility of pertussis toxoid

(**Note** This test is not necessary for the product obtained by genetic modification.) Use three groups each of not less than five histamine-sensitive mice. Inject intraperitoneally into the first group twice the single human dose of the vaccine stored at 2° to 8°. Inject intraperitoneally into the second group twice the single human dose of the vaccine incubated at 37° for 4 weeks. Inject diluent into the third group of mice. After 5 days, inject into each mouse 2 mg of histamine base intraperitoneally in a volume not exceeding 0.5 ml and observe for 24 hours. The test is invalid if one or more control mice die following histamine challenge. The vaccine complies with the test if no animal in the first or second group dies following histamine challenge. If one mouse dies in either or both of the first and second groups, the test may be repeated with the same number of mice or with a greater number and the results of valid tests combined; the vaccine complies with the test if, in both of the groups given the vaccine, not more than 5 per cent of the total number of mice die following histamine challenge.

The histamine sensitivity of the strain of mice used is verified at suitable intervals as follows: inject intravenously threefold dilutions of a reference pertussis toxin preparation in phosphate-buffered saline solution containing 0.2 per cent w/v of *gelatin* and challenge with histamine as above; the strain is suitable if more than 50 per cent of the animals are sensitized by 50 ng of pertussis toxin and none of the control animals injected with only diluent and challenged similarly with histamine show symptoms of sensitization.

Antimicrobial preservative Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The content is not less than the minimum amount shown to be effective and is not more than 115 per cent of the quantity stated on the label.

Specific toxicity Complies with the test for Specific toxicity described under *Diphtheria Vaccine, Adsorbed*, p. 245, and *Tetanus Vaccine, Adsorbed*, p. 273.

Assay

Diphtheria component Carry out the "Biological Assay of Adsorbed Diphtheria Vaccine" (Appendix 15.3.1). The lower confidence limit ($P = 0.95$) of the estimated potency is not less than the minimum potency stated on the label. Unless otherwise justified and authorized, the minimum potency stated on the label is 30 IU per single human dose (0.5 ml).

Tetanus component Carry out the "Biological Assay of Adsorbed Tetanus Vaccine" (Appendix 15.3.3). The lower confidence limit ($P = 0.95$) of the estimated potency is not less than 40 IU per single human dose (0.5 ml).

Pertussis component Complies with the Assay under *Pertussis Vaccine (Acellular Component), Adsorbed*, p. 264.

TETANUS AND DIPHTHERIA VACCINE FOR ADULT USE, ADSORBED

Adsorbed Diphtheria and Tetanus Vaccine for Adults and Aldolescents; Tetanus and Diphtheria Toxoids Adsorbed for Adults Use; dT Vaccine; Td Vaccine

Category Active immunizing agent.

Adsorbed Tetanus and Diphtheria Vaccine for Adult Use is a sterile suspension of suitable adjuvant(s) such as aluminium hydroxide, aluminium phosphate, onto which purified diphtheria toxoid and tetanus toxoid are adsorbed. The diphtheria toxoid and tetanus toxoid are prepared from diphtheria toxin and tetanus toxin produced by the growth in suitable media of *Corynebacterium diphtheriae* and *Clostridium tetani*, respectively, by treatment with formaldehyde. Each 0.5 ml contains not less than 2 IU of adsorbed diphtheria toxoid and 20 IU of adsorbed tetanus toxoid.

The vaccine complies with the requirements stated under Vaccines, with the following modifications.

Description Light colour suspension, free from evident clumps after shaking.

Strength available 20 IU of adsorbed tetanus toxoid and 2 IU of adsorbed diphtheria toxoid per 0.5 ml.

Dose *Intramuscular*, preferably into the deltoid or the midlateral muscles of the thigh, 0.5 ml.

Contra-indication

1. It is contra-indicated in individuals hypersensitive to any ingredient in the formulations.
2. It is contra-indicated in individuals who have had immediate anaphylactic reaction to a previous dose.
3. It is not for subcutaneous or intravenous administration.

Warning

1. Patients who experienced severe Arthus-type hypersensitivity reaction following a prior dose of tetanus toxoid usually have high serum tetanus antitoxin levels and should not be given even emergency doses of Adsorbed Tetanus and Diphtheria Vaccine for Adult Use more frequently than every 10 years, even if they have a wound that is neither clean nor minor.
2. It should be used with caution to individuals with thrombocytopenia or any coagulation disorder that would contra-indicate intramuscular injection unless the potential benefits outweigh the risk of administration.
3. It may cause mild to moderate local reactions at the injection site, including tenderness, erythema, and induration, which may persist for several days.
4. Fever, chills, malaise, fatigue, arthralgias or generalized aches and pains, nausea and vomiting, erythema multiforme or other rash, flushing, generalized urticaria or pruritus, tachycardia, dizziness, and hypotension may occur.
5. Patients receiving immunosuppresive therapy, including radiation, corticosteroids, antimetabolites, alkylating agents, and cytotoxic drugs, or with other immunodeficiencies may have diminished antibody response to active immunization.
6. It may be administered simultaneously at a separate site with *H. influenzae* type b (Hib) conjugate vaccines, hepatitis B vaccine, poliovirus vaccine inactivated (IPV), measles virus vaccine live, mumps virus vaccine live, and/or rubella virus vaccine live.

Additional information

1. The concentration of diphtheria toxoid in Adsorbed Tetanus and Diphtheria Vaccine for Adult Use, which is intended for use in patients 7 years of age and older, is lower than that of the concentration of diphtheria toxoid in Adsorbed Diphtheria and Tetanus Vaccine.
2. It is recommended that adults and children 7 years of age and older receive Adsorbed Tetanus and Diphtheria Vaccine for Adult Use rather than the single-entity tetanus toxoid for the primary immunizing series, all booster doses, and active tetanus immunization in wound management. This is to help ensure protection against diphtheria infection, since a large proportion of adults is susceptible to diphtheria infection.
3. The primary immunizing series of Adsorbed Tetanus and Diphtheria Vaccine for Adult Use consists of 3 doses (2 initial and reinforcing) for adults and children 7 years of age and older.
4. More frequent administration of Adsorbed Tetanus and Diphtheria Vaccine for Adult Use is not recommended except under circumstances of wound management or diphtheria prophylaxis since it may be associated with increased incidence and severity of adverse reactions.

Expiration date When stored under the prescribed conditions, the expiration date is not later than 2 years from the date of the last satisfactory test for potency.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition the label on the container states (1) the number of IU in each single human dose; (2) the name and the amount of the adjuvant(s); (3) that it is not to be frozen; (4) that it must be well shaken before use.

Identification Complies with the test for Identification of *Tetanus Vaccine, Adsorbed*, p. 273, and of *Diphtheria Vaccine, Adsorbed*, p. 245.

pH 6.0 to 7.0 (Appendix 4.11).

Antimicrobial preservative Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not more than 115 per cent of the quantity stated on the label.

Phenol Phenol should not be added to Adsorbed Tetanus and Diphtheria Vaccine for Adult Use, since it has been shown to have deleterious effects on antigenic properties of the toxoids.

Specific toxicity Complies with the test for Specific toxicity described under *Diphtheria Vaccine, Adsorbed*, p. 245, and under *Tetanus Vaccine, Adsorbed*, p. 273. For these two tests, the same animals are used and are observed for 6 weeks in order to cover the observation period specified for diphtheria.

Assay

A. Carry out the "Biological Assay of Adsorbed Diphtheria Vaccine" (Appendix 15.3.1). The lower confidence limit ($P = 0.95$) of the estimated potency is not less than 2 IU per single human dose (0.5 ml).

B. Carry out the "Biological Assay of Adsorbed Tetanus Vaccine" (Appendix 15.3.3). The lower confidence limit ($P = 0.95$) of the estimated potency is not less than 20 IU per single human dose (0.5 ml).

TYPHOID POLYSACCHARIDE VACCINE

Category Active immunizing agent.

Typhoid Polysaccharide Vaccine is a preparation of purified capsular Vi polysaccharide obtained from *Salmonella typhi* Ty 2 strain or some other suitable strain that has the capacity to produce Vi polysaccharide.

Capsular Vi polysaccharide consists of partly 3-*O*-acetylated repeated units of 2-acetylamino-2-deoxy-D-galactopyranuronic acid with α-(1 → 4) linkages.

The vaccine complies with the requirements stated under Vaccines, with the following modification.

Description Clear, colourless liquid, free from visible particles.

Strength available 25 μg of the Vi polysaccharide of *Salmonella typhi* per 0.5 ml.

Dose Adults and children 2 years of age and over: *Intramuscular* or *subcutaneous*, 0.5 ml.

Contra-indication It is not for intravenous administration.

Warning

1. It may cause pain, tenderness, erythema and induration at the injection site.

2. Fever, malaise, headache, myalgia, nausea, diarrhea, feverishness or vomiting may occur.

Expiration date When stored under the prescribed conditions, the expiration date is not later than 3 years after the date of the last satisfactory tests.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition the label on the container states (1) the number of micrograms of polysaccharide per human dose; (2) the total quantity of polysaccharide in the container.

Identification Carry out an identification test using a suitable immunochemical method (Appendix 14.5).

pH 6.5 to 7.5 (Appendix 4.11).

Content of *O*-acetyl groups Not less than 75 per cent and not more than 125 per cent of 0.085 μmol per dose (25 μg of polysaccharide).

Standard acetylcholine chloride solution Prepare a solution containing 15 mg of *acetylcholine chloride* per ml.

Standard acetylcholine curve Immediately before use, dilute 1.0 ml of Standard acetylcholine chloride solution to 50.0 ml with *water* for working dilution which contains 0.3 mg per ml of *acetylcholine chloride*. From this solution, accurately transfer 0.05, 0.1, 0.2, 0.4, and 0.8 ml, each in duplicate (one for reaction solution and the other for the correction solution) to 10 test-tubes. Dilute with *water* to 1.0 ml and mix. Prepare a blank using 1.0 ml of *water*. Add 1.0 ml of a mixture of 2 volumes of *water* and 1 volume of *hydrochloric acid* to each of the correction tubes and to the blank. Add 2.0 ml of *alkaline hydroxylamine TS* to each tube. Allow the reaction to proceed for exactly 2 minutes and add 1.0 ml of a mixture of 2 volumes of *water* and 1 volume of *hydrochloric acid* to each of the reaction tubes. Add 1.0 ml of a 10 per cent w/v solution of *iron(III) chloride* in 0.1 M *hydrochloric acid* to each tube, stopper the tubes and shake vigorously to remove bubbles.

Measure the absorbances of the acetylcholine chloride-containing solutions relative to the blank at 540 nm (Appendix 2.2). For each reaction solution, subtract the absorbance of the corresponding correction

solution. Draw a calibration curve from the corrected absorbances for the five reference solutions and the corresponding contents of acetylcholine chloride.

Procedure Place 1 ml of the vaccine in each of three tubes (two reaction solutions and one correction solution). Repeat the same procedure as directed under Standard acetylcholine curve beginning with "Prepare a blank...". Measure the absorbances of the resulting solutions and, by reference to the Standard acetylcholine chloride curve, calculate the content of acetylcholine. Read from the curve the content of acetylcholine chloride in the vaccine being examined.

Calculation Calculate the mean of the two values. One mole of acetylcholine chloride (181.66 g) is equivalent to 1 mole of *O*-acetyl (43.05 g).

Antimicrobial preservative Where applicable, determine the amount of antimicrobial preservative by a suitable physicochemical method. The content is not less than the minimum amount shown to be effective and not more than 115 per cent of the content stated on the label.

Assay Determine Vi polysaccharide by a suitable immunochemical method (Appendix 14.5), using a reference purified polysaccharide.

The estimated amount of polysaccharide per dose is not less than 80 per cent and not more than 120 per cent of the content stated on the label. The confidence limits ($P = 0.95$) of the estimated amount of polysaccharide are not less than 80 per cent and not more than 120 per cent.

TYPHOID VACCINE, ORAL

Typhoid (Strain Ty 21a) Vaccine, Live (Oral)

Category Active immunizing agent.

Oral Typhoid Vaccine is a freeze-dried preparation of live *Salmonella typhi* strain Ty 21a grown in a suitable medium.

The vaccine complies with the requirements stated under Vaccines, with the following modification.

Description Dull whitish powder.

Strengths available Two to 6×10^9 CFU of viable *Salmonella typhi* strain Ty 21a per capsule.

Dose Adults and children 6 years of age and older: one capsule.

Contra-indication It is contra-indicated in individuals who are immunosuppressed and in acute gastro-intestinal illness.

Warning

1. Mild gastro-intestinal disturbances or a transitory exanthema may occur in high-risk areas.

2. Caution should be excercised when it is to be used concomitantly with antibacterials or antimalarials.

Additional information Swallow the vaccine capsule about 1 hour before a meal with a cold or lukewarm drink not exceeding 37°. Do not chew the vaccine capsule; swallow as soon as possible.

Expiration date When stored under the prescribed conditions, the expiration date is not later than 1 year from the date of the last satisfactory tests.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition the label on the container states (1) the minimum number of live bacteria per dosage unit; (2) that the vaccine is for oral use only.

Identification Culture bacteria from the vaccine to be examined on an agar medium containing 1 per cent of galactose and bromothymol blue. Light blue, concave colonies, transparent due to lysis of cells, are formed. No yellow colonies (galactose-fermenting) are found.

Microbial purity Carry out the test described under "Microbial Limit Tests" (Appendix 10.2), using suitable selective media. Determine the total aerobic microbial count (TAMC) and total combined yeasts and moulds count (TYMC), using the plate-count method. The number of contaminating micro-organisms per dosage unit is not more than 2×10^2 CFU of TAMC and 20 CFU of TYMC. No pathogenic bacterium, particularly *Escherichia coli, Staphylococcus aureus*, *Pseudomonas aeruginosa,* and no salmonella other than strain Ty 21a are found.

Water 1.5 to 4.0 per cent w/w (Karl Fischer Method, Appendix 4.12), determined on the contents of the capsule.

Number of live bacteria Carry out the test using not fewer than five dosage units of vaccine. Homogenize the contents of the dosage units in *saline TS* at 4° using a mixer in a cold room with sufficient glass beads to emerge from the liquid. Immediately after homogenization prepare a suitable dilution of the suspension using cooled diluent and inoculate on brain heart infusion agar; incubate at 36°±1° for 20 to 36 hours. The vaccine contains not less than 2×10^9 live *S. typhi* Ty 21a bacteria per dosage unit.

Other requirements Complies with the requirements described under "Capsules" (Appendix 1.16).

VARICELLA VACCINE, LIVE

Category Active immunizing agent.

Live Varicella Vaccine is a freeze-dried preparation of a suitable attenuated strain of *Herpesvirus varicellae*, propagated in human diploid cells.

The vaccine, reconstituted as stated on the label, complies with the requirements stated under Vaccines, with the following modifications.

Description The dried vaccine is a white powder or pellet; when reconstituted, it becomes a clear liquid that may be coloured owing to the presence of a pH indicator.

Strengths available Not less than 1000, 1350 or 2000 PFU per 0.5 ml.

Dose *Subcutaneous*, at deltoid region, 0.5 ml.

Contra-indication

1. It is contra-indicated in pregnant women; patients with a history of hypersensitivity reactions to this vaccine or any of its components; patients receiving immunosuppressive therapy; patients with blood dyscrasia, leukemia, lymphoma of any type, or other malignant neoplasms affecting the bone marrow or lymphatic systems; patients with primary or acquired immunodeficiency, or active untreated tuberculosis; patients with a family history of congenital or hereditary immunodeficiency.
2. It is not for intravenous administration.

Warning

1. It may cause pain, redness, soreness, swelling or induration at the injection site.
2. Fever, varicella-like maculopapular and/or papulovesicular rash composed of only a few lesions or vesicles may occur.
3. Risk-benefit should be considered if it is to be used in patients with cardiovascular, kidney, liver or hematological disease.
4. It is not recommended for infants.

Precaution

1. Avoid pregnancy for 3 months following vaccination.
2. Individuals with current thrombocytopenia may develop more severe thrombocytopenia following vaccination.

Additional information The vaccine may not be effective in those receiving blood or gammaglobulin preparation within 3 months before or after vaccination.

Expiration date When stored under the prescribed conditions, the expiration date is not later than 2 years from the date of the last satisfactory test for potency.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition the label on the container states (1) the strain of varicella virus used for the preparation; (2) the type and origin of cells used for the preparation; (3) the nature and quantity of any residual antibiotic present in the vaccine; (4) the minimum virus concentration; (5) that the vaccine is not to be administered to pregnant women; (6) the time within which the vaccine must be used after reconstitution.

Identification When the vaccine reconstituted as stated on the label is mixed with specific *Herpesvirus varicellae* antibodies, it is no longer able to infect susceptible cell cultures.

Bovine serum albumin Not more than 0.5 μg per single human dose. Carry out the "Immunochemical methods" (Appendix 14.5).

Assay Titrate for infective virus in a suitable cell culture, using at least 10 wells of 96 well plate for each fourfold dilution or by a technique of equal precision. The virus titration is carried out in parallel with the National or International Reference Preparation of Live Varicella Vaccine for comparison. The virus concentration is not less than the minimum stated on the label.

YELLOW FEVER VACCINE, LIVE

Category Active immunizing agent.

Live Yellow Fever Vaccine is a freeze-dried preparation of live attenuated 17D strain of yellow fever virus grown in embryonated chicken eggs.

The vaccine, reconstituted as stated on the label, complies with the requirements stated under Vaccines, with the following modifications.

Description Slightly dull, light orange-coloured, flaky or crustlike, desiccated mass. When reconstituted, it is clear or slightly opalescent and light orange liquid.

Strength available Not less than 1×10^3 mouse LD_{50} or its equivalent in PFU per 0.5 ml.

Dose *Subcutaneous*, 0.5 ml.

Contra-indication

1. It is contra-indicated in individuals with a history of anaphylactic hypersensitivity to eggs or chicken protein.

2. It is contra-indicated in infant younger than 4 months of age.

Warning

1. It is not recommended in infants under 6 months of age except in high-risk areas.

2. Risk-benefit should be considered if it is to be administered during pregnancy.

3. Risk-benefit should be considered if it is to be administered in individuals with history of hypersensitivity to eggs or chicken protein.

Expiration date When stored under the prescribed conditions, the expiration date is not later than 2 years after the date of the last satisfactory test for virus titre.

Packaging and storage Yellow Fever Vaccine should preferably be stored at all time at the temperature below 5°, protected from light. Do not freeze.

When reconstituted, the vaccine should be used immediately.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition the label on the container states (1) the strain of virus used in preparation of the vaccine; (2) that the vaccine has been prepared in chick embryos; (3) the minimum virus concentration; (4) that contact with disinfectants is to be avoided; (5) the period of time within which the vaccine is to be used after reconstitution.

Identification When the vaccine is mixed with an appropriate amount of specific yellow fever antiserum, there is a significant reduction in its ability to infect susceptible cell cultures.

Ovalbumin Not more than 5 µg of ovalbumin per human dose, determined by a suitable immunochemical method (Appendix 14.5).

Bacterial endotoxins When tested as described in the "Test for Bacterial Endotoxins" (Appendix 8.5), it contains less than 5 Endotoxin Units per human dose.

Thermal stability Maintain not less than three vials of the vaccine in the dry state at 37° for 14 days. Determine the virus concentration as described under *Assay* in parallel for the heated vaccine and for unheated vaccine. The difference in the virus concentration between unheated and heated vaccine does not exceed 1.0 log and the virus concentration of the heated vaccine is not less than the number of PFU equivalent to 1×10^3 mouse LD_{50} per human dose.

Assay

Titrate for infective virus in cell cultures. Use an appropriate virus reference preparation to validate each assay.

The virus concentration is not less than the equivalent in PFU of 1×10^3 mouse LD_{50} per human dose.

The relationship between mouse LD_{50} and PFU is established by each laboratory and approved by the competent authority.

Before assay, the reconstituted vaccine shall stand at a temperature between 20° and 30° for 20 minutes. Appropriate serial dilutions of the reconstituted vaccine are made in diluent for yellow fever virus which has been demonstrated to be free of yellow fever virus inhibitors.

The method shown below, or another suitable technique, may be used to determine the mouse LD_{50} and the PFU.

MOUSE LD_{50} TECHNIQUE

A strain of mice highly susceptible to yellow fever virus, aged 4 to 6 weeks, are injected intracerebrally with 0.03 ml of vaccine dilution. Groups of at least six mice are used for each dilution. The series of dilutions to be used should result in mortality rates which span from the range of 0 to 100 per cent. Inoculation of the mice should be performed immediately after the dilutions have been made. All deaths are recorded during a period of 21-day observations. Mice dying from unrelated causes are removed from both the numerator and denominator of mortality calculations. Mice paralyzed on day 21 are counted as alive.

The mouse LD_{50} is the quantity of virus suspension estimated to produce fatal, specific encephalitis in 50 per cent of intracerebrally inoculated mice.

CELL-CULTURE TECHNIQUE

Use either Method I or II.

Method I Monolayers of Vero or other suitable cells are prepared in multi-well plates. At least three wells are inoculated with a virus dilution. After incubation for 1 hours at 37°±1°, the virus dilution is replaced by a suitable overlay medium consisting of Leibovitz medium No. 15[1] or Minimum Essential Medium (MEM)[1], 5 per cent of *fetal bovine serum*, and 1.6 per cent of *carboxymethylcellulose* (low viscosity sodium salt) or other suitable medium. The plates are incubated in a 5±1 per cent carbon dioxide incubator at 37°±1° for 5 days. On the sixth day, the plates are drained, washed

[1]Use a commercially available medium.

with *saline TS*, stained with a 1 per cent w/v solution of *naphthalene black* or any other suitable stains and thoroughly rinsed with tap water. The plaques are then counted and virus titre is calculated.

Method I Equal amounts (0.2 ml) of a Vero-cell suspension (approximately 6×10^5 cells/ml) and virus dilution in Leibovitz medium No. 15 or MEM or other suitable medium are placed in each of the 16-mm flat-bottomed wells in sterile trays suitable for cell culture. The trays are sealed and incubated for 4 hours at 37°±1° in a 5±1 per cent carbon dioxide incubator. After incubation, 0.4 ml of overlay medium, consisting of Leibovitz medium No. 15 or MEM medium, 3 per cent of *fetal bovine serum*, and 1.6 per cent of *carboxymethylcellulose* (low viscosity sodium salt), is added to each well. The trays are resealed and incubated at 37°±1° in a 5±1 per cent carbon dioxide incubator for 5 days. On the sixth day, the trays are drained, washed with *saline TS*, stained with a 1 per cent w/v solution of *naphthalene black* or any other suitable stains and thoroughly rinsed with tap water. The plaques are then counted and virus titre is calculated.

ลิขสิทธิ์ กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข
ห้ามทำซ้ำ ดัดแปลง แก้ไข โดยไม่ได้รับอนุญาต

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ห้ามทำซ้ำ ดัดแปลง แก้ไข โดยไม่ได้รับอนุญาต

DIAGNOSTIC PREPARATION

ลิขสิทธิ์ กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข
ห้ามทำซ้ำ ดัดแปลง แก้ไข โดยไม่ได้รับอนุญาต

ลิขสิทธิ์ กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข
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TUBERCULIN PURIFIED PROTEIN DERIVATIVE

Tuberculin PPD

Category Diagnostic preparation.

Tuberculin Purified Protein Derivative (Tuberculin PPD) for human use is a sterile liquid or freeze-dried preparation obtained by precipitation from the heated products of the culture and lysis of *Mycobacterium bovis* and/or *Mycobacterium tuberculosis* and capable of demonstrating a delayed hypersensitivity in an animal sensitized to micro-organisms of the same species.

Description Liquid Tuberculin PPD is a clear colourless or pale-yellow liquid.

Freeze-dried Tuberculin PPD, when reconstituted, becomes a colourless or pale-yellow liquid.

Strength available It contains the PPD equivalent to 5 IU of PPD-S[1] per 0.1 ml.

Dose *Intradermal*, 0.1 ml.

Contra-indication It is not for intravenous, intramuscular or subcutaneous administration.

Warning

1. It should not be administered to persons with severe blistering tuberculin reactions in the past, persons with documented active tuberculosis or a clear history of treatment for tuberculosis infection or disease, and persons with extensive burns or eczema.
2. It may cause vesiculation, ulceration, necrosis, pain, or pruritus at the test site in some tuberculin-sensitive individuals.
3. Nausea, headache, dizziness, malaise, rash, urticaria, edema, and pyrexia may occur.

Additional information To ensure accurate results, Tuberculin PPD should be administered before, simultaneously with, or 4 to 6 weeks or longer after administration of live viral vaccines.

Expiration date When stored under the prescribed conditions, the expiration date is not later than 6 months for the liquid form, and not later than 2 years for the freeze-dried form, from the date of the last satisfactory test for potency.

[1]The first international standard preparation for Purified Protein Derivative (PPD) of *M. tuberculosis* (human strain) tuberculin, established in 1951 for WHO, by the Henry Phipps Institute, Philadelphia, Pa., USA.

Packaging and storage Tuberculin PPD shall be stored at a temperature of 2° to 8°, protected from light; avoid freezing.

Labelling Complies with the "General Information for Biological Products", p. 177. In addition, the label on the container states (1) the number of International Units or its equivalent per container; (2) the species of mycobacteria used to prepare the product; (3) the name andquantity of any antimicrobial preservative or other substances added to the preparation; (4) for freeze-dried products, a statement that the product is to be reconstituted using the liquid provided by the manufacturer. (**Note** If the package does not contain a leaflet warning that the inhalation of concentrated tuberculin PPD may produce toxic effects, this warning must be shown on the label on the container together with a statement that the powder must be handled with care.)

Identification The assay may serve as identification.

pH 6.5 to 7.5 (Appendix 4.11).

Sterility Complies with the "Sterility Test" (Appendix 10.1).

Live mycobacteria This test may be omitted if it has been carried out on concentrated bulk.

Carry out the following methods for live mycobacteria.

Animal inoculation method: Inject 5.0 ml intraperitoneally or subcutaneously into each of two guinea-pigs weighing 300 to 400 g. Observe the animals for not less than 42 days. Kill the animals and carry out an autopsy. If no guinea-pig shows sign of infection with mycobacteria, the preparation complies with the test.

Culture method: If the sample to be examined may be contaminated by micro-organisms other than mycobacteria, treat it with a suitable decontamination solution, such as acetylcysteine-sodium hydroxide solution or sodium laurylsulfate solution. Inoculate 0.2 ml of the sample in triplicate onto each of two suitable solid media (Löwenstein-Jensen medium and Middlebrook 7H 10 medium are considered suitable). Inoculate 0.5 ml in triplicate into a suitable liquid medium. Incubate all media at 37° for 56 days.

The growth promotion of the media in the presence of the preparation shall be examined by inoculation of a suitable strain of *Mycobacterium* sp. and if necessary use a suitable neutralizing substance.

If contaminating micro-organisms develop during the first 8 days of incubation, repeat the test and carry out at the same time a bacteriological sterility test.

If at the end of the incubation time no growth of mycobacteria occurs in any of the test media, the preparation complies with the test.

Antimicrobial preservative Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The content is not less than the minimum amount shown to be effective and not more than 115 per cent of the amount stated on the label. If phenol has been used in the preparation, the concentration is not more than 0.5 per cent w/v when determined by the test described under the "Determination of Phenol", p. 179.

Sensitizing effect of PPD This test may be omitted if it has been carried out on concentrated bulk. Use three guinea-pigs that have not been subjected to any treatment likely to interfere with the test. On three occasions at intervals of 5 days, inject intradermally into each guinea-pig about 500 IU of the preparation to be examined in a volume of 0.1 ml. Two to three weeks after the third injection, administer the same dose intradermally to the same animals and to a control group of three guinea-pigs of the same weight that have not previously received injections of tuberculin. After 24 to 72 hours, the reactions in the two groups of animals are not substantially different.

Assay The potency of tuberculin PPD is determined by comparing the reactions produced by the intradermal injection of increasing doses of the preparation to be examined into sensitized guinea-pigs with the reactions produced by known concentrations of the reference preparation.

Test animals Sensitize not less than six white or albino guinea-pigs weighing not less than 300 g.

Methods of sensitization Inject intradermally, four sites at one time, each of 0.1 ml of suspension containing a suitable amount (0.1 to 0.4 mg per ml) of heat-killed, dried tubercle bacilli in liquid paraffin. Alternatively, 0.1 ml of an aqueous suspension of 0.5 mg of BCG vaccine per ml can be used instead of heat-killed, dried tubercle bacilli.

Carry out the test after the period of time required for optimal sensitization which is usually 4 to 8 weeks, but not later than 6 months after sensitization.

Procedure Depilate the flanks of the animals so that it is possible to make at least three injections on each side but not more than a total of 12 injection points per animal.

Prepare dilutions of the preparation to be examined and of the reference preparation using *phosphate-buffered saline (pH 6.5 to 7.5)* containing 50 mg per litre of *polysorbate 80*. If the preparation to be examined is freeze-dried and does not contain a stabilizer, reconstitute it using the liquid described above. Use at least three different doses of the reference preparation and at least three different doses of the preparation to be examined. For both preparations, use doses such that the highest dose is about 10 times the lowest dose. Choose the doses such that when they are injected the lesions produced have a diameter of not less than 8 mm and not more than 25 mm. In any given test, the order of the dilutions injected at each point is chosen at random in a Latin square design. Inject each dose intradermally in a constant volume of 0.1 ml or 0.2 ml. Measure the diameters of the lesions 24 to 48 hours later and calculate the results of the test by the statistical methods, assuming that the diameters of the lesions are directly proportional to the logarithm of the concentration of the preparation.

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P = 0.95$) are not less than 64 per cent and not more than 156 per cent of the stated potency.

If the preparation fails to meet the requirement for potency, it shall be discarded, or the assay shall be repeated by the same method using a different group of sensitized animals. The estimates of potency and confidence limits shall be made using the results of all the assays. The preparation is of acceptance potency if the combined results of the assays meet the criteria specified above.

APPENDICES

ลิขสิทธิ์ กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข
ห้ามทำซ้ำ ดัดแปลง แก้ไข โดยไม่ได้รับอนุญาต

ลิขสิทธิ์ กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข
ห้ามทำซ้ำ ดัดแปลง แก้ไข โดยไม่ได้รับอนุญาต

Content of the Appendices

ลิขสิทธิ์ กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข
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APPENDIX 1 GENERAL INFORMATION

The specifications given below are strictly for the use of the materials as reagents. The inclusion of a material in this Appendix does not imply that it is suitable for use in medicines. Exceptionally, a trademark or supplier may be indicated for certain reagents whose availability is limited. It is however acceptable to use reagents from another source provided that they comply with the standards of the Pharmacopoeia.

1.1 REAGENTS

The name of a substance or solution indicates a reagent included in the following list. The specifications given for reagents do not necessarily guarantee their quality for use in medicines.

Some of the reagents included may be injurious to health. Important cautions have been stated for these reagents. They should be handled in accordance with good laboratory practice and any relevant regulations.

Reagents in aqueous solution are prepared using *water*. Reagent solutions used in the limit tests for barium, calcium and sulfates are prepared using *distilled water*. Where the name of the solvent is not stated, an aqueous solution is intended.

Unless otherwise specified, the reagents and reagent solutions are to be stored in well-closed containers. The labelling should comply with the relevant national legislation.

Acetic Acid To *glacial acetic acid*, add sufficient *water* to produce a solution containing 33 per cent w/w of $C_2H_4O_2$.

Acetic Acid, Dilute To *glacial acetic acid*, add sufficient *water* to produce a solution containing 6 per cent w/w of $C_2H_4O_2$.

Acetic Acid, Glacial Use Glacial Acetic Acid (TP monograph).

Acetic Acid, Glacial, Anhydrous $C_2H_4O_2$ = 60.05
Use Glacial Acetic Acid (TP monograph).

Acetic Anhydride $C_4H_6O_3$ = 102.09

Use analytical reagent grade of commerce containing not less than 97.0 per cent w/v of $C_4H_6O_3$.

DESCRIPTION Colourless liquid.

BOILING RANGE 136° to 142° (Appendix 4.5).

Acetylacetone (Diacetylmethane) $C_5H_8O_2$ = 100.12

Use analytical reagent grade of commerce.

DESCRIPTION Colourless or slightly yellow, easily flammable liquid.

SOLUBILITY Soluble in *water*; miscible with *acetone*, with *chloroform*, with *ethanol*, with *ether*, and with *glacial acetic acid*.

BOILING RANGE 138° to 140° (Appendix 4.5).

REFRACTIVE INDEX 1.452 to 1.453, at 20° (Appendix 4.7).

***N*-Acetylneuraminic Acid** (*O*-Sialic Acid) $C_{11}H_{19}NO_9$ = 309.27

DESCRIPTION White, acicular crystals.

SOLUBILITY Soluble in *water* and in *methanol*; slightly soluble in *ethanol*; practically insoluble in *acetone*, in *chloroform* and in *ether*.

MELTING TEMPERATURE About 186°, with decomposition (Appendix 4.3).

SPECIFIC ROTATION About –36°, at 20°, determined in a 1 per cent w/v solution (Appendix 4.8).

Acid Blue 83 (Coomassie Brilliant Blue R250; Brilliant Blue R) $C_{45}H_{44}N_3NaO_7S_2$ = 826.07

Use general reagent grade of commerce.

DESCRIPTION Brown powder.

Acrylamide C_3H_5NO = 71.08

Use general reagent grade of commerce.

MELTING TEMPERATURE About 84° (Appendix 4.3).

Agar The dried extract from *Gelidium* sp. and other algae belonging to the class Rhodophyceae.

Use microbiological reagent grade of commerce.

DESCRIPTION White, light yellowish orange, or yellowish grey to pale yellow; rectangular columns, strings, strips, flakes, or granules; semitranslucent and somewhat lustrous; almost odourless; tasteless and mucilaginous. A 1 per cent w/v boiling solution of *agar* is neutral.

SOLUBILITY Soluble in boiling *water*; insoluble in cold *water* and in organic solvents.

(**Note** When used for microbiological purposes, it is to be dried to a water content of not more than 20 per cent w/w.)

Agarose for Chromatography, Cross-linked

Use chromatographic reagent grade of commerce.

Prepared from agarose by reaction with 2,3-dibromopropanol in strongly alkaline conditions.

Swollen beads 60 to 140 µm in diameter presented as a 4 per cent suspension in water. It is used in size-exclusion chromatography for the separation of proteins with relative molecular weights of 6×10^4 to 20×10^6 and of polysaccharides with relative molecular weights of 3×10^3 to 5×10^6.

Agarose for Chromatography 1, Cross-linked

Use chromatographic reagent grade of commerce.

Prepared from agarose by reaction with 2,3-dibromopropanol in strongly alkaline conditions.

Swollen beads 60 to 140 µm in diameter presented as a 4 per cent suspension in water. It is used in size-exclusion chromatography for the separation of proteins

with relative molecular weights of 7×10^4 to 40×10^6 and of polysaccharides with relative molecular weights of 1×10^5 to 2×10^7.

Albumin, Bovine Serum Bovine Serum Albumin (Cohn fraction V) containing about 96 per cent w/w of protein, that has been shown to be pyrogen-free and also shown to be free from proteolytic activity by a suitable means, for example, using *chromogenic substrate*.

DESCRIPTION White to light tan powder.

WATER Not more than 3.0 per cent w/w (Karl Fischer Method, Appendix 4.12).

Store at a temperature between 2° and 8°.

Albumin, Human Human serum albumin containing not less than 96 per cent of albumin.

Alizarin Complexone Dihydrate (Alizarin Fluorine Blue) $C_{19}H_{15}NO_8.2H_2O$ = 421.36

DESCRIPTION Fine, ochre to orange-brown powder.

MELTING TEMPERATURE About 185° (Appendix 4.3).

LOSS ON DRYING Not more than 10.0 per cent w/w after drying at 105° to constant weight (Appendix 4.15). Use 1 g.

Aluminium Nitrate $Al(NO_3)_3.9H_2O$ = 375.13

Use analytical reagent grade of commerce.

DESCRIPTION Deliquescent crystals.

SOLUBILITY Very soluble in *water* and in *ethanol*; very slightly soluble in *acetone*; practically insoluble in *ethyl acetate* and in *pyridine*.

Store in tightly closed containers.

Aluminium Oxide, Activated Acid Al_2O_3 = 101.96

Activated by heating at 200° to 250° for 3 hours. Mean particle size, 50 to 200 μm.

DESCRIPTION Almost white, fine, granular powder. Very hygroscopic.

Aluminium Potassium Sulfate (Potash Alum; Alum) $KAl(SO_4)_2.12H_2O$ = 474.38

Use analytical reagent grade of commerce.

DESCRIPTION Colourless, transparent crystalline masses or granular powder; odourless.

SOLUBILITY Soluble in 7.5 parts of *water*, in 0.3 part of boiling *water* and in 3 parts of *glycerol*; practically insoluble in *ethanol*.

4-Aminophenazone (Aminopyrazolone; 4-Amino-antipyrine) $C_{11}H_{13}N_3O$ = 203.3

Use general reagent grade of commerce.

DESCRIPTION Light yellow needles or powder.

MELTING TEMPERATURE About 108° (Appendix 4.3).

Ammonia NH_3 = 17.03

For 18 M and 13.5 M *ammonia* use analytical reagent grade solutions of commerce containing 35 per cent and 25 per cent w/w of NH_3, and weighing 0.88 g and 0.91 g per ml, respectively.

Solutions of molarity xM should be prepared by diluting 75x ml of 13.5 M *ammonia* or 56x ml of 18 M *ammonia* to 1000 ml with *water*.

When *ammonia* is specified and the strength is not stated, use a reagent prepared by diluting 67 g of 13.5 M *ammonia* to 100 ml with *water*.

Ammonia Solution, Dilute Use *Ammonia TS*.

Ammonia Solution, Strong Use Strong Ammonia Solution (TP monograph).

Ammonium Acetate $C_2H_7NO_2$ = 77.08

Use analytical reagent grade of commerce.

Store in well-closed containers.

Ammonium Chloride NH_4Cl = 53.49

Use analytical reagent grade of commerce.

DESCRIPTION Colourless crystals or white, fine or coarse, crystalline powder; odourless.

SOLUBILITY Freely soluble in *water* and in *glycerol* and even more soluble in boiling *water*; sparingly soluble in *ethanol*.

Ammonium Dihydrogenphosphate (Ammonium Phosphate, Monobasic) $(NH_4)H_2PO_4$ = 115.03

Use analytical reagent grade of commerce.

pH About 4.2, in a 2.3 per cent w/v solution (Appendix 4.11).

Ammonium Iron(III) Sulfate (Ferric Ammonium Sulfate) $NH_4Fe(SO_4)_2.12H_2O$ = 482.18

Use analytical reagent grade of commerce.

DESCRIPTION Pale violet crystals, or almost colourless, crystalline powder.

SOLUBILITY Soluble in *water*, yielding a clear yellow or brown solution.

Ammonium Metavanadate (Ammonium Vanadate) NH_4VO_3 = 116.98

Use analytical reagent grade of commerce.

DESCRIPTION White, crystalline powder.

SOLUBILITY Slightly soluble in cold *water*; soluble in *ammonia TS* and in hot *water*.

Ammonium Molybdate $(NH_4)_6Mo_7O_{24}.4H_2O$ = 1235.86

Use analytical reagent grade of commerce.

Ammonium Nitrate NH_4NO_3 = 80.04

Use analytical reagent grade of commerce.

Store in tightly closed containers.

Ammonium Oxalate $C_2H_8N_2O_4.H_2O$ = 142.11

Use analytical reagent grade of commerce.

DESCRIPTION Colourless crystals.

SOLUBILITY Soluble in *water*.

Ammonium Peroxydisulfate (Ammonium Persulfate) $(NH_4)_2S_2O_8$ = 228.19

Use analytical reagent grade of commerce.

DESCRIPTION White granular crystals or crystalline powder.

SOLUBILITY Soluble in *water*.

Ammonium Sulfate $(NH_4)_2SO_4$ = 132.13

Use analytical reagent grade of commerce.

Ammonium Thiocyanate NH_4SCN = 76.12

Use analytical reagent grade of commerce.

DESCRIPTION Colourless crystals.

SOLUBILITY Very soluble in *water*; readily soluble in ethanol.

Store in tightly closed containers.

Amyl Alcohol (Isoamyl Alcohol; Isopentyl Alcohol; 3-Methyl-1-butanol) $C_5H_{12}O$ = 88.15

Use general reagent grade of commerce.

DESCRIPTION Clear, colourless liquid; odour, characteristic.

SOLUBILITY Slightly soluble in *water*. Miscible with most organic solvents.

BOILING TEMPERATURE About 130° (Appendix 4.6).

REFRACTIVE INDEX About 1.406, at 20° (Appendix 4.7).

WEIGHT PER MILLILITRE About 0.81 g (Appendix 4.9).

Aniline C_6H_7N = 93.13

DESCRIPTION Colourless or pale yellow, oily liquid, readily discolouring on exposure to air and light.

BOILING RANGE Not less than 95 per cent distils between 182° and 184° (Appendix 4.5).

WEIGHT PER MILLILITRE 1.020 to 1.025 g (Appendix 4.9).

ACID-INSOLUBLE MATTER Dissolve 5 ml in 25 ml of *dilute hydrochloric acid*: the solution is clear or not more than slightly turbid.

Anisaldehyde (4-Methoxybenzaldehyde) $C_8H_8O_2$ = 136.15

Use general reagent grade of commerce.

DESCRIPTION Colourless to pale yellow, oily liquid; odour, aromatic.

SOLUBILITY Slightly soluble in *water*; miscible with *ethanol* and with *ether*.

BOILING TEMPERATURE About 248° (Appendix 4.6).

WEIGHT PER MILLILITRE About 1.125 g (Appendix 4.9).

Antithrombin III

Use general reagent grade of commerce.

Antithrombin III is purified from human plasma by heparin agarose chromatography and should have a specific activity of at least 6 IU per mg.

Argon Ar = 39.95

Use laboratory cylinder grade of commerce containing not less than 99.995 per cent v/v of Ar.

Arsenic Trioxide As_2O_3 = 197.84

Use analytical reagent grade of commerce.

DESCRIPTION Heavy, white powder.

SOLUBILITY Very slowly soluble in 60 parts of *water*; more readily soluble in *water* on the addition of *hydrochloric acid*, or solutions of alkali hydroxides or carbonates.

Ascorbic Acid (L-Ascorbic Acid) $C_6H_8O_6$ = 176.13

Use analytical reagent grade of commerce.

DESCRIPTION White, crystalline powder.

SOLUBILITY Freely soluble in *water*; sparingly soluble in *ethanol*; insoluble in *benzene*, in *chloroform* and in *ether*.

MELTING TEMPERATURE About 190° (Appendix 4.3).

SPECIFIC ROTATION About +22°, at 20°, determined in a 2 per cent w/v solution (Appendix 4.8). Measure the angle of rotation immediately after preparing the solution.

Barbital (Barbitone) $C_8H_{12}N_2O_3$ = 184.20

Use general reagent grade of commerce.

MELTING TEMPERATURE About 190° (Appendix 4.3).

Barbital Sodium (Barbitone Sodium) $C_8H_{11}N_2NaO_3$ = 206.18

Use general reagent grade of commerce.

DESCRIPTION Colourless crystals or white crystalline powder; odourless.

SOLUBILITY Freely soluble in *water*; slightly soluble in *ethanol*; practically insoluble in *chloroform* and in *ether*.

Barbituric Acid $C_4H_4N_2O_3$ = 128.09

Use general reagent grade of commerce.

DESCRIPTION White or almost white powder.

MELTING TEMPERATURE About 253° (Appendix 4.3).

Benzyl Alcohol C_7H_8O = 108.14

Use general reagent grade of commerce.

DESCRIPTION Colourless liquid.

SOLUBILITY Sparingly soluble in *water*; freely soluble in *ethanol (50 per cent)*; miscible with *chloroform*, with *ethanol*, and with *ether*.

BOILING TEMPERATURE About 204° (Appendix 4.6).

WEIGHT PER MILLILITRE About 1.05 g (Appendix 4.9).

Barium Chloride $BaCl_2.2H_2O$ = 244.27

Use analytical reagent grade of commerce.

Benzene C_6H_6 = 78.11

Use analytical reagent grade of commerce.

DESCRIPTION Flammable, colourless liquid.

BOILING TEMPERATURE About 80° (Appendix 4.6).

Benzoic Acid $C_7H_6O_2$ = 122.12

Use analytical reagent grade of commerce.

DESCRIPTION Colourless, light, feathery crystals or white powder; odour, slight and characteristic.

SOLUBILITY Slightly soluble in *water*; freely soluble in *chloroform*, in *ethanol* and in *ether*.

MELTING RANGE 121.5° to 123.5° (Appendix 4.3).

Benzoyl Chloride C_7H_5ClO = 140.57

Use analytical reagent grade of commerce.

DESCRIPTION Colourless, lachrymatory liquid, fuming in moist air.

RELATIVE DENSITY About 1.21 (Appendix 4.9).

BOILING TEMPERATURE About 197° (Appendix 4.6).

Bismuth Oxynitrate (Bismuth Subnitrate) $Bi_5O(OH)_9(NO_3)_4$ = 1461.99

DESCRIPTION White, microcrystalline powder.

SOLUBILITY Insoluble in *water* and in *ethanol*; readily soluble in *dilute nitric acid* and in *dilute hydrochloric acid*.

Boric Acid H_3BO_3 = 61.83

Use analytical reagent grade of commerce.

DESCRIPTION Colourless, scales of a somewhat pearly lustre, or crystals, or white powder that is unctuous to the touch.

SOLUBILITY Soluble in *water* and in *ethanol*; freely soluble in boiling *ethanol*, in *glycerol* and in boiling *water*.

Bovine Coagulation Factor Xa An enzyme which converts prothrombin to thrombin. The semi-purified preparation is obtained from liquid bovine plasma and it may be prepared by activation of the zymogen factor X with a suitable activator such as Russell's viper venom.

Bromelains A concentrate of proteolytic enzymes derived from *Ananas comosus* Merrill.

DESCRIPTION Pale yellow-brown powder.

ACTIVITY A 1-g quantity liberates about 1.2 g of amino nitrogen from a standard gelatin solution in 20 minutes at 45° and pH 4.5.

Bromine Br_2 = 159.81

Use analytical reagent grade of commerce.

DESCRIPTION Heavy, brownish red, fuming highly corrosive liquid.

SOLUBILITY Slightly soluble in *water*; soluble in most organic solvents.

REFRACTIVE INDEX About 3.1 at 20° (Appendix 4.9).

To prepare 0.05 M *bromine*, dissolve 3 g of *potassium bromate* and 15 g of *potassium bromide* in sufficient *water* to produce 1000 ml. Weaker solutions should be prepared using proportionately lesser amounts of reagents or by appropriate dilution.

5-Bromo-2'-deoxyuridine $C_9H_{11}BrN_2O_5$ = 307.10

Use general reagent grade of commerce.

MELTING TEMPERATURE About 194° (Appendix 4.3).

HOMOGENEITY Examine under the conditions prescribed in the test for Related substances in the monograph for Idoxuridine applying to the plate 5 µl of a 0.025 per cent w/v solution. The chromatogram shows only one principal spot.

1-Butanol (*n*-Butyl Alcohol) $C_4H_{10}O$ = 74.12

Use analytical reagent grade of commerce.

DESCRIPTION Clear, colourless liquid.

SOLUBILITY Soluble in 11 parts of water, at 15.5°.

BOILING RANGE Not less than 95 per cent distils between 116° and 119° (Appendix 4.5).

WEIGHT PER MILLILITRE 0.807 to 0.810 g (Appendix 4.9).

2-Butanone (Ethyl Methyl Ketone) C_4H_8O = 72.11

Use chromatographic reagent grade of commerce.

DESCRIPTION Colourless liquid; odour, characteristic. Flammable.

BOILING TEMPERATURE About 79° (Appendix 4.6).

RELATIVE DENSITY About 0.81 (Appendix 4.9).

Butylated Hydroxyanisole $C_{11}H_{16}O_2$ = 180.25

Use general reagent grade of commerce.

DESCRIPTION White or slightly yellow, waxy solid or white or almost white, crystalline powder; odour, faint, characteristic.

SOLUBILITY Insoluble in *water*; freely soluble in *chloroform*, in *ethanol*, in *ether*, and in *propylene glycol*.

MELTING TEMPERATURE About 61° (Appendix 4.3).

Cadmium Iodide CdI_2 = 366.22

Use analytical reagent grade of commerce.

DESCRIPTION Pearly white flakes or crystalline powder.

SOLUBILITY Very soluble in *water*.

Caffeine $C_8H_{10}N_4O_2$ = 194.19

Use general reagent grade of commerce.

MELTING TEMPERATURE About 236° (Appendix 4.3).

Calcium Carbonate $CaCO_3$ = 100.09

Use analytical reagent grade of commerce.

DESCRIPTION White microcrystalline powder; odourless.

SOLUBILITY Practically insoluble in *water*.

Calcium Carbonate, Chelometric Standard $CaCO_3$ = 100.09

Use analytical reagent grade of commerce.

DESCRIPTION White, microcrystalline powder; odourless.

SOLUBILITY Practically insoluble in *water*.

Calcium Chloride $CaCl_2.2H_2O$ = 147.02

Use analytical reagent grade of commerce.

Calcium Chloride, Anhydrous $CaCl_2$ = 110.99

Use a suitable grade.

DESCRIPTION Deliquescent, white granules.

Store in tightly closed containers.

Calcium Hydroxide $Ca(OH)_2$ = 74.09

Use analytical reagent grade of commerce.

DESCRIPTION Soft, white powder.

SOLUBILITY Slightly soluble in *water*; soluble in aqueous solution of *glycerol* and sugars.

Calcon (Solochrome Dark Blue; Mordant Black 17) $C_{20}H_{13}N_2NaO_5S$ = 416.38

Use general reagent grade of commerce.

DESCRIPTION Brownish black powder with a violet sheen.

SOLUBILITY Sparingly soluble in *water*; freely soluble in *acetone* and in *ethanol*.

Gives a purple-red colour with calcium ions in alkaline solutions. When metal ions are absent, for example, in the presence of an excess of disodium edetate, the solution is blue.

Calcon Mixture (Solochrome Dark Blue Mixture; Mordant Black 17 Mixture) A mixture of 1 part of *calcon* with 99 parts of freshly ignited *anhydrous sodium sulfate*.

SENSITIVITY TO CALCIUM Dissolve 200 mg in 5 ml of *water*. To 1 ml of the solution add 50 ml of *water*, 10 ml of 1 M *sodium hydroxide* and 1 ml of a 1 per cent w/v solution of *magnesium sulfate*: the solution is blue. Add 0.1 ml of a 0.15 per cent w/v solution of *calcium chloride*: the solution becomes violet and, on the subsequent addition of 0.1 ml of 0.01 M *disodium edetate*, turns to pure blue.

Calconcarboxylic Acid (Patton and Reeder's Reagent) $C_{21}H_{14}N_2O_7S$ = 438.37

Use general reagent grade of commerce.

DESCRIPTION Brownish black powder.

Gives a sharp colour change from red to blue in titrations of calcium with disodium edetate.

Calconcarboxylic Acid Mixture A mixture of 1 part of *calconcarboxylic acid* and 99 parts of *sodium chloride*.

SENSITIVITY TO CALCIUM Dissolve 50 mg in a mixture of 100 ml of *water* and 2 ml of 10 M *sodium hydroxide*: the solution is blue. Add 1 ml of a 1 per cent w/v solution of *magnesium sulfate* and 0.1 ml of a 0.15 per cent w/v solution of *calcium chloride*: the solution becomes violet. Add 0.15 ml of 0.010 M *disodium edetate*: the solution becomes pure blue.

***dl*-10-Camphorsulfonic Acid** $C_{10}H_{16}O_4S$ = 232.29

DESCRIPTION White to off-white, crystals or powder; optically inactive.

MELTING TEMPERATURE About 199°, with decomposition (Appendix 4.3).

Carbomer A cross-linked polymer of acrylic acid containing a large proportion (56 per cent to 68 per cent) of carboxylic acid (COOH) groups after drying in *vacuum* at 80° for 1 hour.

Use general reagent grade of commerce.

Average molecular weight, about 3×10^6.

Carbon Dioxide CO_2 = 44.01

Use laboratory cylinder grade of commerce.

Carbon Disulfide CS_2 = 76.14

Use analytical reagent grade of commerce.

DESCRIPTION Colourless, volatile, flammable liquid; odour, unpleasant.

BOILING TEMPERATURE About 46° (Appendix 4.6).

WEIGHT PER MILLILITRE About 1.26 g (Appendix 4.9).

Carbon Tetrachloride CCl_4 = 153.82

DESCRIPTION Clear, colourless, volatile liquid; odour, characteristic.

SOLUBILITY Practically insoluble in *water*; miscible with *absolute ethanol* and with *ether*.

BOILING RANGE Not less than 95 per cent distils between 76° and 77° (Appendix 4.5).

WEIGHT PER MILLILITRE 1.592 to 1.595 g (Appendix 4.9).

Casein A mixture of related phosphoproteins obtained from milk.

Use general reagent grade of commerce.

DESCRIPTION White, amorphous powder or granules.

SOLUBILITY Insoluble in *water* and in neutral solvents; readily dissolved by *ammonia TS* and by solutions of alkali hydroxides, usually forming a cloudy solution.

Cerium(III) Nitrate (Cerous Nitrate) $Ce(NO_3)_3.6H_2O$ = 434.23

Use general reagent grade of commerce.

DESCRIPTION Colourless or pale yellow, crystalline powder.

SOLUBILITY Soluble in *water*.

Cetostearyl Alcohol A mixture of solid aliphatic alcohols consisting chiefly of stearyl and cetyl alcohols. It may be obtained by the reduction of the appropriate fatty acids.

Use a suitable grade.

DESCRIPTION White or cream-coloured unctuous mass or almost white flakes or granules; when heated, melts to a clear, colourless or pale yellow liquid free from cloudiness or suspended matters; odour, faint.

Cetrimide $C_{19}H_{42}ClN$ = 320.00

Use a suitable grade.

DESCRIPTION White to creamy-white, voluminous, free-flowing powder; odour, slight and characteristic.

Charcoal, Decolorizing

DESCRIPTION Black, light powder free from grittiness.

SOLUBILITY Practically insoluble in all usual solvents.

DECOLORIZING POWER Dissolve 100 mg of *strychnine sulfate* in 50 ml of *water*, add 1 g of the test substance, shake during 5 minutes, and pass through a dry filter, rejecting the first 10 ml of the filtrate. To a 10-ml portion of the subsequent filtrate add 1 drop of *hydrochloric acid* and 5 drops of *mercury(II) iodide TS*: no turbidity is produced.

ACID-SOLUBLE MATTER Not more than 3 per cent w/w. To 1.0 g add 25 ml of *dilute nitric acid* and boil for 5 minutes. Filter whilst hot through a sintered-glass filter of porosity of 4 to 10 µm and wash with 10 ml of hot *water*. Evaporate the combined filtrate and washings to dryness on a water-bath, add to the residue 1 ml of *hydrochloric acid*, evaporate to dryness again and dry the residue to constant weight at 100° to 105°. The residue weighs not more than 30 mg.

SULFATED ASH Not more than 5.0 per cent w/w (Appendix 5.3).

Chlorine Cl_2 = 70.91

Use analytical reagent grade of commerce.

Chloroacetic Acid $C_2H_3ClO_2$ = 94.50

Use analytical reagent grade of commerce.

DESCRIPTION Colourless, deliquescent crystals.

MELTING TEMPERATURE About 62° (Appendix 4.3).

Store in tightly closed containers.

Chloroform $CHCl_3$ = 119.38

Caution Care should be taken not to vaporize chloroform in the presence of a flame, because of the production of harmful gases.

Use analytical reagent grade of commerce containing 0.4 to 1.0 per cent w/w of ethanol.

DESCRIPTION Colourless, volatile liquid; odour, characteristic.

SOLUBILITY Slightly soluble in *water*; miscible with *absolute ethanol*, with *ether*, with fixed and volatile oils, and with most organic solvents.

BOILING TEMPERATURE About 60° (Appendix 4.6).

RELATIVE DENSITY 1.475 to 1.481 (Appendix 4.6).

Store protected from light.

Chloroform Water Shake 2.5 ml of *chloroform* with 900 ml of *water* until dissolved and dilute with *water* to 1000 ml.

Chloroplatinic(IV) Acid (Platinic Chloride; Hexachloroplatinic(IV) Acid Hexahydrate) $H_2PtCl_6.6H_2O$ = 518.00

Use general reagent grade of commerce.

DESCRIPTION Brown, crystalline masses. Deliquescent.

SOLUBILITY Very soluble in *water* and in *ethanol*.

Chromic Acid Cleansing Mixture

Caution Wear safety goggles. Prepare this mixture in a hard, borosilicate-glass, 2000-ml beaker, since the heat produced may cause soft-glass containers to break. Chromic Acid Cleansing Mixture is extremely corrosive and hygroscopic, and should be stored in glass-stoppered bottles in a safe place. When the mixture acquires a green colour, it should not be returned to the storage bottle, but should be discarded under continuously flowing water.

Dissolve 200 g of *sodium dichromate* in 100 ml of *water*, and, slowly and cautiously, add 1500 ml of *sulfuric acid* with stirring.

Chromotropic Acid Sodium Salt $C_{10}H_6Na_2O_8S_2.2H_2O$ = 400.28

Use general reagent grade of commerce.

DESCRIPTION Pale brown powder.

Cinchonidine $C_{19}H_{22}N_2O$ = 294.40

Use general reagent grade of commerce.

DESCRIPTION White, crystalline powder.

SOLUBILITY Practically insoluble in *water*; soluble in *chloroform* and in *ethanol*.

MELTING TEMPERATURE About 208°, with decomposition (Appendix 4.3).

SPECIFIC ROTATION –105° to –110°, determined in a 5 per cent w/v solution in *ethanol* (Appendix 4.8).

Store protected from light.

Cineole (Eucalyptol) $C_{10}H_{18}O$ = 154.25

Use a grade of commerce specially supplied for *o*-cresol determinations.

DESCRIPTION Colourless liquid; odour, camphoraceous.

BOILING TEMPERATURE About 176° (Appendix 4.6).

REFRACTIVE INDEX 1.456 to 1.459, at 20° (Appendix 4.7).

Cineole used in gas chromatography complies with the following test.

ASSAY Carry out the determination as described in the "Gas Chromatography" (Appendix 3.4), using the normalization procedure.

Reference solution Dissolve 20 µl of *cineole* in *hexane* and dilute to 10.0 ml with the same solvent.

Test solution Dissolve 20 µl of the test substance in *hexane* and dilute to 10.0 ml with the same solvent.

Chromatographic system A chromatographic procedure may be carried out using (a) a fused-silica column (60 m × 0.25 mm) coated with *macrogol 20000* (film thickness 0.25 µm), (b) *helium* as the carrier gas at a flow rate of about 1.5 ml per minute, and (c) a flame ionization detector, with the following temperature programme:

	Time (Minute)	Temperature (°)
Column	0 – 10	60
	10 – 70	60 – 180
	70 – 75	180
Injection port		200
Detector		220

Procedure Separately inject equal volumes (about 1 µl) of *Reference solution* and *Test solution* into the chromatograph, record the chromatograms, and measure the responses for the peaks. The area of the principal peak is not less than 98 per cent of the total area of the peaks.

Citric Acid $C_6H_8O_7.H_2O$ = 210.14

Use analytical reagent grade of commerce.

When used in the "Limit Test for Iron" (Appendix 5.3), complies with the following requirement. Dissolve 0.5 g in 10 ml of *water*, add 0.1 ml of *mercaptoacetic acid*, mix, make alkaline with *strong ammonia solution*, and add sufficient *water* to produce 20 ml. No pink colour is produced.

Cobalt(II) Chloride (Cobalt Chloride; Cobaltous Chloride) $CoCl_2.6H_2O$ = 237.93

Use analytical reagent grade of commerce.

DESCRIPTION Red, crystalline powder or deep red crystals.

SOLUBILITY Very soluble in *water* and in *ethanol*; soluble in *acetone* and in *glycerol*.

Cobalt(II) Nitrate (Cobalt(II) Nitrate Hexahydrate) $Co(NO_3)_2.6H_2O$ = 291.0

Use analytical reagent grade of commerce.

DESCRIPTION Small, garnet red crystals.

Copper Cu = 63.55

Use general reagent grade of commerce.

DESCRIPTION In the form of wire, foil, turnings, powder, granules or gauze.

SOLUBILITY Practically insoluble in *hydrochloric acid*; soluble in *nitric acid* and in hot *sulfuric acid*.

Copper(II) Sulfate $CuSO_4.5H_2O$ = 249.68

Use analytical reagent grade of commerce.

Cottonseed Oil Cottonseed Oil is the refined fixed oil obtained from the seed of cultivated plants of various varieties of *Gossypium hirsutum* L. or of other species of *Gossypium* (Family Malvaceae).

Use general reagent grade of commerce.

DESCRIPTION Pale yellow, oily liquid; odourless or almost odourless. At temperatures below 10° particles of solid fat may separate from the Oil, and at about 0° to –5° the Oil becomes solid or nearly so.

***o*-Cresol** (2-Methylphenol) C_7H_8O = 108.14

Use general reagent grade of commerce.

DESCRIPTION Colourless to faintly coloured, crystalline solid or a supercooled liquid; odour, tarry and phenolic.

SOLUBILITY Soluble in about 50 parts of *water*; miscible with *chloroform*, with *ethanol* and with *ether*.

FREEZING TEMPERATURE Not lower than 30.5° (Appendix 4.4).

BOILING TEMPERATURE About 190° (Appendix 4.6).

REFRACTIVE INDEX 1.540 to 1.550, at 20° (Appendix 4.7).

RELATIVE DENSITY About 1.05 (Appendix 4.9).

Store protected from light, moisture and oxygen and distil before use.

Cyclohexane C_6H_{12} = 84.16

DESCRIPTION Clear, colourless liquid.

BOILING RANGE Not less than 95 per cent distils between 80° and 82° (Appendix 4.5).

WEIGHT PER MILLILITRE 0.776 to 0.780 g (Appendix 4.9).

REFRACTIVE INDEX 1.4262 to 1.4265, at 20° (Appendix 4.7).

Cyclohexane UV *Cyclohexane* which complies with the following additional test.

ABSORBANCE Determine the absorbance of the sample throughout the range of 220 to 250 nm (Appendix 2.2) against *water* as the blank. The absorbance should not exceed 0.346 at 220 nm, 0.155 at 235 nm, 0.046 at 240 nm, and 0.009 at 250 nm.

3-Cyclohexylpropionic Acid $C_9H_{16}O_2$ = 156.22

Use general reagent grade of commerce.

RELATIVE DENSITY About 0.998 (Appendix 4.9).

REFRACTIVE INDEX About 1.4648, at 20° (Appendix 4.7).

BOILING TEMPERATURE About 130° (Appendix 4.6).

RELATIVE DENSITY 0.922 to 0.927 (Appendix 4.9).

L-Cystine $C_6H_{12}N_2O_4S_2$ = 240.53

Use general reagent grade of commerce.

DESCRIPTION White, crystalline powder.

SOLUBILITY Very slightly soluble in *water*; soluble in dilute mineral acids and in solutions of alkali hydroxides; insoluble in *ethanol* and in other organic solvents.

SPECIFIC ROTATION –218° to –224° at 20°, calculated on the dried basis, determined in 1 M *hydrochloric acid* (Appendix 4.8).

2′-Deoxyuridine $C_9H_{12}N_2O_5$ = 228.20

Use general reagent grade of commerce.

DESCRIPTION White or almost white, crystalline solid.

MELTING TEMPERATURE About 165° (Appendix 4.3).

HOMOGENEITY Examine under the conditions prescribed in the test for Related substances in the monograph for Idoxuridine applying to the plate 5 µl of a 0.025 per cent w/v solution. The chromatogram shows only one principal spot.

Deuterated Acetone (Deuteroacetone) C_3D_6O = 64.11

Use spectroscopic reagent grade of commerce with a minimum isotopic purity of 99.5 per cent and containing not more than 0.1 per cent of water and deuterium oxide.

BOILING TEMPERATURE About 55° (Appendix 4.6).

REFRACTIVE INDEX About 1.357, at 20° (Appendix 4.7).

RELATIVE DENSITY About 0.87 (Appendix 4.9).

Deuterium Oxide D_2O = 20.03

Use general reagent grade of commerce with a minimum isotopic purity of 99.7 per cent.

DESCRIPTION Colourless liquid.

BOILING TEMPERATURE About 101° (Appendix 4.6).

REFRACTIVE INDEX About 1.328, at 20° (Appendix 4.7).

RELATIVE DENSITY About 1.11 (Appendix 4.9).

Deuterochloroform $CDCl_3$ = 120.38

Use spectroscopic reagent grade of commerce with a minimum isotopic purity of 99.7 per cent and containing not more than 0.05 per cent of water and deuterium oxide.

DESCRIPTION Colourless liquid.

BOILING TEMPERATURE About 60° (Appendix 4.6).

REFRACTIVE INDEX About 1.445, at 20° (Appendix 4.7).

WEIGHT PER MILLILITRE About 1.5 g (Appendix 4.9).

RELATIVE DENSITY About 1.51 (Appendix 4.9).

Dextrose (D-Glucose) $C_6H_{12}O_6$ = 180.16

Use general reagent grade of commerce.

DESCRIPTION White, crystalline or granular powder.

SOLUBILITY Freely soluble in *water*; sparingly soluble in *ethanol*.

SPECIFIC ROTATION About +52.5° at 20°, determined in a 10 per cent w/v solution containing about 0.2 per cent v/v of *ammonia* (Appendix 4.8).

Dextrose Monohydrate (D-Glucose Monohydrate) $C_6H_{12}O_6.H_2O$ = 198.17

Use general reagent grade of commerce.

DESCRIPTION Colourless crystals or white to cream, crystalline powder.

SPECIFIC ROTATION +52.5° to +53.0°, calculated on the anhydrous basis (Appendix 4.8), using a solution prepared in the following manner: Dissolve 10 g in 80 ml of *water* and add 0.05 ml of 6 M *ammonia*, allow to stand for 6 hours and dilute to 100.0 ml with *water*.

2,3-Diaminonaphthalene $C_{10}H_{10}N_2$ = 158.20

Use a suitable grade.

Diaveridine $C_{13}H_{16}N_4O_2$ = 260.30

Use general reagent grade of commerce.

DESCRIPTION Crystals.

MELTING TEMPERATURE About 233° (Appendix 4.3).

Dichlorobenzene $C_6H_4Cl_2$ = 147.0038

Use analytical reagent grade of commerce.

DESCRIPTION Clear, oily liquid.

SOLUBILITY Practically insoluble in *water*; miscible with *ethanol* and with *ether*.

BOILING TEMPERATURE About 180° (Appendix 4.6).

REFRACTIVE INDEX About 1.551, at 20° (Appendix 4.7).

RELATIVE DENSITY About 1.31 (Appendix 4.9).

1,2-Dichloroethane (Ethylene Chloride) $C_2H_4Cl_2$ = 98.96

Use analytical reagent grade of commerce.

DESCRIPTION Colourless liquid.

BOILING RANGE Not less than 95 per cent distils between 82° and 84° (Appendix 4.5).

WEIGHT PER MILLILITRE About 1.25 g (Appendix 4.9).

2,7-Dichlorofluorescein $C_{20}H_{10}Cl_2O_5$ = 401.20

Use adsorption indicator grade of commerce.

DESCRIPTION Yellowish brown to yellowish orange powder.

Dichloromethane (Methylene Chloride) CH_2Cl_2 = 84.93

DESCRIPTION Clear, colourless, mobile liquid.

SOLUBILITY Soluble in 50 parts of *water*; miscible with *ethanol* and with *ether*.

BOILING RANGE Not less than 95 per cent distils between 39° and 41° (Appendix 4.5).

WEIGHT PER MILLILITRE 1.323 to 1.325 g (Appendix 4.9).

NON-VOLATILE MATTER Not more than 50 ppm. When evaporated on a water-bath and dried at 105° to constant weight.

Diethylamine $C_4H_{11}N$ = 73.14

> ***Caution*** May be irritating to skin and mucous membrane.

Use analytical reagent grade of commerce.

DESCRIPTION Volatile, colourless liquid.

BOILING TEMPERATURE About 55° (Appendix 4.6).

WEIGHT PER MILLILITRE About 0.71 g (Appendix 4.9).

Diethylaminoethyldextran Anion exchange resin presented as the hydrochloride.

DESCRIPTION Powder, forming gels with *water*.

Di(2-ethylhexyl) Phthalate (Dioctyl Phthalate) $C_{24}H_{38}O_4$ = 390.56

Use general reagent grade of commerce.

DESCRIPTION Colourless, oily liquid.

SOLUBILITY Practically insoluble in *water*; soluble in organic solvents.

REFRACTIVE INDEX About 1.486 at 20° (Appendix 4.7).

RELATIVE DENSITY About 0.98 (Appendix 4.9).

VISCOSITY At 20°, about 80 mPa. (Appendix 4.10).

SOLUBILITY Slightly soluble in *ethanol*; miscible with *carbon disulfide*, with *chloroform*, with *ether*, and with *petroleum ether*.

SPECIFIC GRAVITY 0.915 to 0.921 (Appendix 4.9).

Store in tightly closed containers, protected from light and excessive heat.

Dihydroquinine (Hydroquinine) $C_{20}H_{26}N_2O_2$ = 326.44

Use general reagent grade of commerce.

DESCRIPTION Needles.

SOLUBILITY Almost insoluble in *water*; freely soluble in *acetone*, in *chloroform* and in *ethanol*.

MELTING TEMPERATURE About 172° (Appendix 4.3).

Dimethylacetamide C_4H_9NO = 87.12

DESCRIPTION Clear, colourless liquid.

SOLUBILITY Miscible with *water* and with many organic solvents.

BOILING TEMPERATURE About 165° (Appendix 4.6).

WEIGHT PER MILLILITRE About 0.94 g (Appendix 4.9).

ULTRAVIOLET ABSORPTION Determine its absorbance throughout the range of 270 to 400 nm, using *water* to set the instrument; the absorbance does not exceed 1.00 at 270 nm, 0.30 at 280 nm, 0.15 at 290 nm, 0.05 at 310 nm, 0.03 at 320 nm, and 0.01 at 360 to 400 nm (Appendix 2.2).

4-Dimethylaminobenzaldehyde $C_9H_{11}NO$ = 149.19

Use analytical reagent grade of commerce.

DESCRIPTION White or pale yellow, crystalline powder.

SOLUBILITY Almost insoluble in *water*; readily soluble in ethanol.

MELTING RANGE 73° to 75° (Appendix 4.3).

SOLUBILITY TEST IN HYDROCHLORIC ACID Dissolve 1.0 mg in 20 ml of *dilute hydrochloric acid*: a clear solution, free from any red colour, is produced.

4-Dimethylaminocinnamaldehyde $C_{11}H_{13}NO$ = 175.23

DESCRIPTION Orange-yellow powder.

SOLUBILITY Soluble in *acetone*, in *benzene*, and in *ethanol*.

MELTING RANGE 132° to 136° (Appendix 4.3).

***N*,*N*-Dimethylaniline** $C_8H_{11}N$ = 121.18

Use analytical reagent grade of commerce.

DESCRIPTION Light yellow liquid. Clear, oily liquid, almost colourless when freshly distilled, darkening to a reddish brown colour on storage.

SOLUBILITY Insoluble in *water*; soluble in *chloroform*, in *ethanol*, in *ether*, and in dilute mineral acids.

BOILING RANGE Not less than 95 per cent distils between 192° and 194° (Appendix 4.5).

REFRACTIVE INDEX About 1.568, at 20° (Appendix 4.7).

1,1-Dimethylethylamine (*tert*-Butylamine, 2-amino-2-methylpropane) $C_4H_{11}N$ = 73.14

Use general reagent grade of commerce.

DESCRIPTION Liquid.

REFRACTIVE INDEX 1.3770 to 1.3790, at 20° (Appendix 4.7).

RELATIVE DENSITY About 0.694 (Appendix 4.9).

BOILING TEMPERATURE About 46° (Appendix 4.5).

Dimethylformamide C_3H_7NO = 73.09

DESCRIPTION Clear, colourless liquid.

SOLUBILITY Slightly soluble in *water*; miscible with *ethanol*, with *ether* and with cold *water*.

BOILING TEMPERATURE About 153° (Appendix 4.5).

WEIGHT PER MILLILITRE About 0.95 g (Appendix 4.9).

Dimethyl Sulfoxide C_2H_6OS = 78.13

DESCRIPTION Clear, colourless, viscous liquid. Hygroscopic.

SOLUBILITY Soluble in *water*, in *ethanol*, and in *ether*.

BOILING RANGE Not less than 95 per cent distils between 189° and 192° (Appendix 4.5).

WEIGHT PER MILLILITRE 1.100 to 1.103 g (Appendix 4.9).

2,4-Dinitrofluorobenzene (1-Fluoro-2,4-dinitrobenzene) $C_6H_3FN_2O_4$ = 186.10

Use general reagent grade of commerce.

DESCRIPTION Pale yellow, vesicatory crystals, lumps or liquid with a lachrymatory vapour.

MELTING TEMPERATURE About 29° (Appendix 4.3).

WEIGHT PER MILLILITRE About 1.48 g (Appendix 4.9).

REFRACTIVE INDEX About 1.569, at 20° (Appendix 4.7).

Diphenylamine $C_{12}H_{11}N$ = 169.23

Use analytical reagent grade of commerce.

DESCRIPTION White crystals; odour, characteristic.

MELTING TEMPERATURE About 55° (Appendix 4.3).

Store protected from light.

Diphenylbenzidine (*N,N'*-Diphenylbenzidine) $C_{24}H_{20}N_2$ = 336.44

Use general reagent grade of commerce.

DESCRIPTION White or faintly grey-coloured, crystalline powder.

SOLUBILITY Insoluble in *water*; slightly soluble in *acetone* and in *ethanol*.

MELTING RANGE 246° to 250° (Appendix 4.3).

Store protected from light.

Dipotassium Edetate (Dipotassium Dihydrogen Ethylenediaminetetra-acetate) $C_{10}H_{14}N_2K_2O_8.2H_2O$ = 404.46

Use general reagent grade of commerce.

Dipotassium Hydrogenphosphate (Potassium Phosphate, Dibasic) K_2HPO_4 = 174.18

Use general reagent grade of commerce.

DESCRIPTION White, crystalline powder.

SOLUBILITY Soluble in *water*.

Disodium Edetate $C_{10}H_{14}N_2Na_2O_8.2H_2O$ = 372.24

Use analytical reagent grade of commerce.

Disodium Hydrogenphosphate $Na_2HPO_4.12H_2O$ = 358.14

Use analytical reagent grade of commerce.

DESCRIPTION Translucent crystals or granules.

SOLUBILITY Freely soluble in water; practically insoluble in *ethanol*.

Disodium Hydrogenphosphate, Anhydrous Na_2HPO_4 = 141.96

Use analytical reagent grade of commerce.

Disodium Hydrogenphosphate Dihydrate (Disodium Hydrogen Orthophosphate Dihydrate) $Na_2HPO_4.2H_2O$ = 178.00

Use analytical reagent grade of commerce.

Store in tightly closed containers.

Dithiothreitol (Cleland's Reagent) $C_4H_{10}O_2S_2$ = 154.24

Use general reagent grade of commerce.

DESCRIPTION Slightly hygroscopic needles.

SOLUBILITY Freely soluble in *water*, in *acetone*, in *ethanol*, in *ether*, and in *ethyl acetate*.

MELTING RANGE Between 42° and 44° (Appendix 4.3).

Store in tightly closed containers.

Dithizone (Phenylazothioformic Acid 2-Phenylhydrazide; Diphenylthiocarbazone) $C_{13}H_{12}N_4S$ = 256.32

Use analytical reagent grade of commerce.

DESCRIPTION Almost black powder.

Store protected from light.

Edetic Acid (Ethylenediaminetetra-acetic Acid) $C_{10}H_{16}N_2O_8$ = 292.25

Use general reagent grade of commerce.

DESCRIPTION White, crystalline powder.

SOLUBILITY Very slightly soluble in *water*; soluble in solutions of alkali hydroxides.

MELTING TEMPERATURE Above 220°, with decomposition (Appendix 4.3).

Eosin Y (Acid Red 87; Eosin Yellowish Y) $C_{20}H_6Br_4Na_2O_5$ = 691.86

Use general reagent grade of commerce.

DESCRIPTION Red to brownish red pieces or powder; dissolves in *water* to yield yellow purplish red solution with a greenish yellow fluorescence.

SOLUBILITY Soluble in water and in ethanol.

Ethanol Use Ethanol (95 Per Cent) (TP monograph).

Ethanol, Absolute C_2H_6O = 46.07

Use analytical reagent grade of commerce containing not less than 99.5 per cent v/v of C_2H_6O.

DESCRIPTION Colourless, clear, mobile and volatile liquid; odour, characteristic and spirituous. Flammable, burning with a blue, smokeless flame. Hygroscopic.

SOLUBILITY Miscible with *water*, with *chloroform* and with *ether*.

BOILING RANGE 78° to 79° (Appendix 4.5).

RELATIVE DENSITY 0.791 to 0.794 (Appendix 4.9).

Store protected from light at a temperature not exceeding 30°.

Ethanol, Aldehyde-free (Aldehyde-free Alcohol) Mix 1200 ml of *ethanol* with 5 ml of a 40 per cent w/v solution of *silver nitrate* and 10 ml of a cooled 50 per cent w/v solution of *potassium hydroxide*. Shake, allow to stand for a few days and filter. Distil the filtrate immediately before use.

Ethanol, Diluted Prepare by diluting the volumes of *ethanol* indicated in the following table with *water* to 1000 ml.

Strength per cent v/v	Volume of *ethanol* ml	Weight per ml (approx.) g
90	947	0.83
80	842	0.86
70	737	0.89
60	632	0.91
50	526	0.93
45	474	0.94
25	263	0.97
20	210	0.975

Ethanol, Neutralized To a suitable quantity of *ethanol* add 2 or 3 drops of *phenolphthalein TS* and just sufficient 0.02 M or 0.1 M *sodium hydroxide* to produce a faint pink colour.

Prepare neutralized ethanol just prior to use.

Ether $C_4H_{10}O$ = 74.12

> ***Caution*** Ether tends to form explosive peroxides especially when anhydrous.

Use analytical reagent grade of commerce.

DESCRIPTION Clear, colourless, volatile, very mobile liquid; odour, characteristic. Highly flammable; mixtures of its vapour with oxygen, air, or nitrous oxide in certain concentrations are explosive.

SOLUBILITY Soluble in 10 parts of *water*; miscible with *benzene*, with *chloroform*, with *dichloromethane*, with *ethanol*, with fixed oils, with *petroleum ether*, and with volatile oils.

PEROXIDES Transfer 8 ml of *potassium iodide and starch TS* to a 12-ml ground-glass-stoppered cylinder about 1.5 cm in diameter. Fill completely with the test substance, shake vigorously and allow to stand in the dark for 30 minutes. No colour is produced.

Store protected from light at a temperature not exceeding 15°. The name and concentration of any added stabilizer are stated on the label.

Ether, Peroxide-free $(C_2H_5)_2O$ = 74.12

Shake 1000 ml of *ether* with 20 ml of a solution of 30 g of *iron(II) sulfate* in 55 ml of *water* and 3 ml of *sulfuric acid*. Continue shaking until a small sample from upper layer no longer produces a blue colour when shaken with an equal volume of a 2 per cent w/v solution of potassium iodide and 1 drop of *starch TS*. Discard the aqueous layer.

Ethyl Acetate $C_4H_8O_2$ = 88.11

Use analytical reagent grade of commerce.

DESCRIPTION Colourless liquid; odour, fruity-like.

BOILING RANGE 76° to 78° (Appendix 4.5).

WEIGHT PER MILLILITRE 0.901 to 0.904 g (Appendix 4.9).

Ethylbenzene C_8H_{10} = 106.17

Use general reagent grade of commerce containing not less than 99.5 per cent w/w of C_8H_{10} when determined by gas chromatography.

DESCRIPTION Colourless, flammable liquid.

SOLUBILITY Practically insoluble in water; miscible with the usual organic solvents.

BOILING TEMPERATURE About 135° (Appendix 4.6).

REFRACTIVE INDEX About 1.496, at 20° (Appendix 4.7).

RELATIVE DENSITY About 0.87 (Appendix 4.9).

Ethylene Oxide (Oxirane) C_2H_4O = 44.05

Use general reagent grade of commerce.

DESCRIPTION Colourless gas.

2-Ethylhexanoic Acid (2-Ethylhexoic Acid) $C_8H_{16}O_2$ = 144.21

Use analytical reagent grade of commerce.

DESCRIPTION Colourless liquid.

RELATIVE DENSITY About 0.91, at 20° (Appendix 4.9).

REFRACTIVE INDEX About 1.425 (Appendix 4.7).

Formaldehyde Solution (Formalin) CH_2O = 30.03

Use analytical grade of commerce containing not less than 34.0 per cent w/v and not more than 37.0 per cent w/v of CH_2O.

DESCRIPTION Colourless, aqueous solution with a lachrymatory vapour.

WEIGHT PER MILLILITRE About 1.08 g (Appendix 4.9).

Store at a temperature between 15° and 25°.

Formamide CH_3NO = 45.04

Use analytical reagent grade of commerce.

DESCRIPTION Colourless, oily liquid.

SOLUBILITY Soluble in *water* and in *ethanol*.

WEIGHT PER MILLILITRE About 1.13 g (Appendix 4.9).

Store in tightly closed containers.

Formic Acid CH_2O_2 = 46.03

Use analytical reagent grade of commerce containing about 90 per cent w/w of CH_2O_2 and about 23.6 M in strength.

DESCRIPTION Colourless, corrosive liquid; odour, pungent.

WEIGHT PER MILLILITRE About 1.20 g (Appendix 4.9).

Formic Acid, Anhydrous CH_2O_2 = 46.03

Use analytical reagent grade formic acid of commerce containing not less than 98.0 per cent w/w of CH_2O_2.

DESCRIPTION Colourless, corrosive liquid; odour, pungent.

RELATIVE DENSITY About 1.22 (Appendix 4.9).

Fructose (Levulose) $C_6H_{12}O_6$ = 180.16

Use general reagent grade of commerce.

DESCRIPTION Colourless crystals or white, crystalline powder; odourless.

SOLUBILITY Freely soluble in *water*; soluble in *ethanol* and in *methanol*.

MELTING TEMPERATURE About 103°, with decomposition (Appendix 4.3).

SPECIFIC ROTATION About –92° at 20°, determined in a 10 per cent w/v solution containing 0.03 per cent v/v of *ammonia* (Appendix 4.8).

D-Galactose $C_6H_{12}O_6$ = 180.16

Use general reagent grade of commerce.

DESCRIPTION White crystalline or finely granulated powder.

SOLUBILITY Soluble in *water*; very slightly soluble in *ethanol*.

MELTING TEMPERATURE About 164°, with decomposition (Appendix 4.3).

SPECIFIC ROTATION About +80° at 20°, determined in a 10 per cent w/v solution containing about 0.05 per cent v/v of *ammonia* (Appendix 4.8).

Gelatin

Use general reagent grade of commerce.

DESCRIPTION Colourless or slightly yellow, transparent, brittle, tasteless sheets, flakes, or powder; odourless.

SOLUBILITY Soluble in hot *water*, *acetic acid* and *glycerol*; insoluble in organic solvents.

Glutaraldehyde $C_5H_8O_2$ = 100.12

Use general reagent grade of commerce.

DESCRIPTION Oily liquid.

SOLUBILITY Freely soluble in *water*, in *benzene*, in *ethanol*, and in *ether*.

BOILING TEMPERATURE About 188° (Appendix 4.6).

REFRACTIVE INDEX About 1.434 (Appendix 4.7).

Glycerol (Glycerin) $C_3H_8O_3$ = 92.09

Use analytical reagent grade of commerce.

DESCRIPTION Colourless, viscous liquid.

WEIGHT PER MILLILITRE About 1.26 g (Appendix 4.9).

Glycerol (85 Per Cent) *Glycerol* containing 12.0 to 16.0 per cent w/w of water.

WEIGHT PER MILLILITRE 1.22 to 1.24 g (Appendix 4.9).

Glyoxal Bis(2-hydroxyanil) $C_{14}H_{12}N_2O$ = 240.26

Use general reagent grade of commerce.

DESCRIPTION White crystals.

SOLUBILITY Soluble in hot *ethanol*.

MELTING RANGE 203° to 205° (Appendix 4.3).

Guaiacol (2-Methoxyphenol) $C_7H_8O_2$ = 124.14

Use analytical reagent grade of commerce.

DESCRIPTION Crystalline mass or colourless or yellowish liquid. Hygroscopic.

SOLUBILITY Slightly soluble in *water*; very soluble in *dichloromethane*; freely soluble in *ethanol*.

MELTING TEMPERATURE About 28°. (Appendix 4.3).

BOILING TEMPERATURE About 205°. (Appendix 4.6).

Store protected from light.

Guanine $C_5H_5N_5O$ = 151.13

DESCRIPTION White powder.

SOLUBILITY Practically insoluble in *water*, freely soluble in *potassium hydroxide* solution and in dilute acids; sparingly soluble in *ethanol* and in *ether*.

Helium He = 4.00

Use laboratory cylinder grade of commerce containing not less than 99.995 per cent v/v of He.

***n*-Heptane** C_7H_{16} = 100.20

Use general reagent grade of commerce.

DESCRIPTION Clear, colourless, volatile, flammable liquid; odour, characteristic.

SOLUBILITY Practically insoluble in *water*; soluble in *absolute ethanol*; miscible with *chloroform*, with *ether* and with most fixed and volatile oils.

BOILING RANGE 94.5° to 99.0° (Appendix 4.5).

REFRACTIVE INDEX 1.387 to 1.388, at 20° (Appendix 4.7).

WEIGHT PER MILLILITRE 0.683 to 0.686 g (Appendix 4.9).

Hexadimethrine Bromide $(C_{13}H_{30}Br_2N_2)_n$

Use general reagent grade of commerce.

DESCRIPTION White to off-white, amorphous powder. Hygroscopic.

SOLUBILITY Soluble in *water* up to 10 per cent to give a colourless to light yellow solution.

Store in tightly closed containers.

Holmium Oxide Ho_2O_3 = 377.86

Use general reagent grade of commerce.

DESCRIPTION Yellowish powder.

SOLUBILITY Insoluble in *water*.

Hydrazine Sulfate (Hydrazinium Sulfate) $H_6N_2O_4S$ = 130.12

Use analytical reagent grade of commerce.

DESCRIPTION Colourless crystals or white, crystalline powder.

SOLUBILITY Soluble in about 40 parts of *water*; practically insoluble in *ethanol (25 per cent)*.

Hydriodic Acid HI = 127.91

> ***Caution*** To avoid possible explosions this acid should be distilled only in inert atmosphere.

Use analytical reagent grade of commerce containing about 55 per cent w/w of HI (about 7.5 M in strength).

DESCRIPTION Almost colourless liquid when freshly prepared, but rapidly becoming yellow or brown owing to the liberation of iodine.

SOLUBILITY Miscible with *water* and with *ethanol*.

WEIGHT PER MILLILITRE About 1.7 g (Appendix 4.9).

Hydrochloric Acid HCl = 36.46

Use analytical reagent grade of commerce.

Where no molarity is indicated use analytical reagent grade of commerce with a relative density of about 1.18, containing not less than 35 per cent w/w and not more than 38 per cent w/w of HCl and about 11.5 M in strength.

DESCRIPTION Clear, colourless, fuming liquid; odour, pungent.

SOLUBILITY Miscible with *water*.

Solutions of molarity *x* M should be prepared by diluting 85*x* ml of *hydrochloric acid* to 1000 ml with *water*.

Store in a container of polyethylene or other non-reacting material at a temperature not exceeding 30°.

Hydrochloric Acid, Dilute A 10 per cent w/v solution. Prepare by mixing 226 ml of *hydrochloric acid* with *water* to produce 1000 ml.

Hydrochloric Acid, 0.1 M Methanolic Dilute 8.5 ml of *hydrochloric acid* in sufficient *methanol* to produce 1000 ml.

Hydrogen Peroxide Solution, Strong H_2O_2 = 34.01

Use analytical reagent grade of commerce containing about 30 per cent w/v of H_2O_2.

DESCRIPTION Colourless liquid.

WEIGHT PER MILLILITRE About 1.10 g (Appendix 4.9).

Hydrogen Sulfide H_2S = 34.08

Prepared by the action of *hydrochloric acid*, diluted with an equal volume of *water* on *iron sulfide*, the resulting gas is washed by passing it through *water*.

DESCRIPTION Colourless, poisonous gas; odour, characteristic and unpleasant.

Hydroxylamine Hydrochloride (Hydroxylammonium Chloride) $NH_2OH.HCl$ = 69.49

Use analytical reagent grade of commerce.

DESCRIPTION Colourless crystals or white, crystalline powder.

SOLUBILITY Very soluble in *water*; soluble in *ethanol*.

5-Hydroxymethylfurfural $C_6H_6O_3$ = 126.11

Use general reagent grade of commerce.

MELTING TEMPERATURE About 32° (Appendix 4.3).

8-Hydroxyquinoline (8-Quinolinol) C_9H_7NO = 145.16

Use analytical reagent grade of commerce.

DESCRIPTION White to yellowish white, crystalline powder.

MELTING TEMPERATURE About 74° (Appendix 4.3).

Hydroxy Naphthol Blue $C_{20}H_{12}N_2O_{11}S_3Na_2$ = 598.48 (598.48162)

Use general reagent grade of commerce containing 1 per cent w/w of hydroxy naphthol blue deposited on sodium chloride.

DESCRIPTION Small blue crystals.

SOLUBILITY Freely soluble in *water*.

Imidazole (Glyoxaline) $C_3H_4N_2$ = 68.08

Use purified grade of commerce.

DESCRIPTION White, crystalline powder.

SOLUBILITY Soluble in *water* and in *ethanol*.

MELTING TEMPERATURE About 90° (Appendix 4.6).

Indophenol Blue $C_{18}H_{16}N_2O$ = 276.34

DESCRIPTION Dark purple powder.

SOLUBILITY Soluble in *ethanol* and in *toluene*, giving dark purple solutions.

MELTING RANGE 168° to 170° (Appendix 4.3).

Iodine I_2 = 253.81

Use analytical reagent grade of commerce.

Iodine Monobromide (Iodine Bromide) IBr = 206.81

Use general reagent grade of commerce.

DESCRIPTION Bluish black or brownish black crystals with a lachrymatory vapour.

MELTING TEMPERATURE About 40° (Appendix 4.3).

BOILING TEMPERATURE About 116° (Appendix 4.6).

Store in a cool place, protected from light.

Iodine Monochloride ICl = 162.36 (162.3575)

Use general reagent grade of commerce.

DESCRIPTION Black crystals.

SOLUBILITY Soluble in *water*, in *acetic acid* and in *ethanol*.

BOILING TEMPERATURE About 97.4° (Appendix 4.6).

5-Iodouracil $C_4H_3IN_2O_2$ = 237.98

Use general reagent grade of commerce.

DESCRIPTION White or almost white crystalline powder.

MELTING TEMPERATURE About 276°, with decomposition (Appendix 4.3).

HOMOGENEITY Examine under the conditions prescribed in the test for Related substances in the monograph for Idoxuridine applying to the plate 5 µl of a 0.025 per cent w/v solution. The chromatogram shows only one principal spot.

Iron(III) Chloride (Ferric Chloride) $FeCl_3.6H_2O$ = 270.30

Use analytical grade of commerce.

DESCRIPTION Yellowish orange or brownish, crystalline masses; deliquescent.

Store in well-closed containers.

Iron(III) Sulfate $Fe_2(SO_4)_3.xH_2O$

Use general reagent grade of commerce.

DESCRIPTION White to yellow, hygroscopic powder which decomposes in air.

Store protected from light.

Isopropyl Myristate (Isopropyl Tetradecanoate) $C_{17}H_{34}O_2$ = 270.45

Use general reagent grade of commerce.

For use as a solvent in sterility test procedures, Isopropyl Myristate conforms to the following additional specification:

pH OF WATER EXTRACT Transfer 100 ml to a 250-ml centrifuge bottle, add 10 ml of twice-distilled *water*, close the bottle with a suitable closure, and shake vigorously for 60 minutes. Centrifuge the mixture at 1800 rpm for 20 minutes, aspirate the upper (isopropyl myristate) layer, and determine the pH of the residual water layer: the pH is not less than 6.5.

Isopropyl Myristate not conforming to the test for pH of Water Extract may be rendered suitable for use in sterility test procedures as follows:

Using a 20-mm × 20-cm glass column, add *activated alumina*, and tamp down to a height of 15 cm. Pass 500 ml of the isopropyl myristate through the column, using a slight positive pressure to maintain an even flow, and use the eluate collected directly in the sterility test procedure.

Kaolin, Light A purified native hydrated aluminium silicate containing a suitable dispersing agent.

Use general reagent grade of commerce.

DESCRIPTION Light, white powder free from gritty particles, unctuous to the touch.

SOLUBILITY Practically insoluble in *water* and in mineral acids.

COARSE PARTICLES Transfer 5.0 g to a stoppered cylinder about 35 mm in diameter and about 16 cm in length, add 60 ml of a 1 per cent w/v solution of *sodium pyrophosphate*, shake thoroughly, and allow to stand for 5 minutes. By means of a pipette, draw off 50 ml from a point about 5 cm below the surface of the liquid. To the liquid remaining add 50 ml of *water*, shake, allow to stand for 5 minutes, and draw off 50 ml in the same way as before. Repeat the operation until a total of 400 ml of the suspension has been drawn off under the prescribed conditions. Transfer the remainder to an evaporating dish, and evaporate to dryness on a water-bath. The residue, after drying at 105°, weighs not more than 25 mg.

FINE PARTICLES Disperse 5.0 g in 250 ml of *water* by shaking vigorously for 2 minutes in a stoppered flask, immediately pour into a glass cylinder 5 cm in diameter, and transfer 20 ml by means of a pipette to a glass dish; evaporate to dryness and dry at 105° to constant weight. Allow the remainder of the suspension to stand for 4 hours at 20° and withdraw a second 20-ml portion, using a pipette with its tip exactly 5 cm below the surface and without disturbing the sediment. Transfer the second portion to a glass dish, evaporate to dryness, and dry at 105° to constant weight. The weight of the residue from the second portion is not less than 70 per cent of the weight of the residue from the first portion.

Lactose $C_{12}H_{22}O_{11}.H_2O$ = 360.31

Use analytical reagent grade of commerce.

DESCRIPTION White, free-flowing powder.

SOLUBILITY Freely but slowly soluble in *water*; practically insoluble in *ethanol*.

SPECIFIC ROTATION About +52.4° at 20°, determined in a 10 per cent w/v solution (Appendix 4.8).

Lanthanum Nitrate $La(NO_3)_3.6H_2O$ = 433.01

Use atomic absorption spectroscopic grade of commerce.

DESCRIPTION Colourless crystals. Deliquescent.

SOLUBILITY Freely soluble in *water*.

Store in tightly closed containers.

Lead(II) Acetate $C_4H_6O_4Pb.3H_2O$ = 379.34

Use analytical reagent grade of commerce.

DESCRIPTION Small, white, transparent, monoclinic prisms or heavy, crystalline masses; odour, acetous. Efflorescent in warm air. Becomes basic when heated.

SOLUBILITY Soluble in 2 parts of *water* and in 63 parts of *ethanol*; freely soluble in *glycerol*.

Lead(II) Nitrate $Pb(NO_3)_2$ = 331.21

Use analytical reagent grade of commerce.

DESCRIPTION Colourless or white crystals, or white crystalline powder.

SOLUBILITY Soluble in *water*, forming a clear, colourless solution.

Lead(IV) Oxide (Lead Dioxide) PbO_2 = 239.20

Use analytical reagent grade of commerce.

DESCRIPTION Dark brown powder.

SOLUBILITY Practically insoluble in *water*; soluble in *hydrochloric acid* with evolution of chlorine; soluble in hot concentrated alkali hydroxide solutions.

Lithium Hydroxide $LiOH.H_2O$ = 41.96

Use analytical reagent grade of commerce.

Store in tightly closed containers.

Magenta, Basic (Basic Fuchsin) $C_{20}H_{19}N_3.HCl$ = 337.85

A mixture of rosaniline and pararosaniline hydrochlorides.

DESCRIPTION Dark green powder or greenish glistening crystalline fragments, having a bronze-like lustre; odour, not more than a faint odour.

SOLUBILITY Soluble in *water*, in *amyl alcohol*, and in *ethanol*; insoluble in *ether*.

SENSITIVITY To 10 ml of a solution (1 in 500) add 10 ml of *ammonia TS* and 500 mg of *zinc powder*, and agitate the mixture: the solution becomes colourless. Place a few drops of the decolorized solution on filter paper and nearby, on the same paper, place a few drops of *dilute hydrochloric acid*: a red colour develops at the zone of contact.

LOSS ON DRYING Not more than 5.0 per cent w/w after drying at 105° to constant weight (Appendix 4.15).

SULFATED ASH Not more than 0.3 per cent w/w (Appendix 5.3).

Store protected from light.

Magnesium Oxide MgO = 40.30

Use general reagent grade of commerce.

DESCRIPTION White, fine powder.

SOLUBILITY Very slightly soluble in *water*; insoluble in *ethanol*, soluble in dilute acids with at most slight effervescence.

Magnesium Sulfate (Epsom Salts) $MgSO_4.7H_2O$ = 246.47

Use analytical reagent grade of commerce.

DESCRIPTION Brilliant, colourless crystals or white, crystalline powder; odourless. It efflorescent in warm, dry air.

SOLUBILITY Soluble in 1.5 parts of *water*; very soluble in boiling *water*; practically insoluble in *ethanol*.

Manganese(IV) Oxide (Manganese Dioxide) MnO_2 = 86.94

Use analytical reagent grade of commerce.

DESCRIPTION Black or brownish black powder.

Manganese(II) Sulfate $MnSO_4.H_2O$ = 169.01

Use analytical reagent grade of commerce.

DESCRIPTION Pale red, slightly efflorescent powder; odourless.

SOLUBILITY Soluble in *water*; insoluble in *ethanol*.

Store in tightly closed containers.

D-Mannose (Mannose) $C_6H_{12}O_6$ = 180.16

Use general reagent grade of commerce.

DESCRIPTION Colourless crystals or white, crystalline powder.

MELTING TEMPERATURE About 132° with decomposition (Appendix 4.3).

SPECIFIC ROTATION About +13.7° to +14.2° at 20°, determined in a 20 per cent w/v in *water* containing about 0.05 per cent w/v of NH_3 (Appendix 4.8).

2-Mercaptoethanol C_2H_6OS = 78.13

Use general reagent grade of commerce.

RELATIVE DENSITY About 1.116 (Appendix 4.9).

BOILING TEMPERATURE About 157° (Appendix 4.6).

Mercury(I) Nitrate (Mercurous Nitrate Dihydrate) $Hg_2(NO_3)_2.2H_2O$ = 561.22

Use analytical reagent grade of commerce.

DESCRIPTION Colourless crystals; odourless or slight nitric acid odour.

SOLUBILITY Soluble in *water*.

MELTING TEMPERATURE About 70° (Appendix 4.3).

Mercury(II) Chloride $HgCl_2$ = 271.50

Use analytical reagent grade of commerce.

DESCRIPTION Heavy, colourless or white, crystalline masses, or white, crystalline powder.

SOLUBILITY Soluble in 15 parts of *water* and in 3 parts of *ethanol*.

Mercury(II) Iodide HgI_2 = 454.40

Use general reagent grade of commerce.

DESCRIPTION Dense, scarlet, crystalline powder.

SOLUBILITY Slightly soluble in *water* and in *chloroform*; soluble in an excess of *potassium iodide TS*; sparingly soluble in *acetone*, in *ethanol* and in *ether*.

Store protected from light.

Mercury(II) Oxide, Yellow (Mercuric Oxide) HgO = 216.59

Use commercial grade.

DESCRIPTION Orange-yellow, amorphous powder.

Store protected from light.

Metalphthalein (Phthalein Purple) $C_{32}H_{32}N_2O_{12}.xH_2O$

Use indicator grade of commerce.

DESCRIPTION Creamy white to brown powder.

SENSITIVITY Dissolve 10 mg in 1 ml of 13.5 M *ammonia* and dilute with *water* to 100 ml. To 5 ml of the solution add 95 ml of *water*, 4 ml of 13.5 M *ammonia*, 50 ml of *ethanol* and 0.1 ml of 0.10 M *barium chloride*; the solution is bluish violet. Add 0.15 ml of 0.10 M *disodium edetate*: the solution becomes colourless.

Methanesulfonic Acid CH_4O_3S = 96.10

Use general reagent grade of commerce.

DESCRIPTION Colourless, corrosive liquid.

REFRACTIVE INDEX About 1.430 (Appendix 4.7).

WEIGHT PER MILLILITRE About 1.48 g (Appendix 4.9).

Methanol (Methyl Alcohol) CH_4O = 32.04

Use analytical reagent grade of commerce.

DESCRIPTION Colourless liquid.

BOILING RANGE 64° to 65° (Appendix 4.6).

RELATIVE DENSITY 0.791 to 0.793 (Appendix 4.9).

Methanol, Aldehyde-free *Methanol* which complies with the following additional test.

ALDEHYDES AND KETONES Not more than 0.001 per cent w/v. Dissolve 25 g of *iodine* in 1000 ml of *methanol*. Add this solution, with constant stirring, to 400 ml of 1 M *sodium hydroxide* and add 150 ml of *water*. Allow to stand for 16 hours, filter and boil under a reflux condenser until the odour of iodoform is no longer detectable. Distil the resulting solution by fractional distillation.

Methanol, Anhydrous *Methanol* which complies with the following additional test.

WATER Not more than 0.1 per cent w/w (Appendix 4.12).

Methenamine (Hexamine) $C_6H_{12}N_4$ = 140.19

Use analytical reagent grade of commerce containing not less than 99.0 per cent w/w of $C_6H_{12}N_4$.

DESCRIPTION White, crystalline powder or colourless crystals.

SOLUBILITY Freely soluble in *water*; soluble in *dichloromethane* and in *ethanol*.

Methylcellulose 450

Use general reagent grade of commerce. The nominal viscosity is 450 mPa·s.

DESCRIPTION White, yellowish white or greyish white powder or granules; almost odourless.

Methyl Red Sodium Salt $C_{15}H_{14}N_3NaO_2$ = 291.28

Use analytical reagent grade of commerce.

2-Methoxyethanol (Ethylene Glycol Monomethyl Ether) $C_3H_8O_2$ = 76.10

Use chromatographic reagent grade of commerce.

DESCRIPTION Clear, colourless liquid.

SOLUBILITY Miscible with *water*; with *ethanol* and with *ether*.

BOILING TEMPERATURE About 125° (Appendix 4.6).

REFRACTIVE INDEX About 1.406, at 20° (Appendix 4.7).

RELATIVE DENSITY About 0.93 (Appendix 4.9).

2-Methyl-1-Propanol (Isobutyl Alcohol) $C_4H_{10}O$ = 74.12

Use analytical reagent grade of commerce.

DESCRIPTION Colourless liquid; odour, characteristic.

BOILING TEMPERATURE About 107° (Appendix 4.6).

REFRACTIVE INDEX 1.397 to 1.399, at 15° (Appendix 4.7).

RELATIVE DENSITY About 0.80 (Appendix 4.9).

3-Methyl-2-benzothiazolinone Hydrazone Hydrochloride Hydrate $C_8H_9N_3S.HCl.H_2O$ = 233.70

MELTING TEMPERATURE About 270° (Appendix 4.3).

SUITABILITY FOR THE DETERMINATION OF ALDEHYDES To 2 ml of *aldehyde-free methanol* add 60 µl of a 0.1 per cent w/v solution of *propionaldehyde* in *aldehyde-free methanol* and 5 ml of a 0.4 per cent w/v solution of the reagent being examined, mix and allow to stand for 30 minutes. Add 25 ml of a 0.2 per cent w/v solution of *iron(III) chloride*, dilute to 100 ml with *acetone* and mix. The absorbance of the resulting solution at 660 nm is not less than 0.62 (Appendix 2.2). Use in the reference cell a solution prepared at the same time and in the same manner but without the propionaldehyde solution.

4-Methyl-2-pentanone (Methyl Isobutyl Ketone) $C_6H_{12}O$ = 100.16

DESCRIPTION Clear, colourless, stable liquid; odour, characteristic.

SOLUBILITY Slightly soluble in *water*; miscible with most organic solvents.

WEIGHT PER MILLILITRE 0.799 to 0.802 g (Appendix 4.9).

BOILING TEMPERATURE About 115° (Appendix 4.5).

Methylene Blue $C_{16}H_{18}ClN_3S.3H_2O$ = 373.90

Use a redox indicator grade of commerce.

DESCRIPTION Dark green crystals or crystalline powder, having a bronze-like lustre.

SOLUBILITY Soluble in 25 parts of *water* and in 65 parts of *ethanol*; soluble in *chloroform*.

***N,N′*-Methylenebisacrylamide** $C_7H_{10}N_2O_2$ = 154.17

Use general reagent grade of commerce.

DESCRIPTION White, fine powder.

***N*-Methylformamide** C_2H_5NO = 59.07

Use analytical reagent grade of commerce containing not less than 99 per cent v/v of C_2H_5NO when determined by gas chromatography.

Mordant Black 11 (Eriochrome Black T; Solochrome Black) $C_{20}H_{12}N_3NaO_7S$ = 461.38

Use general reagent grade of commerce.

DESCRIPTION Brownish black powder having a faint, metallic sheen.

SOLUBILITY Soluble in hot *water*, in *ethanol* and in *methanol*.

SENSITIVITY To 10 ml of a 0.0005 per cent w/v solution in a mixture of equal volumes of *methanol* and *water*, add 0.25 M *sodium hydroxide* until the pH is 10: the solution is pure blue in colour and free from cloudiness. Add 0.01 mg of magnesium ion (Mg): the colour of the solution changes to red-violet, and with the continued addition of magnesium ion it becomes wine-red.

Mordant Black 11 Mixture Grind 200 mg of *mordant black 11* to a fine powder with 20 g of *potassium chloride*.

Naphthalene $C_{10}H_8$ = 128.17

DESCRIPTION Monoclinic prismatic plates, or white scales or powder. A solution in petroleum ether shows a purple fluorescence under light from a mercury-arc lamp. Sublimes at temperatures above the melting temperature.

SOLUBILITY Insoluble in *water*; very soluble in *ether*, and in fixed and volatile oils; freely soluble in *carbon disulfide*, in *chloroform*, in *olive oil*, and in *toluene*; soluble in *ethanol* and in *methanol*.

MELTING RANGE 80° to 81° (Appendix 4.3).

BOILING RANGE 217° to 219° (Appendix 4.5).

Naphthalene Black 12B (Amido Black 10B; Acid Black 1) $C_{22}H_{14}N_4Na_2O_9S_2$ = 588.47

Use general reagent grade of commerce.

DESCRIPTION Dark brown powder.

1,3-Naphthalenediol (Naphthoresorcinol) $C_{10}H_8O_2$ = 160.17

Use general reagent grade of commerce.

DESCRIPTION Greyish white to tan crystals or powder.

SOLUBILITY Sparingly soluble in *water*, in *ethanol* and in *ether*; freely soluble in *methanol*.

MELTING RANGE 122° to 127° (Appendix 4.3).

SOLUBILITY TEST IN METHANOL Dissolve 500 mg in 50 ml of *methanol*: the solution is clear and complete.

1-Naphthol (α-Naphthol) $C_{10}H_8O$ = 144.17

Use analytical reagent grade of commerce.

DESCRIPTION Colourless or slightly pinkish crystals or crystalline powder.

SOLUBILITY Insoluble in *water*; soluble in *ethanol* and in *ether*. A 20 per cent w/v solution in *ethanol* yields a not more than slightly opalescent, colourless or almost colourless solution, with no pink tint.

MELTING TEMPERATURE About 95° (Appendix 4.3).

Store protected from light.

2-Naphthol (β-Naphthol) $C_{10}H_8O$ = 144.17

Use analytical reagent grade of commerce.

DESCRIPTION Crystalline; odour, faint phenolic.

MELTING TEMPERATURE About 122° (Appendix 4.3).

Store protected from light.

***N*-(1-Naphthyl)ethylenediamine Dihydrochloride** $C_{12}H_{14}N_2.2HCl$ = 259.18

Use general reagent grade of commerce which may contain methanol of crystallization.

DESCRIPTION White or cream powder.

SOLUBILITY Soluble in *water*.

MELTING TEMPREATURE Not less than 188° (Appendix 4.3).

Ninhydrin (Indane-1,2,3-trione) $C_9H_4O_3.H_2O$ = 178.14

Use analytical reagent grade of commerce.

DESCRIPTION A very pale yellow, crystalline powder.

MELTING TEMPERATURE About 255° (Appendix 4.3).

Store protected from light.

Nitric Acid HNO_3 = 63.01

When no molarity is indicated, use analytical reagent grade of commerce containing about 70.0 per cent w/w of HNO_3 and about 16 M in strength.

DESCRIPTION Corrosive, fuming liquid.

WEIGHT PER MILLILITRE About 1.42 g (Appendix 4.9).

When solutions of molarity xM are required, they should be prepared by diluting 63x ml of *nitric acid* with *water* to 1000 ml.

Store protected from light.

Nitric Acid, Dilute Mix 106 ml of *nitric acid* with sufficient *water* to produce 1000 ml (approximately 10 per cent w/w of HNO_3).

Nitrogen N_2 = 28.01

Use laboratory cylinder grade of commerce, washed with *water* and dried.

Nitrogen for Chromatography *Nitrogen* containing not less than 99.95 per cent v/v of N_2.

Nitromethane CH_3NO_2 = 61.04

> ***Caution*** Nitromethane forms explosive compounds with amines and strong bases.

Use general reagent grade of commerce.

DESCRIPTION Colourless liquid.

SOLUBILITY Slightly soluble in *water*; miscible with *ethanol* and with *ether*.

BOILING TEMPERATURE About 102° (Appendix 4.6).

REFRACTIVE INDEX 1.381 to 1.383, at 20° (Appendix 4.7).

RELATIVE DENSITY 1.132 to 1.134 (Appendix 4.9).

Octoxynol 10 (Octoxinol 10) $C_{34}H_{62}O_{11}$ (average) = 647

Use general reagent grade of commerce (Triton X-100 or equivalent is suitable).

DESCRIPTION Clear, pale yellow, viscous liquid.

Store in tightly closed containers.

Oxalic Acid $C_2H_2O_4.2H_2O$ = 126.07

Use general reagent grade of commerce.

DESCRIPTION Colourless crystals.

SOLUBILITY Soluble in *water* and in *ethanol*.

Oxygen O_2 = 32.00

Use general reagent grade of commerce containing not less than 99 per cent v/v of O_2.

DESCRIPTION Colourless gas; odourless. One litre at 0° and at a pressure of 101.3 kPa (about 760 Torr) weighs about 1.429 g.

SOLUBILITY One volume dissolves in about 32 volumes of *water* and in about 7 volumes of *ethanol* at 20° and at a pressure of 101.3 kPa (about 760 Torr).

Papain

Use a suitable grade.

DESCRIPTION White to light tan, amorphous powder.

SOLUBILITY Soluble in *water*; the solution being colourless to light yellow and more or less opalescent; practically insoluble in *chloroform*, in *ethanol* and in *ether*.

Pentane (*n*-Pentane) C_5H_{12} = 72.15

DESCRIPTION Clear, colourless, flammable liquid.

SOLUBILITY Very slightly soluble in *water*; miscible with *ethanol*, with *ether* and with many organic solvents.

BOILING RANGE Not less than 95 per cent distils between 34° and 36° (Appendix 4.5).

WEIGHT PER MILLILITRE About 0.63 g (Appendix 4.9).

Pepsin

A substance containing a proteolytic enzyme of the gastric secretion of animals, diluted, if necessary, by admixture with lactose or sucrose. Use a grade of commerce capable of digesting 2500 times its own weight of coagulated egg albumen.

DESCRIPTION Colourless, or light buff-coloured, amorphous powder, or translucent scales; odour, faintly meaty.

SOLUBILITY Soluble in *water*, yielding an opalescent solution; insoluble in *ethanol* and in *ether*.

Perchloric Acid $HClO_4$ = 100.46

When no molarity is indicated, use analytical reagent grade of commerce containing not less than 70.0 per cent and not more than 73.0 per cent w/w of $HClO_4$ and about 12 M in strength.

DESCRIPTION Corrosive liquid.

WEIGHT PER MILLILITRE About 1.7 g (Appendix 4.9).

Petroleum Ether (Light Petroleum)

> ***Caution*** Petroleum Ether is dangerously flammable. Keep away from flames and store in tightly closed containers in a cool place.

DESCRIPTION Colourless, very volatile, highly flammable liquid, obtained from petroleum, consisting of a mixture of the lower members of the paraffin series of hydrocarbons supplied in the following fractions:
boiling range, 30° to 40°; weight per ml, about 0.63 g
boiling range, 40° to 60°; weight per ml, about 0.64 g
boiling range, 50° to 70°; weight per ml, about 0.66 g
boiling range, 60° to 80°; weight per ml, about 0.67 g
boiling range, 80° to 100°; weight per ml, about 0.70 g
boiling range, 100° to 120°; weight per ml, about 0.72 g
boiling range, 120° to 160°; weight per ml, about 0.75 g.

Phenol C_6H_6O = 94.11

> ***Caution*** Phenol is caustic, and blanches the skin and mucous membranes.

Use analytical reagent grade of commerce.

DESCRIPTION Deliquescent crystals; odour, characteristic.

FREEZING TEMPERATURE Not less than 40° (Appendix 4.4).

BOILING TEMPERATURE About 180° (Appendix 4.6).

Store protected from light at a temperature not exceeding 15°.

2-Phenoxyethanol $C_8H_{10}O_2$ = 138.17

DESCRIPTION Colourless, slightly viscous liquid.

SOLUBILITY Soluble in *water*. Miscible with *acetone*, with *ethanol* and with *glycerol*.

FREEZING TEMPERATURE Not less than 12.0° (Appendix 4.4).

REFRACTIVE INDEX 1.536 to 1.538 (Appendix 4.7).

WEIGHT PER MILLILITRE 1.105 to 1.110 g (Appendix 4.9).

Phospholipid Wash a quantity of human or bovine brain freed from meninges and blood vessels and macerate in a suitable blender. Weigh 1000 to 1300 g of the macerate and measure its volume (*v* ml). Extract with three 4*v*-ml portions of *acetone*, filter by suction and dry the precipitate at 37° for 18 hours. Extract the dried precipitate with two 2*v*-ml portions of a mixture of 2 volumes of *petroleum ether (boiling range, 30° to 40°)* and 3 volumes of *petroleum ether (boiling range, 40° to 60°)*, filtering each extract through a filter paper previously washed with the petroleum ether mixture. Combine the extracts and evaporate to dryness at 45° at a pressure not exceeding 0.7 kPa (about 5 Torr). Dissolve the residue in 0.2*v* ml of *ether* and allow to stand at 4° until a deposit is produced. Centrifuge and evaporate the clear supernatant liquid under reduced pressure until the volume is about 100 ml per kg of the original macerate. Allow to stand at 4° until a precipitate is produced (12 to 24 hours) and centrifuge. To the clear supernatant liquid add 5 volumes of *acetone*, centrifuge, discard the supernatant liquid, and dry the precipitate.

Store protected from light in a vacuum desiccator.

Phosphomolybdic Acid $H_3PO_4.12MoO_3.24H_2O$ = 2257.62

Use analytical reagent grade of commerce.

DESCRIPTION Fine, orange-yellow crystals.

SOLUBILITY Very soluble in *water*.

Phosphoric Acid (Orthophosphoric Acid) H_3PO_4 = 98.00

> ***Caution*** Avoid contact, as phosphoric acid rapidly destroys tissues.

Use analytical reagent grade of commerce containing not less than 84 per cent w/w of H_3PO_4 and about 15.7 M in strength.

DESCRIPTION Corrosive, clear colourless, syrupy liquid.

SOLUBILITY Miscible with *water* and with *ethanol*.

WEIGHT PER MILLILITRE About 1.75 g (Appendix 4.9).

Phosphorus Pentoxide Desiccant (Diphosphorus Pentoxide) P_2O_5 = 141.94

Use a grade specially supplied for use in desiccators.

DESCRIPTION White, amorphous, deliquescent powder hydrated by water with the evolution of heat.

Store in well-closed containers.

Piperazine Hydrate (Piperazine Hexahydrate; Diethylenediamine) $C_4H_{10}N_2.6H_2O$ = 194.23

Use general reagent grade of commerce.

DESCRIPTION Colourless, glossy, deliquescent crystals.

MELTING TEMPERATURE About 44° (Appendix 4.3).

Plasma, Platelet-poor Withdraw 45 ml of human blood into a 50-ml plastic syringe containing 5 ml of a sterile 3.8 per cent w/v solution of *sodium citrate*. Immediately centrifuge the citrated whole blood at 1150 × *g* for 30 minutes at 4°. Remove the upper two thirds of the supernatant plasma using a plastic syringe and immediately centrifuge at 3500 × *g* for 30 minutes at 4°. Remove the upper two thirds of the liquid and freeze it rapidly in suitable quantities in plastic tubes at a temperature of –40° or below. Use plastic or siliconized equipment throughout.

Plasma Substrate 2 Prepare from human blood containing less than 1 per cent of the normal amount of factor IX. Collect 9 volumes of the blood into 1 volume of a 3.8 per cent w/v solution of *sodium citrate.*

Store in small amounts in plastic tubes at a temperature of –30° or below.

Platelet Substitute To 0.5 to 1 g of *phospholipids* add 20 ml of *acetone* and allow to stand for 2 hours with frequent shaking. Centrifuge for 2 minutes and discard the supernatant liquid. Dry the residue using a water pump, mix with 20 ml of *chloroform* and shake for 2 hours. Filter under vacuum and suspend the residue obtained in 5 to 10 ml of *saline TS*.

Prepare a dilution in *saline TS* so that it will give clotting time differences between consecutive dilutions of the reference preparation used in the "Biological Assay of Human Coagulation Factor IX" (Appendix 15.1.5) of about 10 seconds.

Store the dilute suspensions at –30° and use within 6 weeks.

2-Propanol (Isopropanol) C_3H_8O = 60.10

Use analytical reagent grade of commerce.

DESCRIPTION Colourless liquid; odour, characteristic.

BOILING RANGE 81° to 83° (Appendix 4.5).

WEIGHT PER MILLILITRE About 0.785 g (Appendix 4.9).

Protamine Sulfate

Use general reagent grade of commerce.

Polyethyleneglycol 400 (Macrogol 400)

Use general reagent grade of commerce.

DESCRIPTION Clear, colourless, viscous liquid.

SOLUBILITY Soluble in *water* and in *ethanol*.

FREEZING TEMPERATURE About 6° (Appendix 4.4).

WEIGHT PER MILLILITRE About 1.13 g (Appendix 4.9).

VISCOSITY At 20°, about 130 mPa.s (Appendix 4.10).

Polymethylphenylsiloxane Average molecular weight, 4000.

Use chromatographic grade of commerce containing about 50 per cent of methyl groups and 50 per cent of phenyl groups.

DESCRIPTION Very viscous liquid (about 1300 mPa·s).

WEIGHT PER MILLILITRE About 1.09 g (Appendix 4.9).

Polysorbate 20 (Polyoxyethylene(20) Sorbitan Monolaurate)

Use general reagent grade of commerce (Tween 20 is suitable).

DESCRIPTION Yellow or brownish yellow, oily liquid.

WEIGHT PER MILLILITRE 1.073 to 1.078 g (Appendix 4.9).

Polysorbate 80 (Polyoxyethylene(20) Sorbitan Monooleate)

Use general reagent grade of commerce (Tween 80 is suitable).

DESCRIPTION Yellow to amber coloured, oily liquid.

WEIGHT PER MILLILITRE About 1.10 g (Appendix 4.9).

Polyvinyl Alcohol $(C_2H_4O)_n$

DESCRIPTION White to cream-coloured granules, or white to cream-coloured powder; odourless.

SOLUBILITY Freely soluble in *water* at room temperature. Solutions may be effected more rapidly at somewhat higher temperatures.

Store in tightly closed containers.

Potassium Bromide KBr = 119.00

Use analytical reagent grade of commerce.

DESCRIPTION White, cubical crystals or granular powder; odourless.

SOLUBILITY Soluble in *water*; slightly soluble in *ethanol*.

Potassium bromide used for infrared absorption spectrophotometry complies with the following requirement.

INFRARED ABSORPTION The spectrum of a disc prepared from the material, previously dried at 250° for 1 hour, has a substantially flat baseline over the range of 4000 to 620 cm^{-1}; it exhibits no maxima with an absorbance greater than 0.02 above the baseline with the exception of maxima due to water at 3440 and 1630 cm^{-1} (Appendix 2.1).

Potassium Carbonate, Anhydrous K_2CO_3 = 138.20

Use analytical reagent grade of commerce.

DESCRIPTION White, granular powder. Hygroscopic.

SOLUBILITY Very soluble in *water*.

Store in tightly closed containers.

Potassium Chloride KCl = 74.55

Use analytical reagent grade of commerce.

DESCRIPTION Colourless crystals or white, granular powder; odourless.

SOLUBILITY Very soluble in *water*; slightly soluble in *ethanol*.

Potassium chloride used for infrared absorption spectrophotometry complies with the following requirement.

INFRARED ABSORPTION The spectrum of a disc prepared from the material, previously dried at 250° for 1 hour, has a substantially flat baseline over the range of 4000 to 620 cm^{-1}; it exhibits no maxima with an absorbance greater than 0.02 above the baseline with the exception of maxima due to water at 3440 and 1630 cm^{-1} (Appendix 2.1).

Potassium Chromate K_2CrO_4 = 194.19

Use analytical reagent grade of commerce.

DESCRIPTION Yellow crystals.

SOLUBILITY Very soluble in *water*.

Potassium Cyanide KCN = 65.12

> ***Caution*** Because of the extremely poisonous nature of cyanides, all tests are to be carried out under a hood with a strong draught. In measuring solutions use a burette NOT a pipette.

Use analytical reagent grade of commerce.

DESCRIPTION White, crystalline powder or white mass or crystals, gradually decomposing on exposure to air.

SOLUBILITY Very soluble in *water*, forming a clear, colourless solution.

Potassium Dichromate (Dipotassium Dichromate) $K_2Cr_2O_7$ = 294.18

Use analytical reagent grade of commerce.

DESCRIPTION Orange-red crystals or crystalline powder.

SOLUBILITY Soluble in *water*; practically insoluble in *ethanol*.

Potassium dichromate used for the calibration of spectrophotometers contains not less than 99.9 per cent w/w of $K_2Cr_2O_7$, calculated with reference to the substance dried at 130° for 1 hour.

Potassium Dihydrogencitrate $C_6H_7KO_7$ = 230.22

Use general reagent grade of commerce.

Potassium Dihydrogenphosphate (Potassium Dihydrogen Orthophosphate) KH_2PO_4 = 136.09

Use analytical reagent grade of commerce.

DESCRIPTION Colourless crystals or white, granular or crystalline powder; odourless.

SOLUBILITY Freely soluble in *water*; practically insoluble in *ethanol*.

Potassium Hexacyanoferrate(II) (Potassium Ferrocyanide) $K_4Fe(CN)_6.3H_2O$ = 422.39

Use analytical reagent grade of commerce.

DESCRIPTION Yellow, crystalline powder.

SOLUBILITY Soluble in *water*.

Potassium Hexacyanoferrate(III) (Potassium Ferricyanide) $K_3Fe(CN)_6$ = 329.25

Use analytical reagent grade of commerce.

DESCRIPTION Ruby-red crystals.

SOLUBILITY Very soluble in *water*.

Potassium Hydrogentartrate $C_4H_5KO_6$ = 188.18

Use analytical reagent grade of commerce.

DESCRIPTION White, crystalline powder or colourless, slightly opaque crystals.

SOLUBILITY Soluble in 165 parts of *water* and in 16 parts of boiling *water*; very slightly soluble in *ethanol*.

Potassium Hydroxide KOH = 56.11

Use analytical reagent grade of commerce.

DESCRIPTION White or practically white, fused masses, or small pellets, or flakes, or sticks, or other forms.

SOLUBILITY Freely soluble in water, in *ethanol* and in *glycerol*; very soluble in boiling *ethanol*.

Store in tightly closed containers.

Potassium Hydroxide, 0.5 M Ethanolic Dissolve about 34 g of *potassium hydroxide* in 20 ml of *water*, and add sufficient *aldehyde-free ethanol* to make 1000 ml. Allow to stand for 24 hours and decant the clear supernatant liquid.

Store in tightly closed containers.

Potassium Iodide KI = 166.0

Use analytical reagent grade of commerce.

DESCRIPTION White crystalline powder.

Potassium Nitrate KNO_3 = 101.10

Use analytical reagent grade of commerce.

DESCRIPTION Colourless crystals, or white crystalline powder; odourless.

SOLUBILITY Soluble in 3.3 parts of *water*; very soluble in boiling *water*; soluble in *glycerol*; practically insoluble in *ethanol*.

Potassium Permanganate $KMnO_4$ = 158.03

Use analytical grade of commerce.

Potassium Sodium Tartrate $C_4H_4KNaO_6.4H_2O$ = 282.22

Use analytical reagent grade of commerce.

DESCRIPTION Colourless crystals or white, crystalline powder; odourless.

SOLUBILITY Soluble in *water*; almost insoluble in *ethanol*.

Potassium Sulfate K_2SO_4 = 174.26

Use analytical reagent grade of commerce.

DESCRIPTION Hard, colourless crystals, or white granules or powder.

SOLUBILITY Soluble in *water*; practically insoluble in *ethanol*.

Potassium Tetroxalate (Potassium Trihydrogen Dioxalate) $C_4H_3KO_8.2H_2O$ = 254.2

Use analytical reagent grade of commerce.

DESCRIPTION White, crystalline powder.

SOLUBILITY Soluble in 60 parts of *water* and in 12 parts of boiling *water*; slightly soluble in *ethanol*.

1-Propanol (*n*-Propyl Alcohol) C_3H_8O = 60.10

DESCRIPTION Clear, colourless liquid.

SOLUBILITY Miscible with *water* and with *ethanol*.

BOILING RANGE Not less than 95 per cent distils between 96° and 99° (Appendix 4.5).

Propionaldehyde (Propanal) C_3H_6O = 58.08

Use general reagent grade of commerce.

DESCRIPTION Liquid; odour, suffocating.

SOLUBILITY Soluble at 20° in 5 parts of *water*. Miscible with *ethanol* and *ether*.

MELTING TEMPERATURE About –81° (Appendix 4.3).

BOILING TEMPERATURE About 49° (Appendix 4.6).

REFRACTIVE INDEX 1.365 at 20° (Appendix 4.7).

WEIGHT PER MILLILITRE About 0.81 g (Appendix 4.9).

Propylene Glycol (Propane-1,2-diol) $C_3H_8O_2$ = 76.10

Use general reagent grade of commerce.

DESCRIPTION Colourless, hygroscopic, viscous liquid.

SOLUBILITY Miscible with *water*, with *chloroform* and with *ethanol*; soluble in 6 parts of *ether*; immiscible with *petroleum ether* and with fixed oils.

BOILING TEMPERATURE About 187° (Appendix 4.6).

WEIGHT PER MILLILITRE About 1.04 g (Appendix 4.9).

Pyridylazonaphthol (1-(2-Pyridylazo)-2-naphthol; PAN) $C_{15}H_{11}N_3O$ = 249.27

DESCRIPTION Stable, orange-red crystals.

SOLUBILITY Slightly soluble in *water*; soluble in *ethanol* and in hot solutions of dilute Alkalines.

MELTING RANGE 140° to 142° (Appendix 4.3).

SENSITIVITY Add 0.1 ml of a 0.1 per cent w/v solution in *ethanol* to a mixture of 10 ml of *water* and 1 ml of a buffer

solution prepared by mixing 80 ml of 0.2 M *acetic acid* and 20 ml of an 8.2 per cent w/v solution of *sodium acetate*, and mix. To this solution add 1 ml of a mixture of 1 ml of *copper(II) sulfate TS* and 2 ml of water, and mix; the colour changes from yellow to red.

Pyridine C_5H_5N = 79.10

Use analytical reagent grade of commerce.

DESCRIPTION Colourless liquid; odour, unpleasant.

BOILING TEMPERATURE About 115° (Appendix 4.6).

Store in well-closed containers.

Pyridine, Anhydrous Dry *pyridine* over *anhydrous sodium carbonate*, filter and distil.

WATER Not more than 0.01 per cent w/w (Karl Fischer Method, Appendix 4.12).

Quinine Sulfate $(C_{20}H_{24}N_2O_2)_2.H_2SO_4.2H_2O$ = 782.95

Use general reagent grade of commerce.

DESCRIPTION White or off-white, efflorescent, crystalline solid.

SOLUBILITY Slightly soluble in *water*, in *chloroform* and in *ethanol*; freely soluble in *ethanol* at 80° and in a mixture of 2 parts of *chloroform* and 1 part of *dehydrated ethanol*; sparingly soluble in *water* at 100°; very slightly soluble in *ether*.

MELTING TEMPERATURE About 234°, with decomposition (Appendix 4.3).

SPECIFIC ROTATION About –220°, determined in a 0.5 per cent w/v solution in 0.5 M *hydrochloric acid* (Appendix 4.8).

Store protected from light.

Rabies Antiserum, Fluorescein-conjugated Immunoglobulin fraction with a high rabies antibody titre, prepared from the sera of suitable animals that have been immunized with inactivated rabies virus. The immunoglobulin is conjugated with fluorescein isothiocyanate.

Resorcinol (Benzene-1,3-diol) $C_6H_6O_2$ = 110.11

Use analytical reagent grade of commerce.

DESCRIPTION Colourless crystals or crystalline powder.

MELTING TEMPERATURE About 111° (Appendix 4.3).

Salicylic Acid $C_7H_6O_3$ = 138.12

Use analytical reagent grade of commerce.

DESCRIPTION Colourless crystals, usually in fine needles, or fluffy, white, crystalline powder.

SOLUBILITY Slightly soluble in *water* and in *benzene*; freely soluble in *ethanol* and in *ether*; soluble in boiling *water*; sparingly soluble in *chloroform*.

MELTING RANGE 158° to 161° (Appendix 4.3).

Selenium Se = 78.96

Use a suitable grade with a content of not less than 99.99 per cent.

DESCRIPTION Dark-red amorphous, or bluish black, crystalline powder.

SOLUBILITY Insoluble in *water*. Soluble in solutions of sodium and potassium hydroxides or sulfides.

Sesame Oil Sesame Oil is the refined fixed oil obtained from the seed of one or more cultivated varieties of *Sesamum indicum* L. (Family Pedaliaceae).

Use general reagent grade of commerce.

DESCRIPTION Pale yellow, oily liquid; almost odourless.

SOLUBILITY Slightly soluble in *ethanol*; miscible with *carbon disulfide*, with *chloroform*, with *ether*, and with *petroleum ether*.

SPECIFIC GRAVITY 0.916 to 0.921 (Appendix 4.9)

Store in tightly closed containers, protected from light and excessive heat.

Silica Gel, Self-Indicating

DESCRIPTION Amorphous, partly hydrated SiO_2, occurring in glassy granules of varying sizes. It is frequently coated with a substance that changes colour when the capacity to absorb water is exhausted. Such coloured products may be regenerated (i.e., may regain their capacity to absorb water) by heating at 110° until the gel assumes the original colour.

LOSS ON IGNITION Not more than 6.0 per cent w/w (Appendix 4.16). Ignite 2 g, accurately weighed, at 950°±50° to constant weight.

WATER ABSORPTION Not less than 31.0 per cent w/w. Place about 10 g in a tared weighing bottle and weigh. Then place the bottle, with the cover removed, for 24 hours in a closed container in which the atmosphere is maintained at 80 per cent relative humidity by being in equilibrium with sulfuric acid having a specific gravity of 1.19. Weigh and calculate the weight increase.

Silver Diethyldithiocarbamate $C_5H_{10}AgNS_2$ = 256.13

Use general reagent grade of commerce.

DESCRIPTION Pale yellow powder.

SOLUTION IN PYRIDINE A 1.0 per cent w/v solution in *pyridine* is clear and yellow.

Store protected from light.

Silver Nitrate $AgNO_3$ = 169.87

Use analytical reagent grade of commerce.

Silver Oxide Ag_2O = 231.74

Use general reagent grade of commerce.

DESCRIPTION Brownish black, heavy powder.

SOLUBILITY Practically insoluble in *water*; freely soluble in *ammonia TS* and in *dilute nitric acid*; insoluble in *ethanol*.

Store protected from light and avoid exposing to ammonia fumes or easily oxidizable substances.

Sinapic Acid (Sinapinic Acid) $C_{11}H_{12}O_5$ = 224.21

Use general reagent grade of commerce.

DESCRIPTION Slightly yellow matrix substance.

MELTING TEMPERATURE About 202° (Appendix 4.3).

Sodium Acetate $C_2H_3O_2Na.3H_2O$ = 136.08

Use analytical reagent grade of commerce.

Sodium Azide NaN_3 = 65.01

> ***Caution*** Sodium azide is a potent poison. Its conjugate acid HN_3 is more toxic than hydrogen cyanide and is readily liberated from neutral aqueous solutions. Contact of NaH_3 or hydrazoic acid (HN_3) with certain metals may produce explosive salts. Work in a well-ventilated hood, and handle the sample with care.

Use analytical reagent grade of commerce.

Sodium Carbonate $Na_2CO_3.10H_2O$ = 286.14

Use analytical reagent grade of commerce.

MELTING TEMPERATURE Greater than 300° (Appendix 4.3).

Sodium Carbonate, Anhydrous Na_2CO_3 = 105.99

Use analytical reagent grade of commerce.

DESCRIPTION White, hygroscopic powder which loses not more than 1 per cent of its weight on heating to about 300°.

SOLUBILITY Slowly soluble in *water*.

Store in tightly closed containers.

Sodium Chloride NaCl = 58.44

Use analytical reagent grade of commerce.

Sodium Citrate Use *Sodium Citrate Dihydrate* (see under "Reagents").

Sodium Citrate Dihydrate (Trisodium Citrate Dihydrate) $C_6H_5O_7.3Na.2H_2O$ = 294.10

Use analytical reagent grade of commerce.

Sodium Diethyldithiocarbamate $C_5H_{10}NNaS_2.3H_2O$ = 225.30

Use analytical reagent grade of commerce.

DESCRIPTION White or colourless crystals.

SOLUBILITY Very soluble in *water*, yielding a colourless solution.

Sodium Dihydrogenphosphate $NaH_2PO_4.2H_2O$ = 156.01

Use analytical reagent grade of commerce.

Sodium Dihydrogenphosphate Monohydrate (Sodium Dihydrogen Orthophosphate Monohydrate) $NaH_2PO_4.H_2O$ = 137.99

Use analytical reagent grade of commerce.

Store in tightly closed containers.

Sodium Dodecyl Sulfate (Sodium Lauryl Sulfate) $C_{12}H_{25}NaO_4S$ = 288.38

Use purified grade of commerce containing not less than 99.0 per cent w/w of $C_{12}H_{25}NaO_4S$.

DESCRIPTION White, crystalline flakes.

Sodium Fluoride NaF = 41.99

Use analytical reagent grade of commerce. When used in the assay of preparations containing sodium fluoride, use a grade containing not less than 99.9 per cent w/w of NaF.

DESCRIPTION White powder or colourless crystals.

SOLUBILITY Soluble in 25 parts of *water*; practically insoluble in *ethanol*.

Sodium 1-Heptanesulfonate (1-Heptanesulfonic Acid Sodium Salt) $C_7H_{15}NaO_3S$ = 202.24

Use chromatographic reagent grade of commerce containing not less than 96.0 per cent w/w of $C_7H_{15}NaO_3S$.

Sodium Hydrogencarbonate (Sodium Bicarbonate) $NaHCO_3$ = 84.01

Use analytical reagent grade of commerce.

Sodium Hydrosulfite (Sodium Dithionite) $Na_2S_2O_4$ = 174.10

Use general reagent grade of commerce.

DESCRIPTION White or greyish white, crystalline powder.

SOLUBILITY Soluble in *water*; slightly soluble in *ethanol*.

Store in well-closed containers.

Sodium Hydroxide NaOH = 40.00

Use analytical reagent grade of commerce.

DESCRIPTION White or practically white, fused masses, in small pellets, in flakes, or sticks, and in other forms.

SOLUBILITY Freely soluble in *water* and in *ethanol*.

Store in tightly closed containers.

Sodium Hydroxide, Ethanolic Solutions of the requisite molarity may be obtained by dissolving the appropriate amount of *sodium hydroxide* in sufficient *ethanol* to produce 1000 ml.

Sodium Hypochlorite Solution

> ***Caution*** This solution is corrosive and may evolve gases that are corrosive and toxic. It is a powerful oxidant that can react violently with reducing agents. It is irritating and corrosive to skin and mucous membranes.

Use general reagent grade of commerce containing 10 to 14 per cent w/v of available chlorine.

DESCRIPTION Colourless to pale yellow, strongly caustic solution.

Sodium Hypophosphite (Sodium Phosphinate Monohydrate) $NaH_2PO_2.H_2O$ = 105.99

> ***Caution*** It explodes when triturated with chlorates or other oxidizing agents

Use general reagent grade of commerce.

DESCRIPTION White, deliquescent granules; odourless.

SOLUBILITY Very soluble in boiling *water*; freely soluble in *water*, in *glycerol* and in *boiling ethanol*; soluble in cold *ethanol*; slightly soluble in *absolute ethanol*; insoluble in *ether*.

Store in tightly closed containers.

Sodium Metabisulfite (Sodium Pyrosulfite) $Na_2S_2O_5$ = 190.10

Use analytical reagent grade of commerce containing not less than 95.0 per cent w/w of $Na_2S_2O_5$.

DESCRIPTION Colourless, prismatic crystals or white or creamy white powder; odour, sulfurous.

SOLUBILITY Soluble in 2 parts of *water*; freely soluble in *ethanol*.

Store in well-closed containers. On exposure to air and moisture, it is slowly oxidized to sulfate, with disintegration of the crystals.

Sodium Molybdate $Na_2MoO_4.2H_2O$ = 241.95

Use analytical reagent grade of commerce.

Sodium Nitrite $NaNO_2$ = 69.00

Use analytical reagent grade of commerce.

DESCRIPTION White to slightly yellow, granular powder, or white or practically white, opaque, fused masses or sticks. It is deliquescent in air.

SOLUBILITY Freely soluble in *water*; sparingly soluble in *ethanol*.

Sodium Nitroferricyanide (Sodium Nitroprusside) $Na_2[Fe(CN)_5(NO)].2H_2O$ = 297.95

Use analytical reagent grade of commerce.

DESCRIPTION Reddish brown powder or crystals.

SOLUBILITY Readily soluble in *water*.

Sodium Oxalate $C_2Na_2O_4$ = 134.00

Use analytical reagent grade of commerce.

DESCRIPTION White crystalline powder.

SOLUBILITY Slightly soluble in *water*.

Sodium 1-Pentanesulfonate (1-Pentanesulfonic Acid Sodium Salt) $C_5H_{11}NaO_3S$ = 174.19

Use a suitable grade.

Sodium Perchlorate $NaClO_4.H_2O$ = 140.46

Use analytical reagent grade of commerce containing not less than 99.0 per cent w/w of $NaClO_4.H_2O$.

DESCRIPTION White, deliquescent crystals.

Sodium Sulfate, Anhydrous Na_2SO_4 = 142.04

DESCRIPTION White, crystalline powder or granules. Hygroscopic.

SOLUBILITY Soluble in 6 parts of *water*; insoluble in *ethanol*.

ACIDITY OR ALKALINITY Dissolve 4 g in 100 ml of *carbon dioxide-free water*. The solution requires for neutralization to the green colour of *bromothymol blue TS* indicative of pH 7 not more than 0.50 ml of either 0.10 M *sodium hydroxide* or 0.10 M *hydrochloric acid*.

LOSS ON IGNITION Not more than 0.5 per cent w/w (Appendix 4.16). Weigh accurately about 2 g, and ignite at a low red heat in a tared dish.

CHLORIDE Not more than 0.07 per cent w/w (Appendix 5.2). A 500-mg sample shows no more chloride than that corresponds to 0.50 ml of 0.020 M *hydrochloric acid*.

IRON Not more than 20 ppm. Dissolve 500 mg in 40 ml of *water* and 2 ml of *hydrochloric acid*. The solution complies with the "Limit Test for Iron" (Appendix 5.2).

Sodium Sulfide $Na_2S.9H_2O$ = 240.18

Use analytical reagent grade of commerce.

DESCRIPTION Moist, colourless crystals. Deliquescent.

SOLUBILITY Readily soluble in *water*.

Store in tightly closed containers.

Sodium Tartrate $C_4H_4O_6Na_2.2H_2O$ = 230.08

Use analytical reagent grade of commerce.

DESCRIPTION Transparent crystals.

SOLUBILITY Very soluble in *water*; practically insoluble in *ethanol*.

Sodium Tetraborate (Borax; Disodium Tetraborate) $Na_2B_4O_7.10H_2O$ = 381.37

Use analytical reagent grade of commerce.

DESCRIPTION Colourless crystals, crystalline masses or white crystalline powder, efflorescent; odourless.

SOLUBILITY Soluble in *water*; very soluble in boiling *water*; freely soluble in *glycerol*.

Sodium Tetradeuteriodimethylsilapentanoate $C_6H_9D_4NaO_2Si$ = 172.27

Use general reagent grade of commerce with an isotopic purity of not less than 99 per cent and containing not more than 0.5 per cent of water and deuterium oxide.

DESCRIPTION White, crystalline powder.

SOLUBILITY Freely soluble in *water*, in *ethanol* and in *methanol*.

MELTING TEMPERATURE About 300° (Appendix 4.3).

Sodium Tetraphenylborate $C_{24}H_{20}BNa$ = 342.22

Use general reagent grade of commerce.

DESCRIPTION White or slightly yellowish, bulky powder.

SOLUBILITY Freely soluble in *water* and in *acetone*; insoluble in *petroleum ether*.

Sodium Thioglycolate (Sodium Mercaptoacetate) $C_2H_3NaO_2S$ = 114.09

Use general reagent grade of commerce.

DESCRIPTION White, granular powder.

SOLUBILITY Very soluble in *water*; soluble in *ethanol*.

Store in tightly closed containers, protected from light.

Sodium Thiosulfate $Na_2S_2O_3.5H_2O$ = 248.17

Use analytical reagent grade of commerce.

Sodium Tungstate $Na_2WO_4.2H_2O$ = 329.86

Use analytical reagent grade of commerce.

DESCRIPTION Colourless crystals, or white crystalline powder.

SOLUBILITY Readily soluble in *water*.

Starch, Corn

Corn Starch is obtained from the mature grain of *Zea mays* L. (Family Gramineae).

DESCRIPTION *Microscopical* Angular polyhedral granules, 2 to 23 µm in size, or rounded granules, 25 to 32 µm in size. Central hilum consisting of a distinct cavity or 2- to 5-rayed cleft; no concentric striations. Viewed under polarized light, a distinct black cross is seen, intersecting at the hilum.

LOSS ON DRYING Not more than 14.0 per cent w/w after drying at 105° to constant weight (Appendix 4.15).

ACIDITY OR ALKALINITY Triturate about 500 mg with 5 ml of *water*; the mixture is neutral to *litmus paper*.

SULFATED ASH Not more than 0.5 per cent w/w (Appendix 5.3).

Starch, Potato

Potato Starch is obtained from the tuber of *Solanum tuberosum* L. (Family Solanaceae).

DESCRIPTION *Microscopical* Single granules either irregular, ovoid, 30 to 100 µm in size, or rounded, 10 to 35 µm in size, compound granules rare, consisting of groups of two to four elements. Eccentric hilum, with clearly visible concentric striations. Viewed under polarized light, a distinct black cross is seen, intersecting at the hilum.

LOSS ON DRYING Not more than 20.0 per cent w/w after drying at 105° to constant weight (Appendix 4.15).

ACIDITY OR ALKALINITY; SULFATED ASH Complies with the requirements described under *Corn Starch*, p. 318.

Starch, Rice

Rice Starch is obtained from caryopsis of *Oryza sativa* L. (Family Gramineae).

DESCRIPTION *Microscopical* Polyhedral, simple or occasionally compound, granules, 2 to 10 µm in size. Central hilum, poorly visible; no concentric striations. Viewed under polarized light, a distinct black cross is seen, intersecting at the hilum.

LOSS ON DRYING Not more than 15.0 per cent w/w after drying at 105° to constant weight (Appendix 4.15).

SULFATED ASH Not more than 0.8 per cent w/w (Appendix 5.3).

Starch, Soluble Starch which has been treated with hydrochloric acid until, after being washed, it forms an almost clear limpid solution in hot water.

DESCRIPTION Fine, white powder.

SOLUBILITY Soluble in hot *water*, usually forming a slightly turbid solution.

ACIDITY OR ALKALINITY Shake 2.0 g with 20 ml of *water* for 3 minutes and filter: the filtrate is not alkaline, or more than faintly acid, to *litmus paper*.

SENSITIVITY Mix 1.0 g with a little cold *water* and add 200 ml of boiling *water*. Add 5 ml of this solution to 100 ml of *water* and add 0.50 ml of 0.0050 M *iodine*. The deep blue colour is discharged by 0.50 ml of 0.010 M *sodium thiosulfate*.

LOSS ON DRYING Not more than 15.0 per cent w/w after drying at 100° to constant weight (Appendix 4.15).

TOTAL ASH Not more than 0.3 per cent w/w (Appendix 7.7).

Starches Use *Corn Starch*, *Potato Starch* or *Rice Starch*, which complies with the following additional requirement.

SENSITIVITY Prepare a paste of 1.0 g with a small amount of cold *water*, and add to it, with stirring, 200 ml of boiling *water*. Cool, add 5 ml of the cooled solution to

100 ml of *water* containing about 50 mg of *potassium iodide*, mix, and then add 0.50 ml of 0.0050 M *iodine*: a deep blue colour that is discharged by 0.50 ml of 0.010 M *sodium thiosulfate* is produced.

Starch Iodide Paper Impregnate filter paper with a solution of 500 mg of *potassium iodide* in 100 ml of freshly prepared *starch TS*.

Strychnine Sulfate $(C_{21}H_{22}N_2O_2)2.H_2SO_4.5H_2O$ = 856.99

DESCRIPTION Colourless or white crystals, or white, crystalline powder. Its solutions are levorotatory.

SOLUBILITY Soluble in 35 parts of *water*, in about 220 parts of *chloroform* and in 85 parts of *ethanol*; insoluble in *ether*.

SOLUBILITY TEST A solution of 500 mg in 25 ml of *water* is complete, clear and colourless.

SULFATED ASH Not more than 0.1 per cent w/w (Appendix 5.3).

BRUCINE To 100 mg add 1 ml of a 50 per cent v/v solution of *nitric acid*: a yellow colour may be observed, but not a red or reddish brown colour.

Sucrose $C_{12}H_{22}O_{11}$ = 342.30

Use analytical reagent grade of commerce.

DESCRIPTION Lustrous, dry, colourless crystals or white crystalline powder; odourless.

SOLUBILITY Soluble in 0.5 part of *water*; slightly soluble in *ethanol*.

(**Note** When sucrose is used for polarimetry, it must be kept dry in a sealed ampoule.)

Sulfamic Acid H_3NO_3S = 97.09

Use general reagent grade of commerce.

DESCRIPTION White crystals or crystalline powder.

SOLUBILITY Very soluble in *water*.

MELTING RANGE 203° to 205°, with decomposition (Appendix 4.3).

Sulfanilamide $C_6H_8N_2O_2S$ = 172.20

Use general reagent grade of commerce.

DESCRIPTION White, crystalline powder.

SOLUBILITY Slightly soluble in *water*; soluble in *ethanol*, in dilute acid and in alkaline solutions.

MELTING RANGE 165° to 167° (Appendix 4.3).

Sulfathiazole $C_6H_8N_2O_2S$ = 172.20

Use general reagent grade of commerce.

DESCRIPTION White or yellowish white crystals or powder; odourless or almost odourless.

SOLUBILITY Very slightly soluble in *water*; soluble in *acetone*, in dilute mineral acids, in solutions of alkali hydroxides, and in 6 M *ammonium hydroxide*; slightly soluble in *ethanol*.

MELTING RANGE 200° to 203° (Appendix 4.3).

Sulfur Dioxide SO_2 = 64.06

Caution Sulfur Dioxide is poisonous.

Use laboratory cylinder grade of commerce.

DESCRIPTION Colourless gas; odour, penetrating and acrid. When passed into *water*, yields a clear colourless solution which is strongly acid to *litmus paper*.

Sulfuric Acid H_2SO_4 = 98.07

When no molarity is indicated, use analytical reagent grade of commerce containing about 96 per cent w/w of sulfuric acid and about 18 M in strength.

DESCRIPTION Colourless, oily, corrosive liquid.

WEIGHT PER MILLILITRE About 1.84 g (Appendix 4.9).

When solutions of molarity x*M* are required, they should be prepared by carefully adding 54*x* ml of *sulfuric acid* to an equal volume of *water* and diluting to 1000 ml with *water*.

When "sulfuric acid" is followed by a percentage figure, an instruction to add, carefully, *sulfuric acid* to *water* to produce the specified percentage v/v (or, if required, w/w) proportion of sulfuric acid is implied.

Sulfuric Acid, Dilute Add 5.5 ml of *sulfuric acid* to 60 ml of *water*, allow to cool and add sufficient *water* to produce 100 ml. It contains 9.8 per cent w/v of H_2SO_4 and about 1 M in strength.

Sulfuric Acid, Ethanolic Solutions of the requisite molarity may be obtained by mixing *sulfuric acid* with *ethanol* as directed under *Sulfuric Acid*.

When "*ethanolic sulfuric acid*" is followed by a percentage figure, an instruction to use *sulfuric acid* diluted with *ethanol* to produce the specified percentage v/v proportion of sulfuric acid is implied. Prepare by cooling separately the required amount to about –5°, carefully adding the acid to the ethanol. Keep the solution as cool as possible and mix gently.

Sulfuric Acid, Nitrogen-free

Use nitrogen-free sulfuric acid of commerce containing about 96 per cent w/w of H_2SO_4.

Complies with the following additional test.

NITRATES To 5 ml of *water* add carefully 45 ml of the reagent being examined, allow to cool to 40° and add 8 mg of N,N′-*diphenylbenzidine*. The solution is faint pink or very pale blue.

Talc

Use purified grade of commerce.

DESCRIPTION Very fine, white or greyish white, crystalline powder. It is unctuous, adheres readily to the skin, and is free from grittiness.

Tartaric Acid $C_4H_6O_6$ = 150.09

Use analytical reagent grade of commerce.

DESCRIPTION Colourless or translucent crystals or white, fine to granular, crystalline powder; odourless.

SOLUBILITY Very soluble in *water*; freely soluble in *ethanol*.

Tetrabutylammonium Iodide $C_{16}H_{36}IN$ = 369.37

Use general reagent grade of commerce.

DESCRIPTION White, shiny crystalline flakes.

SOLUBILITY Slightly soluble in *water*; soluble in *ethanol* and in *ether*.

***n*-Tetradecane** $C_{14}H_3O$ = 198.39

DESCRIPTION Clear, colourless liquid.

SOLUBILITY Miscible with *ethanol*.

REFRACTIVE INDEX 1.428 to 1.429, at 20° (Appendix 4.7).

WEIGHT PER MILLILITRE About 0.76 g (Appendix 4.9).

Tetradecylammonium Bromide $C_{40}H_{84}BrN$ = 659.02

Use chromatographic reagent grade of commerce.

MELTING RANGE About 88° to 89° (Appendix 4.3).

Tetraheptylammonium Bromide $C_{28}H_{60}BrN$ = 490.70

Use chromatographic reagent grade of commerce.

MELTING RANGE 89° to 91° (Appendix 4.3).

Tetrahydrofuran (Tetramethylene Oxide) C_4H_8O = 72.11

Use analytical reagent grade of commerce.

DESCRIPTION Clear, colourless, flammable liquid.

BOILING TEMPERATURE About 66° (Appendix 4.5).

WEIGHT PER MILLILITRE About 0.89 g (Appendix 4.9).

Do not distil until it complies with the following test.

PEROXIDES Place 8 ml of *starch-iodide TS* in a ground-glass-stoppered cylinder with a capacity of 12 ml and about 1.5 cm in diameter and add sufficient of the substance being examined to fill the cylinder completely, shake vigorously and allow to stand for 30 minutes protected from light. No colour is produced.

Tetrahydrofuran used in spectrophotometry complies with the following additional requirement.

TRANSMITTANCE Not less than 20 per cent at 255 nm, 80 per cent at 270 nm and 98 per cent at 310 nm, determined using *water* in the reference cell.

***N,N,N′,N′*-Tetramethylethylenediamine** $C_6H_{16}N_2$ = 116.21

Use general reagent grade of commerce.

DESCRIPTION Colourless liquid.

BOILING TEMPERTURE About 121° (Appendix 4.6).

RELATIVE DENSITY About 0.78 (Appendix 4.9).

Tetramethylsilane $(CH_3)_4Si$ = 88.23

Use spectroscopic reagent grade of commerce.

DESCRIPTION Colourless liquid.

BOILING TEMPERATURE About 26° (Appendix 4.6).

REFRACTIVE INDEX About 1.358, at 20° (Appendix 4.7).

RELATIVE DENSITY About 0.64 (Appendix 4.9).

When used in nuclear magnetic resonance spectrometry, complies with the following test.

NUCLEAR MAGNETIC RESONANCE In the NMR spectrum of a 10 per cent v/v solution of the reagent being examined in *deuterochloroform*, the intensity of any foreign signal, excluding those due to spinning side bands and to chloroform, is not greater than the intensity of the ^{13}C-satellite signals located at a distance of 59.1 Hz on each side of the principal signal of tetramethylsilane.

Store at a temperature between 2° and 8°.

Tetrazolium Blue (Blue Tetrazolium Salt) $C_{40}H_{32}Cl_2N_8O_2$ = 727.65

Use general reagent grade of commerce.

DESCRIPTION Yellow crystals.

MELING TEMPERATURE About 245°, with decomposition (Appendix 4.3).

Thioacetamide $C_2H_sN_S$ = 75.13

Use general reagent grade of commerce.

DESCRIPTION White crystals or crystalline powder.

SOLUBILITY Freely soluble in *water* and in *ethanol*.

MELTING RANGE 112° to 115° (Appendix 4.3).

Thioactamide Reagent See under "Limit Test for Heavy Metals" (Appendix 5.2).

Thioglycolic Acid (Mercaptoacetic Acid) $C_2H_4O_2S$ = 92.12

Use general reagent grade of commerce.

DESCRIPTION Colourless or almost colourless liquid; odour, strong and unpleasant.

SOLUBILITY Miscible with *water*; soluble in *ethanol*.

WEIGHT PER MILLILITRE About 1.33 g (Appendix 4.9).

Thiourea $(NH_2)_2CS$ = 76.12

Use analytical reagent grade of commerce.

DESCRIPTION White crystals or white, crystalline powder; odourless.

SOLUBILITY Soluble in *water* and in *ethanol*.

MELTING RANGE 176° to 182° (Appendix 4.3).

Thorin (Naphtharson) $C_{16}H_{11}AsN_2Na_2O_{10}S_2$ = 576.29

Use indicator reagent grade of commerce.

DESCRIPTION Red powder.

SOLUBILITY Soluble in *water*.

Thrombin, Bovine A preparation of the enzyme that converts fibrinogen into fibrin.

Use general reagent grade of commerce.

Thrombin (Human Thrombin)

Use general reagent grade of commerce.

DESCRIPTION Yellowish white powder.

Store in sealed, sterile containers under nitrogen, protected from light and at a temperature below 25°.

Thymol $C_{10}H_{14}O$ = 150.22

DESCRIPTION Colourless, often large, crystals, or white, crystalline powder.

SOLUBILITY Soluble in about 1000 parts of *water*, in 1 part of *ethanol*, in 1 part of *chloroform*, in 1.5 parts of *ether*, and in about 2 parts of *olive oil*.

MELTING RANGE 48° to 51°, but when melted it remains liquid at a considerably lower temperature (Appendix 4.3).

NON-VOLATILE MATTER Volatilize 2 g on a water-bath and dry at 105° to constant weight. The residue weighs not more than 1 mg.

Store in tightly closed containers, protected from light.

Toluene (Methylbenzene) C_7H_8 = 92.14

Use analytical grade of commerce.

DESCRIPTION Clear, colourless liquid; odour, characteristic. Flammable.

SOLUBILITY Miscible with *water* and with *ethanol*.

WEIGHT PER MILLILITRE 0.865 to 0.870 g (Appendix 4.9).

BOILING TEMPERATURE About 110° (Appendix 4.5).

Tin(II) Chloride (Stannous Chloride) $SnCl_2.2H_2O$ = 225.63

Use analytical reagent grade of commerce.

DESCRIPTION Colourless crystals.

SOLUBILITY Soluble in *dilute hydrochloric acid*.

***p*-Toluidine** C_7H_9N = 107.15

Use general reagent grade of commerce.

DESCRIPTION Lustrous plates or flakes.

SOLUBILITY Slightly soluble in *water*; freely soluble in *ethanol*, in *acetone*, in *methanol*, and in dilute acids.

MELTING TEMPERATURE About 44° (Appendix 4.3).

Trichloroacetic Acid $C_2HCl_3O_2$ = 163.39

> ***Caution*** Trichloroacetic Acid is highly corrosive to the skin and mucous membrane.

Use analytical reagent grade of commerce.

DESCRIPTION Colourless, deliquescent crystals; odour, pungent.

SOLUBILITY Very soluble in *water*, in *chloroform* and in *ethanol*.

Store in tightly closed containers.

Triethylamine $C_6H_{15}N$ = 101.19

DESCRIPTION Colourless liquid.

SOLUBILITY Slightly soluble in *water*; miscible with *ethanol*, with *ether* and with cold *water*.

BOILING RANGE 89° to 90° (Appendix 4.5).

ABSORBANCE To 1.0 ml in a 50-ml volumetric flask add 10 ml of *methanol* and 1 ml of *hydrochloric acid*, and add *chloroform* to volume. The absorbance of this solution, determined at the wavelength of maximum absorbance at about 285 nm, with a suitable spectrophotometer, does not exceed 0.01. (**Note** If the absorbance exceeds 0.01, purify the triethylamine as follows. Reflux 100 ml with 20 ml of *water* and 2 g of *sodium hydrosulfite* for not less than 8 hours, wash with *water*, dry by refluxing, using a Dean-Stark trap, and distil, collecting only the first 75 ml of the filtrate. Store over *anhydrous sodium carbonate* or *anhydrous potassium carbonate*.)

2,2,4-Trimethylpentane (Iso-octane; Trimethylpentane) C_8H_{18} = 114.23

Use general reagent grade of commerce.

DESCRIPTION Colourless liquid; flammable.

RELATIVE DENSITY 0.691 to 0.696 (Appendix 4.9).

REFRACTIVE INDEX 1.391 to 1.393 (Appendix 4.7).

DISTILLATION RANGE Not less than 95 per cent distils between 98° and 100° (Appendix 4.5).

2,2,4-Trimethylpentane used in spectrophotometry complies with the following additional requirement.

TRANSMITTANCE Not less than 98 per cent between 250 and 420 nm (Appendix 2.2), using *water* as the blank.

2,4,6-Trinitrophenol (Picric Acid) $C_6H_3N_3O_7$ = 229.11

> ***Caution*** Explodes when heated rapidly or subjected to percussion. Store moistened with water. Moistened with an equal weight of water for safety.

Use analytical reagent grade of commerce.

DESCRIPTION Yellow prisms or plates.

Tris(hydroxymethyl)methylamine (BP p.A126) (Trometamol; Tromethamine; Tris(hydroxymethyl) aminomethane) $C_4H_{11}NO_3$ = 121.14
Use analytical reagent grade of commerce.

MELTING TEMPERATURE About 170° (Appendix 4.3).

Urea CH_4N_2O = 60.06

Use analytical reagent grade of commerce.

DESCRIPTION White crystals or crystalline powder.

SOLUBILITY Freely soluble in *water* and in boiling *ethanol*; practically insoluble in *chloroform* and in *ether*.

MELTING TEMPERATURE About 133° (Appendix 4.3).

Water Use Water (TP monograph).

Water, Carbon Dioxide-free *Water* that has been boiled vigorously for a few minutes and protected from the atmosphere during cooling and storage.

Water, Particle-free Filter *water* through a membrane with a pore size of 0.22 µm.

Xanthydrol $C_{13}H_{10}O_2$ = 198.22

DESCRIPTION Pale yellow, crystalline powder.

SOLUBILITY Insoluble in *water*; soluble in *ethanol*, in *chloroform* and in *ether*. Soluble in *glacial acetic acid*, forming a practically colourless solution; but when the powder is treated with *dilute hydrochloric acid*: a lemon-yellow colour is produced.

MELTING RANGE 121° to 123° (Appendix 4.3).

SULFATED ASH Not more than 2.0 per cent w/w (Appendix 5.3).

Xylene Cyanol FF (Xylene Cyanole FF; Acid Blue 147) $C_{25}H_{27}N_2NaO_6S_2$ = 538.61

DESCRIPTION Grey-blue to dark blue powder.

SOLUBILITY Soluble in *water*.

MELTING TEMPERATURE About 295°, with decomposition (Appendix 4.3).

Xylenol Orange $C_{31}H_{28}N_2Na_4O_{13}S$ = 760.59

Use general reagent grade of commerce.

DESCRIPTION Reddish brown, crystalline powder.

SOLUBILITY Soluble in *water* and in *ethanol*.

In acid solution, it is yellow in colour and its metal complexes are intensely red. It yields a distinct end-point where a metal such as bismuth, cadmium, lanthanum, lead, mercury, scandium, thorium, or zinc is titrated with disodium edetate.

Xylenol Orange Mixture Triturate 1 part of *xylenol orange* with 99 parts of *potassium nitrate*.

SENSITIVITY Add 50 mg to a mixture of 50 ml of *water*, 1 ml of 2 M *acetic acid* and 0.05 ml of 0.10 M *lead(II) nitrate*. Add sufficient *methenamine* to change the colour from yellow to violet, and add 0.10 ml of 0.10 M *disodium edetate*: the colour changes to yellow.

Zinc Zn = 65.38

Use analytical reagent grade of commerce.

DESCRIPTION Bright silver-grey, metallic granules, or powder.

Zinc, Granulated Use *Zinc* (see under "Reagents").

Zinc Powder Zn = 65.38

Use analytical reagent grade of commerce containing not less than 90.0 per cent of Zn.

1.2 VOLUMETRIC SOLUTIONS

In this Pharmacopoeia, all concentrations of volumetric solutions are expressed in terms of molarity. The molarity of a solution is stated as the number of moles of substance contained in 1000 ml of the solution. A solution which contains x moles of substance per 1000 ml is designated as x molar (x M).

Volumetric solutions do not differ from the prescribed strength by more than 10 per cent. The molarity of the volumetric solutions is determined by an appropriate number of titrations. The repeatability does not exceed 0.2 per cent (relative standard deviation).

Throughout the Pharmacopoeia, molar solutions to be standardized before use in assays and other quantitative tests are designated by appending the letters *VS* to the name of the reagent.

Preparation and Standardization

For each solution the preparation and standardization of the most commonly used strength is described. Stronger or weaker solutions than those described are prepared and standardized using proportionate amounts of the reagents or by making an exact dilution of a stronger solution with *carbon dioxide-free water*. Solutions of molarity below 0.01 M are freshly prepared using *carbon dioxide-free water*.

The water used in preparing volumetric solutions complies with the requirements of the monograph on Purified Water. When used for the preparation of unstable solutions such as potassium permanganate and sodium thiosulfate, it should be freshly boiled and cooled. When a solution is to be used in an assay in which the end-point is determined by an electrochemical process, the solution must be standardized in the same way. All volumetric solutions, if practicable, are to be prepared, standardized and used at the same temperature.

Blank Determinations

Where it is directed that "any necessary correction" be made by a blank determination, the determination is to be conducted with the use of the same quantities of the same reagents treated in the same manner as the solution or mixture containing the portion of the substance under assay or test, but with the substance itself omitted. Appropriate blank corrections are to be made for all Pharmacopoeial titrimetric assays.

All Pharmacopoeial assays that are volumetric in nature indicate the weight of the substance being assayed to which each ml of the primary volumetric solution is equivalent. In general, these equivalents may be derived by simple calculation from the respective molecular formulae and weights.

Disodium Edetate, Twentieth-Molar (0.05 M)

$C_{10}H_{14}N_2Na_2O_8.2H_2O$ = 372.24

Dissolve 18.6 g of *disodium edetate* in sufficient *water* to produce 1000 ml.

Standardization: Weigh accurately about 200 mg of *chelometric standard calcium carbonate*, previously dried at 110° for 2 hours and cooled in a desiccator, transfer to a 400-ml beaker, add 10 ml of *water*, and swirl to form a slurry. Cover the beaker with a watch glass, and introduce 2 ml of dilute *hydrochloric acid* from a pipette inserted between the lip of the beaker and the edge of the watch glass. Swirl the beaker to dissolve the calcium carbonate. Wash down the sides of the beaker, the outer surface of the pipette, and the watch glass with *water*, and dilute with *water* to about 100 ml. While stirring the solution, preferably with a magnetic stirrer, add about 30 ml of the disodium edetate solution from a 50-ml burette. Add 15 ml of *sodium hydroxide TS* and 300 mg of *hydroxy naphthol blue mixture*; 100 mg of *calcon mixture* or *calconcarboxylic acid mixture* may be used in place of *hydroxy naphthol blue mixture*. Continue the titration with the disodium edetate solution to a blue end-point. Each ml of 0.05 M *disodium edetate* is equivalent to 5.004 mg of $CaCO_3$.

Hydrochloric Acid, Molar (1 M)

HCl = 36.46

Dilute 85 ml of *hydrochloric acid* in sufficient *water* to produce 1000 ml.

Standardization: Weigh accurately about 1.5 g of *anhydrous sodium carbonate* that previously has been heated at about 270° for 1 hour. Dissolve it in 100 ml of *water* and add 0.1 ml of *methyl red TS*. Add the acid slowly from a burette, with constant stirring until the solution becomes faintly pink. Heat the solution to boiling, cool, and continue the titration. Heat again to boiling, and titrate further as necessary until the faint pink colour is no longer affected by continued boiling. Each ml of 1 M *hydrochloric acid* is equivalent to 52.99 mg of Na_2CO_3.

Iodine, Twentieth-Molar (0.05 M)

I_2 = 253.81

Dissolve 14 g of *iodine* in a solution of 36 g of *potassium iodide* in 100 ml of *water*, and add 0.15 ml of *hydrochloric acid*. Dilute slowly, with continuous stirring, to 1000 ml with *water*.

Standardization: Weigh accurately about 150 mg of *arsenic trioxide*, previously dried at 105° for 1 hour, and dissolve in 20 ml of 1 M *sodium hydroxide* by warming if necessary. Dilute with 40 ml of *water*, add 0.1 ml of *methyl orange TS*, and follow with *dilute hydrochloric acid* until the yellow colour is changed to pink. Then add 2 g of *sodium hydrogencarbonate*, dilute with 50 ml of *water*, and add 3 ml of *starch TS*. Slowly titrate with the iodine solution until a permanent blue colour is produced. Each ml of 0.05 M *iodine* is equivalent to 4.946 mg of As_2O_3.

Preserve in amber-coloured, glass-stoppered bottles.

Lead Nitrate, Twentieth-Molar (0.05 M)

$Pb(NO_3)_2$ = 331.21

Dissolve 16.6 g of *lead(II) nitrate* in sufficient *water* to produce 1000 ml.

Standardization: Dilute 25.0 ml of the lead nitrate solution with 200 ml of *water*, add 10 ml of *ammonia buffer pH 10.9* and about 20 mg of *mordant black 11 mixture*. Titrate with 0.05 M *disodium edetate VS*. Calculate the molarity.

Perchloric Acid, Tenth-Molar (0.1 M)

$HClO_4$ = 100.46

> ***Caution*** The perchloric acid must be well diluted with acetic acid before adding the acetic anhydride. Failure to observe this precaution leads to formation of the explosive acetylperchlorate.

Mix thoroughly 8.5 ml of *perchloric acid* with 500 ml of *anhydrous glacial acetic* and then add 21 ml of *acetic anhydride*; cool, and add *anhydrous glacial acetic acid* to make 1000 ml. Allow to stand for 24 hours.

Determine the water content by the Karl Fischer Method, Appendix 4.12. If necessary, add sufficient *water* or *acetic anhydride* to adjust the water content to between 0.02 per cent and 0.05 per cent, and allow to stand for a further 24 hours.

Standardization: Weigh accurately about 700 mg of *potassium hydrogenphthalate* previously crushed lightly and dried at 120° for 2 hours. Dissolve in 50 ml of *anhydrous glacial acetic acid* in a 250-ml flask. Add 0.1 ml of *crystal violet TS*, and titrate with the perchloric acid solution until the violet colour changes to blue-green. Deduct the volume of the perchloric acid consumed by 50 ml of the anhydrous glacial acetic acid. Each ml of 0.1 M *perchloric acid* is equivalent to 20.42 mg of $C_8H_5KO_4$.

Potassium Hydroxide, Molar (1 M)

KOH = 56.11

Dissolve 68 g of *potassium hydroxide* in sufficient *carbon dioxide-free water* to produce 1000 ml.

Standardization: Titrate 20.0 ml of the solution with 1 M *hydrochloric acid VS* using 0.5 ml of *phenolphthalein TS* as indicator. Perform a blank determination, and make any necessary correction. Calculate the molarity.

Potassium Hydroxide, Tenth-Molar (0.1 M)

Dissolve 6.8 g of *potassium hydroxide* in sufficient *carbon dioxide-free water* to produce 1000 ml.

Standardization: Titrate 20.0 ml of the solution with 0.1 M *hydrochloric acid VS* using 0.5 ml of *phenolphthalein TS* as indicator. Perform a blank determination, and make any necessary correction. Calculate the molarity.

Potassium Hydroxide, Ethanolic, Half-Molar (0.5 M)

Dissolve 34 g of *potassium hydroxide* in 20 ml of *water*, and dilute with *aldehyde-free ethanol* to produce 1000 ml. Allow the solution to stand in a tightly stoppered bottle for 24 hours, and then quickly decant the clear supernatant liquid into a suitable, tight container.

Standardization: Dilute 25.0 ml of 0.5 M *hydrochloric acid VS* with 50 ml of *water*, add 0.1 ml of *phenolphthalein TS* and titrate with the ethanolic potassium hydroxide solution until a permanent, pale pink colour is produced. Perform a blank determination, and make any necessary correction. Calculate the molarity.

Store protected from light.

Potassium Permanganate, Fiftieth-Molar (0.02 M)

$KMnO_4 = 158.03$

Dissolve about 3.3 g of *potassium permanganate* in 1000 ml of *water* in a flask, and boil the solution for about 15 minutes. Insert the stopper in the flask, allow it to stand for at least 2 days, and filter through a fine-porosity, sintered-glass crucible. If necessary, the bottom of the sintered-glass crucible may be lined with a pledget of glass wool. Standardize the solution as follows.

Standardization: Weigh accurately about 200 mg of *sodium oxalate*, previously dried at 110° to constant weight, and dissolve it in 250 ml of *water*. Add 7 ml of *sulfuric acid*, heat to about 70°, and then slowly add the permanganate solution from a burette, with constant stirring, until a pale pink colour, which persists for 15 seconds, is produced. The temperature at the conclusion of the titration should be not less than 60°. Perform a blank determination, and make any necessary correction. Each ml of 0.02 M *potassium permanganate* is equivalent to 6.700 mg of $C_2Na_2O_4$.

Note Since potassium permanganate is reduced on contact with organic substances such as rubber, the solution must be handled in apparatus entirely of glass or other suitably inert material. It should be frequently restandardized.

Store in glass-stoppered, amber-coloured bottles.

Silver Nitrate, Tenth-Molar (0.1 M)

$AgNO_3 = 169.87$

Dissolve 17.5 g of *silver nitrate* in sufficient *water* to produce 1000 ml.

Standardization: Transfer about 100 mg, accurately weighed, of *sodium chloride*, previously dried at 110° for 2 hours, to a 150-ml beaker. Dissolve in 5 ml of *water* and add 5 ml of *acetic acid*, 50 ml of *methanol* and 0.15 ml of *eosin Y TS*. Stir, preferably with a magnetic stirrer, and titrate with the silver nitrate solution until a purplish red colour appears. Each ml of 0.1 M *silver nitrate* is equivalent to 5.844 mg of NaCl.

Sodium Hydroxide, Molar (1 M)

$NaOH = 40.00$

Dissolve 162 g of *sodium hydroxide* in 150 ml of *carbon dioxide-free water*, cool the solution to room temperature and filter through hardened filter paper. Dilute 54.5 ml of the clear filtrate with *carbon dioxide-free* water to 1000 ml.

Standardization: Weigh accurately about 5 g of *potassium hydrogenphthalate*, previously crushed lightly and dried at 120° for 2 hours, and dissolve in 75 ml of *carbon dioxide-free water*. Add 0.1 ml of *phenolphthalein TS*, and titrate with the sodium hydroxide solution to the production of a permanent pink colour. Each ml of 1 M *sodium hydroxide* is equivalent to 204.2 mg of $C_8H_5KO_4$.

Note 1. Solutions of alkali hydroxides absorb carbon dioxide when exposed to air. They should therefore be stored in bottles with suitable non-glass, well-fitting stoppers, provided with a tube filled with soda lime.

2. Prepare solutions of lower concentration (e.g., 0.1 M, 0.01 M) by quantitatively diluting accurately measured volumes of the 1 M solution with sufficient *carbon dioxide-free water* to yield the desired concentration.)

Restandardize the solution frequently.

Sodium Hydroxide, Ethanolic, Tenth-Molar (0.1 M)

Dissolve 4.2 g of *sodium hydroxide* in 10 ml of *water*, and dilute with *aldehyde-free ethanol* to produce 1000 ml. Allow the solution to stand in a tightly stoppered bottle for 24 hours, and then quickly decant the clear supernatant liquid into a suitable, tight container.

Standardization: Weigh accurately about 200 mg of *benzoic acid*, previously dried, and dissolve in a mixture of 10 ml of *ethanol* and 2 ml of *water* and titrate with the ethanolic sodium hydroxide solution, using *phenolphthalein TS* as indicator. Perform a blank determination, and make any necessary correction. Each ml of 0.1 M *ethanolic sodium hydroxide* is equivalent to 12.21 mg of $C_7H_6O_2$.

Sodium Thiosulfate, Tenth-Molar (0.1 M)

$Na_2S_2O_3.5H_2O = 248.17$

Dissolve 25 g of *sodium thiosulfate* and 200 mg of *sodium carbonate* in sufficient recently boiled and cooled *water* to produce 1000 ml.

Standardization: Weigh accurately about 210 mg of *potassium dichromate*, previously pulverized and dried at 120° for 4 hours, and dissolve in 100 ml of *water* in a 500-ml iodine flask. Swirl to dissolve the solid, remove the stopper, and quickly add 3 g of *potassium iodide*, 2 g of *sodium hydrogencarbonate* and 5 ml of *hydrochloric acid*. Insert the stopper in the flask, swirl to mix, and allow to stand in the dark for 10 minutes. Rinse the stopper and the inner walls of the flask with *water*. Titrate the liberated iodine with the sodium thiosulfate

solution until the solution is yellowish green in colour. Add 3 ml of *starch TS*, and continue the titration to the discharge of the blue colour. Each ml of 0.1 M *sodium thiosulfate* is equivalent to 4.903 mg of $K_2Cr_2O_7$.

Restandardize the solution frequently.

Sulfuric Acid, Half-Molar (0.5 M)

Carefully add 28 ml of *sulfuric acid* to *water*, and dilute to 1000 ml with the same solvent.

Standardization: Standardize the solution as described under *Hydrochloric Acid, Molar (1 M)*.

Sulfuric Acid, Twentieth-Molar (0.05 M)

Dilute 100 ml of 0.5 M *sulfuric acid VS* to 1000 ml with *water*.

Standardization: Standardize the solution as described under *Hydrochloric Acid, Molar (1 M)* using about 100 mg of *anhydrous sodium carbonate*, accurately weighed and dissolved in 20 ml of *water*.

Tetrabutylammonium Hydroxide, Tenth-Molar (0.1 M)

$(C_4H_9)_4NOH = 259.48$

Dissolve 40 g of *tetrabutylammonium iodide* in 90 ml of *anhydrous methanol* in a glass-stoppered flask. Place in an ice-bath, add 20 g of powdered *silver oxide*, insert the stopper in the flask, and shake vigorously for 60 minutes. Centrifuge a few ml, and test the supernatant liquid for iodide (Appendix 5.1). If the test is positive, add an additional 2 g of *silver oxide*, and continue to allow to stand for 30 minutes within termittent shaking. When all of the iodide has reacted, filter through a fine-porosity, sintered-glass filter. Rinse the flask and the filter with three 50-ml portions of *anhydrous toluene*, adding the rinsings to the filtrate. Dilute with a mixture of 3 volumes of anhydrous toluene and 1 volume of *anhydrous methanol* to 1000 ml, and flush the solution for 10 minutes with dry *carbon dioxide-free nitrogen*.

Store in a reservoir protected from carbon dioxide and moisture and discard after 60 days.

Alternatively, the solution may be prepared by diluting a suitable volume of commercially available tetrabutylammonium hydroxide solution in methanol with a mixture of 4 volumes of *anhydrous toluene* and 1 volume of *anhydrous methanol*.

Standardization: Weigh accurately about 400 mg of *benzoic acid*, previously dried, and dissolve in 80 ml of *dimethylformamide*. Add 0.15 ml of a 1 per cent w/v solution of *thymol blue* in *dimethylformamide*, and titrate to a blue end-point with the tetrabutylammonium hydroxide solution, delivering the titrant from a burette equipped with a carbon dioxide absorption trap. Perform a blank determination, and make any necessary correction. Each ml of 0.1 M *tetrabutylammonium hydroxide* is equivalent to 12.21 mg of $C_7H_6O_2$.

Note 1. If necessary to obtain a clear solution, further small quantities of *anhydrous methanol* may be added.

2. Tenth-Molar Tetrabutylammonium Hydroxide VS must be standardized immediately before use.

Zinc Sulfate, Twentieth-Molar (0.05 M)

$ZnSO_4.7H_2O = 287.54$

Dissolve 14.4 g of *zinc sulfate* in sufficient *water* to produce 1000 ml.

Standardization: To 10.0 ml of 0.05 M *disodium edetate* VS, add, in the order given, 10 ml of *acetic acid-ammonium acetate buffer TS*, 50 ml of *ethanol*, and 2 ml of *dithizone TS*. Titrate with the zinc sulfate solution to a clear, rose-pink colour. Calculate the molarity.

1.3 STANDARD SOLUTIONS

The following solutions are used as reference standards in limit tests, and should, unless experience has shown it to be unnecessary, be prepared immediately before use.

Aluminium Standard Solution (2 ppm Al) Dilute 1.0 ml of a 0.352 per cent w/v solution of *aluminium potassium sulfate* in 0.1 M *sulfuric acid* to 100.0 ml with *water*.

Aluminium Standard Solution (10 ppm Al) Dilute 1 volume of a 1.39 per cent w/v solution of *aluminium nitrate* to 100 volumes with *water* immediately before use.

Ammonium Standard Solution (1 ppm NH_4) Dilute 40.0 ml of *ammonium standard solution (2.5 ppm NH_4)* to 100.0 ml with *water*.

Ammonium Standard Solution (2.5 ppm NH4) Dilute 1.0 ml of a 0.0741 per cent w/v solution of *ammonium chloride* with *water* to 100.0 ml.

Ammonium Standard Solution (10 ppm NH4) Dilute 1.0 ml of a 0.297 per cent w/v solution of *ammonium chloride* with *water* to 100.0 ml.

Arsenic Standard Solution (1 ppm As) Dilute 10.0 ml of *arsenic standard solution (10 ppm As)* with *water* to 100.0 ml.

Arsenic Standard Solution (10 ppm As) Dissolve 330.0 mg of *arsenic trioxide* in 5 ml of 2 M *sodium hydroxide* and dilute to 250.0 ml with *water*. Dilute 1.0 ml of this solution to 100.0 ml with *water*.

Calcium Standard Solution (100 ppm Ca), Ethanolic Dissolve 250.0 mg of dried *calcium carbonate* in 1.2 ml of 5 M *acetic acid* and dilute with *water* to 100.0 ml. Dilute 10.0 ml of the resulting solution to 100 ml with *ethanol*.

Fluorine Standard Solution (400 ppm F) Dissolve 442 mg of *sodium fluoride* in sufficient 1 M *sodium hydroxide* to produce 500.0 ml.

Store in polyethylene bottles and keep for 3 days only.

Iron Standard Solution (10 ppm Fe) Dissolve 863.4 mg of *ammonium iron(III) sulfate* $[FeNH_4(SO_4)_2.12H_2O]$ in

water, add 10 ml of 1 M *sulfuric acid* and dilute with *water* to 100.0 ml. Transfer 10.0 ml of this solution into a 1000-ml volumetric flask, add 10 ml of 1 M *sulfuric acid*, dilute with *water* to volume, and mix.)

Lead Nitrate Stock Solution (100 ppm Pb) Dissolve 159.8 mg of *lead(II) nitrate* [$Pb(NO_3)_2$] in 100 ml of *water* to which has been added 1 ml of *nitric acid*, then dilute with *water* to 1000.0 ml.

Prepare and store this solution in glass containers free from soluble lead salts.

Lead Standard Solution (1 ppm Pb) Dilute 10.0 ml of *lead standard solution (10 ppm Pb)* with *water* to 100.0 ml.

Lead Standard Solution (2 ppm Pb) Dilute 10.0 ml of *lead standard solution (10 ppm Pb)* with *water* to 50.0 ml.

Lead Standard Solution (10 ppm Pb) On the day of use, dilute 10.0 ml of *lead nitrate stock solution (100 ppm Pb)* with *water* to 100.0 ml.

Selenium Standard Solution (1 ppm Se) Dissolve 40.0 mg of metallic *selenium* in 100 ml of a 50 per cent v/v solution of *nitric acid* in a 1000-ml volumetric flask, warming gently on a water-bath if necessary to effect solution, add *water* to volume, and mix. Pipette 5 ml of this solution into a 200-ml volumetric flask, add *water* to volume, and mix.)

1.4 pH INDICATORS

Alizarin Red S (Mordant Red 3; Alizarin S) $C_{14}H_7NaO_7S.H_2O$ = 360.27

Use general reagent grade of commerce.

DESCRIPTION Yellowish brown or orange-yellow powder. Freely soluble in *water* with yellow colour; soluble in *ethanol*. Transition interval: from pH 3.7 to 5.2. Colour change: from yellow to red.

SENSITIVITY TO BARIUM To 5.0 ml of 0.05 M *sulfuric acid* add 5 ml of *water*, 50 ml of *acetate buffer pH 3.7* and 0.5 ml of a 0.1 per cent w/v solution of the test substance. Add, dropwise, 0.05 M *barium perchlorate*: the colour changes from yellow to orange-red.

Alizarin Red TS A 0.1 per cent w/v solution of *alizarin red S*.

Alizarin Yellow GG $C_{13}H_8N_3NaO_5$ = 309.21

Yellow powder. Slightly soluble in *water*. Transition interval: from pH 10.0 to 12.0. Colour change: from colourless to yellow.

Bromocresol Green (Bromocresol Blue) $C_{21}H_{14}Br_4O_5S$ = 698.01

White or pale buff-coloured powder. Slightly soluble in *water*; soluble in *ethanol* and in solutions of alkali hydroxides. Transition interval: from pH 4.0 to 5.4. Colour change: from yellow to blue.

Bromocresol Green TS Dissolve 50 mg of *bromocresol green* in 100 ml of *ethanol*, and filter if necessary.

Bromocresol Purple $C_{21}H_{16}Br_2O_5S$ = 540.22

White to pink, crystalline powder. Insoluble in *water*; soluble in *ethanol* and in solutions of alkali hydroxides. Transition interval: from pH 5.2 to 6.8. Colour change: from yellow to purple.

Bromophenol Blue $C_{19}H_{10}Br_4O_5S$ = 669.96

Pinkish crystals. Insoluble in *water*; soluble in *ethanol* and in solutions of alkali hydroxides. Transition interval: from pH 2.8 to 4.6. Colour change: from yellow to blue.

Bromothymol Blue $C_{27}H_{28}Br_2O_5S$ = 624.38

Cream-coloured powder. Insoluble in *water*; soluble in *ethanol* and in solutions of alkali hydroxides. Transition interval: from pH 6.0 to 7.6. Colour change: from yellow to blue.

Bromothymol Blue TS Dissolve 100 mg of *bromothymol blue* in 100 ml of *ethanol (50 per cent)*, and filter if necessary.

Congo Red $C_{32}H_{22}N_6Na_2O_6S_2$ = 696.66

Dark red or reddish brown powder. Soluble in 30 parts of *water*; slightly soluble in *ethanol*. Transition interval: from pH 3.0 to 5.0. Colour change: from blue to red.

Congo Red Paper Immerse strips of filter paper for a few minutes in a solution prepared by dissolving 0.1 g of *congo red* in a mixture of 20 ml of *ethanol* and 30 ml of *water* and adding sufficient *water* to produce 100 ml and allow to dry at room temperature. Transition interval: from pH 3.0 to 5.0. Colour change: from violet to orange.

Cresol Red (*o*-Cresolsulfonphthalein) $C_{12}H_{18}O_5S$ = 382.43

Reddish brown powder. Slightly soluble in *water*; soluble in *ethanol* and in dilute solutions of alkali hydroxides. Transition interval: from pH 7.2 to 8.8. Colour change: from yellow to red.

Cresol Red TS Warm 20 mg of *cresol red* with 1.06 ml of 0.05 M *sodium hydroxide* and 2 ml of *ethanol (90 per cent)*; after solution is effected, add sufficient *ethanol (20 per cent)* to produce 100 ml. The solution complies with the following test.

SENSITIVITY A mixture of 0.1 ml of the solution and 100 ml of *carbon dioxide-free water* to which 0.15 ml of 0.020 M *sodium hydroxide* has been added is purplish red; not more than 0.15 ml of 0.020 M *hydrochloric acid* is required to change the colour to yellow.

Crystal Violet $C_{25}H_{30}ClN_3$ = 407.98

Dark-green crystals. Slightly soluble in *water*; sparingly soluble in *ethanol* and in *anhydrous glacial acetic acid*. Its solutions are deep violet in colour.

SENSITIVITY Dissolve 100 mg in 100 ml of *anhydrous glacial acetic acid*, and mix. Pipette 1 ml of the solution into a 100-ml volumetric flask, and dilute with *anhydrous glacial acetic acid* to volume: the solution is violet-blue in colour and does not show a reddish tint. Pipette 20 ml of the diluted solution into a beaker, and titrate with 0.10 M *perchloric acid*, adding the perchloric acid slowly from a microburette: not more than 0.10 ml of 0.10 M *perchloric acid* is required to produce an emerald-green colour.

Crystal Violet TS Dissolve 500 mg of *crystal violet* in 100 ml of *anhydrous glacial acetic acid*.

Dimethyl Yellow $C_{14}H_{15}N_3$ = 225.28

Yellow crystals. Sparingly soluble in *water*. Transition interval: from pH 2.8 to 4.6. Colour change: from red to yellow.

Dimethyl Yellow TS A 0.2 per cent w/v solution of *dimethyl yellow* in *ethanol (90 per cent)*. The solution complies with the following test.

SENSITIVITY A solution containing 2 g of *ammonium chloride* in 25 ml of *carbon dioxide-free water*, to which is added 0.1 ml of the dimethyl yellow solution, is yellow. Not more than 0.10 ml of 0.10 M *hydrochloric acid* is required to change the colour to red.

Indigo Carmine (Indigotindisulfonate; Acid Blue 74) $C_{16}H_8N_2Na_2O_8S_2$ = 466.35

Deep blue powder or blue granules with a coppery lustre.

Indigo Carmine TS To a mixture of 10 ml of *hydrochloric acid* and 990 ml of a 20 per cent w/v solution of *nitrogen-free sulfuric acid* in *water*, add 0.2 g of *indigo carmine* to produce a solution that complies with the following test.

Add 10 ml to a solution of 1.0 mg of *potassium nitrate* in 10 ml of *water*, rapidly add 20 ml of *nitrogen-free sulfuric acid* and heat to boiling. The blue colour is discharged within 1 minute.

Litmus Fragments of indigo blue pigment prepared from various species of *Rocella, Lecanora* or other lichen. The pigment has a faint, characteristic odour. Blue powder, cubes or pieces; odour, faint, characteristic. Partly soluble in *water* and in *ethanol*. Transition interval: from approximately pH 4.5 to 8.0. Colour change: from red to blue.

Litmus Paper Use *red litmus paper* or *blue litmus paper*, as appropriate.

Litmus Paper, Blue Boil 10 parts of coarsely powdered *litmus* under reflux for 1 hour with 100 parts of *ethanol*, decant the ethanol and discard. Add to the residue a mixture of 45 parts of *ethanol* and 55 parts of *water*. After 2 days decant the clear liquid. Impregnate strips of filter paper with the extract and allow to dry.

SENSITIVITY Immerse a strip measuring 10 mm × 60 mm in 100 ml of a mixture of 10 ml of 0.02 M *hydrochloric acid* and 90 ml of *water*. On shaking the paper turns red within 45 seconds.

Litmus Paper, Red To the extract obtained in the preparation of Blue litmus paper add 2 M *hydrochloric acid* dropwise until the blue colour becomes red. Impregnate strips of filter paper with the solution and allow to dry.

SENSITIVITY Immerse a strip measuring 10 mm × 60 mm in 100 ml of 0.002 M *sodium hydroxide*. On shaking the paper turns blue within 45 seconds.

Litmus TS Boil 25 g of coarsely powdered *litmus* with 100 ml of *ethanol (90 per cent)* under a reflux condenser for 1 hour, and pour away the clear liquid; repeat this operation using two 75-ml portions of *ethanol (90 per cent)*. Digest the extracted litmus with 250 ml of *water*, and filter.

Malachite Green (Basic Green 4) $C_{23}H_{25}ClN_2$ = 364.92

Green crystals with a metallic lustre. Very soluble in *water*, soluble in *ethanol*, in *methanol* and in *amyl alcohol*. Transition interval: from pH 0.2 to 1.8. Colour change: from yellow to blue-green.

Malachite Green TS A 0.5 per cent w/v solution of *malachite green* in *anhydrous acetic acid*.

Metacresol Purple $C_{21}H_{18}O_5S$ = 382.43

Dark brown glittering crystals or olive green powder. Very slightly soluble in *water* and in *ethanol*; soluble in *methanol*. Acid-transition interval: from pH 1.2 to 2.8. Colour change: from red to yellow. Alkaline-transition interval: from pH 7.4 to 9.0. Colour change: from yellow to purple.

Metacresol Purple TS A 0.1 per cent w/v solution of *metacresol purple* in 0.001 M *sodium hydroxide*.

Metanil Yellow $C_{18}H_{14}N_3NaO_3S$ = 375.38

Brownish yellow powder. Soluble in *water* and in *ethanol*; sparingly soluble in *ether*; slightly soluble in *acetone*. Transition interval: from pH 1.2 to 2.4 Colour change: from red to yellow.

Metanil Yellow TS A 0.1 per cent w/v solution of *metanil yellow* in *methanol*. The solution complies with the following test.

SENSITIVITY A solution containing 0.1 ml in 50 ml of *anhydrous acetic acid* is pinkish red. Add 0.05 ml of 0.1 M *perchloric acid VS*; the colour of the solution changes to violet.

Methylene Blue TS Dissolve 125 mg of *methylene blue* in 100 ml of *ethanol*, and dilute with the same solvent to 250 ml.

Methyl Orange (Helianthin; Tropaeolin D) $C_{14}H_{14}N_3NaO_3S$ = 327.33

Colour Changes of pH Indicators

B = Blue G = Green Gr = Grey O = Orange P = Purple Pk = Pink R = Red V = Violet Y = Yellow

ลิขสิทธิ์กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข
ห้ามทำซ้ำ ดัดแปลง แก้ไข โดยไม่ได้รับอนุญาต

Orange-yellow powder or crystalline scales. Slightly soluble in cold *water*; readily soluble in hot *water*; insoluble in *ethanol*. Transition interval: from pH 3.0 to 4.4. Colour change: from red to yellow.

Methyl Orange TS Dissolve 0.1 g of *methyl orange* in 80 ml of *water* and add sufficient *ethanol* to produce 100 ml. The solution complies with the following test.

SENSITIVITY A mixture of 0.1 ml of the solution and 100 ml of *carbon dioxide-free water* is yellow; not more than 0.10 ml of 1 M *hydrochloric acid* is required to change the colour of the solution to red.

Methyl Orange-Xylene Cyanol FF TS Dissolve 100 mg of *methyl orange* and 260 mg of *xylene cyanol FF* in 50 ml of *ethanol* and add *sufficient water* to produce 100 ml.

Methyl Red $C_{15}H_{16}ClN_3O_2$ = 305.76

Dark red powder or violet crystals. Sparingly soluble in *water*; soluble in *ethanol*. Transition interval: from pH 4.2 to 6.3. Colour change: from red to yellow.

Methyl Red TS Dissolve 100 mg of *methyl red* in 100 ml of *ethanol*, and filter if necessary.

Methyl Red-Methylene Blue TS (Methyl Purple TS) Add 10 ml of *methyl red TS* to 10 ml of *methylene blue TS*, and mix.

1-Naphtholbenzein (*p*-Naphtholbenzein) $C_{27}H_{20}O_3$ = 392.45

Brilliant brownish black crystals or brownish red powder. Practically insoluble in *water*; soluble in *ethanol* and in *glacial acetic acid*. Transition interval: from pH 8.8 to 10.0. Colour change: from orange to green.

When used for titration in non-aqueous media, it changes from blue or green-blue (alkaline) through orange (neutral) to dark green (acidic).

1-Naphtholbenzein TS Dissolve 200 mg of *1-naphtholbenzein* in 100 ml of *anhydrous glacial acetic acid*. The solution complies with the following test.

SENSITIVITY Add 0.25 ml of the solution to 50 ml of *glacial acetic acid*. Not more than 0.05 ml of 0.10 M *perchloric acid* is required to change the colour of the solution from brownish yellow to green.

Neutral Red $C_{15}H_{17}ClN_4$ = 288.78

Reddish to olive-green, coarse powder. Sparingly soluble in *water* and in *ethanol*. Transition interval: from pH 6.8 to 8.0. Colour change: from red to orange.

Neutral Red TS A 0.1 per cent w/v solution of *neutral red* in *ethanol*.

Phenolphthalein $C_{20}H_{14}O_4$ = 318.33 (TP p. 349)

White or faintly yellowish white, crystalline powder. Insoluble in *water*; soluble in *ethanol*. Transition interval: from pH 8.0 to 10.0. Colour change: from colourless to red.

Phenolphthalein TS Dissolve 1 g of *phenolphthalein* in 100 ml of *ethanol (50 per cent)*.

Phenolphthalein TS, Dilute A 0.1 per cent w/v solution of *phenolphthalein* in *ethanol (80 per cent)*. The solution complies with the following test.

SENSITIVITY A mixture of 0.1 ml and 100 ml of *carbon dioxide-free water* is colourless. Not more than 0.20 ml of 0.020 M *sodium hydroxide* is required to change the colour to pink.

Phenol Red $C_{19}H_{14}O_5S$ = 354.38

Crystalline powder, varying in colour from bright to dark red. Very slightly soluble in *water*; freely soluble in solutions of alkali carbonates and hydroxides; slightly soluble in *ethanol*. Transition interval: from pH 6.8 to 8.2. Colour change: from yellow to red.

Phenol Red TS Warm 100 mg of *phenol red* with 1.42 ml of 0.2 M *sodium hydroxide* and 5 ml of *ethanol* (90 per cent); after solution is effected, add sufficient *ethanol (20 per cent)* to produce 250 ml. The solution complies with the following test.

SENSITIVITY A mixture of 0.1 ml and 100 ml of *carbon dioxide-free water* is yellow. Not more than 0.10 ml of 0.020 M *sodium hydroxide* is required to change the colour of the solution to reddish violet.

Resazurin (Sodium) $C_{12}H_6NNaO_4$ = 251.17

Brownish purple, crystalline powder. Soluble in 100 parts of *water*, forming a deep violet-coloured solution. Transition interval: from pH 3.8 to 6.4. Colour change: from orange to violet.

Hydrogen sulfide and other compounds containing the thiol group decolourize solutions of resazurin sodium, forming dihydroresorufin. When the decolorized solution is shaken in the presence of air, a rose colour develops as a result of the formation of resorufin.

Thymolphthalein $C_{28}H_{30}O_4$ = 430.54

White to slightly yellow, crystalline powder. Insoluble in *water*; soluble in *ethanol* and in solutions of alkali hydroxides. Transition interval: from pH 9.3 to 10.5. Colour change: from colourless to blue.

Thymolphthalein TS Dissolve 100 mg of *thymolphthalein* in 100 ml of *ethanol*, and filter if necessary.

Thymol Blue (Thymolsulfonphthalein) $C_{27}H_{30}O_5S$ = 466.59

Dark coloured, crystalline powder. Slightly soluble in *water*; soluble in *ethanol* and in dilute alkali solutions. Acid-transition interval: from pH 1.2 to 2.8. Colour change: from red to yellow. Alkaline-transition interval: from pH 8.0 to 9.2. Colour change: from yellow to blue.

Titan Yellow (Thiazole Yellow) $C_{28}H_{19}N_5Na_2O_6S_4$ = 695.7

Yellowish brown powder. Soluble in *water* and in *ethanol* to yield in each instance a yellow solution; soluble in dilute alkali to yield a brownish red solution.

Transition interval: from pH 12.0 to 13.0. Colour change: from yellow to red.

SENSITIVITY TO MAGNESIUM Add 0.2 ml of a 0.01 per cent w/v solution and 2 ml of 1 M *sodium hydroxide* to a mixture of 9.5 ml of *water* and 0.5 ml of a solution prepared by dissolving 1.014 g of clear crystals of s*magnesium sulfate* in *water*, diluting with *water* to 100 ml, and then diluting 10 ml of the resulting solution with *water* to 1000 ml: a distinct pink colour is produced within 10 minutes.

Store protected from light.

Titan Yellow TS A 0.05 per cent w/v solution of *titan yellow.*

Titan Yellow Paper Impregnate filter paper with *titan yellow TS.*

Tropaeolin O (Acid Orange 6) $C_{12}H_9N_2NaO_5S$ = 316.26

Brown powder. Soluble in water and in ethanol. Transition interval: from pH 11.0 to 12.7. Colour change: from yellow to orange.

Tropaeolin OO $C_{18}H_{14}N_3NaO_3S$ = 375.38

Yellow powder or orange-yellow scales. Transition interval: from pH 1.3 to 3.2. Colour change: from red to yellow.

Tropaeolin OO TS A 0.1 per cent w/v solution of *tropaeolin OO*.

1.5 BUFFER SOLUTIONS

The successful completion of many Pharmacopoeial tests and assays requires adjustment to, or maintenance of, a specified pH by the addition of buffer solutions. The following text sets out the compositions of various buffer solutions required. For convenience, however, the preparations of several single-purpose solutions are described in individual appendices.

Standard solutions of definite pH are readily available in buffer solutions prepared from the appropriate reagents. In addition, buffer solutions, buffer tablets, and buffer solids may be obtained from commercial sources in convenient prepackaged form. Such preparations are available for the entire working range in pharmaceutical analysis, but are not recommended for pH meter standardization.

The required reagents are described in Appendix 1.1. All crystalline reagents, except boric acid, should previously be dried at 110 to 120° for 1 hour. Buffer solutions should be prepared using *carbon dioxide-free water* and stored in bottles made of alkali-free glass, and must not be used later than 3 months after preparation.

Ammonia Buffer pH 10.0 Dissolve 5.4 g of *ammonium chloride* in 70 ml of 5 M *ammonia* and dilute with *water* to 100 ml.

Acetate Buffer pH 3.0 Dissolve 12 g of *sodium acetate* in *water*, add 6 ml of *glacial acetic acid* and dilute with sufficient *water* to 100 ml.

Acetate Buffer pH 3.5 Dissolve 25.0 g of *ammonium acetate* in 25 ml of *water* and add 38.0 ml of 7 M *hydrochloric acid*. Adjust the pH to 3.5 with either 2 M *hydrochloric acid* or 5 M *ammonia* and dilute with *water* to 100 ml.)

Acetate Buffer pH 4.4 Dissolve 136 g of *sodium acetate* and 77 g of *ammonium acetate* in *water* and dilute to 1000 ml with *water*. Add 250 ml of *glacial acetic acid* and mix.

Acetate Buffer pH 4.6 Dissolve 5.4 g of *sodium acetate* in 50 ml of *water*, add 2.4 g of *glacial acetic acid* and dilute to 100 ml with *water*. Adjust the pH if necessary.

Acetate Buffer pH 6.0 Dissolve 100 g of *ammonium acetate* in 300 ml of *water*, add 4.1 ml of *glacial acetic acid*, adjust the pH, if necessary, using 10 M *ammonia* or 5 M *acetic acid* and dilute to 500 ml with *water*.

Acetic-Ammonia Buffer pH 3.7, Ethanolic To 15 ml of 5 M *acetic acid* add 60 ml of *ethanol* and 24 ml of *water*. Adjust the pH of the solution to 3.7 with 10 M *ammonia* and dilute with *water* to 100 ml.

Barbital Buffer pH 8.6, Mixed Dissolve 1.38 g of *barbital*, 8.76 g of *barbital sodium* and 380 mg of *calcium lactate* in sufficient *water* to produce 1000 ml.

Borate Buffer pH 9.0 To 50 ml of a solution containing 618.9 mg of *boric acid* and 745.6 mg of *potassium chloride*, add 21.30 ml of 0.20 M *sodium hydroxide* and dilute with *water* to 200.0 ml. At 20°, the solution may be used as a solution of standard pH.

Imidazole Buffer Solution pH 7.3 Dissolve 340 mg of *imidazole* and 580 mg of *sodium chloride* in sufficient *water*, add 18.6 ml of 0.1 M *hydrochloric acid* and dilute to 100 ml with *water*. Adjust the pH if necessary.

Phosphate Buffers Solutions from pH 5.8 to pH 8.0 may be prepared by mixing 50 ml of a solution of 0.2 M *potassium dihydrogenphosphate* with the quantities of 0.20 M *sodium hydroxide* specified in the following table, and diluting with *water* to 200.0 ml. At 20°, the solutions may be used as solutions of standard pH.

pH	5.8	6.0	6.2	6.4	6.6	6.8
ml of 0.20 M *sodium hydroxide*	3.72	5.70	8.60	12.60	17.80	23.65
pH	7.0	7.2	7.4	7.6	7.8	8.0
ml of 0.20 M *sodium hydroxide*	29.63	35.00	39.50	42.80	45.20	46.80

Phosphate Buffer pH 6.8, Mixed Dissolve 2.880 g of *disodium hydrogenphosphate* and 1.145 g of *potassium dihydrogenphosphate* in sufficient *water* to produce 100 ml.

Phosphate Buffer pH 7.0, Mixed, 0.067 M Dissolve 908 mg of *potassium dihydrogenphosphate* in sufficient *water* to produce 100 ml (Solution A). Dissolve 2.38 g of *disodium hydrogenphosphate* in sufficient *water* to produce 100 ml (Solution B). Mix 38.9 ml of solution A with

61.1 ml of solution B.

Saline pH 7.4, Phosphate-buffered Dissolve 2.38 g of *disodium hydrogenphosphate*, 0.19 g of *potassium dihydrogenphosphate* and 8.0 g of *sodium chloride* in sufficient *water* to produce 1000 ml and adjust the pH if necessary.

***Tris*-chloride Buffer pH 7.5** Dissolve 727 mg of *tris (hydroxymethyl)methylamine* and 527 mg of *sodium chloride* in 95 ml of *water*, adjust the pH to 7.5 with 2 M *hydrochloric acid* and dilute to 100 ml with *water*.

***Tris*-EDTA BSA Buffer Solution pH 8.4** Dissolve 6.1 g of *tris(hydroxymethyl)methylamine*, 2.8 g of *disodium edetate*, 10.2 g of *sodium chloride* and 10 g of *bovine serum albumin* in *water*, adjust to pH 8.4 using 1 M *hydrochloric acid* and dilute to 1000 ml with *water*.

1.6 TEST SOLUTIONS

Acetic Anhydride-Pyridine TS Dissolve 25 ml of *acetic anhydride* in *anhydrous pyridine* and dilute to 100 ml with *anhydrous pyridine*.

Prepare freshly before use.

Acetic-Bromine TS Dissolve 100 g of *potassium acetate* in *glacial acetic acid*, and add 4 ml of *bromine* and sufficient *glacial acetic acid* to produce 1000 ml.

Alizarin Complexone Dihydrate TS Dissolve 38 mg of *alizarin complexone dihydrate* in a solution of 30 mg of *sodium hydroxide* in 25 ml of *water*. Dilute with *water* to about 150 ml and add 50 mg of *sodium acetate* and 2 M *hydrochloric acid* until a thin layer of the solution is pale pink in colour. Add sufficient *water* to produce 200 ml.

Aminophenazone TS (Aminopyrazolone TS) A 0.1 per cent w/v solution of *4-aminophenazone* in *borate buffer pH 9.0*.

Ammonium Iron(III) Sulfate TS A freshly prepared 10 per cent w/v solution of *ammonium iron(III) sulfate*.

Gives a deep red colour with *ammonium thiocyanate* in acid solutions.

Ammonia TS It contains 9.5 to 10.5 per cent w/w of NH_3. Prepare by diluting 400 ml of *strong ammonia solution* with *water* to make 1000 ml.

Store in well-closed containers, at a temperature not exceeding 20°.

Ammonium Chloride TS A 10.7 per cent w/v solution of *ammonium chloride*.

Ammonium Mercurithiocyanate TS Dissolve 30 g of *ammonium thiocyanate* and 27 g of *mercury(II) chloride* in sufficient *water* to produce 1000 ml.

Ammonium Molybdate TS A 10.0 per cent w/v solution of *ammonium molybdate*.

Ammonium Molybdate with Ascorbic Acid TS Mix in the following order 1 volume of a 2.5 per cent w/v solution of *ammonium molybdate*, 1 volume of a 10 per cent w/v solution of *ascorbic acid* and 1 volume of 3 M *sulfuric acid* and add 2 volumes of *water*.

Use within 1 day.

Ammonium Sulfide TS Saturate *ammonia TS* with *hydrogen sulfide* by bubbling hydrogen sulfide gas through the solution for 1 minute. The solution is not rendered turbid either by *magnesium sulfate TS* or by *calcium chloride TS*. This solution is unstable for use if an abundant precipitate of sulfur is present.

Prepare freshly before use.

Ammonium Thiocyanate TS Dissolve 8 g of *ammonium thiocyanate* in *water* to make 100 ml.

Anisaldehyde TS Mix, in order, 0.5 ml of *anisaldehyde*, 10 ml of *glacial acetic acid*, 85 ml of *methanol* and 5 ml of *sulfuric acid*.

Barium Chloride TS Dissolve 10 g of *barium chloride* in *water* to make 100 ml.

Bromelains TS A 1.0 per cent w/v solution of *bromelains* in a mixture of 1 volume of *mixed phosphate buffer pH 5.5* and 9 volumes of *saline TS*.

Bromine TS (Bromine Water) A saturated solution of *bromine*, prepared by agitating 2 to 3 ml of *bromine* with 100 ml of cold *water* to a glass-stoppered bottle, the stopper of which should be lubricated with petrolatum.

Store in a cool place, protected from light.

Bromophenol Blue TS Dissolve 100 mg of *bromophenol blue* in 100 ml of *ethanol (50 per cent)*, and filter if necessary.

Bromophenol Blue TS, Aqueous Dissolve 50 mg of *bromophenol blue* with gentle heating in 3.73 ml of 0.02 M *sodium hydroxide* and dilute to 100 ml with *water*.

Cadmium Iodide TS A 5.0 per cent w/v solution of *cadmium iodide*.

Calcium Chloride TS Dissolve 7.5 g of *calcium chloride* in *water* to make 100 ml.

Calcium Hydroxide TS A freshly prepared saturated solution which may be prepared in the following manner. Shake 10 g of *calcium hydroxide* repeatedly with 1000 ml of *water* and allow to stand until clear.

Cephalin TS Solvents used to prepare this solution should contain a suitable antioxidant such as *butylated hydroxyanisole* at a concentration of 0.1 mM.

To 0.5 to 1 g of acetone-dried ox brain add 20 ml of *acetone* and leave for 2 hours. Centrifuge for 2 minutes at 500 × *g* and decant the supernatant liquid. Dry the residue at a pressure of 2 kPa (about 15 Torr) and extract the dried material with 20 ml of *chloroform* for 2 hours, shaking the mixture frequently. After removal of the solid material by filtration or centrifugation, evaporate the chloroform from the extract at a pressure of 2 kPa (about 15 Torr). Suspend the residue in 5 to 10 ml of *saline TS*. This stock emulsion may be stored frozen or freeze-dried for 3 months.

Cerium(III) Nitrate TS Dissolve 220 mg of *cerium(III) nitrate* in 50 ml of *water*, add 0.1 ml of *nitric acid* and 50 mg of *hydroxylamine hydrochloride* and dilute with *water* to 1000 ml.

Chlorine TS (Chlorine Water) A freshly prepared, saturated solution of *chlorine* in *water*.

Copper(II) Sulfate TS Dissolve 12.5 g of *copper(II) sulfate* in *water* to make 100 ml.

Copper-Citric TS Dissolve 25 g of *copper(II) sulfate*, 50 g of *citric acid* and 144 g of *anhydrous sodium carbonate* in sufficient *water* to produce 1000 ml.

Diazobenzenesulfonic Acid TS Heat 200 mg of *sulfanilic acid* with 20 ml of 1 M *hydrochloric acid* until dissolved, cool to about 4°, and add dropwise, with continuous swirling, 2.2 ml of a 4 per cent w/v solution of *sodium nitrite*. Allow to stand in ice water for 10 minutes and add 1 ml of a 5 per cent w/v *solution* of *sulfamic acid*.

Diphenylamine TS A 1.0 per cent w/v solution of *diphenylamine* in *sulfuric acid*. The solution must becolourless.

Store protected from light.

Diphenylamine TS1 Dissolve 1 g of *diphenylamine* in 100 ml of *glacial acetic acid* and add 2.75 ml of *sulfuric acid*.

Use immediately after preparation.

Dithizone TS Dissolve 25.6 mg of *dithizone* in 100 ml of *ethanol*.

Store in a cold place, and use within 2 months.

Eosin Y TS A 0.5 per cent w/v solution of *eosin Y*.

Guaiacol TS A 5 per cent w/v solution of *guaiacol* in *ethanol*.

Store protected from light.

Hydrogen Peroxide TS (10 volumes) (Dilute Hydrogen Peroxide Solution)

Dilute *hydrogen peroxide TS (20 volumes)* with an equal volume of *water*.

Hydrogen Peroxide TS (20 volumes)

Use analytical reagent grade of commerce containing about 6 per cent w/v of H_2O_2 or dilute 1 volume of *strong hydrogen peroxide solution* with 4 volumes of *water*.

DESCRIPTION Colourless liquid.

WEIGHT PER MILLILITRE About 1.02 g (Appendix 4.9).

Hydrogen Peroxide TS (100 volumes)

Use *Strong hydrogen Peroxide Solution* (See under "Reagents").

Hydrogen Sulfide TS A freshly prepared, saturated solution of *hydrogen sulfide* in *water*. It contains about 0.45 per cent w/v of H_2S at 20°.

Hydroxylamine TS Dissolve 1 g of *hydroxylamine hydrochloride* in 50 ml of *water*, add 50 ml of *ethanol* and 1 ml of *bromophenol blue TS*, and then add 0.1 M *sodium hydroxide* until the solution becomes green.

Hydroxylamine in Ethanol (60 per cent) TS Dissolve 3.5 g of *hydroxylamine hydrochloride* in 95 ml of *ethanol (60 per cent)*, add 0.5 ml of a 0.2 per cent w/v solution of *methyl orange* in *ethanol (60 per cent)*, and then add 0.5 M *potassium hydroxide* in *ethanol (60 per cent)* until the full yellow colour is obtained. Add sufficient *ethanol (60 per cent)* to produce 100 ml. The solution complies with the following test.

To 10 ml add 0.05 ml of 0.5 M *potassium hydroxide* in *ethanol (60 per cent)*: no change in colour is produced. To a further 10 ml add 0.05 ml of 0.5 M *hydrochloric acid*: the colour changes slightly towards orange.

Hydroxylamine in Ethanol (90 per cent) TS Dissolve 7.0 g of *hydroxylamine hydrochloride* in 90 ml of *ethanol (90 per cent)*, warming gently if necessary, and add 1.6 ml of *dimethyl yellow TS* and sufficient 1 M *potassium hydroxide* in *ethanol (90 per cent)* to give a full yellow colour. Dilute with *ethanol (90 per cent)* to 100 ml.

Hypophosphorous TS Dissolve, by heating gently, 10 g of *sodium hypophosphite* in 20 ml of *water* and dilute to 100 ml with *hydrochloric acid*. Allow to settle and decant or filter through glass wool.

Iodine TS Use 0.05 M *iodine VS*.

Iodine and Potassium Iodide TS Dissolve 500 mg of *iodine* in a solution of 1.5 g of *potassium iodide* in 5 ml of *water*, and dilute with *water* to 25 ml.

Store protected from light.

Iodine Bromide TS (Iodobromide TS) A 2 per cent w/v solution of *iodine monobromide* in *glacial acetic acid*.

Store in glass containers, protected from light.

Iodine Chloride TS (Iodochloride TS) Dissolve 1.65 g of *iodine monochloride* in 100.0 ml of *glacial acetic acid*.

Store protected from light.

Iron(III) Chloride TS Dissolve 9 g of *iron(III) chloride* in *water* to make 100 ml.

Iron(III) Chloride-Sulfamic Acid TS A solution containing 1.0 per cent w/v of *iron(III) chloride* and 1.6 per cent w/v of *sulfamic acid*.

Lead Acetate TS A 9.5 per cent w/v solution of *lead(II) acetate* in *carbon dioxide-free water*.

Store in well-closed containers.

Magenta TS, Decolorized (Fuchsin TS, Decolorized) Dissolve 0.1 g of *basic magenta* in 60 ml of *water* and add 1 g of *anhydrous sodium sulfite* dissolved in 10 ml of *water*. Slowly add 2 ml of *hydrochloric acid*, stirring continuously, and dilute to 100 ml with *water*. Allow to stand protected from light for at least 12 hours. Shake with sufficient *decolorizing charcoal* (0.2 to 0.3 g) to

remove the colour and then filter immediately. If the solution becomes cloudy, filter before use. If on standing the solution becomes violet, decolorize again by adding *decolorizing charcoal*. The solution complies with the following test.

SENSITIVITY TO FORMALDEHYDE To 1.0 ml of the solution add 1.0 ml of *water* and 0.1 ml of *aldehyde-free ethanol*. Add 0.2 ml of a solution containing 0.01 per cent w/v of formaldehyde. A pale pink colour is produced within 5 minutes.

Store protected from light.

Magnesium Sulfate TS A 12.3 per cent w/v solution of *magnesium sulfate*.

Mercuric-Potassium Iodide TS (Mayer's Reagent) Dissolve 1.358 g of *mercury(II) chloride* in 60 ml of *water*. Dissolve 5 g of *potassium iodide* in 10 ml of *water*. Mix the two solutions, and dilute with *water* to 100 ml.

Mercuric-Potassium Iodide TS, Alkaline (Nessler's Reagent) Dissolve 143 g of *sodium hydroxide* in 700 ml of *water*. Dissolve 50 g of red *mercury(II) iodide* and 40 g of *potassium iodide* in 200 ml of *water*. Pour the iodide solution into the hydroxide solution, and dilute with *water* to 1000 ml. Allow to settle, and use the clear supernatant.

Mercury(I) Nitrate TS Dissolve 15 g of *mercury(I) nitrate* in a mixture of 90 ml of *water* and 10 ml of *dilute nitric acid*.

Store in dark, amber-coloured bottles in which a small globule of mercury has been placed.

Mercury(II) Sulfate TS (Denigès' Reagent) Mix 5 g of *yellow mercury(II) oxide* with 40 ml of *water*, and, while stirring slowly, add 20 ml of *sulfuric acid*; add 40 ml of *water* and stir until completely dissolved.

2-Naphthol TS Dissolve 5 g of *2-naphthol*, freshly recrystallized, in 40 ml of 2 M *sodium hydroxide* and add sufficient *water* to produce 100 ml.

Prepare immediately before use.

1-Naphthol TS, Dilute Dissolve 100 mg of *1-naphthol* in 3 ml of a 15 per cent w/v solution of *sodium hydroxide* and dilute to 100 ml with *water*.

Prepare freshly before use.

Ninhydrin TS (Triketohydrindene Hydrate TS) Dissolve 200 mg of *ninhydrin* in *water* to make 10 ml.

Prepare freshly before use.

Nitro-vanado-molybdic TS Solution A Dissolve 10 g of *ammonium molybdate* in *water*, add 1 ml of 10 M *ammonia* and dilute to 100 ml with *water*.

Solution B Dissolve 0.5 g of *ammonium metavanadate* in hot *water*, add 2.8 ml of *nitric acid* and dilute to 100 ml with *water*.

To 100 ml of *solution A* and 100 ml of *solution B* add 96 ml of *nitric acid* and dilute to 500 ml with *water*.

Oxalic Acid TS Dissolve 6.3 g of *oxalic acid* in *water* to make 100 ml.

Prepare freshly before use.

Potassium Chromate TS A 5.0 per cent w/v solution of *potassium chromate*. Gives a red precipitate with silver nitrate in neutral solutions.

Potassium Dichromate TS Dissolve 7.5 g of *potassium dichromate* in *water* to make 100 ml.

Potassium Hexacyanoferrate(II) TS A 5.3 per cent w/v solution of *potassium hexacyanoferrate(II)*.

Potassium Hexacyanoferrate(III) TS Wash about 1 g of *potassium hexacyanoferrate(III)*, in crystals, with a little *water*, and dissolve the washed crystals in 100 ml of *water*. Gives a blue colour with solutions of iron(II) salts.

Prepare freshly before use.

Potassium Hydroxide TS, Ethanolic Dissolve 3 g of *potassium hydroxide* in 5 ml of *water* and add *sufficient aldehyde-free ethanol* to produce 100 ml. Allow the solution to stand for 1 hour and decant the clear solution.

Potassium Iodide TS Dissolve 16.5 g of *potassium iodide* in *water* to make 100 ml.

Store protected from light.

Potassium Iodide and Starch Solution Dissolve 750 mg of *potassium iodide* in 100 ml of *water*, heat to boiling and add, while stirring, a *solution* of 500 mg of *soluble starch* in 35 ml of *water*. Boil for 2 minutes and allow to cool.

SENSITIVITY TO IODINE To 15 ml of the solution add 0.05 ml of *glacial acetic acid* and 0.30 ml of 0.00050 M *iodine*. A blue colour is produced.

Prepare freshly before use.

Potassium Iodobismuthate TS Dissolve 10 g of *tartaric acid* in 40 ml of *water* and add 850 mg of *bismuth oxynitrate*. Shake during 1 hour, add 20 ml of a 40 per cent w/v solution of *potassium iodide*, and shake well. Allow to stand for 24 hours and filter.

Store protected from light.

Potassium Iodobismuthate TS, Acetic Dissolve 8 g of *potassium iodide* in 20 ml of *water* and add the solution to a mixture of 850 mg of *bismuth oxynitrate*, 40 ml of *water* and 10 ml of *glacial acetic acid*.

Potassium Iodobismuthate TS, Dilute Dissolve 10 g of *tartaric acid* in 50 ml of *water* and add 5 ml of *potassium iodobismuthate TS*.

Potassium Iodoplatinate TS Add 50 ml of a 5 per cent w/v solution of *chloroplatinic(IV) acid* to 45 ml of *potassium iodide TS* and dilute to 100 ml with *water*.

Store in amber glass containers.

Potassium Permanganate TS Use 0.02 M *potassium permanganate VS*.

Potassium Permanganate and Phosphoric Acid TS Dissolve 3 g of *potassium permanganate* in a mixture of 15 ml of *phosphoric acid* and 70 ml of *water* and add sufficient *water* to produce 100 ml.

Pyridylazonaphthol TS A 0.1 per cent w/v solution of *pyridylazonaphthol* in *absolute ethanol*.

Resorcinol with Copper(II) Sulfate TS To 80 ml of *hydrochloric acid* add 10 ml of a 2 per cent w/v solution of *resorcinol* and 0.25 ml of a 2.5 per cent w/v solution of *copper(II) sulfate* and dilute to 100 ml with *water*. Prepare the solution at least 4 hours before use.

Store at 2° to 8° and use within 1 week.

Saline TS A 0.9 per cent w/v solution of *sodium chloride*, sterilized by "Heating in an Autoclave" (Appendix 12).

Saline TS, Pyrogen-free *Saline TS* which complies with the requirements of "Pyrogen Test" (Appendix 8.2).

Silver Nitrate TS Use 0.1 M *Silver Nitrate VS*.

Silver Diethyldithiocarbamate TS Dissolve 1 g of *silver diethyldithiocarbamate* in 200 ml of a freshly opened bottle or recently distilled *pyridine*.

Store in light-resistant containers and use within 1 month.

Simulated Gastric Fluid TS Dissolve 2.0 g of *sodium chloride* and 3.2 g of *pepsin* in 7.0 ml of *hydrochloric acid* and sufficient *water* to make 1000 ml. This test solution has a pH of about 1.2.

Sodium Hexanitrocobaltate(III) TS Dissolve 5 g of *cobalt(II) nitrate in water*, add 1.5 ml of *nitric acid* and dilute to 100 ml with *water*. Dissolve 30 g of *sodium nitrite* in *water* and dilute to 100 ml with *water*. Mix the two solutions, allow to stand and use the clear solution.

Prepare immediately before use.

Sodium Hydroxide TS Dissolve 4.0 g of *sodium hydroxide* in *water* to make 100 ml (about 1 M).

Sodium Hypochlorite TS Use *Sodium Hypochlorite Solution* (see under "Reagents").

Sodium Hypochlorite TS, Dilute Dilute 35 ml of *sodium hypochlorite solution* with *water* to 100 ml.

Prepare immediately before use.

Simulated Intestinal Fluid TS Dissolve 6.8 g of *potassium dihydrogenphosphate* in 250 ml of *water*, mix, and add 77 ml of 0.2 M *sodium hydroxide* and 500 ml of *water*. Add 10.0 g of *pancreatin*, mix, and adjust the resulting solution with either 0.2 M *sodium hydroxide* or 0.2 M *hydrochloric acid* to a pH of 6.8±0.1. Dilute with *water* to 1000 ml.

Sodium Metabisulfide TS Dissolve 10 g of *sodium metabisulfite* to make 30 ml.

Prepare freshly before use.

Sodium Molybdotungstophosphate TS Boil under a reflux condenser 350 ml of *water*, 50 g of *sodiumt tungstate*, 12 g of *phosphomolybdic acid* and 25 ml of *phosphoric acid* for 2 hours. Cool and add sufficient *water* to produce 500 ml.

Store at 2° to 8°, protected from light.

Sodium Phosphate-Sodium Chloride Buffer TS Dissolve 90.0 g of *sodium chloride*, 10.9 g of *anhydrous disodium hydrogenphosphate* and 2.45 g of *monosodium dihydrogenphosphate dihydrate* in sufficient *water* to produce 1000.0 ml.

Store in a cold place. Any crystals produced should be dissolved by warming. Shake the solution before use.

Sodium Nitrite TS A freshly prepared 10 per cent w/v solution of *sodium nitrite*.

Prepare immediately before use.

Sodium Sulfide TS Dissolve 1 g of *sodium sulfide* in *water* to make 10 ml.

Prepare freshly before use.

Sodium Sulfide TS1 Dissolve, with heating, 12 g of *sodium sulfide* in 45 ml of a mixture of 10 volumes of *water* and 29 volumes of *glycerol (85 per cent)*, allow to cool and dilute to 100 ml with the same mixture. It should be colourless.

Sodium Tetraphenylborate TS Dissolve 1.2 g of *sodium tetraphenylborate* in *water* to make 200 ml. If necessary, filter before use.

Starch TS Triturate 1 g of *starch* or *soluble starch* with 5 ml of *water* and add with continuous stirring to 100 ml of boiling *water*, and boil for 1 minute. Cool and use only the clear solution.

Prepare freshly before use.

Sulfuric Acid-Formaldehyde TS Mix 2 ml of *formaldehyde TS* with 100 ml of *sulfuric acid*.

Tetrazolium Blue TS, Alkaline Immediately before use mix 1 volume of a 0.2 per cent w/v solution of *tetrazolium blue* in *methanol* with 3 volumes of a 12 per cent w/v solution of *sodium hydroxide* in *methanol*.

Tin(II) Chloride TS, Stronger Acid Dissolve 40 g of *tin(II) chloride* in 100 ml of *hydrochloric acid*.

Store in glass containers and use within 3 months.

Trinitrophenol TS Add 0.25 ml of 10 M *sodium hydroxide* to 100 ml of a saturated solution of *2,4,6-trinitrophenol* in *water*.

1.7 MATERIALS FOR CHROMATOGRAPHY

Gas Chromatography

SUPPORTS

Diatomaceous Support (Diatomaceous Earth; Siliceous Earth) White or almost white, fine granular powder made up of siliceous frustules of fossil diatoms or debris of fossil diatoms. It may be identified by microscopic examination with a magnification of ×500.

Diatomaceous Support, Acid-washed Diatomaceous support that has been purified by treatment with hydrochloric acid and washed with *water* to remove metallic impurities, and to reduce surface activity and peak-tailing.

Diatomaceous Support, Alkali-washed Diatomaceous support that has been treated with potassium hydroxide solution to reduce peak-tailing of basic compounds.

Diatomaceous Support, Silanized Diatomaceous earth for gas chromatography, silanized Acid-washed diatomaceous support that has been silanized with dimethyldichlorosilane or other suitable silanizing agents.

STATIONARY PHASES

A wide range of chemical substances is used, including polyethylene glycols, high-molecular weight esters and amides, hydrocarbons, silicone gums and fluids (polysiloxanes often substituted by methyl, phenyl, nitrilo, vinyl, or fluoroalkyl groups, or mixtures of these), and microporous cross-linked polyaromatic beads. Care should be taken to select grades specifically intended for use in gas chromatography. In most cases reference is made to a particular commercial brand which has been found to be suitable for the determination in question, but such statements do not imply that a different but equivalent commercial brand may not be used.

INTERNAL STANDARDS

Reagents used as internal standards should not contain any impurity which would produce a peak likely to interfere in the determination described in the monograph.

Thin-layer Chromatography

The coating substances described below are used to prepare thin-layer chromatoplates in accordance with the procedure described in Appendix 3.1. Prepare suspensions of the coating substances as recommended by the manufacturer unless otherwise prescribed. Commercial pre-coated chromatoplates may be used for Pharmacopoeial tests provided they comply with the test for chromatographic separation described for the corresponding coating substance.

Kieselguhr G A fine, greyish white powder, the grey colour becoming more pronounced on triturating with water. The average particle size is between 10 and 40 µm. It consists of natural kieselguhr which has been extracted with hydrochloric acid and calcined, and to which about 15 per cent w/w of calcium sulfate hemihydrate has been added. It complies with the following requirements.

CONTENT OF CALCIUM SULFATE Carry out the test described under Silica gel G.

SEPARATING POWER Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using a mixture of 65 volumes of *ethyl acetate*, 23 volumes of *2-propanol* and 12 volumes of *water* as the mobile phase. Prepare the chromatoplates using a slurry of the sample in 0.02 M *sodium acetate*. Apply to the plate, 5 µl of a solution in *pyridine* containing 0.01 per cent w/v of each of *lactose*, *sucrose*, *fructose*, *D-glucose*, and *D-galactose*. After removal of the plate, dry it at 105° to 110°, and allow to cool. Spray the plate with about 10 ml of *anisaldehyde TS* and heat at 105° to 110° for 5 to 10 minutes; the chromatogram shows five, well-defined, well-separated spots with no tailing.

ALKALINITY pH of a suspension prepared by shaking 1 g with 10 ml of *carbon dioxide-free water* for 5 minutes, 7.0 to 8.0 (Appendix 4.11).

Silica gel G A fine, white, homogeneous powder of an average particle size between 10 and 40 µm containing about 13 per cent w/w of calcium sulfate hemihydrate, and complying with the following requirements.

CONTENT OF CALCIUM SULFATE To about 250 mg, accurately weighed, add 3 ml of 2 M *hydrochloric acid* and 100 ml of *water* and shake vigorously for 30 minutes. Filter, wash the residue with *water* and carry out the "Complexometric Titration of Calcium" (Appendix 6.3) on the combined filtrate and washings. Each ml of 0.1 M *disodium edetate VS* is equivalent to 14.51 mg of $CaSO_4 . ½H_2O$.

SEPARATING POWER Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *toluene* as the mobile phase. Apply to the plate, 10 µl of a solution in *dichloromethane* containing 0.1 mg per ml of each of *indophenol blue*, *sudan red G* and *dimethyl yellow* in *toluene*. Allow the mobile phase to ascend 10 cm. The chromatogram shows three clearly separated spots of the indophenol blue, sudan red G and dimethyl yellow in order of increasing R_f value.

ALKALINITY pH of a suspension prepared by shaking 1 g with 10 ml of *carbon dioxide-free water* for 5 minutes, about 7 (Appendix 4.11).

Silica gel GF254 A fine, white, homogeneous powder of an average particle size between 10 and 40 µm containing about 13 per cent w/w of calcium sulfate hemihydrate and about 1.5 per cent w/w of a fluorescent indicator having a maximum intensity at 254 nm. It complies with the tests for Content of Calcium Sulfate, Alkalinity and Separating Power stated under Silica gel G and with the following test.

FLUORESCENCE Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using a mixture of 90 volumes of 2-*propanol* and 10 volumes of *anhydrous formic acid* as the mobile phase. Apply separately to the plate, increasing quantities from 1 to 10 µl of a 1 mg per ml solution of *benzoic acid* in the same solvent mixture. Develop the plate and dry in a current of warm air. Examine the chromatogram under ultraviolet light at 254 nm. The benzoic acid appears as dark spots on a fluorescent background in the upper third of the chromatogram at levels of 2 µg and greater.

Silica gel H A fine, white, homogenous powder of an average particle size between 10 and 40 µm. It is free from calcium sulfate hemihydrate and complies with the tests for Separating Power and Alkalinity stated under Silica gel G.

Silica gel HF254 A fine, white homogeneous powder of an average particle size between 10 and 40 µm containing about 1.5 per cent w/w of a fluorescent indicator having a maximum intensity at about 254 nm. It is free from calcium sulfate hemihydrate and complies with the tests for Alkalinity and Separating Power stated under Silica gel G, and with the test for Fluorescence stated under Silica gel GF254.

1.8 REFERENCE SUBSTANCE

A Reference Substance is an authenticated uniform material that is intended for use in specified chemical, physical and biological tests, by which the properties of the product under examination are compared.

Reference Substances are needed for certain analytical procedures in many monographs of the Thai Pharmacopoeia including:

(1) infrared identification;
(2) spectrophotometric or photometric methods of assay;
(3) chromatographic tests and assays;
(4) biological tests and assays including microbiological assays; and
(5) others.

Where a test or an assay calls for the use of a Reference Substance, the ASEAN Reference Substances (ARS) or other recognized reference substances may be used. The ASEAN Reference Substances are available from Bureau of Drug and Narcotic (BDN), Department of Medical Sciences (DMSc), Nonthaburi, Thailand.

Steps in Establishing an ASEAN Reference Substance

The ARS project was initiated in 1980 under the Technical Cooperation among ASEAN countries on Pharmaceuticals in order to produce reference substances for utilization within ASEAN region. DMSc, Thailand was appointed as the coordinator of the project with the cooperation of four ASEAN member countries including Indonesia, Malaysia, the Philippines, and Singapore. Vietnam, Lao PDR, Myanmar and Cambodia joined later in 1995 and 1997. The project was supported by United Nations Development Program (UNDP) and World Health Organization (WHO) from 1982 to 1991 and Japan Pharmaceutical Manufacturers Association (JPMA) from 1992 to present.

ARS are established following the guidelines entitled "WHO General Guidelines for the Establishment, Maintenance and Distribution of Chemical Reference Substances, Part B Secondary Chemical Reference Substances" and "Guidelines for the Establishment, Handling, Storage and Use of ASEAN Reference Substances". ARS are recognized as secondary reference standards according to the definition of WHO Technical Report Series No. 943, 2007. To date, 189 ARS have been produced and distributed for utilization in pharmaceutical quality control laboratories of the ASEAN member countries.

The establishment of new ARS is proposed by each ASEAN member country in the meeting of ARS Working Group attended by each head of reference standard section/unit from National Drug Control Laboratory. The responsible countries generate a procurement specifications and a testing protocol. A bulk material is obtained from its major manufacturer or distributor(s) in each country. A preliminary test of a candidate material is performed by the responsible country to verify its compliance with the requirements of the monograph. The assignment of content is made by collaborative study organized by the responsible country under supervision of DMSc. The results are evaluated by the responsible country and DMSc; additional testing or investigation are performed where necessary. The reports are compiled and presented for a review and approval to the meeting of ARS Working Group. After approval, the material is subdivided (if not packaged prior to the collaborative study) and labelled. Quality checks are performed before distributing to other ASEAN member countries.

Collaborative Study for the Evaluation of an ASEAN Reference Substances Candidate Material

The objectives of the evaluation study are to confirm the identity and assess the purity of the material, to determine its suitability for use in the official applications and to provide the user with all the necessary information and directions for use.

The model of the testing protocol is set up by DMSc and approved by the ARS Working Group. The details of testing protocol include types of tests, number of tests, number of collaborators, number of replicates, and references to the procedures to be used.

The testing protocol may comprise visual and microscopic evaluation, identification tests (e.g., infrared absorption, UV-absorption), determination of physico-chemical constant (e.g., melting range, specific rotation, specific gravity), chromatographic purity test, inorganic contaminants determination, volatile substances test (e.g., water, solvents), functional group analysis (e.g., titrations, UV absorptivity), thermal

analysis (e.g., differential scanning calorimetry), and assay against another well-characterized standard (e.g., USPRS, BPCRS, EPCRS, and ICRS).

The number of collaborators is generally not less than three; one laboratory of the responsible country and at least two laboratories in other ASEAN member countries. Where appropriate, statistical control is exercised in the design of the evaluation study and in the analysis of the results. The assigned value for reference substances used in assays are labelled in per cent with two decimal places.

Label Text

The label text is designed to provide the user with the information needed for the correct storage and usage of the reference substances in monograph application(s). The label includes a calculation value for reference substances with quantitative applications as well as storage condition. ARS are accompanied by certificate of analysis with more details of testing results, intended use, direction for use, and storage conditions.

Packaging and Storage

ARS are packaged in individual units designed to maintain the integrity of reference substances. The most common containers for solid material are colourless or amber glass vial with plastic inner and screw cap. The packaging environment is determined by the sensitivity of the material to light, oxidation, or atmospheric humidity. Where appropriate, containers are filled in a glove box under inert gas and in conditions of controlled low residual humidity.

To serve its intended purpose, ARS must be properly stored, handled and used. Generally, reference substances should be stored in their original containers away from heat and protected from light. Avoid humid storage areas in particular.

Continued Suitability for Use Program

To ensure that the reference substances maintain the properties determined at the initial evaluation, the responsible country maintains a continued suitability for use program. The retesting intervals and protocols are a function of the uses and properties of the substance and of the information available about its stability. Abbreviated protocols use the stability-indicating methodology employed in the initial characterization of the material to confirm the consistency of attributes such as appearance, chromatographic purity, or volatiles content.

Proper Use

The reference substances are not intended for use as drugs. ARS do not carry an expiration date as long as they are in distribution. An updated version of catalogue can be found on the BDN website at www.dmsc.moph.go.th/webroot/drug/products/ars.stm.

Whenever the directions for use specified in the certificate of analysis require a preliminary drying or a correction for volatiles, it should be performed "at the time" of use. Further experimental details should be controlled by the user's Standard Operating Procedures and Good Laboratory Practices.

1.9 VOLUMETRIC APPARATUS

Most of the volumetric apparatus used in the analytical operation is calibrated at 20°, although the temperature specified generally for Pharmacopoeial tests and assays is 25°. This discrepancy is inconsequential provided the room temperature is reasonably constant.

Use To attain the degree of precision required in many Pharmacopoeial assays involving accurate volumetric measurements, the apparatus must be chosen and used with care. A burette should be of such size that the titrant volume represents not less than 30 per cent of the nominal volume. Where less than 10 ml of titrant is to be measured, a 10-ml burette or a microburette generally is required.

The design of volumetric apparatus is an important factor in assuring accuracy. For example, the length of the graduated portions of graduated cylinders should be not less than five times the inside diameter, and the tips of burettes and pipettes should restrict the outflow rate to not more than 500 µl per second.

Standard of accuracy The capacity tolerances for volumetric flasks, transfer and graduated pipettes, and burettes are those accepted by the International Organization for Standardization (ISO) as indicated in the accompanying tables.

Volumetric Flasks

Nominal Capacity (ml)	Capacity Tolerances (± ml)
5	0.025
10	0.025
25	0.04
50	0.06
100	0.10
200	0.15
250	0.15
500	0.25
1000	0.40
2000	0.60

Transfer and graduated pipettes calibrated "to deliver" should be drained in a vertical position and then touched against the wall of the receiving vessel to drain the tips. Volume readings on burettes should be estimated to the nearest 0.01 ml for 25- and 50-ml burettes, and to the nearest 0.005 ml for 5- and 10-ml burettes. Pipettes calibrated "to contain" are called for in special cases, generally for measuring viscous fluids like syrups; however, a volumetric flask may be substituted for a "to contain" pipette. In such cases, the pipette or flask should be washed clean, after draining, and the washings added to the measured portion.

Transfer Pipettes

Nominal Capacity (ml)	Capacity Tolerances (± ml)
0.5	0.005
1	0.008
2	0.01
5	0.015
10	0.02
20	0.03
25	0.03
50	0.05
100	0.08
200	0.1

Graduated Pipettes

Nominal Capacity (ml)	Capacity Tolerances (± ml)
1	0.006
2	0.01
5	0.03
10	0.05
25	0.1

Burettes

Nominal Capacity (ml)	Capacity Tolerances (± ml)
10	0.02
25	0.03
50	0.05
100	0.1

1.10 WEIGHTS AND BALANCES

Pharmacopoeial tests and assays require the use of balance of capacity and sensitivity corresponding to the degree of accuracy sought.

When weighing quantities of 50 mg or more that are to be "accurately weighed", an analytical balance of 100 to 200 g capacity and 0.1 mg sensitivity is required. When weighing quantities of less than 50 mg that are to be "accurately weighed", an analytical balance of 20 g capacity and 0.001 mg sensitivity, usually called an analytical microbalance, is required.

Apparatus

Analytical balances should possess adequate capacity and sensitivity. They may be either of the equalarm type, requiring the use of a set of calibrated weights, or of any other suitable type (for example, analytical microbalances using magnetic measurement) provided their performance is periodically checked by means of a reference set of calibrated weights.

The analytical balance should be so constructed as to support its full capacity without developing undue stress and its sensitivity should not be altered by repeated weighing of the full-capacity load. The type of analytical balance having constant sensitivity over the whole capacity range is the constant-load, single-pan balance. It has a set of weights suspended from a counterpoised beam; in the process of weighing, these are removed from the beam by a manually operated mechanical device until equilibrium is reached.

The analytical balance should be constructed in a proper housing with suitable openings to permit the placement of the material to be weighed and to exclude air currents. Desiccants may be placed inside the housing, such as silica gel, for the maintenance of a relatively dry atmosphere.

Sets of calibrated weights used with balances that require manual placement of weights and sets of weights used to check the sensitivity of balances of another type should be kept in a case made of suitable material and properly lined.

Placement of Balance

The analytical balance should be placed upon a firm foundation that is as free from mechanical vibration as possible, preferably on an antivibration table of proper design. Alternatively, it may be placed on a concrete slab resting upon piers that are either sunk into the ground or connected to the construction elements of the building; or it may be placed upon a stout table or shelf protected by shock absorbers, such as cork mats or sheet rubber.

The balance should also be protected from humidity and acid fumes, preferably by placing it in a separate room of the laboratory. It should not be placed in a current of air or in direct sunlight.

The balance should be equipped with a levelling device and an indicator of proper position. Proper adjustment of levelling should be frequently checked.

Checking of Sensitivity

The sensitivity of the balance should be periodically checked by a qualified expert.

Checking the Stability of the Equilibrium Position

Before the balance is used, its equilibrium position without load should be checked several times. After each test, the balance has to be arrested.

The equilibrium position of the balance under load should also be determined from time to time, for example, with one-tenth of the full load and with the full load. The difference between equilibrium positions found in two successive determinations made with equal loads should not exceed 0.1 mg for analytical balances and 0.001 mg for analytical microbalances.

Operation of the Balance

When the balance is not in use, the balance beam and pan supports should be raised. The doors of the housing should always be kept closed.

To release the balance, the beam and pans should be lowered very carefully.

Objects to be weighed must be allowed to attain the temperature of the balance before weighing is started. The object to be weighed, as well as the weights, should always be placed on the pan as centrally as possible. During a weighing or on any occasion when objects are

being added to or removed from the pans, both the beam arrests and the pan supports must be raised. Substances must be weighed in suitable containers such as beakers, weighing bottles, or crucibles. Liquids and volatile or hygroscopic solids must be weighed in tightly closed vessels, such as stoppered weighing bottles. No chemicals or objects that might injure the balance pans should be placed directly upon them.

When small quantities of a substance (for example, the sulfated ash) must be weighed in a large vessel and a fairly long period elapses between the two weighings, atmospheric pressure and temperature may alter sufficiently to affect the buoyancy and thus cause an appreciable error. In two-pan balances, this error may be eliminated by using another vessel of similar shape and weight for taring.

The pans of the balance should be periodically lightly brushed with a camel-hair or similar brush to remove any dust that may have collected.

The weights should be handled only by means of a pair of forceps, which should possess tips covered with suitable material.

Calibration

If necessary, turn on the power, and allow the balance to equilibrate for at least 1 hour before proceeding with the calibration. (Microbalances may require up to 24 hours to reach equilibrium.) If the balance power has gone off and then has come back on, as in a power outage, certain types of balance may display a message indicating that the balance must be calibrated before a weighing is made. If the operator touches the balance bar, the message may be cleared and the balance may display zeros; however, the balance will not give the correct weighing until it has been calibrated. Electronic analytical balances have an internal calibration system based on an applied load. The calibration applies for the current ambient temperature.

1.11 POWDER FINENESS AND SIEVES

Powders

The degree of coarseness or fineness of a powder is differentiated and expressed by reference to the nominal mesh aperture size of the sieves used.

The following terms are used in the description of powders:

COARSE POWDER A powder all the particles of which pass through a sieve with a nominal mesh aperture of 1.70 mm and not more than 40.0 per cent through a sieve with a nominal mesh aperture of 355 μm.

MODERATELY COARSE POWDER A powder all the particles of which pass through a sieve with a nominal mesh aperture of 710 μm and not more than 40.0 per cent through a sieve with a nominal mesh aperture of 250 μm.

MODERATELY FINE POWDER A powder all the particles of which pass through a sieve with a nominal mesh aperture of 355 μm and not more than 40.0 per cent through a sieve with a nominal mesh aperture of 180 μm.

FINE POWDER A powder all the particles of which pass through a sieve with a nominal mesh aperture of 180 μm.

VERY FINE POWDER A powder all the particles of which pass through a sieve with a nominal mesh aperture of 125 μm.

When the fineness of a powder is described by means of a number, it is intended that all the particles of the powder shall pass through a sieve of which the nominal mesh aperture, in μm, is equal to that number.

When a batch of a vegetable drug is being ground and sifted, no portion of the drug shall be rejected, but it is permissible, except in the case of assays, to withhold the final tailings, if an approximately equal amount of tailings from a preceding batch of the same drug has been added before grinding.

When the use of sieves is inappropriate, the definition is expressed in terms of the particle size as determined by suitable microscopical examination.

Sieves

Wire mesh sieves used in sifting powdered drugs are identified by numbers indicating the nominal mesh aperture.

The sieves should be made of wires of uniform circular cross-section. The wires may be of stainless steel or of other suitable material except that plated wire is not permitted. Sieves should conform to the specifications which are concordant with the recommended International Standard ISO 3310-1: 2000 (E), shown in the following table.

Calibration and recalibration of test sieves is in accordance with the most current edition of ISO 3310-1. Sieves should be carefully examined for gross distortions and fractures, especially at their screen frame joints, before use. Sieves may be calibrated optically to estimate the average opening size, and opening variability, of the sieve mesh. Alternatively, for the evaluation of the effective opening of test sieves in the size range of 212 to 850 μm, Standard Glass Spheres are available from the national or international organization, e.g. NIST[1]. Unless otherwise specified in the individual monograph, perform the sieve analysis at controlled room temperature and a relative humidity between 20 and 70 per cent.

CLEANING TEST SIEVES Ideally, test sieves should be cleaned using only an air jet or a liquid stream. If some apertures remain blocked by test particles, careful gentle brushing may be used as a last resort. Washing sieves in hot water is not recommended since the sieves can distort and rupture during heating and cooling. If it is necessary to use water, it should be used at ambient temperature and the sieve dried by first using a volatile water-miscible solvent to remove the water and then a

[1]US National Institute of Standards and Technology

low-pressure air jet to remove the solvent. This procedure should be carried out in a fume hood or cabinet that conforms to local regulations.

Method for Determining Powder Fineness

Place the specified quantity of the test powder upon the appropriate sieve having a close-fitting receiving pan and cover. Shake the sieve in a rotary horizontal direction and vertically by tapping on a hard surface for not less than the specified time or until sifting is practically complete. Avoid prolonged shaking that would result in increasing the fineness of the powder during the testing. In the case of oily or other powders that tend to clog the openings, carefully brush the screen at intervals during the test. Breaking up lumps that form during the sifting. Weigh accurately the amount remaining on the sieve and in the receiving pan.

The fineness of a powdered drug or chemical may be determined also by screening through the sieves in a mechanical sieve shaker, which reproduces the circular and tapping motion given to testing sieves in hand sifting but with a uniform mechanical action, following the directions provided by the manufacturer of the shaker.

Number of Sieve*	Nominal Mesh Aperture Size	Preferred Average Wire Diameter	Percentage Sieving Area	US Sieve No.**
μm	mm	mm		
4000	**4.00**	**1.40**	**55**	**5**
3350	3.35			6
2800	**2.80**	**1.12**	**51**	**7**
2360	2.36	1.00	49	8
2000	**2.00**	**0.90**	**48**	**10**
1700	1.70	0.80	46	12
1400	**1.40**	**0.71**	**44**	**14**
1180	1.18	0.63	43	16
1000	**1.00**	**0.56**	**41**	**18**
μm	μm	μm		
850	850			20
710	**710**	**450**	**37**	**25**
600	600	400	36	30
500	**500**	**315**	**38**	**35**
425	425	280	36	40
355	**355**	**224**	**38**	**45**
300	300	200	36	50
250	**250**	**160**	**37**	**60**
212	212	140	36	70
180	**180**	**125**	**35**	**80**
150	150	100	36	100
125	**125**	**90**	**34**	**120**
106	106	71	36	140
90	**90**	**63**	**35**	**170**
75	75	50	36	200
63	**63**	**45**	**34**	**230**
53	53	36	35	270
45	**45**	**32**	**34**	**325**

*Entries in bold area ISO "principal sizes".

**The list of United States standard sieves is included for information purposes.

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1.13 NAMES, SYMBOLS AND ATOMIC WEIGHTS OF ELEMENTS

[Scaled to A_r (^{12}C) = 12, where ^{12}C is a neutral atom in its nuclear and electronic ground state.]

The atomic weights have been revised in accordance with the figures recommended by IUPAC[1]. Elements that lack a characteristic terrestrial isotopic composition have not been included in this table. The standard values of atomic weights and the uncertainties (in parentheses, following the last significant figure to which they are attributed) are given.

Name	Symbol	Atomic Weight	Name	Symbol	Atomic Weight
Aluminium (Aluminum)	Al	26.9815386(8)	Molybdenum	Mo	95.94(2)
Antimony (Stibium)	Sb	121.760(1)	Neodymium	Nd	144.242(3)
Argon	Ar	39.948(1)	Neon	Ne	20.1797(6)
Arsenic	As	74.92160(2)	Nickel	Ni	58.6934(2)
Barium	Ba	137.327(7)	Niobium	Nb	92.90638(2)
Beryllium	Be	9.012182(3)	Nitrogen	N	14.0067(2)
Bismuth	Bi	208.98040(1)	Osmium	Os	190.23(3)
Boron	B	10.811(7)	Oxygen	O	15.9994(3)
Bromine	Br	79.904(1)	Palladium	Pd	106.42(1)
Cadmium	Cd	112.411(8)	Phosphorus	P	30.973762(2)
Caesium (Cesium)	Cs	132.9054519(2)	Platinum	Pt	195.084(9)
Calcium	Ca	40.078(4)	Potassium (Kalium)	K	39.0983(1)
Carbon	C	12.0107(8)	Praseodymium	Pr	140.90765(2)
Cerium	Ce	140.116(1)	Protactinium	Pa	231.03588(2)
Chlorine	Cl	35.453(2)	Rhenium	Re	186.207(1)
Chromium	Cr	51.9961(6)	Rhodium	Rh	102.90550(2)
Cobalt	Co	58.933195(5)	Rubidium	Rb	85.4678(3)
Copper (Cuprum)	Cu	63.546(3)	Ruthenium	Ru	101.07(2)
Dysprosium	Dy	162.500(1)	Samarium	Sm	150.36(2)
Erbium	Er	167.259(3)	Scandium	Sc	44.955912(6)
Europium	Eu	151.964(1)	Selenium	Se	78.96(3)
Fluorine	F	18.9984032(5)	Silicon	Si	28.0855(3)
Gadolinium	Gd	157.25(3)	Silver (Argentum)	Ag	107.8682(2)
Gallium	Ga	69.723(1)	Sodium (Natrium)	Na	22.98976928(2)
Germanium	Ge	72.64 (1)	Strontium	Sr	87.62(1)
Gold (Aurum)	Au	196.966569(4)	Sulfur	S	32.065(5)
Hafnium	Hf	178.49(2)	Tantalum	Ta	180.94788(2)
Helium	He	4.002602(2)	Tellurium	Te	127.60(3)
Holmium	Ho	164.93032(2)	Terbium	Tb	158.92535(2)
Hydrogen	H	1.00794(7)	Thallium	Tl	204.3833(2)
Indium	In	114.818(3)	Thorium	Th	232.03806(2)
Iodine	I	126.90447(3)	Thulium	Tm	168.93421(2)
Iridium	Ir	192.217(3)	Tin (Stannum)	Sn	118.710(7)
Iron (Ferrum)	Fe	55.845(2)	Titanium	Ti	47.867(1)
Krypton	Kr	83.798(2)	Tungsten (Wolfram)	W	183.84(1)
Lanthanum	La	138.90547(7)	Uranium	U	238.02891(3)
Lead (Plumbum)	Pb	207.2(1)	Vanadium	V	50.9415(1)
Lithium	Li	6.941(2)	Xenon	Xe	131.293(6)
Lutetium	Lu	174.967(1)	Ytterbium	Yb	173.04(3)
Magnesium	Mg	24.3050(6)	Yttrium	Y	88.90585(2)
Manganese	Mn	54.938045(5)	Zinc	Zn	65.409(4)
Mercury (Hydrargyrum)	Hg	200.59(2)	Zirconium	Zr	91.224(2)

[1]From M. E. Wieser, Atomic weights of the elements 2005 (IUPAC Technical Report), *Pure Appl. Chem.*, 2006, *78*(11), pp. 2054-2056.

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1.14 WEIGHTS AND MEASURES: SI UNITS

The International System of Units (SI) is used in this Pharmacopoeia and comprises three categories of units, namely basic units, derived units and supplementary units. The basic units are set out in Table 1.

The derived units may be formed by combining the basic units according to certain algebraic relationships between the corresponding quantities. Some of these derived units have special names and symbols. The SI units used in this Pharmacopoeia are shown in Table 2.

Certain units of the SI have not yet been classified as basic or derived; they are known as supplementary units and are shown in Table 3.

Some important and widely used units outside the international system are shown in Table 4.

The prefixes shown in Table 5 are used to form the names and symbols of the decimal multiples and sub-multiples of SI units.

Table 1 Basic Units

Quantity	Name of Basic SI Unit	Symbol
Length	metre	m
Mass	kilogram	kg
Time	second	s
Electric current	ampere	A
Thermodynamic temperature	kelvin	K
Amount of substance	mole	mol
Luminous intensity	candela	cd

Table 2 Derived SI Units Used in the Thai Pharmacopoeia and Their Equivalence with Other Units

Quantity	Name of Derived SI Unit	Symbol	Expression in Basic SI Units	Equivalence with Other Units
Absorbed dose of ionizing radiation	gray	Gy	m^2s^{-2}	1 Gy = 1 joule per kg = 100 rad
Energy, work, quantity of heat	joule	J	kgm^2s^{-2}	1 J = 10^7 ergs
Electrical potential, potential difference, electromotive force	volt	V	$kgm^2A^{-1}s^{-3}$	
Electric resistance	ohm	Ω	$kgm^2A^{-2}s^{-3}$	
Quantity of electricity	Coulomb	C	As	
Force	newton	N	$kgms^{-2}$	1 N = 10^5 dynes
Frequency	hertz	Hz	s^{-1}	1 Hz = 1 cycle per second
Power	watt	W	kgm^2s^{-3}	
Pressure	pascal	Pa	$kgm^{-1}s^{-2}$	1 kPa = 7.5 mm Hg = 7.5 Torr = 1.45 lb/in^2
Radioactivity	becquerel	Bq	s^{-1}	1 Bq = 2.703×10^{-11} curies

Table 3 Supplementary Units

Quantity	Name of Supplementary SI Unit	Symbol
Plane angle	radian	rad
Solid angle	steradian	sr

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Table 4 Units Used with the International System

Quantity	Unit		Value in SI Units
	Name	Symbol	
Gravity (in centrifugation)	gravity	*G*, *g*	$1\ g = 6.672 \times 10^{-11}$ N. m^2/kg^2
Time	minute	min	1 min = 60 s
	hour	h	1 h = 60 min = 3,600 s
	day	d	1 d = 24 h = 86,400 s
Plane angle	degree	°	$1^\circ = (\pi/180)$ rad
	minute	'	$1' = (1/60)^\circ = (\pi/10{,}800)$ rad
	second	"	$1'' = (1/60)' = (\pi/648{,}000)$ rad
Volume	litre	l	$1\ l = 1\ dm^3 = 10^{-3} m^3$
Light	lux	lx	1 lx = 1 lumen m^2
	lumen	lm	1 lm = luminous flux emitted per unit solid angle from a uniform source of 1 candela
Mass	tonne	t	$1\ t = 10^3$ kg
	gram	g	$1\ g = 10^{-3}$ kg

Table 5 Decimal Multiples and Sub-multiples

Factor	Prefix	Symbol
10^{18}	exa	E
10^{15}	peta	P
10^{12}	tera	T
10^{9}	giga	G
10^{6}	mega	M
10^{3}	kilo	k
10^{2}	hecto	h
10^{1}	deca	da
10^{-1}	deci	d
10^{-2}	centi	c
10^{-3}	milli	m
10^{-6}	micro	μ
10^{-9}	nano	n
10^{-12}	pico	p
10^{-15}	femto	f
10^{-18}	atto	a

1.15 MEDICINE DROPPER

The Pharmacopoeial medicine dropper consists of a tube made of glass or other suitable transparent or translucent material that generally is fitted with a collapsible bulb and, while varying in capacity, is constructed at the delivery end to a round opening having an external diameter of about 3 mm. The dropper, when held vertically, delivers *water* in drops each of which weighs between 45 mg and 55 mg.

In using a medicine dropper, one should keep in mind that few medicinal liquids have the same surface and flow characteristics as *water*, and therefore the size of drops varies materially from one preparation to another.

Where accuracy of dosage is important, a dropper that has been calibrated especially for the preparation with which it is supplied should be employed. The volume error incurred in measuring any liquid by means of a calibrated dropper should not exceed 15 per cent under normal use conditions.

1.16 PHARMACEUTICAL DOSAGE FORMS

Dosage forms are provided for most of the Pharmacopoeial drug substances, but the processes for the preparation of many of them are, in general, beyond the scope of the Pharmacopoeia. In addition to defining the dosage forms, this section presents general requirements of some of them. Besides these requirements, the pharmaceutical products should be designed to possess certain desirable properties of bioavailability and stability.

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Bioavailability

Bioavailability[1] is the rate and extent of absorption of a drug from a dosage form as determined by its concentration/time curve in the systemic circulation or by its excretion in urine. A variety of factors are known to affect absorption. They are, for example, the method of manufacture or method of compounding; the particle size and crystal form or polymorph of the drug substance; and the diluents and excipients used in formulating the dosage form, including fillers, binders, disintegrating agents, lubricants, coatings, solvents, suspending agents, and dyes. Lubricants and coatings are foremost among these. The maintenance of a demonstrably high degree of bioavailability requires particular attention to all aspects of production and quality control that may affect the nature of the finished dosage form.

Stability

Stability of a pharmaceutical dosage form[2] refers to the capability of the dosage unit, in a specific container/closure system, to remain within its physical, chemical, microbiological, therapeutic, and toxicological specifications. The shelf-life of the dosage form is the period of time during which a product is expected, if stored under recommended conditions, to remain within the specification as determined by stability studies on a number of batches of the product. The shelf-life is used to establish the expiration date.

The stability parameters of a pharmaceutical dosage form can be influenced by environmental conditions of storage (temperature, light, air, and humidity), as well as the package components. Pharmacopoeial articles should include required storage conditions on their labelling. These are the conditions under which the expiration date shall apply. The storage requirements specified in the labelling for the article must be observed throughout the distribution of the article (for example, beyond the time it leaves the manufacturer up and including its handling by the dispenser or seller of the article to the consumer).

AEROSOLS

Aerosols are presented in special containers under pressure of a gas and contain one or more active ingredients. The preparations are released from the container, upon actuation of an appropriate valve, in the form of an aerosol (dispersion of solid or liquid particles in a gas, the size of the particles being adapted to the intended use). The pressure for the release is generated by suitable propellants. The preparations consist of a solution, an emulsion or a suspension and are intended for local application to the skin or the mucous membranes of various body orifices or for inhalation.

The term "aerosol" refers to the fine mist of spray that results from most pressurized systems. However, the term has been broadly misapplied to all self-contained pressurized products, some of which deliver foams or semisolid fluids. The basic components of an aerosol system are the container, the propellant, the concentrate containing the active ingredient(s), the valve, and the actuator. Suitable auxiliary substances may also be used, for example solvents, solubilizers, emulsifying agents, suspending agents and lubricants for the valve to prevent clogging.

Propellants The propellants are either gases liquefied under pressure or compressed gases or low-boiling liquids. Liquefied gases are, for example, halogenated hydrocarbons (especially chloro-fluoro-derivatives of methane and ethane) and low-molecular-mass hydrocarbons (such as propane and butane). Compressed gases are, for example, carbon dioxide, nitrogen and nitrous oxide. Mixtures of these propellants may be used to obtain optimal solution properties and desirable pressure, delivery and spray characteristics.

Valves A suitable valve keeps the container tightly closed when not in use and regulates the delivery of the contents during use. The spray characteristics are influenced by the type of spraying device, in particular by the dimensions, number and location of orifices. Some valves provide a continuous release, others ("metering dose valves") deliver a defined quantity of product upon each valve actuation. The various valve materials in contact with the contents are compatible with them.

Actuators An actuator is the fitting attached to an aerosol valve stem which, when depressed or moved, opens the valve, and directs the spray containing the drug preparation to the desired area. The actuator usually indicates the direction in which the preparation is dispensed and protects the hand or finger from the refrigerant effects of the propellant. Actuators incorporate an orifice which may vary widely in size and shape. The size of this orifice, the expansion chamber design, and the nature of the propellant and formulation influence the physical characteristics of the spray foam, or stream of solid particles dispensed. For inhalation or oral dose aerosols, an actuator capable of delivering the medication in the proper particle size range is utilized.

Production Aerosols are prepared by one of two general processes. In the "cold-fill" process, the concentrate (generally cooled to a temperature below 0°) and the refrigerated propellant are measured into open containers (usually chilled). The valve-actuator assembly is then crimped onto the container to form a pres-

[1]Further information on bioavailability may be obtained from (1) "ASEAN Guidelines for the Conduct of Bioavailability and Bioequivalence Studies", issued by ASEAN Pharmaceutical Product Working Group (ASEAN PPWG), 2005; (2) "Instruction for the *In Vivo* Bioequivalence Study Protocol Development", notified by Drug Control Division, Food and Drug Administration, Ministry of Public Health, Thailand, 2006.

[2]The stability study requirements may be obtained from (1) "ASEAN Guideline on Stability Study on Drug Product", issued by ASEAN Pharmaceutical Product Working Group (ASEAN PPWG), 2005; (2) "Guidelines for Stability Testing of Pharmaceutical Products", published by Food and Drug Administration and Department of Medical Sciences, Ministry of Public Health, Thailand, 2004.

sure-tight seal. During the interval between propellant addition and crimping, sufficient volatilization of propellant occurs to displace air from the container. In the "pressure-fill" method, the concentrate is placed in the container, and either the propellant is forced under pressure through the valve orifice after the valve is sealed, or the propellant is allowed to flow under the valve cap and then the valve assembly is sealed ("under-the-cap" filling).

In both cases of the "pressure-fill" method, provision must be made for evacuation of air by means of vacuum or displacement with a small amount of propellant. Manufacturing process controls usually include monitoring or proper formulation and propellant fill weight, and pressure testing and leak testing and valve function testing of the finished aerosol. Microbiological attributes should also be controlled.

Leak testing Aerosols comply with the Leak testing under "Test for Aerosols" (Appendix 4.19).

Minimum fill Aerosols comply with the test described in the "Minimum Fill" (Appendix 4.26).

Packaging and storage The containers are tight and resistant to the internal pressure and may be made of metal, glass, plastic or combinations of these materials. They are compatible with their contents. Suitable metals include stainless steel, aluminium, and tin-plated steel. Glass containers are protected with a plastic coating. Aerosols shall be stored at a temperature not exceeding 50° and protected from frost.

Labelling The label of aerosols states (1) the method of use, and, if necessary, that the container should be shaken before use; (2) if necessary, the precautions to be taken, for example, avoid inhaling, avoid contact with the eyes and other mucous membranes; (3) that the container should not be exposed to or stored at a temperature above 50° and should not be exposed to direct sunlight; (4) for a container with a metering dose valve, the amount of active ingredient in a unit spray; (5) that the container should not be punctured or incinerated.

AROMATIC WATERS

Aromatic waters are clear saturated solutions of volatile oils or other aromatic substances in water, usually employed for their flavouring rather than their medicinal properties. Aromatic waters prepared as described below contain a small amount of Ethanol.

Production Aromatic waters are prepared by (1) dilution of a concentrated, ethanolic solution of the aromatic substance with water; (2) solution of the aromatic substance, with or without the use of a dispersing agent; or (3) distillation of the aromatic substance.

Packaging and storage Aromatic waters should be kept in tightly closed containers, protected from intense light and excessive heat.

CAPSULES

Capsules are solid dosage forms with hard or soft shells. They are of various shapes and sizes, and contain a single dose of one or more active ingredients. They are intended for oral administration, but preparations for alternative applications may require a special formulation, method of manufacture, or form of presentation, appropriate to their particular use. For this reason they may not comply with certain sections of this monograph. Starch capsules (often known as cachets) are not described in this monograph.

The different categories of capsules that exist include hard, soft, and modified-release capsules. Their surfaces may bear symbols or other markings. They should be sufficiently robust to withstand handling, including packaging, storage, and transportation, without cracking or breaking. They should be packaged and stored in a manner that protects them from microbial contamination.

Capsule shells are made of gelatin or other substances, the consistency of which may be modified by the addition of substances such as glycerol and sorbitol. Preservatives may also be necessary. The shell should disintegrate in the presence of digestive fluids so that the contents are released. The contents of capsules may be solid, liquid or of a paste-like consistency. Capsule shells and contents may contain excipients such as diluents, sweeteners, colouring matters, flavouring substances, disintegrating agents, glidants, lubricants, and substances capable of modifying the behaviour of the active ingredient(s) in the gastro-intestinal tract. The contents should not cause deterioration of the shell.

When excipients are used, it is necessary to ensure that they do not adversely affect the stability, dissolution rate, bioavailability, safety, or efficacy of the active ingredient(s); there must be no incompatibility between any of the components of the dosage form.

Disintegration Capsules comply with the "Disintegration Test for Tablets and Capsules" (Appendix 4.23). For those capsules for which a dissolution requirement is included in the individual monograph, omission of the requirement for disintegration is considered justifiable and is therefore authorized.

Uniformity of dosage units Unless otherwise prescribed in the individual monographs, capsules comply with the "Uniformity of Dosage Units" (Appendix 4.28).

Visual inspection Unpack and inspect at least 20 capsules. They should be smooth and undamaged. Evidence of physical instability is demonstrated by gross changes in physical appearance, including hardening or softening, cracking, swelling, mottling, or discoloration of the shell.

Packaging and storage Capsules should be kept in well-closed containers at a temperature not exceeding 30° and protected from light, excessive moisture, or dryness.

Hard Capsules

Hard capsules have shells consisting of two prefabricated cylindrical sections that fit together. One end of each section is rounded and closed, and the other is open. The contents of hard capsules are usually in solid form (powder or granules); in certain cases the contents may be in the form of encapsulated powders or micropellets.

Production Hard capsules are prepared by mixing the active ingredient(s) with a number of excipients. Sometimes, the physical characteristics of the mixture allow it to be directly filled into the shell, but it may occasionally be necessary to granulate before filling. Normally the granulate needs to be mixed with lubricants and/or disintegrating agents.

A uniform mass of the capsule mixture is volumetrically fed into the narrower lower section of the shell body which is then closed by slipping the larger section or cap over it. The security of the closure may be ensured by suitable means.

Soft Capsules

Soft capsules have thicker shells than hard capsules, and preservatives are usually added. The shells are of one piece and various shapes. Partial migration of the contents into the shell may occur (and vice versa) depending on the nature of the materials used.

Production Soft capsules are prepared by mixing the active ingredient(s) with a number of excipients. Soft gelatin capsules are usually formed, filled, and sealed in one operation. However, shells for extemporaneous use are sometimes prefabricated. Liquids may be incorporated directly. Solids are usually dissolved or dispersed in a suitable excipient(s) to give a solution or dispersion of thick consistency.

Modified-Release Capsules

Modified-release capsules are hard or soft capsules in which the contents or the shell or both contain additives or are prepared by special procedures such as micro-encapsulation which, separately or together, are designed to modify the rate of release of the active ingredient(s) in the gastro-intestinal tract.

DELAYED-RELEASE CAPSULES (ENTERIC CAPSULES) Delayed-release capsules are hard or soft capsules prepared in such a manner that either the shell or the contents resist the action of the gastric fluid but release the active ingredient(s) in the presence of the intestinal fluid.

All requirements for these specialized dosage forms are given in the individual monographs.

EXTENDED-RELEASE CAPSULES Extended-release capsules are designed to slow the rate of release of the active ingredient(s) in the gastro-intestinal tract.

All requirements for these specialized dosage forms are given in the individual monographs.

Production Delayed-release capsules are prepared by providing hard or soft capsules with a gastro-resistant shell (enteric capsules) or by filling capsules with either granules or particles covered with a gastro-resistant coating.

See also under Hard Capsules or Soft Capsules.

EAR PREPARATIONS

Ear preparations are liquid, semi-solid or powder preparations usually containing one or more active ingredients in a suitable vehicle. They are intended for instillation, for spraying, for insufflation, for application to the auditory meatus or as an ear wash. Ear preparations may contain auxiliary substances, for example, to adjust tonicity or viscosity, to adjust or stabilize the pH, to increase the solubility of the active ingredients, to stabilize the preparation or to provide adequate antimicrobial properties. Such additives should not adversely affect the intended medicinal action of the preparation, nor, at the concentrations used, cause toxicity or undue local irritation.

Preparations for application to the injured ear, particularly where the ear-drum is perforated, or prior to surgery are sterile, free from antimicrobial preservatives and supplied in single-unit containers.

Unless otherwise justified and authorized, ear preparations supplied in multiple-unit containers contain a suitable antimicrobial preservative in appropriate concentration, except when the preparation itself has adequate antimicrobial properties. The antimicrobial preservatives should be compatible with the other ingredients of the preparation and should remain effective throughout the period of use of the ear preparations. Five categories of ear preparations may be distinguished: (1) ear drops; (2) ear sprays; (3) semi-solid ear preparations; (4) ear powders; (5) ear washes.

Minimum fill Ear preparations comply with the test described in the "Minimum Fill" (Appendix 4.26).

Sterility Where the ear preparations are labelled as sterile, unless otherwise directed in the individual monograph, they comply with the "Sterility Test" (Method I, Appendix 10.1).

Ear Drops

Ear drops are suspensions, emulsions or solutions of one or more active ingredients suspended, dispersed or dissolved in liquids such as water, glycols or fatty oils, suitable for application to the auditory meatus without exerting harmful pressure on the ear-drum. They may also be placed in the auditory meatus by means of a plug impregnated with the liquid. Suspended solids may separate slowly on standing but are easily redispersed on shaking. The size of the dispersed particles should be controlled.

Ear drops are usually supplied in multiple-unit containers fitted with an appropriate applicator.

Containers Ear drops are supplied in containers of glass or suitable plastic that are fitted with an integral dropper or with a screw cap of suitable materials incorporating a dropper and rubber or plastic teat. Alternatively, such a cap assembly is supplied separately.

Labelling The label of ear drops states (1) the name(s) and concentration(s) of the active ingredient(s); (2) the name(s) and concentration(s) of any antimicrobial preservative(s); (3) that they are intended for external use only; (4) where appropriate, that the preparation is sterile.

Ear Sprays

(**Note** When ear sprays are supplied in aerosol containers, these comply with the appropriate requirements for Aerosols.)

Ear sprays are suspensions, emulsions or solutions of one or more active ingredients suspended, dispersed or dissolved in liquids suitable for spraying to auditory meatus without exerting harmful pressure on the eardrum. The special requirements may be necessary for the selection of propellants, for particle size for the single-dose delivered by the metering valves.

See also under Ear Drops.

Packaging and storage Ear sprays are supplied in multiple-unit containers fitted with an appropriate applicator.

Labelling When ear sprays are supplied in aerosol containers, the label shall state (1) the method of use and, if necessary, that the container should be shaken before use; (2) if necessary, the precautions to be taken, for example, avoid inhaling; (3) that the container shall not be exposed to or stored at a temperature above 50° and should not be exposed to direct sunlight; (4) for a container with a metering dose valve, the amount of active ingredient in a unit-spray; (5) that the container should not be punctured or incinerated.

See also under Ear Drops.

Semi-Solid Ear Preparations

(**Note** Semi-solid ear preparations comply with the appropriate requirements for Topical Semi-solid Preparations.)

Semi-solid ear preparations are semi-solid dosage forms such as creams, gels, or ointments, etc. intended for application to the external auditory meatus, if necessary by means of a plug impregnated with the preparation. Semi-solid ear preparations are supplied in containers fitted with a suitable applicator.

Labelling See under Ear Drops.

Ear Powders

(**Note** Ear powders comply with the appropriate requirements for powders.)

Ear powders are fine powders intended for application or insufflation to the external auditory meatus.

Containers Ear powders are supplied in containers fitted with a suitable device for application or insufflation.

Labelling See under Ear Drops.

Ear Washes

Ear washes are solutions intended to cleanse the external auditory meatus. They are usually aqueous solutions with a pH within physiological limits.

See also under Ear Drops.

Containers Ear washes are supplied in containers fitted with a suitable applicator.

Labelling See under Ear Drops.

EXTRACTS

Extracts are preparations of liquid, solid or semi-solid consistency, obtained from herbal or animal matter, which is usually dried. Extracts may be subjected to purification processes that increase the content of characterized constituents with respect to the content of dry extractable matter from that which would be expected from extraction with the stated solvent: such extracts are termed "enriched".

Three types of extract can be distinguished:

Type A Type A extracts (standardized extracts) are adjusted to a defined range of therapeutically active constituents. Standardization is achieved by adjustment of the extract with inert material or by blending extracts.

Type B Type B extracts (quantified extracts) are adjusted to a defined range of active constituents. Adjustments are made either by blending batches of extracts or by blending batches of herbal or animal matter prior to extraction.

Type C Type C extracts are essentially defined by the production process (state of the matter to be extracted, solvent, extraction conditions). Constituents considered to be relevant markers may be determined.

Production Extracts are prepared by maceration, percolation or other suitable validated methods using ethanol or other suitable solvent. The matter to be extracted may undergo a preliminary treatment, for example, inactivation of enzymes, grinding or defatting. In addition, unwanted matter may be removed, if necessary, after extraction. Herbal drugs, animal matters and organic solvents used for the preparation of extracts comply with any relevant monograph of the Pharmacopoeia. For soft and dry extracts where the organic solvent is removed by evaporation, recovered or recycled solvent may be used, provided that the recovery procedures are controlled and monitored to ensure that solvents meet appropriate standards before reuse or admixture with other approved materials.

Water used for the preparation of extracts is of suitable quality. Except for the test for bacterial endotoxins, water complying with the section on Purified Water in bulk of the monograph on Purified Water is suitable. Potable water may be suitable if it complies with a defined specification that allows the consistent production of a suitable extract.

Where applicable, concentration to the intended consistency is carried out using suitable methods,

usually under reduced pressure, and at a temperature at which deterioration of the constituents is reduced to a minimum. Volatile oils that have been distilled during processing may be restored to the extracts at an appropriate stage in the manufacturing process. Suitable inert excipients may be added at the various stages of the manufacturing process to improve technological qualities like homogeneity, consistency or stability of active constituents. Where applicable, as a result of analysis of the herbal or animal matter used for the production of extracts, tests for microbiological quality, heavy metals, aflatoxins, and pesticide residues in the extracts have to be carried out.

Labelling For Type A, the label on the container states (1) the herbal or animal matter used; (2) whether the extract is dry, soft or liquid; (3) the composition of the extraction solvent; (4) where applicable, that fresh herbal or animal matter has been used; (5) where applicable, that the extract is "enriched"; (6) the name and amount of any excipient used. For Types B and C, the label on the container states (1) to (6) as for type A; (7) the content of constituents (markers) used for quantification and (8) the range of starting material: final extract (Drug:Extract Ratio or DER).

Liquid Extracts

Liquid extracts are liquid preparations of which, in general, one part by mass or volume is equivalent to one part by mass of the original dried herbal or animal matter. These preparations are adjusted, if necessary, so that they satisfy the requirements for content of solvent, and, where applicable, for constituents or dry residue.

Production Liquid extracts are prepared by using ethanol of suitable concentration or water to extract the stated herbal or animal matter or by dissolving a soft or dry extract (which has been produced using the same strength of extraction solvent as is used in preparing the liquid extract by direct extraction) of the stated herbal or animal matter in either ethanol of suitable concentration or water and filtering, if necessary. A slight sediment may form on standing, which is acceptable as long as the composition of the liquid extract is not changed significantly. Liquid extracts may contain suitable antimicrobial preservatives.

Relative density Where applicable, the liquid extract complies with the limits prescribed in the monograph.

Ethanol content For ethanolic liquid extracts, carry out the "Determination of Ethanol" (Appendix 6.5). The preparation complies with the limits prescribed in the monograph.

Methanol and 2-propanol Not more than 0.05 per cent v/v of methanol and not more than 0.05 per cent v/v of 2-propanol for ethanolic liquid extracts unless otherwise prescribed.

Dry residue In a flat-bottomed dish about 50 mm in diameter and about 30 mm in height, introduce rapidly 2.0 g or 2.0 ml of the extract to be examined. Evaporate to dryness on a water-bath and dry at 105° for 3 hours. Allow to cool in a desiccator over *phosphorus pentoxide desiccant* or *self-indicating silica gel* and weigh. Calculate the result as a percentage or in grams per litre.

Packaging and storage Liquid extracts should be kept in well-closed containers, protected from light.

Labelling The label on the container states in addition to the requirements listed above (1) where applicable, the ethanol content in per cent v/v in the final extract; (2) the concentration of any added antimicrobial preservative.

Soft Extracts

Soft extracts are semi-solid preparations obtained by evaporation of the solvent used for preparation. Soft extracts generally have a dry residue of not less than 70 per cent w/w. They may contain suitable antimicrobial preservatives.

Dry residue In a flat-bottomed dish about 50 mm in diameter and about 30 mm in height, weigh rapidly 2.0 g of the extract to be examined. Heat to dryness on a water-bath and dry at 105° for 3 hours. Allow to cool in a desiccator over *phosphorus pentoxide desiccant* or *self-indicating silica gel* and weigh. Calculate the result as a percentage weight in weight. Where applicable, a monograph on a soft extract prescribes a limit test for the solvent used for extraction.

Packaging and storage Soft extracts should be kept in well-closed containers, protected from light.

Labelling The label on the container states in addition to the requirements listed above (1) where applicable, the ethanol content in per cent v/v in the final extract; (2) the concentration of any added antimicrobial preservative.

Dry Extracts

Dry extracts are solid preparations obtained by evaporation of the solvent used for their production. Dry extracts generally have a dry residue of not less than 95 per cent w/w.

Loss on drying Where applicable, the dry extract complies with the limits prescribed in the monograph. In a flat-bottomed dish about 50 mm in diameter and about 30 mm in height, weigh rapidly 500 mg of the extract to be examined, finely powdered. Dry at 105° for 3 hours. Allow to cool in a desiccator over *phosphorus pentoxide desiccant* or *self-indicating silica gel* and weigh. Calculate the result as a percentage weight in weight. Where applicable, a monograph on a drug extract prescribes a limit test for the solvent used for extraction.

Packaging and storage Dry extracts should be kept in well-closed containers, protected from light.

EYE PREPARATIONS

Eye preparations are sterile liquid, semi-solid or solid preparations intended for administration upon the eyeball and/or to the conjunctiva, or for insertion in the conjunctival sac. Several categories of eye preparations may be distinguished: (1) eye drops; (2) eye lotions; (3) powders for eye drops and powders for eye lotions; (4) semi-solid eye preparations; (5) ocular systems.

Production During the development of an eye preparation, whose formulation contains an antimicrobial preservative, the necessity for and the efficacy of the chosen preservative shall be demonstrated to the satisfaction of the competent authority. A suitable test method together with criteria for judging the preservative properties of the formulation are provided in "Efficacy of Antimicrobial Preservation" (Appendix 10.6).

Eye preparations are prepared using materials and methods designed to ensure sterility and to avoid the introduction of contaminants and the growth of microorganisms; recommendations on this aspect are provided in "Sterilization and terility Assurance" (Appendix 12).

In the manufacture of eye preparations containing dispersed particles, measures are taken to ensure a suitable and controlled particle size with regard to the intended use.

For multiple-unit containers of eye preparations, the period after opening the container, after which the contents must not be used, does not exceed 4 weeks.

Sterility Eye preparations comply with the "Sterility Test" (Appendix 10.1). Applicators supplied separately also comply with the test for sterility. Remove the applicator with aseptic precautions from its package and transfer it to a tube of culture medium so that it is completely immersed. Incubate and interpret the results as described in the test for sterility.

Minimum fill Eye preparations except eye strips and ocular systems comply with the test described in the "Minimum Fill" (Appendix 4.26).

Packaging and storage Eye preparations should be kept in a sterile, tightly closed, tamper-evident container.

Labelling The label of eye preparations states the name(s) of any added antimicrobial preservative(s).

Eye Drops

Eye drops are sterile aqueous or oily solutions, emulsions or suspensions of one or more active substances intended for instillation into the eye. Eye drops may contain excipients, for example, to adjust the tonicity or the viscosity of the preparation, to adjust or stabilize the pH, to increase the solubility of the active substance, or to stabilize the preparation. These substances do not adversely affect the intended medicinal action or, at the concentrations used, cause undue local irritation.

Aqueous preparations supplied in multidose containers contain a suitable antimicrobial preservative in appropriate concentration except when the preparation itself has adequate antimicrobial properties. The antimicrobial preservative chosen must be compatible with the other ingredients of the preparation and must remain effective throughout the period of time during which eye drops are in use.

If eye drops are prescribed without antimicrobial preservatives, they are supplied wherever possible in single-dose containers. Eye drops intended for use in surgical procedures do not contain antimicrobial preservatives and are supplied in single-dose containers.

Eye drops that are solutions, examined under suitable conditions of visibility, are practically clear and practically free from particles.

Eye drops that are suspensions may show a sediment that is readily redispersed on shaking to give a suspension which remains sufficiently stable to enable the correct dose to be delivered.

Multidose preparations are supplied in containers that allow successive drops of the preparation to be administered. The containers contain at most 10 ml of the preparation.

Limit of particle size Unless otherwise specified in the individual monograph, eye drops in the form of a suspension comply with the following test. Introduce a suitable quantity of the suspension into a counting cell or with a micropipette onto a slide, as appropriate, and scan under a microscope an area corresponding to 10 µg of the solid phase. For practical reasons, it is recommended that the whole sample be first scanned at low magnification (e.g., ×50) and particles greater than 25 µm are identified. These larger particles can then be measured at a larger magnification (e.g., ×200 to ×500). For each 10 µg of solid active substance, not more than 20 particles have a maximum dimension greater than 25 µm, and not more than 2 of these particles have a maximum dimension greater than 50 µm. None of the particles has a maximum dimension greater than 90 µm.

Labelling The label of eye drops states the storage condition.

Eye Lotions

Eye lotions are sterile aqueous solutions intended for use in rinsing or bathing the eye or for impregnating eye dressings.

Eye lotions may contain excipients, for example to adjust the tonicity or the viscosity of the preparation or to adjust or stabilize the pH. These substances do not adversely affect the intended action or, at the concentrations used, cause undue local irritation.

Eye lotions supplied in multidose containers contain a suitable antimicrobial preservative in appropriate concentration except when the preparation itself has adequate antimicrobial properties. The antimicrobial preservative chosen is compatible with the other ingre-

dients of the preparation and remains effective throughout the period of time during which the eye lotions are in use.

If eye lotions are prescribed without an antimicrobial preservative, they are supplied in single-dose containers. Eye Lotions intended for use in surgical procedures or in first-aid treatment do not contain an antimicrobial preservative and are supplied in single-dose containers.

Eye lotions, examined under suitable conditions of visibility, are practically clear and practically free from particles.

The containers for multidose preparations do not contain more than 200 ml of eye lotion, unless otherwise specified in the individual monograph.

Labelling See under Eye Drops.

Powders for Eye Drops and Eye Lotions

Powders for the preparation of eye drops and eye lotions are supplied in a dry, sterile form to be dissolved or suspended in an appropriate liquid vehicle at the time of administration. They may contain excipients to facilitate dissolution or dispersion, to prevent caking, to adjust the tonicity, to adjust or stabilize the pH or to stabilize the preparation.

After dissolution or suspension in the prescribed liquid, they comply with the requirements for eye drops or eye lotions, as appropriate.

Uniformity of dosage units Single-unit powders for eye-drops and eye lotions comply with the "Uniformity of Dosage Units" (Appendix 4.28).

Semi-solid Eye Preparations

Semi-solid eye preparations are sterile ointments, creams or gels intended for application to the conjunctiva. They contain one or more active substances dissolved or dispersed in a suitable basis. They have a homogeneous appearance.

Semi-solid eye preparations comply with the requirements of the monograph on Topical Semi-solid Preparations. The basis is non-irritant to the conjunctiva.

Semi-solid eye preparations are packed in small, sterilized collapsible tubes fitted or provided with a sterilized cannula and having a content of not more than 10 g of the preparation. The tubes must be well-closed to prevent microbial contamination. Semi-solid eye preparations may also be packed in suitably designed single-dose containers. The containers, or the nozzles of tubes, are of such a shape as to facilitate administration without contamination.

Limit of particle size Semi-solid eye preparations containing dispersed solid particles comply with the following test: spread gently a quantity of the preparation corresponding to at least 10 µg of solid active substance as a thin layer. Scan under a microscope the whole area of the sample. For practical reasons, it is recommended that the whole sample is first scanned at a small magnification (e.g., ×50) and particles greater than 25 µm are identified. These larger particles can then be measured at a larger magnification (e.g., ×200 to ×500). For each 10 µg of solid active substance, not more than 20 particles have a maximum dimension greater than 25 µm, and not more than 2 of these particles have a maximum dimension greater than 50 µm. None of the particles has a maximum dimension greater than 90 µm.

Labelling See under Eye Drops.

Ocular Systems

See under Systems.

GRANULES

Granules are preparations consisting of solid, dry aggregates of powder particles sufficiently resistant to withstand handling. They are intended for oral administration. Some are swallowed as such, some are chewed and some are dissolved or dispersed in water or another suitable liquid before being administered. Granules contain one or more active ingredients with or without added substances including, where necessary, authorized colouring matter and flavouring agents. Granules are presented as single-unit or multiple-unit preparations. For single-unit preparations each dose is enclosed in an individual container, for example, a sachet, a paper packet or a vial. Each dose of a multiple-unit preparation is administered by means of a device suitable for measuring the quantity prescribed.

Several categories of granules may be distinguished: (1) uncoated granules; (2) granules for the preparation for oral liquids (see under Oral Liquids); (3) coated granules; (4) modified-release granules.

Uniformity of dosage units Unless otherwise prescribed in the individual monographs, granules comply with the "Uniformity of Dosage Units" (Appendix 4.28). The test for Content Uniformity is not required for multivitamin and trace element granules.

Packaging and storage Unless otherwise specified the individual monograph. Granules shall be kept in tightly closed containers.

Labelling For single-unit containers the label states the name(s) and amount(s) of active ingredient(s) per container and for multiple-unit containers the label states the name(s) and amount(s) of active ingredient(s) in a suitable quantity by weight.

Uncoated Granules

Uncoated granules may be plain or effervescent granules.

EFFERVESCENT GRANULES Effervescent granules are uncoated granules generally containing acid substances and either carbonates or bicarbonates which react rapidly in the presence of water to release carbon dioxide. They are intended to be dissolved or dispersed in water before administration.

Disintegration Place a single dose of the granules in a beaker containing 200 ml of *water* at 15° to 25°; numerous gas bubbles are evolved. When the evolution of gas around the individual grains has ceased, the granules have disintegrated, being either dissolved or dispersed in the water. Repeat the operation on a further five doses. The granules comply with the test if each of the six doses used in the test disintegrates within 5 minutes.

Coated Granules

Coated granules are granules covered with one or more layers of mixtures of various substances. Substances used as coatings are usually applied as a solution or suspension in conditions in which evaporation of the vehicle occurs. Coated granules are usually presented as multiple-unit preparations.

Modified-release Granules

DELAYED-RELEASE GRANULES (ENTERIC-COATED GRANULES) Delayed-release granules are intended to resist the gastric fluid and to release the active ingredient(s) in the intestinal fluid. These properties are achieved by covering the granules with a gastro-resistant material (enteric-coated granules) or by other suitable means.

EXTENDED-RELEASE GRANULES Extended-release granules are coated or uncoated granules prepared by using added substances or procedures which, separately or together, are designed to modify the rate or the place at which the active ingredient(s) are released.

HERBAL DRUG PREPARATIONS

Herbal drug preparations are obtained by subjecting herbal drugs to treatments such as extraction, distillation, expression, fractionation, purification, concentration or fermentation. These include comminuted or powdered herbal drugs, tinctures, extracts, essential oils, expressed juices and processed exudates.

Herbal teas comply with the monograph on Herbal teas.

Instant herbal teas consist of powder or granules of one or more herbal drug preparation(s) intended for the preparation of an oral solution immediately before use.

HERBAL TEAS

Herbal teas consist exclusively of one or more herbal drugs intended for oral aqueous preparations by means of decoction, infusion or maceration. The preparation is prepared immediately before use.

Herbal teas are usually supplied in bulk form or in sachets.

The herbal drugs used comply with the appropriate individual monographs.

Recommendations on the microbiological quality of herbal teas under the "Limits for Microbial Contamination" (Category 2 in Table 2, Appendix 10.5) taking into account the prescribed preparation method (use of boiling or non-boiling water).

Identification The identity of herbal drugs present in herbal teas is checked by botanical examinations.

The proportion of herbal drugs present in herbal teas is checked by appropriate methods. Herbal teas in sachets comply with the following test:

Weight variation Determine the average weight of twenty randomly chosen units as follows: weigh a single full sachet of herbal tea, open it without losing any fragments. Empty it completely using a brush. Weigh the empty sachet and calculate the mass of the contents by subtraction. Repeat the operation on the nineteen remaining sachets. Unless otherwise justified not more than two of the twenty individual masses of the contents deviate from the average mass of the contents by more than the percentage deviation shown in the table below and none deviates by more than twice that percentage.

Average Weight	Percentage Deviation
Less than 1.5 g	15 per cent
1.5 g to 2.0 g	10 per cent
More than 2.0 g	7.5 per cent

Packaging and storage Herbal teas should be protected from light.

INFUSIONS

Infusions are dilute solutions that contain the readily soluble constituents of crude drugs. Fresh infusions are made by pouring boiling water onto the drug, in a suitable state of comminution, and macerating for a short time, or they are usually prepared by diluting one volume of a concentrated infusion to ten volumes with water. Concentrated infusions are usually made by maceration of the drug with Ethanol (25 Per Cent).

For dispensing purposes, Infusions should be used within 12 hours of preparation from concentrated infusions.

Packaging and storage Infusions should be kept in well-closed containers.

Labelling The label on the container states the storage conditions.

IRRIGATION SOLUTIONS

Irrigation solutions are sterile solutions of one or more active ingredients intended for irrigation. If the solution is intended to be used for the irrigation of body cavities, for the flushing of wounds or operation cavities or for the irrigation of the urogenital system, it is sterile and apyrogenic. Such solutions are prepared using Water for Irrigation.

Irrigation solutions may contain added substances such as suitable substances to make the preparation isotonic with blood. They are supplied in containers holding sufficient of the solution for use on one occasion only. When viewed under suitable conditions of visibility, they are practically clear and practically free

from particles.

Sterility Unless otherwise directed in the individual monograph, irrigation solutions comply with the "Sterility Test" (Method I, Appendix 10.1).

Packaging and storage Irrigation solutions are supplied in containers made from materials that are sufficiently transparent to permit the visual inspection of the contents and that do not cause deterioration of the preparation as a result of diffusion into or across the material of the container or by yielding foreign substances into the preparation. The containers should be readily distinguishable from containers for preparations intended for parenteral administration. The containers are tamper-evident and are sealed so as to exclude micro-organisms. Unless otherwise specified in the individual monograph, Irrigation Solutions should be stored at a temperature not exceeding 25°.

Labelling The label of irrigation solutions states (1) that the irrigation solution is sterile and, where applicable, that the irrigation solution is apyrogenic; (2) that the irrigation solution is not to be used for injection; (3) that the irrigation solution should be used on one occasion only and that any remainder should be discarded.

LOZENGES

See under Oromucosal Preparations.

MEDICATED FOAMS

Medicated foams are preparations consisting of large volumes of gas dispersed in a liquid generally containing one or more active ingredients, a surfactant ensuring their formation and various other excipients. Medicated foams are usually intended for application to the skin or mucous membranes. Medicated foams are usually formed at the time of administration from a liquid preparation in a pressurized container. The container is equipped with a device consisting of a valve and a push button suitable for the delivery of the foam. Medicated foams intended for use on severely injured skin and on large open wounds are sterile. Medicated foams supplied in pressurized containers comply with the requirements in the monograph for "Aerosols" (Appendix 1.16).

Production Sterile medicated foams are prepared using materials and methods designed to ensure sterility and to avoid the introduction of contaminants and the growth of micro-organisms.

Relative foam density Maintain the container at about 25° for at least 24 hours. Taking care not to warm the container, fit a rigid tube 70 mm to 100 mm long and about 1 mm in internal diameter onto the push button. Shake the container to homogenize the liquid phase of the contents and dispense 5 ml to 10 ml of foam to waste. Tare a flat-bottomed dish of about 60 ml volume and about 35 mm high. Place the end of the rigid tube attached to the push button in the corner of the dish, press the push button and fill the dish uniformly, using a circular motion. After the foam has completely expanded, level off by removing the excess foam with a slide. Weigh. Determine the weight of the same volume of *water* by filling the same dish with *water*. The relative foam density is equivalent to the ratio:

$$\frac{m}{e},$$

where m is the weight of test sample of foam in g and e is the weight of same volume of *water* in g. Carry out three measurements. None of the individual values deviate by more than 20 per cent from the mean value.

Duration of expansion The apparatus (Fig. 1) consists of a 50-ml burette, 15 mm in internal diameter, with 0.1-ml graduations and fitted with a 4-mm single bore stopcock. The graduation corresponding to 30 ml is at least 210 mm from the axis of the stopcock. The lower part of the burette is connected by means of a plastic tube not longer than 50 mm and 4 mm in internal diameter to the foam-generating container equipped with a push button fitted to this connection. Maintain the container at about 25° for at least 24 hours. Shake the container, taken care not to warm it, to homogenize the liquid phase of the contents and dispense 5 ml to 10 ml of the foam to waste.

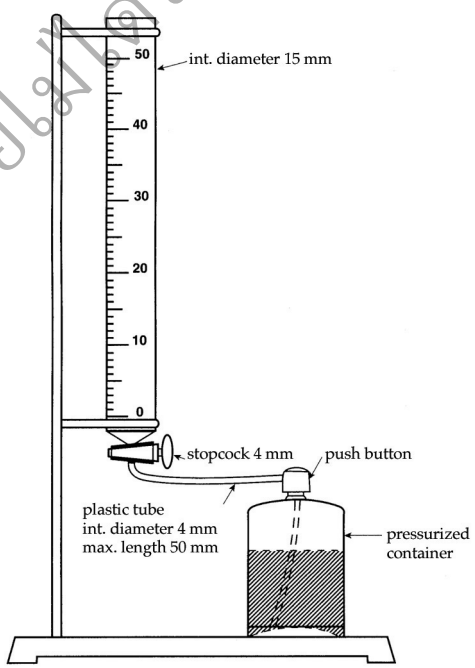

Fig. 1 The Apparatus for Duration of Expansion

Connect the push button to the outlet of the burette. Press the button and introduce about 30 ml of foam in a single delivery. Close the stopcock and at the same time start the chronometer and read the volume of foam in the burette. Every 10 seconds read the growing volume until the maximum volume is reached. Carry out three measurements. None of the times needed to obtain the maximum volume is more than 5 minutes.

Sterility Comply with the "Sterility Test" (Appendix 10.1), when the label indicates that the preparation is sterile.

Labelling The label on the container states (1) that the Medicated Foam is intended for external use only and (2) the storage conditions.

MEDICATED TAMPONS

Medicated tampons are solid, single-dose preparations intended to be inserted into the body cavities for a limited period of time. They consist of a suitable material such as cellulose, collagen or silicone impregnated with one or more active ingredients.

Production In manufacturing, packaging, storage and distribution of medicated tampons, suitable means are taken to ensure their microbial quality.

Microbial limit Comply with the "Limits for Microbial Contamination" (Appendix 10.5).

NASAL PREPARATIONS

Nasal preparations are liquid, semi-solid or solid preparations containing one or more active ingredients. They are intended for administration to the nasal cavities (nostrils) for local or systemic effects. Nasal preparations should as far as possible be non-irritating and should not adversely affect the functions of the nasal mucosa and its cilia. Aqueous Nasal preparations are usually isotonic. Nasal preparations are supplied in multiple-unit or single-unit containers provided, if necessary, with a suitable administration device.

Unless otherwise justified and authorized, aqueous nasal preparations supplied in multiple-unit containers contain a suitable antimicrobial preservative in appropriate concentration, except when the preparation itself has adequate antimicrobial properties.

Five categories of nasal preparations may be distinguished: (1) nasal drops; (2) liquid nasal sprays; (3) nasal powders; (4) semi-solid nasal preparations; (5) nasal washes.

Minimum fill Nasal preparations comply with the test described in the "Minimum Fill" (Appendix 4.26).

Labelling The label of nasal preparations states the instructions for use.

Nasal Drops

Nasal drops are solutions, emulsions or suspensions intended for instillation into the nostrils. Emulsions should not show evidence of phase separation; they have a uniform appearance after shaking. Suspensions may show a sediment which is readily redispersible on shaking to give a suspension which remains sufficiently stable to enable the correct dose to be delivered.

Production Single doses of nasal drops intended for systemic absorption of active ingredients through the nasal mucosa are tested for uniformity of volume, content or weight.

Containers Nasal drops are usually supplied in glass or plastic containers provided with a suitable applicator.

Uniformity of weight Nasal drops that are solutions comply with the following test: weigh individually the contents of ten containers emptied as completely as possible, and determine the average mass. Not more than two of the individual masses deviate by more than 10 per cent from the average mass and none deviates by more than 20 per cent.

Uniformity of content Nasal drops that are suspensions comply with the "Content Uniformity" (Appendix 4.28).

Liquid Nasal Sprays

(**Note** Where liquid nasal sprays are supplied in aerosol containers, these comply with the appropriate requirements for Aerosols.)

Liquid nasal sprays are solutions, emulsions or suspensions intended for spraying into the nostrils.

See also under Nasal Drops.

Production For liquid nasal sprays that are suspensions, the size of the dispersed particles of the spray should be such as to localize their deposition in the nostril.

Single doses of liquid nasal sprays intended for systemic absorption of active ingredients through the nasal mucosa are tested for uniformity of volume, content or weight.

Packaging and storage Liquid nasal sprays are supplied in glass or plastic containers with atomizing devices or in aerosol containers fitted with a suitable adapter and with or without a metering dose valve. They may also be administered by means of suitable inhalers. Aerosol preparations shall be stored at a temperature not exceeding 50° and protected from frost.

Uniformity of weight Metered dose nasal sprays that are solutions comply with the following test: discharge once to waste. Wait for not less than 5 seconds and discharge again to waste. Repeat this procedure for a further three actuations. Weigh the mass of the container, discharge once to waste and weigh the remaining mass of the container. Calculate the difference between the two masses. Repeat the procedure for a further nine containers. They comply with the test if not more than two of the individual values deviate by more than 25 per cent from the average value and none deviates by more than 35 per cent.

Uniformity of delivered dose Metered dose nasal sprays that are suspensions or emulsions comply with the following test: use an apparatus capable of quantitatively retaining the dose leaving the actuator of the atomising device.

Shake a container for 5 seconds and discharge once to waste. Wait for not less than 5 seconds, shake for 5 seconds and discharge again to waste. Repeat this procedure for a further three actuations. After 2 seconds, fire one dose of the metered dose nasal spray into the collecting vessel by actuating the atomizing device.

Collect the contents of the collecting vessel by successive rinses. Determine the content of active ingredient in the combined rinses.

Repeat the procedure for a further nine containers. Unless otherwise justified and authorized, the preparation complies with the test if not more than one of the individual contents is outside the limits of 75 per cent to 125 per cent and none is outside the limits of 65 per cent and 135 per cent of the average content. If two or three individual contents are outside the limits of 75 per cent to 125 per cent but within the limits of 65 per cent to 135 per cent, repeat the test for twenty more containers. The preparation complies with the test if not more than three individual contents of the thirty individual contents are outside the limits of 75 per cent to 125 per cent and none is outside the limits of 65 per cent to 135 per cent of the average content.

Nasal Powders

(**Note** Nasal powders comply with the appropriate requirements for Powders.)

Nasal powders are powders intended for insufflation into the nostrils by means of a suitable device.

Production The size of the particles should be such as to localize their deposition in the nostril. Particle size should be verified by adequate methods of particle-size determination.

Containers See under Nasal Drops.

Semi-solid Nasal Preparations

(**Note** Semi-solid nasal preparations comply with the appropriate requirements for Topical Semi-solid Preparations.)

Semi-solid nasal preparations are semi-solid dosage forms such as creams, gels, or ointments, etc. intended for application into the nostrils.

Containers The containers for semi-solid nasal preparations should be adapted to deliver the product to the site for application.

Nasal Washes

Nasal washes are generally aqueous solutions intended for irrigation of the nostrils. Nasal washes intended for application to injured parts or prior to a surgical operation are sterile.

Sterility Where the nasal washes are labelled as sterile, unless otherwise directed in the individual monograph, they comply with the "Sterility Test" (Method I, Appendix 10.1).

Containers See under Nasal Drops.

Labelling The label of nasal washes shall state, where applicable, a statement that the preparations are sterile.

ORAL LIQUIDS

Oral liquids usually consist of solutions, suspensions or emulsions of one or more active ingredients in a suitable vehicle; some oral liquids may consist of liquid active ingredients as such. They are intended to be swallowed either undiluted or after dilution. Oral liquids may contain suitable antimicrobial preservatives, antioxidants and other auxiliary substances such as dispersing, suspending, thickening, emulsifying, buffering, wetting, solubilizing, stabilizing, flavouring, sweetening agents, and authorized colouring agents. The vehicle for any particular oral liquids should be chosen having regard to the nature of the active ingredient(s) and providing organoleptic characteristics appropriate to the intended use of the preparation.

Oral liquids other than oral emulsions may be supplied as liquids or prepared just before issue for use by diluting concentrated liquid preparations or dissolving or dispersing granules or powder in the liquid stated on the label.

Suspensions may show a sediment that is readily dispersible on shaking. Emulsions may show evidence of phase separation but are easily reformed on shaking. The preparation remains sufficiently stable to enable a homogeneous dose to be withdrawn.

Several categories of oral liquids may be distinguished: (1) elixirs; (2) linctuses; (3) mixtures; (4) oral drops; (5) oral emulsions; (6) oral solutions; (7) oral suspensions; (8) preparations for oral liquids; (9) syrups.

Deliverable volume Oral liquids comply with the test described in the "Deliverable Volume" (Appendix 4.21).

Uniformity of dosage units Oral liquids that are suspensions or solids in single-unit containers comply with the "Uniformity of Dosage Units" (Appendix 4.28).

Packaging and storage Oral liquids should be kept in well-closed containers. They are supplied in multiple-unit or single-unit containers. They are administered either in volumes such as 5 ml, or multiples of 5 ml, or in small volumes (drops). Each dose of a multiple-unit preparation is administered by means of a device suitable for measuring the prescribed volume.

Oral liquids are supplied in containers that comply with the appropriate requirements given in "Containers" (Appendix 11).

Labelling The label of oral liquids states for Oral Emulsions, Oral Suspensions and, where appropriate, for Mixtures, that the bottle should be shaken before use.

Elixirs

Elixirs are clear, flavoured oral liquids containing one or more active ingredients dissolved in a vehicle that usually contains a high proportion of Sucrose or a suitable polyhydric alcohol or alcohols and may also contain Ethanol (95 Per Cent) or Dilute Ethanols.

Linctuses

Linctuses are viscous oral liquids that may contain one or more active ingredients in solution. The vehicle usually contains a high proportion of Sucrose, other

sugars or a suitable polyhydric alcohol or alcohols. Linctuses are intended for use in the treatment or relief of cough, and are sipped and swallowed slowly without the addition of water.

Mixtures

Mixtures are oral liquids containing one or more active ingredients dissolved, suspended or dispersed in a suitable vehicle. Suspended solids may separate slowly on standing but are easily redispersed on shaking.

Oral Drops

Oral drops are oral liquids that are intended to be administered in small volumes with the aid of a suitable measuring device (Appendix 1.15).

Oral Emulsions

Oral emulsions are oral liquids containing one or more active ingredients. They are stabilized oil-in-water dispersions, either or both phases of which may contain dissolved solids. Solids may also be suspended in oral emulsions.

Containers When issued for use, oral emulsions should be supplied in wide-mouthed bottles.

Oral Solutions

Oral solutions are oral liquids containing one or more active ingredients dissolved in a suitable vehicle.

Oral Suspensions

Oral suspensions are oral liquids containing one or more active ingredients suspended in a suitable vehicle. Suspended solids may slowly separate on standing but are easily redispersed.

Preparations for Oral Liquids

Preparations for oral liquids are solids or mixtures of solids intended for the preparations of solutions or suspensions by dissolving or dispersing them in a suitable vehicle. They may contain auxiliary substances in particular to facilitate dispersion or dissolution and to prevent caking. They are designated "for Oral Solution" or "for Oral Suspension" (e.g., Ampicillin for Oral Suspension).

Packaging and storage Preparations for oral liquids shall be kept in tightly closed containers.

Labelling The label of preparations for oral liquids states (1) the directions for preparing the oral liquids including the nature and quantity of liquid to be used; (2) the storage condition; (3) the conditions and the duration of storage after constitution.

Syrups

Syrups are concentrated aqueous solutions of sucrose, other sugars or sweetening agents, to which small quantities of suitable polyhydric alcohols may be added to retard crystallization or to increase the solubility of the other ingredients. Syrups usually contain aromatic or other flavouring materials and may also contain active ingredient(s). They should be recently prepared unless they contain suitable antimicrobial preservatives.

Packaging and storage Syrups should be kept in well-closed containers and stored at temperatures not exceeding 30°.

OROMUCOSAL PREPARATIONS

Oromucosal preparations are solid, semi-solid or liquid preparations, containing one or more active substances intended for administration to the oral cavity and/or the throat to obtain a local or systemic effect. Preparations intended for a local effect may be designed for application to a specific site within the oral cavity such as the gums (gingival preparations) or the throat (oropharyngeal preparations). Preparations intended for a systemic effect are designed to be absorbed primarily at one or more sites on the oral mucosa (e.g., sublingual preparations). Mucoadhesive preparations are intended to be retained in the oral cavity by adhesion to the mucosal epithelium and may modify systemic drug absorption at the site of application. For many oromucosal preparations, it is likely that some proportion of the active substance(s) will be swallowed and may be absorbed via the gastrointestinal tract.

Oromucosal preparations may contain suitable antimicrobial preservatives and other excipients such as dispersing, suspending, thickening, emulsifying, buffering, wetting, solubilizing, stabilizing, flavouring and sweetening agents. Solid preparations may in addition contain glidants, lubricants and excipients capable of modifying the release of the active substance(s).

Several categories of preparations for oromucosal use may be distinguished: (1) gargles; (2) mouthwashes; (3) gingival solutions; (4) oromucosal solutions and oromucosal suspensions; (5) semi-solid oromucosal preparations (including for example gingival gel, gingival paste, oromucosal gel, oromucosal paste); (6) oro-mucosal drops, oromucosal sprays and sublingual sprays (including oropharyngeal sprays); (7) lozenges and pastilles; (8) compressed lozenges; (9) sublingual tablets and buccal tablets; (10) oromucosal capsules; (11) mucoadhesive preparations.

Production During the development of an oromucosal preparation containing an antimicrobial preservative, the effectiveness of the chosen preservative shall be demonstrated to the satisfaction of the competent authority. A suitable test method together with the criteria for judging the preservative properties of the formulation are provided under "Efficacy of Antimicrobial Preservation" (Appendix 10.6).

In the manufacture, packaging, storage and distribution of oromucosal preparations, suitable means are taken to ensure their microbiological quality; recommendations on this aspect are provided under "Limit for Microbial Contamination" (Appendix 10.5).

In the manufacture of semi-solid and liquid oromucosal preparations containing dispersed particles,

measures are taken to ensure a suitable and controlled particle size with regard to the intended use.

Uniformity of dosage units Single-unit oromucosal preparations comply with the "Uniformity of dosage units" (Appendix 4.28).

Gargles

Gargles are aqueous solutions intended for gargling to obtain a local effect. They are not to be swallowed. They are supplied as ready-to-use solutions or concentrated solutions to be diluted. They may also be prepared from powders or tablets to be dissolved in water before use. Gargles may contain excipients to adjust the pH which, as far as possible, is neutral.

Mouthwashes

See under Topical Preparations.

Gingival Solutions

Gingival solutions are intended for administration to the gingivae by means of a suitable applicator.

Oromucosal Solutions and Oromucosal Suspensions

Oromucosal solutions and oromucosal suspensions are liquid preparations intended for administration to the oral cavity by means of a suitable applicator.

Oromucosal suspensions may show a sediment which is readily dispersible on shaking to give a suspension which remains sufficiently stable to enable the correct dose to be delivered.

Semi-solid Oromucosal Preparations

Semi-solid oromucosal preparations are hydrophilic gels or pastes intended for administration to the oral cavity or to a specific part of the oral cavity such as the gingivae (gingival gel, gingival paste). They may be provided as single-dose preparations.

Semi-solid oromucosal preparations comply with the requirements of the monograph for "Topical semi-solid Preparations" (Appendix 1.16).

Oromucosal Drops, Oromucosal Sprays and Sublingual Sprays

Oromucosal drops, oromucosal sprays and sublingual sprays are solutions, emulsions or suspensions intended for local or systemic effect. They are applied by instillation or spraying into the oral cavity or onto a specific part of the oral cavity such as spraying under the tongue (sublingual spray) or into the throat (oropharyngeal spray).

Emulsions may show evidence of phase separation but are readily redispersed on shaking. Suspensions may show a sediment which is readily dispersed on shaking to give a suspension which remains sufficiently stable to enable the correct dose to be delivered.

Liquid oromucosal sprays are supplied in containers with atomizing devices or in pressurized containers having a suitable adaptor, with or without a metering dose valve, which comply with the requirements of the monograph for "Aerosols" (Appendix 1.6).

The size of the droplets of the spray is such as to localize their deposition in the oral cavity or the throat as intended.

Unless otherwise prescribed or justified and authorized, oromucosal drops supplied in single-dose containers, single doses of metered-dose oromucosal sprays and sublingual sprays, all intended for systemic action, comply with the following requirement.

Oromucosal Drops in Single-dose Containers

Uniformity of dosage units Oromucosal drops in single-unit containers comply with the "Uniformity of Dosage Units" (Appendix 4.28).

Metered-dose Oromucosal Sprays and Sublingual Sprays

Uniformity of dosage units Metered-dose oromucosal sprays and sublingual sprays comply with the "Uniformity of Dosage Units" (Appendix 4.28) or, where justified and authorized, with the test for uniformity of weight or the test for uniformity of delivered dose shown below.

IN THE CASE OF METERED-DOSE OROMUCOSAL SPRAYS AND SUBLINGUAL SPRAYS THAT ARE SOLUTIONS, PROCEED AS FOLLOWS Discharge once to waste. Wait for a minimum of 5 seconds, shake for 5 seconds and discharge again to waste. Repeat this procedure for a further 3 actuations. Weigh the container, discharge once to waste and weigh the container again. Calculate the difference between the 2 weights. Repeat the procedure for a further 9 containers. Determine the weight variation (Appendix 4.28).

IN THE CASE OF METERED-DOSE OROMUCOSAL SPRAYS AND SUBLINGUAL SPRAYS THAT ARE SUSPENSIONS OR EMULSIONS PROCEED AS FOLLOWS Use an apparatus capable of quantitatively retaining the dose leaving the actuator of the atomizing device. Shake the container for 5 seconds and discharge once to waste. Wait for a minimum of 5 seconds, shake for 5 seconds and discharge again to waste. Repeat this procedure for a further 3 actuations. After 2 seconds, fire 1 dose of the metered-dose spray into the collecting vessel by actuating the atomising device. Collect the contents of the collecting vessel by successive rinses. Determine the content of active substance in the combined rinses. Repeat the procedure for a further 9 containers. Determine the content uniformity (Appendix 4.28).

Weight variation Metered-dose oromucosal sprays and sublingual sprays that are solutions comply with the following test. Discharge once to waste. Wait for a minimum of 5 seconds, shake for 5 seconds and discharge again to waste. Repeat this procedure for a further 3 actuations. Weigh the container, discharge once to waste and weigh the container again. Calculate the difference between the 2 weights. Repeat the procedure for a further 9 containers.

The preparation complies with the test if maximum 2 of the individual values deviate by more than 25 per cent from the average value and none deviates by more than 35 per cent.

Uniformity of delivered dose Metered-dose oromucosal sprays and sublingual sprays that are suspensions or emulsions comply with the following test. Use an apparatus capable of quantitatively retaining the dose leaving the actuator of the atomizing device. Shake the container for 5 seconds and discharge once to waste. Wait for a minimum of 5 seconds, shake for 5 seconds and discharge again to waste. Repeat this procedure for a further 3 actuations. After 2 seconds, fire 1 dose of the metered-dose spray into the collecting vessel by actuating the atomizing device. Collect the contents of the collecting vessel by successive rinses. Determine the content of active substance in the combined rinses. Repeat the procedure for a further 9 containers.

Unless otherwise justified and authorized, the preparation complies with the test if maximum 1 of the individual contents is outside the limits of 75 per cent to 125 per cent and none is outside the limits of 65 per cent to 135 per cent of the average content.

If 2 or maximum 3 individual contents are outside the limits of 75 per cent to 125 per cent but within the limits of 65 per cent to 135 per cent, repeat the test for 20 more containers. The preparation complies with the test if maximum 3 individual contents of the 30 individual contents are outside the limits of 75 per cent to 125 per cent and none is outside the limits of 65 per cent to 135 per cent of the average content.

Lozenges and Pastilles

Lozenges and pastilles are solid, single-dose preparations intended to be sucked to obtain, usually, a local effect in the oral cavity and the throat. They contain one or more active substances, usually in a flavoured and sweetened base, and are intended to dissolve or disintegrate slowly in the mouth when sucked. Lozenges are hard preparations prepared by moulding. Pastilles are soft, flexible preparations prepared by moulding of mixtures containing natural or synthetic polymers or gums and sweeteners.

Compressed Lozenges

Compressed lozenges are solid, single-dose preparations intended to be sucked to obtain a local or systemic effect. They are prepared by compression and are often rhomboid in shape. Compressed lozenges conform with the general definition of tablets.

Production In the manufacture of compressed lozenges, measures are taken to ensure that they possess suitable mechanical strength to resist handling without crumbling or breaking. This may be demonstrated by examining the "Friability of Uncoated Tablet" (Appendix 4.30) and the "Resistance to Crushing of Tablets" (Appendix 4.31).

Dissolution For compressed lozenges intended for a systemic effect, a suitable test is carried out to demonstrate the appropriate release of the active substance(s).

Sublingual Tablets and Buccal Tablets

Sublingual tablets and buccal tablets are solid, single-dose preparations to be applied under the tongue or to the buccal cavity, respectively, to obtain a systemic effect. They are prepared by compression of mixtures of powders or granulations into tablets with a shape suited for the intended use. Sublingual tablets and buccal tablets conform to the general definition of tablets.

Production In the manufacture of sublingual tablets and buccal tablets, measures are taken to ensure that they possess suitable mechanical strength to resist handling without crumbling or breaking. This may be demonstrated by examining the "Friability of Uncoated Tablet" (Appendix 4.30) and the "Resistance to Crushing of Tablets" (Appendix 4.31).

Dissolution Unless otherwise justified and authorized, a suitable test is carried out to demonstrate the appropriate release of the active substance(s).

Oromucosal Capsules

Oromucosal capsules are soft capsules to be chewed or sucked.

Mucoadhesive Preparations

Mucoadhesive preparations contain one or more active substances intended for systemic absorption through the buccal mucosa over a prolonged period of time. They may be supplied as mucoadhesive buccal tablets or as other mucoadhesive solid or semi-solid preparations.

Mucoadhesive buccal tablets are prepared by compression of mono- or multi-layered tablets. They usually contain hydrophilic polymers, which on wetting with the saliva produce a flexible hydrogel that adheres to the buccal mucosa.

Production In the manufacture of mucoadhesive buccal tablets, measures are taken to ensure that they possess suitable mechanical strength to resist handling without crumbling or breaking. This may be demonstrated by examining the "Friability of Uncoated Tablet" (Appendix 4.30) and the "Resistance to Crushing of Tablets" (Appendix 4.31).

Dissolution Unless otherwise justified and authorized, a suitable test is carried out to demonstrate the appropriate release of the active substance(s).

PARENTERAL PREPARATIONS

Parenteral preparations are sterile preparations intended for administration by injection, infusion or implantation into the body. There are five main forms of these preparations defined as follows: (1) medicaments or solutions or emulsions thereof suitable for injection, bearing titles of the form, ____ Injection; (2) dry solids or liquid concentrates containing active ingredient(s) with or without buffer(s), diluent(s) or other added substances, and which, upon the addition

of suitable solvents, yield solutions conforming in all respects to the requirements for Injections, and which are distinguished by titles of the form, ____ for Injection; (3) solids which are suspended in a suitable fluid medium and which are not to be injected intravenously or into the spinal canal, distinguished by titles of the form, Sterile ____ Suspension; (4) dry solids which, upon the addition of suitable vehicles, yield preparations conforming in all respects to the requirements for sterile suspensions; and which are distinguished by titles of the form, Sterile ____ for Suspension; and (5) sterile solid preparations, of which size and shape are suitable for implantation into body tissues, distinguished by titles of the form, ____ Implant. The term "Injections" may be used for preparations (1) to (4).

Where used in this Pharmacopoeia, the designation *Large-volume intravenous solution* applies to a single-dose injection that is intended for intravenous use and is packaged in containers labelled as containing more than 100 ml. The designation *Small-volume Injection* applies to an injection that is packaged in containers labelled as containing 100 ml or less.

Production Parenteral preparations are prepared by methods designed to ensure their sterility and to avoid the introduction of contaminants, the presence of pyrogens and the growth of micro-organisms.

Water used in the manufacture of injections complies with the requirements for Water for Injections in bulk.

Aqueous vehicles The vehicles for aqueous parenteral preparations comply with the "Pyrogen Test" (Appendix 8.2) or the "Test for Bacterial Endotoxins" (Appendix 8.5), whichever is specified. Water for Injection generally is used as the vehicle, unless otherwise specified in the individual monograph. Sodium Chloride may be added in amounts sufficient to render the resulting solution isotonic; Sodium Chloride Injection, or Ringer's Injection, may be used in whole or in part instead of Water for Injection unless otherwise specified in the individual monograph. For conditions applying to other adjuvants, see Added substances, in this appendix.

Other vehicles Fixed oils used as vehicles for nonaqueous injections are of vegetable origin, are odourless or nearly so, and have no odour or taste suggesting rancidity. They comply with the test for solid paraffin under Liquid Paraffin, the cooling bath being maintained at 10°, have a saponification value of between 185 and 200 (Appendix 5.7), have an iodine value of between 79 and 128 (Iodine Bromide Method, Appendix 5.6), and comply with the requirements of the following tests.

UNSAPONIFIABLE MATTER Reflux on a water-bath 10 ml of the oil with 15 ml of a 16.7 per cent w/v solution of *sodium hydroxide* and 30 ml of *ethanol*, with occasional shaking until the mixture becomes clear. Transfer the solution to a shallow dish, evaporate the ethanol on a water-bath, and mix the residue with 100 ml of *water*: a clear solution results.

ACID VALUE Not more than 0.22 (Appendix 5.4); using 0.020 M *potassium hydroxide* as titrant.

Synthetic mono- or diglycerides of fatty acids may be used as vehicles, provided they are liquid and remain clear when cooled to 10° and have an iodine value of not more than 140 (Iodine Bromide Method, Appendix 5.6).

These and other nonaqueous vehicles may be used, provided they are safe in the volume of parenteral preparations administered, and also provided they do not interfere with the therapeutic efficacy of the preparation or with its response to prescribed assays and tests.

Added substances Suitable substances may be added to parenteral preparations to increase stability or usefulness, unless prescribed in the individual monograph, provided they are harmless in the amounts administered and do not interfere with the therapeutic efficacy or with the responses to the specified assays and tests. No colouring agent may be added, solely for the purpose of colouring the finished preparation, to a solution intended for parenteral administration [see also Added Substances, under General Notices, p. 6, and "Efficacy of Antimicrobial Preservation" (Appendix 10.6)].

Observe special care in the choice and use of added substances in injections that are administered in a volume exceeding 5 ml. The following maximum limits prevail unless otherwise directed: for agents containing mercury and the cationic, surface-active compounds, 0.01 per cent; for those of the types of chlorobutanol, cresol, and phenol, 0.5 per cent; and for sulfur dioxide, or an equivalent amount of the sulfite, bisulfite, or metabisulfite of potassium or sodium, 0.2 per cent.

A suitable substance or mixture of substances to prevent the growth of micro-organisms must be added to preparations intended for injection that are packaged in multiple-dose containers, regardless of the method of sterilization employed, unless otherwise specified in the individual monograph, or unless the active ingredients are themselves antimicrobial. Such substances are used in concentrations that will prevent the growth of or kill micro-organisms in the preparations. Such substances also comply with the "Efficacy of Antimicrobial Preservation" (Appendix 10.6) and "Content of Antimicrobial Agents" (Appendix 6.22). Sterilization processes are employed even though such substances are used [see also Added Substances, under General Notices, p. 6, and "Sterilization and Sterility Assurance" (Appendix 12)]. The air in the container may be evacuated or be displaced by a chemically inert gas. If the injection is oxygen-sensitive, that information must appear in the labelling.

Containers for parenteral preparations Containers, including the closures, for parenteral preparations do not interact physically or chemically with the prepara-

tions in any manner to alter the strength, quality, or purity beyond the official requirements under the ordinary or customary conditions of handling shipment, storage, sale, and use. The container is made of material that permits inspection of the contents. Parenteral preparations are supplied in glass ampoules, bottles or vials or in other containers such as plastic bottles or bags and in prefilled syringes the integrity of which is ensured by suitable means. The type of glass preferable for each parenteral preparation is usually stated in the individual monograph.

For definitions of single-dose and multiple-dose containers, see Containers under General Notices, p. 10. Containers comply with the "Containers" (Appendix 11). Containers are closed by fusion, or by application of suitable closures, in such manner as to prevent contamination or loss of contents. Closures for multiple-dose containers permit the withdrawal of the contents without removal or destruction of the closure. The closure permits penetration by a needle, and, upon withdrawal of the needle, at once recloses the container against contamination.

Containers for sterile solids Containers, including the closures, for dry solids intended for parenteral use do not interact physically or chemically with the preparation in any manner to alter the strength, quality, or purity beyond the official requirements under the ordinary or customary conditions of handling, shipment, storage, sale and use.

A container for a sterile solid permits the addition of a suitable solvent and withdrawal of portions of the resulting solution or suspension in such manner that the sterility of the product is maintained.

Where the Assay in a monograph provides a procedure for Assay preparation in which the total withdraw able contents are to be withdrawn from a single-dose container with a hypodermic needle and syringe, the contents are to be withdrawn as completely as possible into a dry hypodermic syringe of a rated capacity not exceeding three times the volume to be withdrawn and fitted with a 21-gauge needle not less than 2.5 cm in length, care being taken to expel any air bubbles, and discharged into a container for dilution and assay.

Volume in containers Each container of an injection is filled with a volume in slight excess of the labelled size or the volume which is to be withdrawn. The excess volumes recommended in the accompanying table are usually sufficient to permit withdrawal and administration of the labelled volumes.

DETERMINATION OF VOLUME OF INJECTIONS IN CONTAINERS Select one or more containers if the volume is 10 ml or more, three or more if the volume is more than 3 ml and less than 10 ml, or five or more if the volume is 3 ml or less. Take up individually the contents of each container selected into a dry hypodermic syringe of a rated capacity not exceeding three times the volume to be measured, and fitted with a 21-gauge needle not less than 2 cm (1 inch) in length. Expel any air bubbles from the syringe and needle, and then discharge the contents of the syringe, without emptying the needle, into a standardized, dry cylinder (graduated to contain rather than to deliver the designated volumes) of such size that the volume to be measured occupies at least 40 per cent of its rated volume. Alternatively, the contents of the syringe may be discharged into a dry, tared beaker, the volume, in ml, being calculated as the weight, in g, of injections taken divided by its density. The contents of two or three 1-ml or 2-ml containers may be pooled for the measurement, provided that a separate, dry syringe assembly is used for each container. The content of containers holding 10 ml or more may be determined by means of opening them and emptying the contents directly into the graduated cylinder or tared beaker.

The volume is not less than the labelled volume in the case of containers examined individually or, in the case of 1-ml and 2-ml containers, is not less than the sum of the labelled volumes of the containers taken collectively.

For injections in multiple-dose containers labelled to yield a specific number of doses of a stated volume, proceed as directed in the foregoing, using the same number of separate syringes as the number of doses specified. The volume is such that each syringe delivers not less than the stated dose.

For injections containing oil, warm the containers, if necessary, and thoroughly shake them immediately before removing the contents. Cool to 25° before measuring the volume.

Constituted solutions Sterile dosage forms from which constituted solutions are prepared for injection bearing titles of the form, ____ for Injection comply with the "Constituted Solutions" (Appendix 4.20).

Particulate matter All large-volume injections for single-dose infusion, and those small-volume injections for which the monographs specify such requirements, are subject to the particulate matter limits set forth in the "Particulate Matter in Injections" (Appendix 4.27). An article packaged as both a large-volume and a small-volume injection meets the requirements set forth for "Small-volume Injections" where the container is labelled as containing 100 ml or less if the individual monograph includes a test for "Particulate matter"; it meets the requirements set forth for "Large-volume Injections for Single-dose Infusion" where the container is labelled as containing more than 100 ml. Injections packaged and labelled for use as irrigating solutions are exempt from requirements for "Particulate matter".

Pyrogen test; Bacterial endotoxins test Parenteral preparations comply with the "Pyrogen Test" (Appendix 8.2) or the "Test for Bacterial Endotoxins" (Appendix 8.5) as specified in the individual monograph.

Sterility Parenteral preparations comply with the "Sterility Test" (Appendix 10.1). Unless otherwise specified in the individual monograph, carry out the test using Method I.

Uniformity of dosage units Parenteral preparations that are packaged in single-unit containers comply with the "Uniformity of Dosage Units" (Appendix 4.28). The test for Content Uniformity is required for sterile solids containing a unit weight equal to or less than 40 mg but not required for multivitamin and trace element parenteral preparations.

Packaging and storage The volume of the parenteral preparations in single-dose containers provides the amount specified for parenteral administration at one time and in no case is more than sufficient to permit the withdrawal and administration of 1 litre.

Preparations intended for intraspinal, intracisternal, or peridural administration are packaged only in single-dose containers.

Unless otherwise specified in the individual monograph, no multiple-dose container contains a volume of parenteral preparation more than sufficient to permit the withdrawal of 30 ml.

Injections packaged for use as irrigation solutions or for hemofiltration or dialysis or for parenteral nutrition are exempt from the 1-litre restriction of the foregoing requirements relating to packaging. Containers for injections packaged for use as irrigation solutions or for hemofiltration may be designed to empty rapidly and may contain a volume of more than 1 litre.

Labelling The label of injection complies with the Labelling, under General Notices, p. 12. The label also states the name of any added substances and the storage conditions.

The container is so labelled that a sufficient area of the container remains uncovered for its full length or circumference to permit inspection of the contents. In case the area of the container is not sufficient to be labelled as mentioned above, unless otherwise specified in the individual monograph, the label shall state (1) the name of the drug product and (2) the batch or lot number assigned by the manufacturer.

The label of a single-dose parenteral preparation states that any portion of the contents remaining should be discarded.

In the case of a dry preparation or other preparation to which a diluent is intended to be added before use, the label includes the following information: the amount of each ingredient, the composition of recommended diluent(s) [the name(s) alone, if the formula is specified in the individual monograph], the amount to be used to attain a specific concentration of active ingredient and the final volume of solution so obtained, a brief description of the physical appearance of the constituted solution, directions for proper storage of the constituted solution, and an expiration date limiting the period during which the constituted solution may be expected to have the required or labelled potency if it has been stored as directed.

POWDERS

Powders are preparations consisting of solid, loose, dry particles of varying degrees of fineness that contain one or more active ingredients with or without added substances including, where necessary, flavouring agents and authorized colouring matter. Two categories of powders may be distinguished: (1) oral powders; (2) topical powders.

Minimum fill Powders comply with the test described in the "Minimum Fill" (Appendix 4.26).

Uniformity of dosage units Powders comply with the "Uniformity of Dosage Units" (Appendix 4.28). The test for Content Uniformity is not required for multivitamin and trace element powders.

Packaging and storage Powders should be kept in tightly closed containers.

Oral Powders

Oral powders are generally administered in or with water or another suitable liquid. They may also be swallowed directly.

Oral powders are presented as single-unit or multiple-unit preparations. For single-unit powders each dose is enclosed in a separate container, for example, a sachet, a paper packet or a vial. Multiple-unit powders require the provision of a measuring device capable of delivering the quantity prescribed.

Effervescent oral powders are presented as single-unit or multiple-unit powders and generally contain acid substances and either carbonates or bicarbonates that react rapidly in the presence of water to release carbon dioxide. They are intended to be dissolved or dispersed in water before administration.

Labelling For single-unit containers the label states the name(s) and quantity(ies) of active ingredient(s) per container. For multiple-unit containers the label states the name(s) and quantity(ies) of active ingredient(s) in a suitable amount by weight.

Topical Powders

Topical powders are free from grittiness. They are presented as single-unit or multiple-unit preparations. If a topical powder is specifically intended for use on large open wounds or on severely injured skin, it must be sterile.

Multiple-unit topical powders should preferably be dispensed in sifter-top containers or in aerosol containers.

Dusting powders are topical powders consisting of finely divided powders that are intended to be applied to the skin for therapeutic, prophylactic or lubricant purposes. In general, they should be passed through at least a *No. 150 sieve* to assure freedom from grit that could irritate traumatized areas.

Labelling The label of topical powders states, where applicable, that the topical powder is sterile.

PREPARATIONS FOR INHALATION

Preparations for inhalation are solid or liquid preparations that contain one or more active ingredients. They are intended for administration to the lower

respiratory tract for local or systemic effect. Preparations for inhalation should not adversely affect the functions of the mucosa of the respiratory tract and its cilia.

Preparations for inhalation are supplied in multiple-unit or single-unit containers provided with a suitable administration device, if necessary.

Four categories of preparations for inhalation may be distinguished: (1) inhalations; (2) powders for inhalation; (3) inhalation aerosols; (4) inhalants.

Preparations for inhalation that are converted into an aerosol are generally administered by nebulizers, by pressurized metered-dose inhalers or by dry-powder inhalers.

Production During the development of a preparation for inhalation which contains an antimicrobial preservative, the effectiveness of the chosen preservative shall be demonstrated to the satisfaction of the competent authority. A suitable method of test together with the criteria for judging the preservative properties of the formulation are described in the "Efficacy of Antimicrobial Preservation" (Appendix 10.6). The size of particles to be inhaled should be adjusted so as to localize their deposition in the lower respiratory tract and verified by suitable methods of particle-size determination.

For preparations for inhalation that are supplied in single-unit containers provided with a suitable administration device, the single doses released by the administration device are tested for uniformity of volume, weight or content. The content of a unit spray of suspensions for inhalation dispensed in multiple-unit aerosol containers is verified using a suitable number of unit sprays for quantification.

Minimum fill Preparations for inhalation comply with the test described in the "Minimum Fill" (Appendix 4.26).

Packaging and storage Preparations for inhalation should be kept in well-closed containers. Aerosol preparations shall be stored at a temperature not exceeding 50° and should be protected from frost.

Labelling The label of preparations for inhalation states the name(s) and quantity(ies) of any antimicrobial preservative(s).

Inhalations

Inhalations are solutions or suspensions or emulsions of one or more active ingredients administered by the nasal or oral respiratory route for local or systemic effect.

As far as possible, liquids not dispensed in aerosol containers are aqueous, isotonic liquids. The pH of the liquid should be not lower than 3 and not higher than 8.5. Co-solvents or suitable solubilizers may be used to increase the solubility of the active ingredients. Aqueous inhalations supplied in multiple-unit containers should contain a suitable antimicrobial preservative at a suitable concentration except where the preparation itself has adequate antimicrobial properties.

Suspensions are readily dispersible on shaking and the suspension remains sufficiently stable to enable the correct dose to be delivered by the device. Suitable suspension stabilizers may be added.

Inhalations are usually supplied in glass or plastic containers. If inhalations are provided in concentrated form, they should be diluted in the nebulizers to the prescribed volume with the prescribed liquid before use.

Powders for Inhalation

Powders for inhalation are powders containing medicinal substances usually diluted with suitable diluents. These powders are normally dispensed in hard gelatin capsules. They may also be administered by mechanical devices that require manually produced pressure or a deep inhalation by the patient, e.g. *Cromoglycate Sodium for Inhalation*.

Uniformity of dosage units Powders for inhalation comply with the "Uniformity of Dosage Units" (Appendix 4.28).

Labelling The label on the container states that the capsules are intended for use in an inhaler and are not to be swallowed.

Inhalation Aerosols

(**Note** Inhalation aerosols comply with the appropriate requirements for aerosols.)

Inhalation aerosols are metered-dose preparations. They are drugs or solutions or suspensions or emulsions intended to be inhaled in controlled amounts and are delivered by the actuation of an appropriate metering valve. They contain multiple doses, often exceeding several hundreds. The most common single-dose volumes delivered are from 25 to 100 µl (also expressed as mg) per actuation.

Production The formulation of inhalation aerosols and the components of the delivery device (i.e., the aerosol container with its integral metering valve and the actuator) should be designed and, where appropriate, the particle size of the active ingredients should be controlled so that, when the inhalation aerosols are used in accordance with the manufacturer's recommendations, an adequate proportion of the active ingredients is made available for inhalation. A proportion of the active ingredient is deposited on the inner surface of the actuator; the amount available for inhalation is therefore less than the amount released by actuation of the valve.

Inhalation aerosols should be manufactured in conditions designed to minimize microbial and particulate contaminations.

Packaging and storage Inhalation aerosols are supplied in suitable containers fitted with an appropriate metering valve that forms an integral part of the container. The containers are usually supplied with an appropriate actuator. Inhalation aerosols should be protected from extremes of temperature and from undue fluctuations in temperature.

Labelling The label of inhalation aerosols states (1) the quantity(ies) of active ingredient(s) delivered by each actuation of the valve; (2) the instructions for using the inhalation aerosols; (3) any special precautions associated with the use of the inhalation aerosols.

Inhalants

Inhalants are drugs or combination of drugs which may contain an inert, suspended, diffusing agent. They are intended to release volatile constituents for inhalation either when placed on a pad or when added to hot, but not boiling, water.

Labelling The label on the container states that the inhalants are not to be taken by mouth.

RECTAL PREPARATIONS

Rectal preparations are intended for rectal use in order to obtain a systemic or local effect, or they may be intended for diagnostic purposes.

Several categories of rectal preparations may be distinguished: (1) rectal suppositories; (2) rectal capsules; (3) rectal solutions and suspensions (enemas); (4) powders and tablets for rectal solutions and suspensions; (5) rectal creams; (6) rectal gels; (7) rectal ointments; (8) rectal foams; and (9) rectal tampons.

Production During the development of a rectal preparation, the formulation for which contains an antimicrobial preservative, the effectiveness of the chosen preservative shall be demonstrated to the satisfaction of the competent authority. A suitable method of testing together with criteria for judging the preservative of the formulation is provided under "Efficacy of Antimicrobial Preservation" (Appendix 10.6).

In the manufacture, packaging, storage and distribution of rectal preparations, suitable means are taken to ensure their microbial quality; recommendations on this aspect are provided under "Limit for Microbial Contamination" (Appendix 10.5).

In the manufacture of semi-solid and liquid rectal preparations containing dispersed particles, measures are taken to ensure a suitable and controlled particle size with regard to the intended use.

Dissolution A suitable test may be required to demonstrate the appropriate release of the active ingredient(s) from solid, single dose preparations, for example the dissolution test for suppositories and soft capsules (Appendix 4.24). Where a dissolution test is prescribed, a disintegration test may not be required.

Uniformity of dosage units Rectal preparations comply with the "Uniformity of Dosage Units" (Appendix 4.28).

Rectal Suppositories

Rectal suppositories are solid, single-dose preparations. The shape, volume and consistence of suppositories are suitable for rectal administration.

They contain one or more active ingredients dispersed or dissolved in a simple or compound excipient which may be soluble or dispersible in water or may melt at body temperature. Excipients such as diluents, adsorbents, surface-active agents, lubricants, antimicrobial preservatives and colouring matter, authorized by the competent authority, may be added if necessary.

Production Rectal suppositories are prepared by compression or moulding. If necessary, the active ingredient(s) are previously ground and sieved through a suitable sieve. When prepared by moulding, the medicated mass, sufficiently liquified by heating, is poured into suitable moulds. The suppository solidifies on cooling. Various excipients are available for this process, such as hard fat, macrogols, cocoa butter, and various gelatinous mixtures consisting, for example, of gelatin, water and glycerol.

A suitable test is carried out to demonstrate the appropriate release of the active ingredient(s) from suppositories intended for modified release or for prolonged local action.

Disintegration Unless intended for modified release or for prolonged local action, rectal suppositories comply with the "Disintegration Test for Suppositories and Pessaries" (Appendix 4.22). For suppositories with a fatty base, examine after 30 minutes and for suppositories with a water-soluble base after 60 minutes, unless otherwise justified and authorized.

Packaging and storage Rectal suppositories shall be kept in well-closed containers.

Labelling The label on the container states (1) that they are intended for external use only; (2) the storage conditions.

Rectal Capsules

Rectal capsules (shell suppositories) are solid, single-dose preparations generally similar to soft capsules as defined in capsules except that they may have lubricating coatings. They are of elongated shape, are smooth and have a uniform external appearance.

Production A suitable test is carried out to demonstrate the appropriate release of the active ingredient(s) from rectal capsules intended for modified release or prolonged local action.

Disintegration Unless intended for modified release or for prolonged local action, rectal capsules comply with the "Disintegration Test for Suppositories" (Appendix 4.22). Examine the state of the capsules after 30 minutes, unless otherwise justified and authorized.

Rectal Solutions and Suspensions (Enemas)

Rectal solutions and suspensions (enemas) are liquid preparations intended for rectal use in order to obtain a systemic or local effect, or they may be intended for diagnostic purposes.

They are single-dose preparations containing one or more active ingredients dissolved or dispersed in water, glycerol, macrogols or other suitable solvents. Suspensions may show a sediment which is readily dispersible

on shaking to give a suspension which remains sufficiently stable to enable the correct dose to be delivered.

Rectal solutions and suspensions may contain excipients intended, for example, to adjust the viscosity of the preparation, to adjust or stabilize pH, to increase the solubility of the active ingredient(s) or to stabilize the preparation. These substances do not adversely affect the intended medical action or, at the concentrations used, cause undue local irritation.

Rectal solutions and suspensions are supplied in containers containing a volume in the range of 2.5 ml to 2000 ml. The container is adapted to deliver the preparation to the rectum or it is accompanied by a suitable applicator.

Powders and Tablets for Rectal Solutions and Suspensions

Powders and tablets intended for the preparation of rectal solutions or suspensions are single-dose preparations which are dissolved or dispersed in water at the time of administration. They may contain excipients to facilitate dissolution or dispersion or to prevent aggregation of the particles.

After dissolution or suspension, they comply with the requirements for rectal solutions or rectal suspensions, as appropriate.

Disintegration Tablets for rectal solutions or suspensions disintegrate within 3 minutes when tested as described under "Disintegration Test for Suppositories and Pessaries" (Appendix 4.22), but using *water* at 15° to 25°.

Labelling The label on the container states (1) the directions for preparing the rectal solution or suspension; (2) the storage conditions; (3) the period during which the constituted rectal solutions or suspensions may be expected to remain satisfactory for use when prepared and stored in accordance with the manufacturer's recommendations.

Rectal Creams

See under Topical Semi-solid Preparations.

Rectal Gels

See under Topical Semi-solid Preparations.

Rectal Ointments

See under Topical Semi-solid Preparations.

Rectal Foams

See under Medicated Foams.

Rectal Tampons

Rectal tampons are solid, single-dose preparations intended to be inserted into the lower part of the rectum for a limited time.

See also under Medicated Tampons.

SPIRITS

Spirits are ethanolic or hydroethandic solutions of volatile substances usually prepared by simple solution or by admixture of the ingredients. Some spirits serve as flavouring agents while others have medicinal value. Reduction of the high ethanolic content of spirits by admixture with aqueous preparations often causes turbidity.

Packaging and storage Spirits shall be kept in tightly closed containers, protected from light to prevent loss by evaporation and to limit oxidative changes.

SYSTEMS

Systems are preparations that allow for the uniform release or targeting of drugs to the body. These preparations are commonly called drug delivery systems or delivery systems. The most widely used of these are transdermal systems.

Transdermal Systems

Transdermal drug delivery systems are self-contained, discrete dosage forms that, when applied to intact skin, are designed to deliver the drug(s) through the skin to the systemic circulation. Systems typically comprise an outer covering (barrier), a drug reservoir, which may have a rate controlling membrane, a contact adhesive applied to some or all parts of the system and the system/skin interface, and a protective liner that is removed before applying the system. The activity of these systems is defined in terms of the release rate of the drug(s) from the system. The total duration of drug release from the system and the system surface area may also be stated.

Labelling The label of transdermal systems states (1) the dose released per unit time; (2) the storage conditions.

Ocular Systems

Ocular systems are sterile, solid or semi-solid preparations of suitable size and shape, designed to be inserted in the conjunctival cul-de-sac, to produce an ocular effect. They generally consist of a reservoir of active ingredient(s) embedded in a matrix or bounded by a rate-controlling membrane. The active ingredient, which is more or less soluble in physiological fluids, is released over a predetermined period of time. Ocular systems are individually distributed into sterile containers.

Sterility Unless otherwise specified in the individual monograph, ocular systems comply with the "Sterility Test" (Method I, Appendix 10.1).

Drug release pattern Ocular systems comply with the test for drug release pattern in the individual monograph.

Labelling The label of ocular systems states (1) the dose released per unit time; (2) the storage conditions.

Intrauterine Systems

An intrauterine system, based on a similar principle but intended for release of drug over a much longer period of time, i.e. one year, is also available.

TABLETS

Tablets are solid dosage forms containing one or more active ingredients. They are obtained by single or multiple compression (in certain cases they are moulded) and may be uncoated or coated. They are usually intended for oral administration.

The different categories of tablets that exist include soluble tablets, effervescent tablets, tablets for use in the mouth, and modified-release tablets. Tablets are normally circular in shape and their surfaces are flat or convex. Tablets may have lines or break-marks, symbols, or other markings. They should be sufficiently hard to withstand handling, including packaging, storage, and transportation, without crumbling or breaking.

Tablets may contain excipients such as diluents, binders, disintegrating agents, glidants, lubricants, substances capable of modifying the behaviour of the dosage forms and the active ingredient(s) in the gastrointestinal tract, colouring matter, and flavouring substances. When such excipients are used, it is necessary to ensure that they do not adversely affect the stability, dissolution rate, bioavailability, safety, or efficacy of the active ingredient(s); there must be no incompatibility between and of the components of the dosage form.

Preparations for alternative applications, such as solution-tablets for injections, irrigations, or for external use, etc., are also available in this presentation. These preparations may require a special formulation, method of manufacture, or form of presentation, appropriate to their particular use. For this reason they may not comply with certain sections of this monograph.

Production The following information is intended to provide very broad guidelines concerning the main steps to be followed during production. Tablets may be prepared by three general methods: wet granulation, dry granulation (roll compaction or slugging), and direct compression. The purpose of both wet and dry granulation is to improve flow of the mixture and/or to enhance its compressibility. Tablets are compressed by the application of high pressures, utilizing steel punches and dies, to powders or granulations. However, throughout manufacturing, certain procedures should be validated and monitored by carrying out appropriate inprocess controls.

For modified-release tablets, a suitable test is carried out to demonstrate the appropriate release of the active ingredient(s).

Delayed-release tablets are prepared by covering tablets with a gastro-intestinal coating or from granules or particles already covered with a gastro-resistant coating.

In the manufacture of tablet cores, means are taken to ensure that they possess a suitable mechanical strength to withstand handling without crumbling or breaking. This may be demonstrated by examining the "Friability of Uncoated Tablets" (Appendix 4.30) and the "Resistance to Crushing of Tablets" (Appendix 4.31). Chewable tablets are prepared to ensure that they are easily crushed by chewing.

Disintegration Comply with the "Disintegration Test for Tablets and Capsules" (Appendix 4.23). For those tablets for which a dissolution requirement is included in the individual monograph, omission of the requirement for disintegration is considered justifiable and is therefore authorized.

Dissolution Comply with the "Dissolution Test" (Appendix 4.24). Where a dissolution test is specified in the individual monograph, a disintegration test may not be required.

Uniformity of dosage units Comply with the "Uniformity of Dosage Units" (Appendix 4.28). The test for Content Uniformity is not required for multivitamin and trace element tablets.

Visual inspection Unpack and inspect at least 20 tablets. They should be undamaged, smooth and usually of uniform colour.

Evidence of physical instability is demonstrated by:

(1) presence of excessive powder and/or pieces of tablets at the bottom of the container (from abraded, crushed, or broken tablets);

(2) cracks or capping, chipping in the tablet surfaces or coating, swelling, mottling, discoloration, fusion between tablets;

(3) the appearance of crystals on the container walls or on the tablets.

Packaging and storage Tablets should be kept in well-closed containers at a temperature not exceeding 30° and protected from crushing and mechanical shock. Moisture-sensitive forms such as effervescent tablets should be kept in tightly closed containers or moisture-proof packs and may require the use of separate packages containing water-adsorbent agents, such as silica gel.

Uncoated Tablets

The majority of uncoated tablets are made in such a way that the release of active ingredients is unmodified. A broken section, when examined under a lens, shows either a relatively uniform texture (single-layer tablets) or a stratified texture (multi-layer tablets), but no signs of coating.

TABLETS FOR SOLUTIONS (SOLUBLE TABLETS) Tablets for solutions are uncoated tablets that dissolve in water to give a clear solution.

EFFERVESCENT TABLETS Effervescent tablets are uncoated tablets generally containing acid substances and carbonates or hydrogen carbonates that react rapidly in the presence of water to release carbon dioxide. They are intended to be dissolved or dispersed in water before administration.

Labelling The label should state "Not to be swallowed directly".

DISPERSIBLE TABLETS Dispersible tablets are uncoated tablets that produce a uniform dispersion in water.

Uniformity of dispersion Place two tablets in 100 ml of *water* and stir until completely dispersed. A smooth dispersion is produced which passes through a sieve screen with a nominal mesh aperture of 710 µm.

TABLETS FOR USE IN THE MOUTH Tablets for use in the mouth are usually uncoated tablets. They are formulated to effect a slow release and local action of the active ingredient (for example, compressed lozenges) or the release and systemic absorption of the active ingredient under the tongue (sublingual tablets) or in the other parts of the mouth. (Certain tablets for use in the mouth may be referred to as "Lozenges".)

Chewable tablets Chewable tablets are tablets intended to be chewed, producing a pleasant tasting residue in the oral cavity that is easily swallowed and does not leave a bitter or unpleasant after-taste. These tablets have been used in tablet formulations for children, especially multivitamin formulations, and for the administration of antacids and selected antibiotics. Chewable tablets are prepared by compression, usually utilizing mannitol, sorbitol, or sucrose as binders and fillers, and containing colours and flavours to enhance their appearance and taste.

Buccal and sublingual tablets Buccal tablets are tablets intended to be inserted in the buccal pouch, and sublingual tablets are intended to be inserted beneath the tongue, where the active ingredient is absorbed directly through the oral mucosa. Few drugs are readily absorbed in this way, but for those that are (such as nitroglycerin and certain steroid hormones), a number of advantages may result.

Coated Tablets

Coated tablets are tablets covered with one or more layers of mixtures of substances such as natural or synthetic resins, polymers, gums, fillers, sugars, plasticizers, polyhydric alcohols, waxes, colouring matters, flavouring substances, and sometimes also active ingredients. A broken section, when examined under a lens, shows a core which is surrounded by a continuous layer of a different texture.

The tablets may be coated for a variety of reasons such as protection of the active ingredients from air, moisture, or light, masking of unpleasant tastes and odours, or improvement of appearance. The substance used for coating is usually applied as a solution or suspension.

SUGAR-COATED TABLETS Sugar-coated tablets are tablets containing a sugar coating.

FILM-COATED TABLETS Film-coated tablets are tablets covered with a thin layer of resins, polymers, and/or plasticizers capable of forming a film.

Modified-release Tablets

Modified-release tablets are coated, uncoated, or matrix tablets containing excipients or prepared by procedures which, separately or together, are designed to modify the rate of release of the active ingredient(s) in the gastro-intestinal tract.

DELAYED-RELEASE TABLETS Delayed-release tablets are tablets intended to resist gastric fluid but to release the active ingredient(s) in the intestinal fluid. This is achieved by using coating substances such as cellacefate (cellulose acetate phthalate) and anionic copolymers of methacrylic acid and its esters. It is sometimes necessary to apply more than one layer.

All requirements for these specialized dosage forms are given in the individual monographs.

EXTENDED-RELEASE TABLETS Extended-release tablets are tablets designed to slow the rate of release of the active ingredient(s) in the gastro-intestinal tract.

All requirements for these specialized dosage forms are given in the individual monographs.

TINCTURES

Tinctures are ethanolic or hydroethanolic solutions usually obtained from dried vegetable or animal matter or from chemical substances. They are usually obtained either using 1 part of drug and 10 parts of extraction solvent or 1 part of drug and 5 parts of extraction solvent. Tinctures are usually clear; a slight sediment may be produced on standing provided that the composition is not changed significantly.

Production For some preparations, the matter to be extracted must undergo a preliminary treatment, for example, inactivation of enzymes, grinding or defatting. Tinctures are prepared by maceration, percolation or other suitable, justified methods, using Ethanol of suitable concentration. Tinctures may also be obtained by dissolving or diluting extracts in Ethanol of suitable concentration.

PRODUCTION BY PERCOLATION If necessary, the drug is reduced to pieces of suitable size, mixed thoroughly with a portion of extraction solvent and allowed to stand for an appropriate time. The mixture is transferred to a percolator and the percolate allowed to flow slowly ensuring that the drug is always covered with the remaining extraction solvent. The drug residue may be pressed out and the expressed fluid combined with the percolate.

PRODUCTION BY MACERATION Unless otherwise prescribed, the drug is reduced to pieces of suitable size, mixed thoroughly with the prescribed extraction solvent and allowed to stand in a closed container for an appropriate time. The drug residue is separated from the extraction solvent and, if necessary, pressed out. In the latter case, the two liquids obtained are combined.

PRODUCTION FROM EXTRACTS The tincture is prepared by dissolving or diluting an extract, using Ethanol of appropriate concentration. The content of solvent and constituents or, where applicable, the contents of solvent and of dry residue correspond to that of tinc-

tures obtained by maceration or percolation.

When the content of constituents has to be adjusted, such adjustment may be carried out, if necessary, either by adding the extraction solvent of suitable concentration or by adding another tincture of the vegetable or animal matter used for the preparation.

Ethanol Comply with the limits prescribed in the monograph (Appendix 6.5).

Methanol and 2-propanol Not more than 0.05 per cent v/v of methanol or 2-propanol (Appendix 5.15), unless otherwise prescribed in the monograph.

Total solids Comply with the limits prescribed in the monograph (Appendix 7.21).

Weight per millilitre Comply with the limits prescribed in the monograph (Appendix 4.9).

Packaging and storage Tinctures should be kept in tightly closed containers, protected from light, in a cool place.

Labelling The label on the container states (1) the vegetable, animal matter or chemical substances used; (2) where applicable, that fresh vegetable or animal matter was used; (3) the name and concentration of the solvent used for the preparation; (4) the concentration of ethanol in the final tincture; (5) the content of active principle and/or the ratio of starting material to extraction fluid and of starting material to final tincture.

TOPICAL PREPARATIONS

Topical preparations are drugs intended for topical application in a wide variety of dosage forms.

Minimum fill Topical preparations except plasters comply with the test described in the "Minimum Fill" (Appendix 4.26).

Applications

Applications are liquid or semi-liquid preparations containing one or more active ingredients intended for application to the skin. They may contain suitable antimicrobial preservatives, unless the active ingredients or vehicles have sufficient intrinsic antibacterial and antifungal activity. They may contain other suitable auxiliary substances such as stabilizers. They have traditionally been used to administer antiparasitic medications.

Collodions

Collodions are liquid preparations, usually containing pyroxylin in a mixture of ether and ethanol, that are intended for application to the skin. When they are allowed to dry, a flexible film is formed at the site of application.

Packaging and storage Collodions should be kept in well-closed containers, at a temperature not exceeding 25° and remote from fire.

Jellies

See hydrophilic gels under Topical Semi-solid Preparations.

Liniments

Liniments are liquid or semi-liquid preparations containing one or more active ingredients in suitable vehicles. They are intended to be applied to the unbroken skin with friction. They may contain suitable antimicrobial preservatives.

Packaging and storage Liniments should be kept in well-closed containers. Certain plastic containers, such as those made from polystyrene, are unsuitable for liniments.

Lotions

Lotions are liquid or semi-liquid preparations containing one or more active ingredients in suitable vehicles. They are usually intended to be applied to the unbroken skin without friction. Lotions may contain suitable antimicrobial preservatives, unless the active ingredients or vehicles have sufficient intrinsic antibacterial and antifungal activity. They may contain other suitable added substances such as stabilizers. The term "lotion" is applied to solutions or suspensions or emulsions applied topically.

TOPICAL SOLUTIONS Topical solutions are solutions, usually aqueous but often containing other solvents, such as alcohol and polyols, intended for topical application to the skin, or to the oral mucosal surface.

TOPICAL SUSPENSIONS Topical suspensions are liquid preparations containing solid particles dispersed in a liquid vehicle, intended for application to the skin.

TOPICAL EMULSIONS Topical emulsions are liquid preparations in which one liquid is dispersed throughout another liquid in the form of small droplets.

Packaging and storage Lotions should be kept in well-closed containers.

Mouthwashes

Mouthwashes are aqueous solutions containing one or more active ingredients. They are intended for use in contact with the mucous membranes of the oral cavity, usually after dilution with warm water. They may contain added substances such as suitable antimicrobial preservatives.

Packaging and storage Mouthwashes should be kept in well-closed containers.

Labelling The label of mouthwashes states the directions for the dilution of the mouthwash for use, if appropriate.

Paints

Paints are solutions or dispersions of one or more

active ingredients. They are intended for application to the skin or, in some cases, mucous membranes.

Packaging and storage Paints should be kept in tightly closed containers.

Plasters

Plasters are usually solid at ordinary temperature, and are intended for external application. They are used by spreading on cloth, paper, or plastic film and adhering to the skin.

Medicated plaster, long used for local or regional drug delivery, are the prototypical transdermal system.

Topical Emulsions

See under Lotions.

Topical Powders

See under Powders.

Topical Semi-solid Preparations

Topical semi-solid preparations are intended to be applied to the skin or to certain mucous surfaces for local action or percutaneous penetration of medicaments, or for their emollient or protective action. They are of homogeneous appearance.

Topical semi-solid preparations consist of a simple or compound base in which, usually, one or more active substances are dissolved or dispersed. According to its composition, the base may influence the action of the preparation and the release of the active substance(s).

The bases may consist of natural or synthetic substances and may be single-phase or multi-phase systems. According to the nature of the base the preparation may have hydrophilic or hydrophobic (lipophilic) properties; it may contain suitable additives such as antimicrobial preservatives, antioxidants, stabilizers, emulsifiers and thickeners.

If a preparation is specifically intended for use on large open wounds or on severely injured skin, it should be sterile. Preparations required to be sterile must comply with the test for sterility.

If the particle size of the ingredients is of importance for the therapeutic purpose of a topical semi-solid preparation, the test to be applied should be specified.

Topical semi-solid preparations can be distinguished into four categories: (1) creams (hydrophobic or hydrophilic); (2) gels (hydrophobic or hydrophilic); (3) ointments (hydrophobic, water-emulsifying or hydrophilic); (4) pastes.

Sterility Where the preparation is labelled as sterile and unless otherwise directed in the individual monograph, it complies with the "Sterility Test" (Method I, Appendix 10.1).

Packaging and storage Topical semi-solid preparations should be stored in well-closed containers or, if the preparation contains water or other volatile constituents, in a tightly closed container. The containers are preferably collapsible metal tubes from which the preparation may be readily extruded. Other types of container may be used. Containers for preparations for nasal, aural, vaginal, or rectal use should be adapted to deliver the product to the site of application or should be accompanied by a suitable applicator. They should be stored at a temperature not exceeding 30° unless otherwise prescribed. For creams and gels, they shall not be frozen.

Labelling The label of topical semi-solid preparations states (1) the name and concentration of any added antimicrobial preservative(s); (2) where applicable, that the preparation is sterile.

CREAMS Creams are homogeneous, semi-solid preparations consisting of opaque emulsion systems. They are multiphase preparations composed of a lipophilic phase and an aqueous phase.

Hydrophobic creams Hydrophobic creams have the lipophilic phase as the continuouss phase. They contain water-in-oil emulsifying agents such as wool fat, sorbitan esters and monoglycerides.

Hydrophilic creams Hydrophilic creams have the aqueous phase as the continuous phase. They contain oil-in-water emulsifying agents such as sodium or triethanolamine soaps, sulfated fatty alcohols and polysorbates, combined, if necessary, with water-in-oil emulsifying agents.

GELS Gels are usually homogeneous, clear, semi-solid preparations consisting of a liquid phase within a three-dimensional polymeric matrix with physical or sometimes chemical cross-linkage by means of suitable gelling agents.

Hydrophobic gels Hydrophobic gel (oleogel) bases usually consist of liquid paraffin with polyethylene or fatty oils gelled with colloidal silica or aluminium or zinc soaps.

Hydrophilic gels Hydrophilic gel (hydrogel) bases usually consist of water, glycerol, or propylene glycol gelled with suitable agents such as tragacanth, starch, cellulose derivatives, carboxyvinyl polymers and magnesium aluminium silicates.

OINTMENTS Ointments are homogeneous, semi-solid preparations intended for external application to the skin or mucous membranes. They are formulated using hydrophobic, hydrophilic, or water-emulsifying bases to provide preparations that are immiscible, miscible, or emulsifiable with skin secretions. They can also be derived from hydrocarbon (fatty), absorption, water-removable, or water-soluble bases.

Hydrophobic ointments Hydrophobic (lipophilic) ointments are usually anhydrous and can absorb only small amounts of water. Typical bases used for their formulation are water-insoluble hydrocarbons such as hard, soft, and liquid paraffin, vegetable oil, animal fats, waxes, synthetic glycerides, and polyalkylsiloxanes.

Water-emulsifying ointments Water-emulsifying ointments can absorb large amounts of water. They

typically consist of a hydrophobic fatty base in which a water-in-oil agent, such as wool fat, wool alcohols, sorbitan esters, monoglycerides, or fatty alcohols can be incorporated to render them hydrophilic. They may also be water-in-oil emulsions that allow additional quantities of aqueous solutions to be incorporated. Such ointments are used especially when formulating aqueous liquids or solutions.

Hydrophilic ointments Hydrophilic ointment bases are either water-removable or water-soluble bases. They are oil-in-water emulsions such as hydrophilic ointment or greaseless ointment bases comprised of water soluble constituents such as mixtures of liquid and solid polyethyleneglycols (macrogols).

PASTES Pastes are homogeneous, semi-solid preprations usually containing high concentrations of insoluble powdered substances (commonly not less than 20 per cent) dispersed in a suitable base. One class is made from a single phase aqueous gel (e.g., zinc oxide gelatin paste). The other class, the fatty pastes (e.g., zinc oxide paste) consists of thick, stiff ointments that do not ordinarily flow at body temperature. The pastes should adhere well to the skin. In many cases they form a protective film that controls the evaporation of water.

Topical Solutions

See under Lotions.

Topical Suspensions

See under Lotions.

VAGINAL PREPARATIONS

Vaginal preparations are liquid, semi-solid preparations intended for administration to the vagina usually in order to obtain a local effect. They contain one or more active ingredients in a suitable basis. Several categories of vaginal preparations may be distinguished: (1) moulded pessaries; (2) vaginal tablets; (3) vaginal capsules; (4) vaginal foams; and (5) vaginal tampons.

Production In the manufacturing, packaging, storage and distribution of vaginal preparations, suitable means are taken to ensure their microbial quality; recommendations on this aspect are provided under "Limits for Microbial Contamination" (Appendix 10.5).

Disintegration Vaginal preparations comply with the "Disintegration Test for Suppositories" (Appendix 4.22).

Uniformity of dosage units Vaginal preparations comply with the "Uniformity of Dosage Units" (Appendix 4.28).

Moulded Pessaries

Moulded pessaries are solid, single-dose preparations. They have various shapes, usually ovoid, with a volume and consistence suitable for insertion into the vagina. Apart from their shape they conform to rectal suppositories.

Moulded pessaries are prepared using the method and excipients described for rectal suppositories. The active ingredient(s) are dispersed or dissolved in a simple or compound basis, which may be soluble, insoluble but melting at body temperature or dispersible in water.

Vaginal preparations which conform to the definition of moulded pessaries may be prepared by compression. They comply with the requirements for moulded pessaries.

Production A suitable test is carried out to demonstrate the appropriate release of the active ingredient(s) from pessaries intended for modified release or prolonged local action.

Packaging and storage Moulded pessaries shall be kept in well-closed containers.

Labelling The label on the container states (1) that they are intended for external use only; (2) the storage conditions.

Vaginal Tablets

Vaginal tablets (compressed pessaries) are solid single-dose preparations. They generally conform to the definitions of uncoated or film-coated tablets in Tablets.

Production A suitable test is carried out to demonstrate the appropriate release of the active ingredient(s) from vaginal tablets intended for modified release or prolonged local action.

Vaginal Capsules

Vaginal capsules (shell pessaries) are solid, single dose preparations. They are generally similar to soft capsules, differing only in their shape and size. Vaginal capsules have various shapes, usually ovoid. They are smooth and have a uniform external appearance.

Production A suitable test is carried out to demonstrate the appropriate release of the active ingredients(s) from vaginal capsules intended for modified release or prolonged local action.

Vaginal Foams

See under Medicated Foams.

Vaginal Tampons

Vaginal tampons are solid, single-dose preparations intended to be inserted in the vagina for a limited time.

See also under Medicated Tampons.

1.17 ESTIMATION OF BODY SURFACE AREA

The body surface area is the measured or calculated surface of a human body. The body surface area is used in many measurements in medicine, including the calculation of drug dosages and the amount of fluids to be administered intravenously. The body surface area

TABLE OF BODY SURFACE AREA FROM HEIGHT AND WEIGHT (m^2)*

Height (cm) \ Weight (kg)	1.5	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0	11.0	12.0	13.0	14.0	15.0	16.0	17.0	18.0	19.0	20.0	21.0	22.0	23.0	24.0	25.0	26.0	27.0	28.0	29.0
45	0.13	0.15	0.18	0.20	0.22	0.24																							
50	0.15	0.16	0.20	0.22	0.24	0.26																							
55		0.18	0.21	0.24	0.26	0.28	0.30																						
60			0.22	0.25	0.28	0.30	0.32	0.34	0.36	0.37	0.39	0.40																	
65			0.24	0.27	0.29	0.32	0.34	0.36	0.38	0.39	0.41	0.43	0.44	0.45															
70			0.25	0.28	0.31	0.33	0.36	0.38	0.40	0.42	0.43	0.45	0.47	0.48	0.49														
75				0.30	0.33	0.35	0.38	0.40	0.42	0.44	0.46	0.47	0.49	0.50	0.52														
80						0.37	0.39	0.42	0.44	0.45	0.48	0.50	0.51	0.53	0.54	0.56													
85							0.41	0.44	0.46	0.48	0.50	0.52	0.54	0.55	0.57	0.58	0.60	0.61											
90								0.45	0.48	0.50	0.52	0.54	0.56	0.58	0.59	0.61	0.63	0.64	0.66	0.67									
95									0.50	0.52	0.54	0.56	0.58	0.60	0.62	0.63	0.65	0.67	0.68	0.70									
100									0.52	0.54	0.56	0.58	0.60	0.62	0.64	0.66	0.68	0.69	0.71	0.72	0.74	0.75							
105									0.53	0.56	0.58	0.60	0.62	0.64	0.66	0.68	0.70	0.72	0.73	0.75	0.77	0.78	0.80	0.81	0.82	0.84	0.85		
110										0.58	0.60	0.62	0.65	0.67	0.69	0.70	0.72	0.74	0.76	0.78	0.79	0.81	0.82	0.84	0.85	0.87	0.88	0.89	0.91
115												0.64	0.67	0.69	0.71	0.73	0.75	0.77	0.78	0.80	0.82	0.83	0.85	0.86	0.88	0.89	0.91	0.92	0.94
120												0.66	0.69	0.71	0.73	0.75	0.77	0.79	0.81	0.83	0.84	0.86	0.88	0.89	0.91	0.92	0.94	0.95	0.97
125														0.73	0.75	0.77	0.79	0.81	0.83	0.85	0.87	0.89	0.90	0.92	0.93	0.95	0.97	0.98	1.00
130																	0.82	0.84	0.86	0.87	0.89	0.91	0.93	0.95	0.96	0.98	0.99	1.01	1.02
135																	0.84	0.86	0.88	0.90	0.92	0.94	0.95	0.97	0.99	1.01	1.02	1.04	1.05
140																				0.92	0.94	0.96	0.98	1.00	1.01	1.03	1.05	1.07	1.08
145																				0.95	0.97	0.99	1.00	1.02	1.04	1.06	1.08	1.09	1.11
150																				0.97	0.99	1.01	1.03	1.05	1.07	1.08	1.10	1.12	1.14
155																								1.07	1.09	1.11	1.13	1.15	1.16
160																								1.10	1.12	1.14	1.16	1.17	1.19
165																								1.12	1.14	1.16	1.18	1.20	1.22
170																													

*From the formula of DuBois and DuBois (*Arch. Intern. Med.*, 1916, 17, 863).
$S = H^{0.725} \times W^{0.425} \times 71.84 \times 10^{-4}$ (S = body surface in m^2, H = height in cm, W = weight in kg)

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TABLE OF BODY SURFACE AREA FROM HEIGHT AND WEIGHT (m^2)*

Weight (kg)

Height (cm)	30.0	32.5	35.0	37.5	40.0	42.5	45.0	47.5	50.0	52.5	55.0	57.5	60.0	62.5	65.0	67.5	70.0	72.5	75.0	77.5	80.0	85.0	90.0	95.0	100.0	105.0	110.0	115.0	120.0
45																													
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80																													
85																													
90																													
95																													
100																													
105																													
110	0.92																												
115	0.95	0.98	1.02																										
120	0.98	1.01	1.05																										
125	1.01	1.05	1.08	1.11	1.14																								
130	1.04	1.08	1.11	1.14	1.17	1.21	1.23	1.26	1.29																				
135	1.07	1.11	1.14	1.17	1.21	1.24	1.27	1.30	1.33	1.35	1.38																		
140	1.10	1.13	1.17	1.21	1.24	1.27	1.30	1.33	1.36	1.39	1.42	1.45	1.47																
145	1.12	1.16	1.20	1.24	1.27	1.30	1.33	1.37	1.40	1.43	1.46	1.48	1.51	1.54	1.56														
150	1.15	1.19	1.23	1.27	1.30	1.34	1.37	1.40	1.43	1.46	1.49	1.52	1.56	1.58	1.60	1.63	1.65												
155	1.18	1.22	1.26	1.30	1.33	1.37	1.40	1.44	1.47	1.50	1.53	1.56	1.59	1.61	1.64	1.67	1.69	1.72	1.74										
160	1.21	1.25	1.29	1.33	1.37	1.40	1.44	1.47	1.50	1.53	1.56	1.59	1.62	1.65	1.68	1.71	1.73	1.76	1.78	1.81	1.83								
165	1.24	1.28	1.32	1.36	1.40	1.43	1.47	1.50	1.53	1.57	1.60	1.63	1.66	1.69	1.72	1.74	1.77	1.80	1.82	1.85	1.87								
170	1.26	1.31	1.35	1.39	1.43	1.46	1.50	1.53	1.57	1.60	1.63	1.66	1.69	1.72	1.75	1.78	1.81	1.84	1.86	1.89	1.92	1.97							
175			1.38	1.42	1.46	1.49	1.53	1.57	1.60	1.64	1.67	1.70	1.73	1.76	1.79	1.82	1.85	1.88	1.90	1.93	1.96	2.01	2.06	2.10					
180					1.49	1.53	1.56	1.60	1.63	1.67	1.70	1.73	1.77	1.80	1.83	1.86	1.89	1.91	1.94	1.97	2.00	2.05	2.10	2.15	2.20	2.24	2.29	2.33	
185							1.59	1.63	1.67	1.70	1.74	1.77	1.80	1.83	1.86	1.89	1.92	1.95	1.98	2.01	2.04	2.09	2.14	2.19	2.24	2.29	2.33	2.38	2.42
190							1.63	1.66	1.70	1.74	1.77	1.80	1.84	1.87	1.90	1.93	1.96	1.99	2.02	2.05	2.08	2.13	2.18	2.23	2.28	2.33	2.38	2.42	2.47
195									1.73	1.77	1.80	1.84	1.87	1.90	1.94	1.97	2.00	2.03	2.06	2.09	2.12	2.17	2.22	2.28	2.33	2.37	2.42	2.47	2.51
200									1.76	1.80	1.84	1.87	1.91	1.94	1.97	2.00	2.04	2.07	2.10	2.13	2.15	2.21	2.27	2.32	2.37	2.42	2.47	2.51	2.56

*From the formula of DuBois and DuBois (*Arch. Intern. Med.*, 1916, 17, 863).
$S = H^{0.725} \times W^{0.425} \times 71.84 \times 10^{-4}$ (S = body surface in m^2, H = height in cm, W = weight in kg)

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can be calculated by various formulas without direct measurement. When weight and height are known, the formula of DuBois and DuBois (1) published in 1916 is recommended.

(1) The body surface area (m^2) = Height (cm)$^{0.725}$ × Weight (kg)$^{0.425}$ × 71.84 × 10^{-4}

The table of the body surface area from weight and height, which is calculated from the formula of DuBois and DuBois, is represented in the following pages.

A simplified formula, which can be calculated for the body surface area, is commonly known as the Mosteller formula (2) published in 1987.

(2) The body surface area (m^2) = [Weight (kg) × Height (cm)/3600]½

1.18 ESTIMATION OF LEAN BODY WEIGHT

Total body weight has two components, lean body weight and fat weight. Lean body weight includes the muscles, bones, tendons, ligaments and water in the body. Fat weight includes the fat stored in the fat cells and the organs of the body.

The majority of human body composition measurements have used indirect techniques based on different assumptions about the chemical or physical properties of lean body weight. Lean body weight can be estimated as the difference between body weight and total adipose tissue expressed in kilograms. The conversion from litres of total adipose tissue is achieved by multiplying with the mean density (0.923) of human adipose tissue. The algorithm[1] below may be used to obtain estimates of lean body weight for use in calculating doses of drugs with narrow therapeutic index.

Males
Total AT = $1.36 \times W/H - 42.0$

Females
Total AT = $1.61 \times W/H - 38.3$

Males and Females
LBW = BW – (Total AT × 0.923)

in which AT is adipose tissue in litre, BW is body weight in kg, LBW is lean body weight in kg, W/H is weight/height in kg/m.

[1]Modified from A.J. Stunkard, T.A. Wadden, *Obesity: Theory and Therapy*, 2nd ed., New York, Raven Press, 1993, pp.14-19.

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APPENDIX 2 SPECTROSCOPY

2.1 INFRARED SPECTROPHOTOMETRY

Spectrophotometric measurements in the infrared region are used mainly as an identification test. The infrared spectrum is unique for any given chemical compound with the exception of optical isomers, which have identical spectra in solution. Polymorphism and other factors, such as variations in crystal size and orientation, the grinding procedure, and the possible formation of hydrates, may, however, be responsible for a difference in the infrared spectrum of a given compound in the solid state. The infrared spectrum is usually not greatly affected by the presence of small quantities of impurities in the tested substance. For identification purpose the spectrum may be compared with that of a reference substance, concurrently prepared or with a reference spectrum.

Apparatus

Spectrophotometers suitable for use in the infrared region should operate in the range of 4000 to 625 cm^{-1} (2.5 to 16 μm). They should be checked frequently to ensure that they meet the standards of performance laid down by the manufacturer of the instrument, including the reliability of the wavelength scales, which should be checked by use of a polystyrene film.

For the use of the attenuated total reflectance technique, the instrument should be equipped with a suitable attachment, which may be a single-reflection or a multi-reflection one. The attachment consists of a reflecting element and a suitable mounting permitting its alignment in the spectrophotometer for maximum transmission.

Use of Solvents

The solvent used in infrared spectrophotometry must not affect the material, usually sodium chloride, of which the cell is made.

No solvent in appreciable thickness is completely transparent throughout the infrared spectrum. *Carbon tetrachloride* is practically transparent (up to 1 mm in thickness) from 4000 to 1700 cm^{-1} (2.5 to 5.9 μm). *Chloroform*, *dichloromethane*, and *dibromomethane* are other useful solvents. *Carbon disulfide IR* (up to 1 mm in thickness) is suitable as a solvent to 250 cm^{-1} (40 μm), except in the 2400 to 2000 cm^{-1} (4.2 to 5.0 μm) and the 1800 to 1300 cm^{-1} (5.6 to 7.7 -μm) regions, where it has strong absorption. Its weak absorption in the 875 to 845 cm^{-1} (11.4 to 11.8 μm) region should also be noted. Other solvents have relatively narrow regions of transparency.

Preparation of the Sample

Substances are examined in one of the following forms, prepared as directed.

For measurement by transmission or absorption

LIQUIDS Examine a liquid as a thin film held between two plates or in a cell of suitable path-length constructed of material transparent to infrared radiation in the region to be examined.

LIQUIDS OR SOLIDS PREPARED AS SOLUTIONS Prepare a solution in a suitable solvent, and use a concentration and path-length to give a satisfactory spectrum over a sufficiently wide wavelength range. Absorption due to the solvent should be compensated for by placing in the reference beam a similar cell containing the solvent used; it should be noted that absorption bands due to the substance under examination that coincide with strong solvent absorption will not be recorded. Suitable concentrations of the solute will vary with the substance being examined but typical concentrations are 1 to 10 per cent at 0.5 to 0.1 mm path-length.

SOLIDS Examine a solid after dispersion in a suitable liquid (mull) or solid (halide disc).

Mull Triturate 1 to 5 mg of the substance with the minimum amount of *liquid paraffin* or other suitable liquid to give a smooth creamy paste. Compress a portion of the mull between two suitable plates.

Disc Triturate about 1 mg of the substance with approximately 300 mg of dry, finely powdered *potassium bromide IR* or *potassium chloride IR*, as directed. These quantities are usually suitable for a disc 13 mm in diameter. Grind the mixture thoroughly, spread it uniformly in a suitable die and compress under vacuum at a high pressure. Commercial dies are available and the maker's instructions should be followed. Mount the resultant disc in a suitable holder in the spectrophotometer. Several factors, such as inadequate or excessive grinding, moisture or other impurities in the halide carrier, may give rise to unsatisfactory discs. Unless its preparation presents particular difficulties, a disc should be rejected if visual inspection shows lack of uniformity or if the transmittance at about 2000 cm^{-1} (5 μm) in the absence of a specific absorption band is less than 75 per cent without compensation. If the other ingredients of tablets, injections, or other dosage forms are not completely removed from the substance being examined, they may contribute to the spectrum.

GASES Examine gases in a cell transparent to infrared radiation and having an optical path length of about 100 mm. Evacuate the cell and fill to the desired pressure through a stopcock or needle valve using a suitable gas transfer line between the cell and the container of the gas being examined. If necessary, adjust the pressure in the cell to atmospheric pressure using a gas transparent to infrared radiation (for example, *nitrogen* or *argon*). To avoid absorption interferences due to water, carbon dioxide or other atmospheric gases, place in the reference beam an identical cell that is either evacuated or filled with the gas transparent to infrared radiation.

For measurement by diffuse reflectance

SOLIDS Triturate a mixture of the test substance with finely powdered and dried *potassium bromide* or *potassium chloride*. Use a mixture containing approximately

5 per cent of the substance, unless otherwise specified. Grind the mixture, place it in a sample cup and examine the reflectance spectrum. The spectrum of the sample in absorbance mode may be obtained after mathematical treatment of the spectra by the Kubelka-Munk function.

For measurement by attenuated total reflection

Attenuated total reflection (including multiple reflection) involves light being reflected internally by a transmitting medium, typically for a number of reflections. However, several accessories exist where only one reflection occurs. Prepare the substance as follows. Place the test substance in close contact with an internal reflection element (IRE) such as diamond, germanium, zinc selenide, thallium bromide-thallium iodide (KRS-5) or another suitable material of high refractive index. Ensure close and uniform contact between the substance and the whole crystal surface of the internal reflection element, either by applying pressure or by dissolving the substance in an appropriate solvent, then covering the IRE with the obtained solution and evaporating to dryness. Examine the attenuated total reflectance (ATR) spectrum.

Identification by Reference Substances

Prepare the substance being examined and the Reference Substance under the same operational conditions. Some substances are known to exhibit polymorphism which could lead to differences between the two spectra. In such cases, the sample and the reference substance are therefore to be pretreated. The pretreatment is designed to ensure that the substance being examined and the reference substance are isomorphous. Record the spectrum of each from about 4000 to 600 cm^{-1} (2.5 to 16.5 μm) using the same instrumental conditions as were used to demonstrate compliance with the requirement for resolution.

Identification by Reference Spectra

In certain special cases, it may be necessary to use a reference spectrum. The spectrum should be scanned using the same instrumental conditions as were used to demonstrate compliance with the requirement for resolution. To allow for possible differences in wavelength calibration between the instrument on which the reference spectrum was obtained and that on which the spectrum of the substance is to be recorded, suitable reference absorbance maxima of a polystyrene spectrum are superimposed on the reference spectrum. These will normally occur at about 2851 cm^{-1} (3.51 μm), 1601 cm^{-1} (6.25 μm) and 1028 cm^{-1} (9.73 μm), but when there is interference with any of these maxima by a band in the spectrum of the substance being examined, alternative reference maxima will be specified. Similar reference maxima should be superimposed on the spectrum of the substance. With reference to these polystyrene maxima, the positions and relative intensities of the absorbance bands of the substance should be concordant with those of the reference spectrum. When comparing the two spectra, care should be taken to allow for the possibility of differences in resolving power between the instrument on which the reference spectrum was prepared and the instrument being used to examine the substance. A reference spectrum of a polystyrene film recorded on the same instrument as the reference spectrum of the substance is included in the compendium of reference spectra for assessing these differences. It should be noted that the greatest variations due to differences in resolving power are likely to occur in the region of 4000 to 2000 cm^{-1} (2.5 to 5 μm).

NEAR-INFRARED SPECTROPHOTOMETRY

Near-infrared spectrophotometry is a technique particularly useful for identifying organic substances. Although the spectra are restricted to C–H, N–H, O–H and S–H resonances, they usually have a high informative content. However, the spectra depend on a number of parameters such as particle size, polymorphism, residual solvents, humidity which cannot always be controlled.

For this reason, direct comparison of the spectrum obtained with the preparation of sample with the reference spectrum is usually impossible and some suitable validated mathematical treatment of the data is required.

Apparatus

Spectrophotometers for recording spectra in the near-infrared region consist of:

(1) a filter, grating or interferometer system capable of providing the whole range of electromagnetic radiation in the region of about 780 nm to about 2500 nm (12821 cm^{-1} to 4000 cm^{-1});

(2) a means of collecting and measuring the intensity of the transmitted or reflected radiation (transmission or reflection), such as an integration sphere, a fibre optic probe, etc, coupled to an appropriate detector;

(3) a means of mathematical treatment of the spectral data obtained.

Preparation of the Sample

For measurement by transmission This method generally applies to liquids, diluted or undiluted, and to solids in solution. Examine the samples in a cell of suitable path-length (generally 0.5 mm to 4 mm), transparent to near-infrared radiation, or by immersion of a fibre optic probe of a suitable configuration, which yields a spectrum situated in a zone of transmittance compatible with the specifications of the apparatus and appropriate for the intended purpose.

When recording the near-infrared spectrum of a liquid sample, the hazards of temperature dependent perturbations or any other effects of spectral disturbances must be taken into consideration.

In all cases, compensation for background interferences must be made in a manner appropriate to the optical configuration of the apparatus, for example, a reference scan of air (for liquids) or solvent (for solutions) may be subtracted from the sample spectrum.

For measurement by diffuse reflection This method generally applies to solids.

Examine the samples in a suitable device.

When immersing a fibre optic probe in the sample, care must be taken in the positioning of the probe to ensure that it remains stationary during the acquisition of the spectra and that the measuring conditions are as reproducible as possible from one sample to another. In all cases, compensation for background interferences must be made in a manner appropriate to the optical configuration of the instrument, for example, a reference scan of an internal or external reflection standard must be subtracted from the sample spectrum. The particle size and the state of hydration or of solvation must also be taken into consideration.

For measurement by transflection This method generally applies to liquids, diluted or undiluted, and to solids in solution or in suspension. Examine the sample in a cell with a suitable diffuse reflector, made of either metal or of an inert substance (for example titanium oxide), not exhibiting a spectrum in the near-infrared region and introduced at a suitable concentration into the sample. The samples are examined as described above under For measurement by transmission or For measurement by diffuse reflection.

Control of Instrument Performance

Use the apparatus according to the manufacturer's instructions and carry out the prescribed verifications at regular intervals, according to the use of the apparatus and the substances to be tested.

VERIFICATION OF THE WAVELENGTH SCALE (EXCEPT FOR FILTER APPARATUS) Verify the wavelength scale employed, generally in the region between 780 nm and 2500 nm using (a) suitable wavelength standard(s) which has characteristic maxima at the wavelengths under investigation, for example polystyrene or rare-earth oxides.

VERIFICATION OF THE WAVELENGTH REPEATABILITY (EXCEPT FOR FILTER APPARATUS) Verify the wavelength repeatability using (a) suitable standard(s), for example polystyrene or rare-earth oxides. The standard deviation of the wavelengths is consistent with the spectrophotometer specification.

VERIFICATION OF RESPONSE REPEATABILITY Verify the response repeatability using (a) suitable standard(s), for example reflective thermoplastic resins doped with carbon black. The standard deviation of the maxima response is consistent with the spectrophotometer specification.

VERIFICATION OF PHOTOMETRIC NOISE Determine the photometric noise using a suitable reflectance standard, for example white reflective ceramic tiles or reflective thermoplastic resins. Scan the reflection standard in accordance with the spectrophotometer manufacturer's recommendation and calculate the photometric noise, either peak to peak, or for a given wavelength. In the latter case, the photometric noise is represented by the standard deviation of the responses. The photometric noise is consistent with the spectrophotometer specification.

Establishment of a Spectral Reference Library

Record the spectra of a suitable number of batches of the substance which have been fully tested as prescribed in the monograph and which exhibit the variation typical (e.g., manufacturer, particle size,...) of the substance being analyzed. The set of spectra represents the information that defines the similarity border for that substance and is the entry for that substance in the spectral database used to identify the substance. The number of substances in the database depends on the specific application.

The collection of spectra in the database may be represented in different ways defined by the mathematical technique used for identification. These may be:

(1) individual spectra representing the substance;

(2) mean spectra of each substance and a description of the variability.

The selectivity of the database to identify positively a given material and discriminate adequately against other materials in the database is to be established during the validation procedure. This selectivity must be challenged on a regular basis to ensure ongoing validity of the database; this is especially necessary after any major change in a substance, for example: a change of supplier or in the manufacturing process of the material.

This database is then valid for use only on the originating instrument or on a similar instrument provided the transferred database has been demonstrated to remain valid.

Method

Prepare the sample being examined in the same manner as for the establishment of the database. A suitable mathematical transformation of the log (1/T) or log (1/R) spectrum may be calculated for both the sample and the spectral reference library, for example second derivative or multiplicative scatter correction, to facilitate spectral comparison.

Comparison of the transforms of the sample and the spectral reference library involves the use of a suitable chemometric classification technique.

2.2 ULTRAVIOLET AND VISIBLE SPECTROPHOTOMETRY

Absorption spectroscopy in the ultraviolet and visible region is one of the most useful tools for qualitative and quantitative analysis.

When a beam of monochromatic radiation traverses a solution containing an absorbing substance, its radiant power is reduced in relation to the distance that it travels through. It also decreases in relation to the concentration of absorbing molecules or ions encountered in that medium. These two factors determine the proportion of the total incident energy that emerges.

The decrease in power of monochromatic radiation is stated quantitatively by Beer's law:

$$\log (1/T) = A = abc.$$

The terms used in connection with spectrophotometric tests are defined as follows:

Absorbance (A) is the logarithm, to the base 10, of the reciprocal of the transmittance (T). (**Note** Descriptive terms used formerly include optical density, absorbancy and extinction.)

Absorptivity (a) is the quotient of the absorbance (A) per litre, of the substance (c) and the absorption path length in cm (b).

(**Note** It is not to be confused with specific absorbance, specific extinction or extinction coefficient [A (1 per cent, 1 cm) or E (1 per cent, 1 cm)]. These are generally used in different pharmacopoeiae, as the quotient of the absorbance divided by the product of the concentration, expressed in g per 100 ml, of the substance, and the absorption path length in cm, therefore: A (1 per cent, 1 cm) = 10 a.)

Molar absorptivity (ε) is the quotient of the absorbance (A) divided by the product of the concentration, expressed in moles per litre, of the substance (c) and the absorptivity (a) and the molecular weight of the substance. (**Note** Terms formerly used include molar absorbancy index, molar extinction coefficient and molar absorption coefficient.)

Transmittance (T) is the quotient of the radiant power transmitted by a sample (I) divided by the radiant power incident upon the sample (I_0).

Absorption spectrum is a graphic representation of absorbance, or any function of absorbance, plotted against wavelength or function of wavelength.

Where a monograph gives a single value for the position of an absorption maximum, it is understood that the value obtained may differ by not more than ±2 nm.

Apparatus

All types of spectrophotometer are designed to permit substantially monochromatic radiant energy to be passed through the test substance in a suitable form and to allow measurement of the fraction of energy that is transmitted. The spectrophotometer comprises an energy source, a dispersing device with slits for selecting the wavelength band, a cell for holding the test substance, a detector of radiant energy, associated amplifiers, and measuring and recording devices. Some instruments are manually operated, while others are equipped for automatic operation. Instruments are available for use in the visible region of the spectrum, usually 380 nm to about 700 nm, and in the ultraviolet and visible regions of the spectrum, usually 190 nm to about 700 nm.

Both double-beam and single-beam instruments are commercially available and either is suitable. Depending on the type of apparatus used, the results may be displayed on a scale, on a digital counter, or by a recorder or printer.

The apparatus should be maintained in proper working condition. The housing of the optical system should minimize any possibility of errors due to stray light; this is particularly relevant in the short-wave region of the spectrum.

CONTROL OF WAVELENGTHS Verify the wavelength scale using the absorption maxima of Holmium Perchlorate Solution, the line of a hydrogen or deuterium discharge lamp or the lines of a mercury vapour as shown below. The permitted tolerance is ±1 nm for the range 200 to 400 nm and ±3 nm for the range 400 to 600 nm.

241.15 nm (Ho)	404.66 nm (Hg)
253.70 nm (Hg)	435.83 nm (Hg)
287.15 nm (Ho)	486.00 nm (Dβ)
302.25 nm (Hg)	486.10 nm (Hβ)
313.16 nm (Hg)	536.30 nm (Ho)
334.15 nm (Hg)	546.07 nm (Hg)
361.50 nm (Ho)	576.96 nm (Hg)
365.48 nm (Hg)	579.07 nm (Hg)

The wavelength scale may also be calibrated by means of suitable glass filters that have useful absorption bands through the visible and ultraviolet regions. Standard glass containing didymium (a mixture of praseodymium and neodymium) has been widely used. Glass containing holmium is considered superior. The exact values for the position of characteristic maxima in holmium glass filters are 241.5±1, 287.5±1, 360.9±1, and 536.2±3 nm. Holmium glass filters are obtainable from some national institutions and from commercial sources. The performance of an uncertified filter should be checked against one that has been properly certified.

CONTROL OF ABSORBANCE Check the absorbance using Potassium Dichromate Solution UV at the wavelengths indicated in the following table, which gives for each wavelength the exact value of A (1 per cent, 1 cm) and the permitted limits.

Wavelength (nm)	A (1 per cent, 1 cm)	Maximum Tolerance
235	124.5	122.9 to 126.2
257	144.0	142.4 to 145.7
313	48.6	47.0 to 50.3
350	106.6	104.9 to 108.2

LIMIT OF STRAY LIGHT Stray light may be detected at a given wavelength with suitable filters or solutions. For example the absorbance of a 1.2 per cent w/v solution of *potassium chloride* at a path-length of 1 cm should be more than 2 at 200 nm when compared with *water* as reference liquid.

SPECTRAL SLIT WIDTH When measuring the absorbance at an absorption maximum, the spectral slit width must be small compared with the half-width of the absorption band, or erroneously low absorbances will be measured. Particular care is needed for certain

substances and the instrumental slit width used should always be such that further reduction does not result in an increased absorbance reading.

CELLS Cells usually in the spectral range discussed are 1-cm absorption cells with glass or silica windows. Other path lengths may also be used. The cells used for the test solution and the blank should be matched, and must have the same spectral transmittance when containing only the solvent. If this is not the case, an appropriate correction must be applied.

SOLVENTS In measuring the absorbance of a solution at a given wavelength, the absorbance of the solvent cell and its contents shall not exceed 0.4 and is preferably less than 0.2 when measured with reference to air at the same wavelength. The solvent in the solvent cell shall be of the same batch as that used to prepare the solution and must be free from fluorescence at the wavelength of measurement. *Ethanol*, *absolute ethanol*, *methanol* and *cyclohexane UV* used as solvents shall have an absorbance, measured in a 1-cm cell at 240 nm with reference to *water*, not exceeding 0.10. Statements of concentration and thickness of the solution to be used in the determination of light absorption apply to measurements made with photoelectric instruments.

SOLUTIONS FOR USE IN THE CALIBRATION OF WAVELENGTHS AND ABSORBANCE

Holmium Perchlorate Solution Dissolve 40 g of *holmium oxide* in sufficient 1.4 M *perchloric acid* to produce 1000 ml.

Potassium Dichromate Solution UV Dry a quantity of *potassium dichromate* by heating to constant weight at 130°. Weigh accurately a quantity not less than 57.0 mg and not more than 63.0 mg and dissolve it in sufficient 0.005 M *sulfuric acid* to produce 1000.0 ml.

Determination of Absorbance

Unless otherwise prescribed, measure the *absorbance*, A, at the prescribed wavelength using a path-length of 1 cm, and the measurements are carried out with reference to the solvent used to prepare the solution being examined. In certain cases measurements are carried out with reference to a mixture of reagents, details of which are prescribed in the monograph.

When the absorbance is being measured for a quantitative determination, for example, an assay or a limit test, a manually scanning instrument is used. In tests for identification, it is more convenient to use a recording instrument and the concentration of the solution and the path-length are specified accordingly. If these conditions are not appropriate for a particular instrument, the thickness and the concentration of the solution should be varied. A statement in an assay or test of the wavelength at which maximum absorption occurs implies the maximum occurring either precisely at or in the vicinity of the given wavelength.

When an assay or test prescribes the use of a reference substance, the spectrophotometric measurements are made first with the solution prepared from the reference substance and second with the corresponding solution prepared from the substance being examined. The second measurement is carried out as quickly as possible after the first, using the matched cell and the same experimental conditions.

The requirements for light absorption in the Pharmacopoeia apply to the dried, anhydrous, or solvent-free material in all those monographs in which standards for loss on drying, water or solvent content are given. In calculating the result, the loss on drying, water, or solvent content determined by the method specified in the monograph is used.

SECOND DERIVATIVE SPECTROPHOTOMETRY

Derivative spectrophotometry involves the transformation of absorption spectra (zero order) into first, second or higher order derivative spectra. A first derivative spectrum is a plot of the gradient of the absorption curve (rate of change of the absorbance with wavelength, $dA/d\lambda$) against wavelength. A second derivative spectrum is a plot of the curvature of the absorption spectrum ($d^2A/d\lambda^2$) against wavelength. If the absorbance follows the Beer-Lambert relationship, the second derivative at any wavelength, λ, is related to concentration by the following equation:

$$\frac{d^2A}{d\lambda^2} = \frac{d^2A(1\%, 1\ cm)}{d\lambda^2} \times cd$$

where A = the absorbance at wavelength l,

$A\ (1\%, 1\ cm)$ = the specific absorbance at wavelength l,

c = the concentration of the absorbing solute expressed as a percentage w/v, and

d = the thickness of the absorbing layer in cm.

Apparatus

A spectrophotometer complying with the requirements prescribed above and equipped with an analogue resistance-capacitance differentiation module or a digital differentiator or another means of producing second derivative spectra should be used in accordance with the manufacturer's instructions. Some methods of producing second derivative spectra lead to a wavelength shift relative to the zero order spectrum and this should be taken into account, when necessary. Unless otherwise stated in the monograph, the spectral slit width of the spectrophotometer, where variable, should be set as described under Spectral slit width above. The cells used should comply with the statements given under the heading Cells.

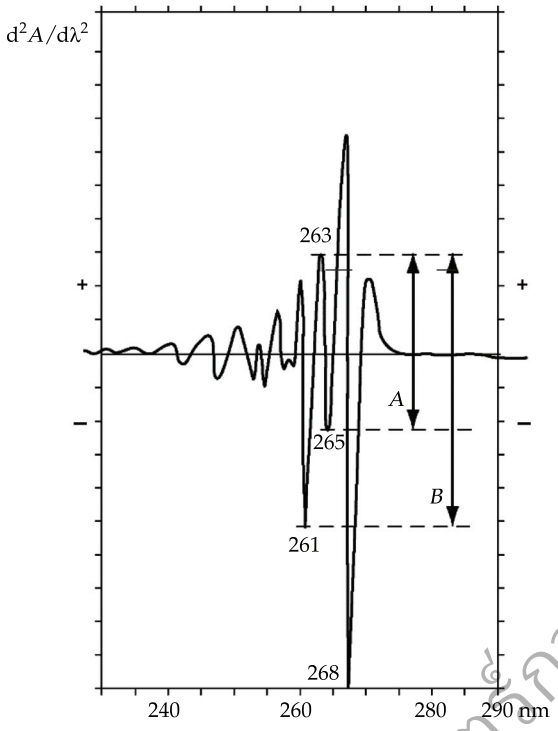
Resolution

When prescribed in a monograph, record the second derivative spectrum in the range 255 to 275 nm of a 0.020 per cent v/v solution of *toluene* in *methanol* using *methanol* in the reference cell. The spectrum shows a small negative extremum (or trough) located between two large negative extrema at 261 nm and 268 nm, respectively as shown in the figure. Unless otherwise

prescribed in the monograph, the ratio A/B (see the figure) is not less than 0.2.

Procedure

Prepare a solution of the substance being examined, adjust the various instrument settings according to the manufacturer's instructions and calculate the amount of the substance being determined as prescribed in the monograph.



2.3 ATOMIC SPECTROMETRY: EMISSION AND ABSORPTION

These techniques are used to determine the concentration of certain metallic ions by measuring the intensity of emission or absorption of light at a particular wavelength by the atomic vapour of the element generated from the substance, for example, by introducing a solution of the substance into a flame.

FOR ATOMIC EMISSION SPECTROMETRY

Atomic emission spectrometry is a method for determining the concentration of an element in a substance by measuring the intensity of one of the emission lines of the atomic vapour of the element generated from the substance. The determination is carried out at the wavelength corresponding to this emission line.

Apparatus

This consists essentially of an atomic generator of the element being determined (flame, plasma, arc, etc), a monochromator and a detector. If the generator is a flame, water is the solvent of choice for preparing test and standard preparations, although organic solvents may also be used if precautions are taken to ensure that the solvent does not interfere with the stability of the flame.

Method

Operate an atomic emission spectrometer in accordance with the manufacturer's instructions at the prescribed wavelength setting. Introduce a blank solution into the atomic generator and just the instrument reading to zero. Introduce the most concentrated standard preparation and adjust the sensitivity to give a suitable reading.

Determinations are made by comparison with standard preparations with known concentrations of the element to be determined either by the method of direct calibration (Method I) or the method of standard additions (Method II).

Use Method I unless otherwise directed.

METHOD I: METHOD OF DIRECT CALIBRATION

Prepare the solution of the substance to be examined (test preparation) as prescribed. Prepare not less than three standard preparations of the element being determined the concentrations of which span the expected value in the test preparation. Any reagents used in the preparation of the test preparation are added to the standard preparations in the same concentration. Introduce the test preparation and each standard preparation into the instrument at least three times and record the steady reading. Rinse the apparatus with blank solution each time and ascertain that the reading returns to its initial blank value. Prepare a calibration curve from the mean of the readings obtained with the standard preparations and determine the concentration of the element in the test preparation from the curve so obtained.

METHOD II: METHOD OF STANDARD ADDITION

Add to at least three similar volumetric flasks equal volumes of the solution of the substance being examined (test preparation) prepared as prescribed. Add to all but one of the flasks progressively larger volumes of a standard preparation containing a known concentration of the element to be determined to produce a series of solutions containing increasing concentrations of that element known to give responses in the linear part of the curve. Dilute the contents of each flask to volume with solvent.

Introduce each of the solutions into the instrument at least three times and record the steady reading. Rinse the apparatus with solvent each time and ascertain that the reading returns to its initial blank value. Calculate the linear equation of the graph using a least-squares fit and derive from it the concentration of the element being determined in the test preparation. Alternatively, plot on a graph the mean of readings against the added quantity of the element being determined. Extrapolate the line joining the points on the graph until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of the element to be determined in the test preparation.

If a solid sampling technique is required, full details of the procedure to be followed are provided in the monograph.

FOR ATOMIC ABSORPTION SPECTROMETRY

Atomic absorption spectrometry is a method for determining the concentration of an element in a substance by measuring the absorption of radiation by atomic vapour of the element generated from the substance. The determination is carried out at the wavelength of one of the absorption lines of the element concerned.

Apparatus

This consists essentially of a source of radiation, an atomic generator of the element to be determined (flame, furnace, etc), a monochromator and a detector.

The method of introducing the substance to be analyzed depends on the type of atomic generator used. If it is flame, substances are nebulized and *water* is the solvent of choice for preparing test and standard preparations although organic solvents may also be used if precautions are taken to ensure that the solvent does not interfere with the stability of the flame. When a furnace is used, substances may be introduced dissolved in *water* or an organic solvent, but with this technique solid sampling is also possible.

The atomic vapour may also be generated outside the spectrometer, for example, the cold vapour method for mercury of certain hydrides. For mercury, atoms are generated by chemical reduction and the atomic vapour is swept by a stream of an inert gas into an adsorption cell mounted in the optical path of the instrument. Hydrides are either mixed with the gas feeding the burner or swept by an inert gas into a heated cell in which they are dissociated into atoms.

Method

Operate an atomic absorption spectrometer in accordance with the manufacturer's instructions at the prescribed wavelength setting. Introduce a blank solution into the atomic generator and adjust the instrument reading so that it indicates maximum transmission. Introduce the most concentrated standard preparation and adjust the sensitivity to obtain a suitable absorbance reading.

Determinations are made by comparison with reference solutions with known concentrations of the element being determined either by the method of direct calibration (Method I) or the method of standard additions (Method II).

Use Method I unless otherwise directed.

METHOD I: METHOD OF DIRECT CALIBRATION

Prepare the solution of the substance being examined (test preparation) as prescribed. Prepare not fewer than three standard preparations of the element to be determined the concentrations of which span the expected value in the test preparation. Any reagents used in the preparation of the test preparation are added to the standard and blank solutions at the same concentration. Introduce the test preparation and each standard preparation into the instrument at least three times and record the steady reading. Rinse the apparatus with blank solution each time and ascertain that the reading returns to its initial blank value. If a furnace is being used, it is fired between readings.

Prepare a calibration curve from the mean of the readings obtained with the standard preparations and determine the concentration of the element in the test preparation from the curve so obtained. If a solid sampling technique is required, full details of the procedure to be followed are provided in the monograph.

METHOD II: METHOD OF STANDARD ADDITION

Add to at least three similar volumetric flasks equal volumes of the solution of the substance being examined (test preparation) prepared as prescribed. Add to all but one of the flasks progressively larger volumes of a standard preparation containing a known concentration of the element being determined to produce a series of solutions containing increasing concentrations of that element known to give responses in the linear part of the curve. Dilute the contents of each flask to volume with solvent.

Introduce each of the solutions into the instrument at least three times and record the steady reading. Rinse the apparatus with solvent each time and ascertain that the reading returns to its initial blank value. If a furnace is being used, it is fired between readings.

Calculate the linear equation of the graph using a least-squares fit and derive from it the concentration of the element to be determined in the test preparation. Alternatively, plot on a graph the mean of readings against the added quantity of the element to be determined. Extrapolate the line joining the points on the graph until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of the element being determined in the test preparation.

If a solid sampling technique is required, full details of the procedure to be followed are provided in the monograph.

2.4 FLUORESCENCE SPECTROPHOTOMETRY

Fluorescence spectrophotometry is the measurement of the emission of light from a chemical substance while it is being exposed to ultraviolet, visible, or other electromagnetic radiation. In general, the light emitted by a fluorescent solution is of maximum intensity at a wavelength longer than that of the exciting radiation, usually by some 20 to 30 nm.

The terms used with fluorescence spectrophotometry are defined as follows:

Fluorescence intensity is an empirical expression of fluorescence activity, commonly given in terms of arbitrary units proportional to detector response.

The *fluorescence emission spectrum* is the relationship between the intensity of the emitted radiation and the

wavelength and is frequently represented in a graphic form.

The *fluorescence excitation spectrum* is the relationship between the maximum intensity of radiation emitted by an activated substance and the wavelength of the incident radiation and is frequently represented in a graphic form.

Apparatus

Measurement of fluorescence intensity can be made with a simple filter fluorimeter. Such an instrument consists of a radiation source, a primary filter, a sample chamber, a secondary filter, and a fluorescence detection system. In most such fluorimeters the detector is placed on an axis at 90° from that of the incident beam. This right-angle geometry permits the incident radiation to pass through the test solution without contaminating the output signal received by the fluorescence detector. Filters are used to eliminate the incident radiation as a result of the inherent scattering properties of the solutions themselves, or if dust or other solids are present. The primary filter selects short wavelength radiation capable of causing excitation of the test substance, while the secondary filter is normally a sharp cut-off filter that allows the longer wavelength fluorescence to be transmitted but blocks the scattered excited radiation.

A fluorescence spectrophotometer differs from a filter fluorimeter in that filters are replaced by monochromators, of either the prism or the grating type.

The cells used in fluorescence measurements may be rectangular cells similar to those used in absorption spectrophotometers, except that they are polished on all 4 vertical sides and on the bottom, or cells in the shape of round tubes with flat polished bottoms may be used. A convenient size is 2 to 3 ml, but some instruments can be fitted with small cells holding 0.1 to 0.3 ml, or with a capillary holder requiring even less solution.

STANDARDIZATION Fluorimeters and fluorescence spectrophotometers should be standardized daily with a stable fluorophore to assure proper reproducibility of response. The changes are due to instrumental variables such as differences in lamp intensity and photomultiplier sensitivity. The fluorophore may be a pure specimen of the fluorescent substance under test or another readily purified fluorescent substance with absorption and fluorescence bands similar to those of the test substance. Quinine in dilute sulfuric acid is often a convenient fluorophore for blue fluorescence, sodium fluorescein for green fluorescence, and rhodamine for red fluorescence.

The wavelength scale of the fluorescence spectrophotometer should be periodically calibrated.

METHOD Dissolve the substance to be examined in the solvent or mixture of solvents prescribed in the monograph. Solutions prepared for fluorescence spectrophotometry are usually 10 to 100 times less concentrated than those used in absorption spectrophotometry. Transfer the solution to the cell or the tube of the fluorimeter and illuminate with an excitant light beam of the wavelength prescribed in the monograph and as nearly monochromatic as possible.

Measure the intensity of the emitted light at an angle of 90° to the excitant beam, after passing it through a filter which transmits predominantly light of the wavelength of the fluorescence.

For quantitative determinations, first introduce into the apparatus the solvent or mixture of solvents used to dissolve the substance to be examined and set the instrument to zero. Introduce the standard solution and adjust the sensitivity of the instrument so that the reading is greater than 50. If the second adjustment is made by altering the width of the slits, a new zero setting must be made and the intensity of the standard must be measured again. Finally introduce the solution of the substance being examined, record the intensity and calculate its concentration.

If the intensity of the fluorescence is not directly proportional to the concentration, the measurement may be effected using a calibration curve.

In some cases, measurement can be made with reference to a fixed standard (for example, a fluorescent glass or a solution of another fluorescent substance). In such cases, the concentration of the substance to be examined must be determined using a previously drawn calibration curve under the same conditions.

2.5 TURBIDIMETRY AND NEPHELOMETRY

Turbidimetry is the measurement of the degree of attenuation of a radiant beam incident on particles suspended in a medium, the measurement being made in the directly transmitted beam. It may be measured with a standard photoelectric filter photometer or spectrophotometer with illumination at an appropriate wavelength.

Nephelometry is the measurement of the light scattered by suspended particles, the measurement usually being made perpendicularly to the incident beam.

Turbidimetry or nephelometry may be useful for the measurement of precipitates formed by the interaction of very dilute solutions of reagents, or other particulate matter, such as suspensions of bacterial cells. In order that consistent results may be achieved, all variables must be carefully controlled. Problems due to birefringence may be encountered particularly with bacterial cells. Where proper control is possible, extremely dilute suspensions may be measured.

The terms used with turbidimetry and nephelometry are defined as follows:

Transmittance (T) is the ratio of the radiant flux transmitted by the test substance to that of the incident radiant flux. Terms formerly used include transmittancy and transmission.

Turbidance (S) is the measure of the light-scattering effect of suspended particles. The amount of suspended

matter may be measured by observation of either the transmitted light (turbidimetry) or the scattered light (nephelometry).

Turbidity (τ) is the measure of the decrease in incident beam intensity per unit length of a given suspension in light-scattering measurements.

Apparatus

Turbidity may be measured with a standard photoelectric filter photometer or spectrophotometer, preferably with illumination in the red-orange region of the spectrum (for example, by using a blue filter).

Nephelometric measurements require an instrument with a photocell placed so as to receive scattered rather than transmitted light; as this geometry applies also to fluorimeters in general, the latter can be used as nephelometers by proper selection of filters.

INSTRUMENTAL MEASUREMENT For instrumental measurement, it is advisable to ensure that settling of the particles being measured will be negligible. This is usually accomplished by including a protective colloid in the liquid suspending medium. It is important that results be interpreted by comparison of readings with those representing known concentrations of suspended matter, produced under exactly the same conditons.

VISUAL COMPARISON Carry out turbidity comparison in tubes that are matched as closely as possible in internal diameter and in all other respects. Flat-bottomed comparison tubes of transparent glass of about 70-ml capacity and about 23-mm internal diameter are suitable. For turbidity comparison, the tubes should be viewed horizontally, against a dark background, with the aid of a light source directed from the sides of the tubes.

2.6 RAMAN SPECTROMETRY

Raman spectrometry (inelastic light scattering) is a light-scattering process in which the specimen under examination is irradiated with intense monochromatic light (usually laser light) and the light scattered from the specimen is analyzed for frequency shifts.

Raman spectrometry is complementary to infrared spectrometry in the sense that the two techniques both probe the molecular vibrations in a material. However, Raman and infrared spectrometry have different relative sensitivities for different functional groups. Raman spectrometry is particularly sensitive to nonpolar bonds (e.g., C–C single or multiple bonds) and less sensitive to polar bonds. Hence, water, which has a strong infrared absorption spectrum, is a weak Raman scatterer and is thus well suited as a solvent for Raman spectrometry.

Apparatus

Spectrometers for recording Raman spectra typically consist of the following components:

(1) a monochromatic light source, typically a laser, with a wavelength in the ultraviolet, visible or nearinfrared region;

(2) suitable optics (lens, mirrors or optical-fibre assembly) which directs the irradiating light to and collects the scattered light from the sample;

(3) an optical device (monochromator or filter) that transmits the frequency-shifted Raman scattering and prevents the intense incident frequency (Rayleigh scattering) from reaching the detector;

(4) a dispersing device (grating or prism monochromator) combined with wavelength-selecting slits and a detector (usually a photomultiplier tube); or a dispersing device (grating or prism) combined with a multichannel detector (usually a charge-coupled device, CCD); an interferometer with a detector that records the intensity of the scattered light over time, and a data-handling device that converts the data to the frequency or wavenumber domain by a Fourier-transform calculation.

Preparation of the Sample

Raman spectra can be obtained from solids, liquids and gases either directly, or in glass containers or tubes, generally without prior sample preparation or dilution.

A major limitation of Raman spectrometry is that impurities may cause fluorescence that interferes with the detection of the much weaker Raman signal. Fluorescence may be avoided by choosing a laser source with a longer wavelength, for example in the near infrared, as the exciting line. The intensity of certain Raman lines may be enhanced in a number of ways, for instance in Resonance Raman (RR) and by Surface Enhanced Raman Spectrometry (SERS). Due to the narrow focus of the irradiating laser beam, the spectrum is typically obtained from only a few microlitres of sample. Hence, the sample in homogeneities must be considered, unless the sample volume is increased, for example by rotation of the sample.

Identification and Quantitation Using Reference Substances

Prepare the substance to be examined and the reference substance by the same procedure and record the spectra under the same operational conditions. The maxima in the spectrum obtained with the substance to be examined correspond in position and relative intensity to those in the spectrum obtained with the reference substance (RS).

When the spectra recorded in the solid state show differences in the positions of the maxima, treat the substance to be examined and the reference substance in the same manner so that they crystallize or are produced in the same form, or proceed as described in the monograph, and then record the spectra.

While Beer-Lambert's law is not valid for Raman spectrometry, Raman intensity is directly proportional to the concentration of the scattering species. As for other spectroscopic techniques, quantitation can be performed using known amounts or concentrations of reference substances. Owing to the small spatial resolution of the technique, care must be taken to ensure representative samples of standards and unknowns, for

example by making sure that they are in the same physical state or by using an internal standard for liquid samples.

Identification and Quantitation Using Spectral Libraries and Statistical Methods for Classification and Calibration

CONTROL OF INSTRUMENT PERFORMANCE Use the apparatus according to the manufacturer's instructions and carry out the prescribed calibrations and system performance tests at regular intervals, depending on the use of the apparatus and the substances to be examined. When using Raman spectrometry for quantitative determinations, or when setting up spectral reference libraries for (chemometric) classification or calibration, particular care should be taken to ensure that corrections are made or measures are taken to control the variability in wavenumber and response-intensity of the instrumentation.

VERIFICATION OF THE WAVENUMBER SCALE Verify the wavenumber scale of the Raman shift (normally expressed in reciprocal centimetres) using a suitable standard which has characteristic maxima at the wavenumbers under investigation, for example, an organic substance, an Ne lamp or Ar^+ plasma lines from an argon-ion laser. The calibration measurement should be matched to the sample type, i.e. a solid calibration sample should be used for solid samples and a liquid calibration sample for liquid samples. Choose a suitable substance (e.g., indene, cyclohexane or naphthalene) for which accurate wavenumber shifts have been established. The indene sample can favourably be placed in an NMR tube, evacuated and sealed under inert gas, and stored cool in the dark to avoid degradation of the sample.

Wavenumber, Shifts (and Acceptable Tolerances) of Cyclohexane, Indene and Naphthalene

Cyclohexane*	Indene**	Naphthalene*
		3056.4 (±1.5)
2938.3 (±1.5)		
2923.8 (±1.5)		
2852.9 (±1.5)		
	1609.7 (±1.0)	1576.6 (±1.0)
1444.4 (±1.0)	1552.6 (±1.0)	1464.5 (±1.0)
1266.4 (±1.0)	1205.2 (±1.0)	1382.2 (±1.0)
1157.6 (±1.0)		1147.2 (±1.0)
1028.3 (±1.0)	1018.6 (±1.0)	1021.6 (±1.0)
801.3 (±1.0)	730.5 (±1.0)	763.8 (±1.0)
	533.9 (±1.0)	513.8 (±1.0)

*Standard guide for Raman shift standards for spectrometer calibration (American Society for Testing and Materials ASTM E 1840).
**D. A. Carter, W. R. Thompson, C. E. Taylor and J. E. "Pemberton", *Applied Spectroscopy*, *49*(11), 1995, pp. 1561-1576.

VERIFICATION OF THE RESPONSE-INTENSITY SCALE The absolute and relative intensities of the Raman bands are affected by several factors including:

(1) the state of polarization of the irradiating light;
(2) the state of polarization of the collection optic;
(3) the intensity of the irradiating light;
(4) differences in instrument response;
(5) differences in focus and geometry at sample;
(6) differences in packing density for solid samples.

Appropriate acceptance criteria will vary with the application but a day-to-day variation of 10 per cent in relative band intensities is achievable in most cases.

ESTABLISHMENT OF A SPECTRAL REFERENCE LIBRARY Record the spectra of a suitable number of materials which have been fully tested (e.g., as prescribed in a monograph) and which exhibit the variation (manufacturer, batch, crystal modification, particle size, etc.) typical of the material to be analyzed. The set of spectra represents the information that defines the similarity border or quantitative limits, which may be used, e.g. to identify the substance or control the amount formed in a manufacturing process. The number of substances in the database depends on the specific application. The collection of spectra in the database may be represented in different ways defined by the mathematical technique used for classification or quantitation.

The selectivity of the database which makes it possible to identify positively a given material and distinguish it adequately from other materials in the database is to be established during the validation procedure. This selectivity must be challenged on a regular basis to ensure ongoing validity of the database; this is especially necessary after any major change in a substance (e.g., change in a supplier or in the manufacturing process of the material) or in the set-up of the Raman instrument (e.g., verification of the wavenumber and response repeatability of the spectrometer).

This database is then valid for use only with the originating instrument, or with a similar instrument, provided the transferred database has been demonstrated to remain valid.

METHOD Prepare and examine the sample in the same manner as for the establishment of the database. A suitable mathematical transformation of the Raman spectrum may be calculated to facilitate spectrum comparison or quantitative prediction.

Comparison of the spectra or transforms of the spectra or quantitative prediction of properties or amounts in the material in question may involve the use of a suitable chemometric or statistical classification or calibration technique.

2.7 X-RAY FLUORESCENCE SPECTROMETRY

Wavelength dispersive X-ray fluorescence spectrometry is a procedure that uses the measurement of the intensity of the fluorescent radiation emitted by an element having an atomic weight between 11 and 92 excited by a continuous primary X-ray radiation. The intensity of the fluorescence produced by a given element depends on the concentration of this element in

the sample but also on the absorption by the matrix of the incident and fluorescent radiation. At trace levels, where the calibration curve is linear, the intensity of the fluorescent radiation emitted by an element in a given matrix, at a given wavelength, is proportional to the concentration of this element and inversely proportional to the mass absorption coefficient of the matrix at this wavelength.

METHOD Set and use the instrument in accordance with the instructions given by the manufacturer. Liquid samples are placed directly in the instrument; solid samples are first compressed into pellets, sometimes after mixing with a suitable binder.

To determine the concentration of an element in a sample, it is necessary to measure the net impulse rate produced by one or several standard preparations containing known amounts of this element in given matrices and to calculate or measure the weight absorption coefficient of the matrix of the sample being analyzed.

CALIBRATION From a calibration solution or a series of dilutions of the element to be analyzed in various matrices, determine the slope of the calibration curve, b_0, from the following equation:

$$b_0 \frac{1}{\mu_M} = \frac{I_C^N}{C},$$

where μ_M = absorption coefficient of the matrix, calulated or measured,
I_C^N = net impulse rate, and
C = concentration of the element being assayed in the standard preparation.

WEIGHT ABSORPTION COEFFICIENT OF THE MATRIX OF THE SAMPLE If the empirical formula of the sample being analyzed is known, calculate its weight absorption coefficient from the known elemental composition and the tabulated elemental weight absorption coefficients. If the elemental composition is unknown, determine the weight absorption coefficient of the sample matrix by measuring the intensity of the scattered X-radiation IU (Compton scattering) from the following equation:

$$\frac{1}{\mu_{MP}} = a + bI_U$$

where μ_{MP} = weight absorption coefficient of the sample, and
I_U = scattered X-radiation.

DETERMINATION OF THE NET IMPULSE RATE OF THE ELEMENT TO BE DETERMINED IN THE SAMPLE Calculate the net impulse rate of the element being determined from the measured intensity of the fluorescence line and the intensity of the background line(s), allowing for any tube contaminants present.

CALCULATION OF THE TRACE CONTENT If the concentration of the element is in the linear part of the calibration curve, it can be calculated using the following equation:

$$C = \frac{I_{EP}^N}{b_0 \frac{1}{\mu_{MP}}} \times f,$$

where f = dilution factor.

2.8 MASS SPECTROMETRY

Mass spectrometry is based on the direct measurement of the ratio of the mass to the number of positive or negative elementary charges of ions (m/z) in the gas phase obtained from the substance being analyzed. This ratio is expressed in atomic mass units (1 a.m.u = one twelfth the mass of ^{12}C) or in daltons (1 Da = the mass of the hydrogen atom).

The ions, produced in the ion source of the apparatus, are accelerated and then separated by the analyzer before reaching the detector. All of these operations take place in a chamber where a pumping system maintains a vacuum of 10^{-3} to 10^{-6} Pa.

The resulting spectrum shows the relative abundance of the various ionic species present as a function of m/z. The signal corresponding to an ion will be represented by several peaks corresponding to the statistical distribution of the various isotopes of that ion. This pattern is called the isotopic profile and (at least for small molecules) the peak representing the most abundant isotopes for each atom is called the monoisotopic peak.

Information obtained in mass spectrometry is essentially qualitative (determination of the molecular mass, information on the structure from the fragments observed) or quantitative (using internal or external standards) with limits of detection ranging from the picomole to the femtomole.

Introduction of the Sample

The very first step of an analysis is the introduction of the sample into the apparatus without overly disturbing the vacuum. In a common method, called direct liquid introduction, the sample is placed on the end of a cylindrical rod (in a quartz crucible, on a filament or on a metal surface). This rod is introduced into the spectrometer after passing through a vacuum lock where a primary intermediate vacuum is maintained between atmospheric pressure and the secondary vacuum of the apparatus.

Other introduction systems allow the components of a mixture to be analyzed as they are separated by an appropriate apparatus connected to the mass spectrometer.

GAS CHROMATOGRAPHY/MASS SPECTROMETRY The use of suitable columns (capillary or semi-capillary) allows the end of the column to be introduced directly into the source of the apparatus without using a separator.

LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY This combination is particularly useful for the analysis of

polar compounds, which are insufficiently volatiole or too heat-labile to be analyzed by gas chromatography coupled with mass spectrometry. This method is complicated by the difficulty of obtaining ions in the gas phase from a liquid phase, which requires very special interfaces such as:

(1) DIRECT LIQUID INTRODUCTION: the mobile phase is nebulized, and the solvent is evaporated in front of the ion source of the apparatus;

(2) PARTICLE-BEAM INTERFACE: the mobile phase, which may flow at a rate of up to 0.6 ml per minute, is nebulized in a desolvation chamber such that only the analytes, in neutral form, reach the ion source of the apparatus; this technique is used for compounds of relatively low polarity with molecular masses of less than 1000 Da;

(3) MOVING-BELT INTERFACE: the mobile phase, which may flow at a rate of up to 1 ml per minute; is applied to the surface of a moving belt; after the solvent evaporates, the components to be analyzed are successively carried to the ion source of the apparatus where they are ionized; this technique is rather poorly suited to very polar or heat-labile compounds.

Other types of coupling (electrospray, thermospray, atmospheric-pressure chemical ionization) are considered to be ionization techniques in their own right and are described in the section on modes of ionization.

SUPERCRITICAL FLUID CHROMATOGRAPHY/MASS SPECTROMETRY The mobile phase, usually consisting of supercritical carbon dioxide enters the gas state after passing a heated restrictor between the column and the ion source.

CAPILLARY ELECTROPHORESIS/MASS SPECTROMETRY The eluent is introduced into the ion source, in some cases after adding another solvent so that flow rates of the order of a few microlitres per minute can be attained. This technique is limited by the small quantities of sample introduced and the need to use volatile buffers.

Modes of Ionization

ELECTRON IMPACT The sample, in the gas state, is ionized by a beam of electrons whose energy (usually 70 eV) is more than the ionization energy of the sample. In addition to the molecular ion M^+, fragments characteristic of the molecular structure are observed. This technique is limited mainly by the need to vaporize the sample. This makes it unsuited to polar, heat-labile or high molecular mass compounds. Electron impact is compatible with the coupling of gas chromatography to mass spectrometry and sometimes with the use of liquid chromatography.

CHEMICAL IONIZATION This type of ionization involves a reagent gas such as methane, ammonia, nitrogen oxide, nitrogen dioxide or oxygen. The spectrum is characterized by ions of the $(M + H)^+$ or $(M - H)^-$ types, or adduct ions formed from the analyte and the gas used. Fewer fragments are produced than with electron impact. A variant of this technique is used when the substance is heat-labile: the sample, applied to a filament, is very rapidly vaporized by the Joule-Thomson effect (desorption chemical ionization).

FAST-ATOM BOMBARDMENT (FAB) OR FAST-ION BOMBARDMENT IONIZATION (LIQUID SECONDARY-ION MASS SPECTROMETRY, LSIMS) The sample, dissolved in a viscous matrix such as glycerol, is applied to a metal surface and ionized by a beam of neutral atoms such as argon or xenon or high-kinetic-energy caesium ions. Ions of the $(M + H)^+$ or $(M - H)^-$ types or adduct ions formed from the matrix or the sample are produced. This type of ionization, well suited to polar and heatlabile compounds, allows molecular masses of up to 10,000 Da to be obtained. The technique can be combined with liquid chromatography by adding 1 to 2 per cent of glycerol to the mobile phase; however, the flow rates must be very low (a few microlitres per minute). These ionization techniques also allow thinlayer chromatography plates to be analyzed by applying a thin layer of matrix to the surface of these plates.

FIELD DESORPTION AND FIELD IONIZATION The sample is vaporized near a tungsten filament covered with microneedles (field ionization) or applied to this filament (field desorption). A voltage of about 10 kV, applied between this filament and a counter-electrode, ionizes the sample. These two techniques mainly produce molecular ions M^+ and $(M + H)^+$ ions and are used for low polarity and/or heat-labile compounds.

MATRIX-ASSISTED LASER DESORPTION IONIZATION (MALDI) The sample, in a suitable matrix and deposited on a metal support, is ionized by a pulsed laser beam whose wavelength may range from UV to IR (impulses lasting from a picosecond to a few nanoseconds). This mode of ionization plays an essential role in the analysis of very high molecular mass compounds (more than 100,000 Da) but is limited to time-of-flight analyzers (see below).

ELECTROSPRAY This mode of ionization is carried out at atmospheric pressure. The samples, in solutions, are introduced into the source through a capillary tube, the end of which has a potential of the order of 5 kV. A gas can be used to facilitate nebulization. Desolvation of the resulting microdroplets produces singly or multiply charged ions in the gas phase. The flow rates vary from a few microlitres per minute to 1 ml per minute. This technique is suited to polar compounds and to the investigation of biomolecules with molecular masses of up to 100,000 Da. It can be coupled to liquid chromatography or capillary electrophoresis.

ATMOSPHERIC-PRESSURE CHEMICAL IONIZATION (APCI) Ionization is carried out at the atmospheric pressure by the action of an electrode maintained at a potential of several kilovolts and placed in the path of the mobile phase, which is nebulized both by thermal effects and by the use of a stream of nitrogen. The resulting ions carry a single charge and are of the $(M + H)^+$ type in the positive mode and of the $(M - H)^-$ types in the negative

mode. The high flow rates that can be used with this mode of ionization (up to 2 ml per min) make this an ideal technique for coupling to liquid chromatography.

THERMOSPRAY The sample, in the mobile phase consisting of water and organic modifiers and containing a volatile electrolyte (generally ammonium acetate), is introduced in nebulized form after having passed through a metal capillary tube at a controlled temperature. Acceptable flow rates are of the order of 1 ml per minute to 2 ml per minute. The ions of the electrolyte ionize the compounds to be analyzed. This ionization process may be replaced or enhanced by an electrical discharge of a bout 800 volts, notably when the solvents are entirely organic. This technique is compatible with the used of liquid chromatography coupled with mass spectrometry.

Analyzers

Differences in the performance of analyzers depend mainly on two parameters:

(1) the range over which m/z ratios can be measured, i.e. the mass range;

(2) their resolving power characterized by the ability to separate two ions of equal intensity with m/z ratios differing by ΔM, and whose overlap is expressed as a give percentage of valley definition; for example, a resolving power (M/ΔM) of 1000 with 10 per cent valley definition allows the separation of m/z ratios of 1000 and 1001 with the intensity returning to 10 per cent a above baseline. However, the resolving power may in some cases (time-of-flight analyzers, quadrupoles, iontrap analyzers) be defined as the ratio between the molecular mass and peak width at half height (50 per cent valley definition).

MAGNETIC AND ELECTROSTATIC ANALYZERS The ions produced in the ion source are accelerated by a voltage V, and focused towards a magnetic analyzer (magnetic field B) or an electrostatic analyzer (electrostatic field E), depending on the configuration of the instrument. They follow a trajectory of radius r according to Laplace's law:

$$m/z = \frac{B^2 r^2}{2V}$$

Two types of scans can be used to collect and measure the various ions produced by the ion source: a scan of B holding V fixed or a scan of V with constant B. The magnetic analyzer is usually followed by an electric sector that acts as a kinetic energy filter and allows the resolving power of the instrument to be increased appreciably. The maximum resolving power of such an instrument (double sector) ranges from 10,000 to 150,000 and in most cases allows the value of m/z ratios to be calculated accurately enough to determine the elemental composition of the corresponding ions. For monocharged ions, the mass range is from 2000 Da to 15,000 Da. Some ions may decompose spontaneously (metastable transitions) or by colliding with a gas (collision-activated dissociation, CAD) in field-free regions between the ion source and the detector. Examination of these decompostitions is very useful for the determination of the structure as well as the characterization of a specific compound in a mixture and involves tandem mass spectrometry. There are many such techniques depending on the region where these decompositions occur:

(1) daughter-ion mode (determination of the decomposition ions of a given parent-ion): B/E = constant, *MIKES* (Mass-analyzed Ion Kinetic Energy Spectroscopy);

(2) parent-ion mode (determination of all ions which by decomposition give an ion with a specific m/z ratio): B^2/E = constant;

(3) neutral-loss mode (detection of all the ions that lose the same fragment):

$$\frac{B}{E(1-E/E_0)^{1/2}} = \text{constant},$$

where E_0 is the basic voltage of the electric sector.

QUADRUPOLES The analyzer consists of four parallel metal rods, which are cylindrical or hyperbolic in cross-section. They are arranged symmetrically with respect to the trajectory of the ions; the pairs diagonally opposed about the axis of symmetry of rods are connected electrically. The potentials to the two pairs of rods are opposed. They are the resultant of a constant component and an alternating component. The ions produced at the ion source are transmitted and separated by varying the voltages applied to the rods so that the ratio of continuous voltage to alternating voltage remains constant. The quadrupoles usually have a mass range of 1 a.m.u. to 2000 a.m.u., but some may range up to 4000 a.m.u. Although they have a lower resolving power than magnetic sector analyzers, they nevertheless allow the monoisotopic profile of single charged ions to be obtained for the entire mass range. It is possible to obtain spectra using three quadrupoles arranged in series, Q_1, Q_2, Q_3 (Q_2 serves as a collision cell and is not really an analyzer; the most commonly used collision gas is argon). The most common types of scans are the following:

(1) daughter-ion: Q_1 selects an m/z ion whose fragments obtained by collision in Q_2 are analyzed by Q_3;

(2) parent-ion mode: Q_3 filters only a specific m/z ratio, while Q_1 scans a given mass range. Only the ions decomposing to give the ion selected by Q_3 are detected;

(3) neutral loss mode: Q_1 and Q_3 scan a certain mass range but at an offset corresponding to the loss of a fragment characteristic of a product or family of compounds.

It is also possible to obtain spectra by combining quadrupole analyzers with magnetic or electrostatic sector instruments; such instruments are called hybrid mass spectrometers.

ION-TRAP ANALYZER The principle is the same as for a quadrupole, this time with the electric fields in three

dimensions. This type of analyzers allows production spectra over several generations (MS^n) to be obtained.

ION-CYCLOTRON RESONANCE ANALYZERS Ions produced in a cell and subjected to a uniform, intense magnetic field move in circular orbits at frequencies which can be directly correlated to their *m*/*z* ratio by applying a Fourier transform algorithm. This phenomenon is called ion-cyclotron resonance. Analyzers of this type consist of superconducting magnets and are capable of very high resolving power (up to 1,000,000 and more) as well as MS^n spectra. However, very low pressures are required (of the order of 10^{-7} Pa).

TIME-OF-FLIGHT ANALYZERS The ions produced at the ion source are accelerated at voltage *V* of 10 to 20 kV. They pass through the analyzer, consisting of a field-free tube, 25 cm to 1.5 m long, generally called a flight tube. The time (*t*) for an ion to travel to the detector is proportional to the square root of the *m*/*z* ratio. Theoret ically, the mass range of such an analyzer is infinite. In practice, it is limited by the ionization or desorption method. Time-of-flight analyzers are mainly used for high molecular mass compounds (up to several hundred thousand daltons). This technique is very sensitive (a few picomoles of product are sufficient). The accuracy of the measurements and the resolving power of such instruments may be improved considerably by using an electrostatic mirror (reflectron).

Signal Acquisition

There are essentially three possible modes.

COMPLETE SPECTRUM MODE The entire signal obtained over a chosen mass range is recorded. The spectrum represents the relative intensity of the different ionic species present as a function of *m*/*z*. The results are essentially qualitative. The use of spectral reference libraries for more rapid identification is possible.

FRAGMENTOMETRIC MODE (SELECTED-ION MONITORING) The acquired signal is limited to one (single-ion monitoring, SIM) or several (multiple-ion monitoring, MIM) ions characteristic of the substance to be analyzed. The limit of detection can be considerably reduced in this mode. Quantitative or semi-quantitative tests can be carried out using external or internal standards (for example, deuterated standards). Such tests cannot be carried out with time-Rf-flight analyzers.

FRAGMENTOMETRIC DOUBLE MASS SPECTROMETRY MODE (MULTIPLE REACTION MONITORING, MRM) The unimolecular or bi-molecular decomposition of a chosen precursor ion characteristic of the substance to be analyzed is followed specifically. The selectivity and the highly specific nature of this mode of acquisition provide excellent sensitivity levels and make it the most appropriate for quantitative studies using suitable internal standards (for example, deuterated standards). This type of analysis can be performed only on an apparatus fitted with three quadrupoles in series, ion-trap analyzers or cyclotron-resonance analyzers.

Calibration

Calibration allows the corresponding *m*/*z* value to be attributed to the detected signal. As a general rule, this is done using a reference substance. This calibration may be external (acquisition file separate from the analysis) or internal (the reference substance(s) are mixed with the substance to be examined and appear on the same acquisition file). The number of ions or points required for reliable calibration depends on the type of analyzers and on the desired accuracy of the measurement, for example, in the case of a magnetic analyzer where the *m*/*z* ratio varies exponentially with the value of the magnetic field, there should be as many points as possible.

Signal Detection and Data Processing

Ions separated by an analyzer are converted into electric signals by a detection system such as a photomultiplier or an electron multiplier. These signals are amplified before being re-converted into digital signals for data processing, allowing various functions such as calibration, reconstruction of spectra, automatic quantification, archiving, creation or use of libraries of mass spectra. The various physical parameters required for the functioning of the apparatus as a whole are controlled by the computer.

2.9 NUCLEAR MAGNETIC RESONANCE SPECTROMETRY

Nuclear magnetic resonance (NMR) spectrometry is based on the fact that nuclei such as ^1H, ^{13}C, ^{19}F and ^{31}P possess a permanent nuclear magnetic moment. When placed in an external magnetic field, (main field), they take certain well-defined orientations with respect to the direction of this field which correspond to distinct energy levels. For a given field value, transitions between neighbouring energy levels take place due to absorption of electromagnetic radiation of characteristic wavelengths at radio frequencies.

The determination of these frequencies may be made either by sequential search of the resonance conditions (continuous-wave spectrometry) or by simultaneous excitation of all transitions with a multi-frequency pulse followed by computer analysis of the free-induction decay of the irradiation emitted as the system returns to the initial state (pulsed spectrometry).

A proton magnetic resonance spectrum appears as a set of signals which correspond to protons and are characteristic of their nuclear and electronic environment within the molecule. The separation between a given signal and that of a reference compound is called a chemical shift (δ) and is expressed in parts per million

(ppm); it characterizes the kind of proton in terms of electronic environment. Signals are frequently split into groups of related peaks, called doublets, triplets... multiplets; this splitting is due to the presence of permanent magnetic fields emanating from adjacent nuclei, particularly from other protons within two to five valence bonds. The intensity of each signal, determined from the area under the signal, is proportional to the number of equivalent protons.

Apparatus

A nuclear magnetic resonance spectrometer for continuous wave spectrometry consists of a magnet, a low-frequency sweep generator, a sample holder, a radio-frequency transmitter and receiver, a recorder and an electronic integrator. A pulsed spectrometer is additionally equipped with a pulse transmitter and a computer for the acquisition, storage and mathematical transformation of the data into a conventional spectrum.

Unless otherwise directed in the monograph, use a nuclear magnetic resonance spectrometer operating at not less than 60 MHz in accordance with the manufacturer's instructions and the following operating conditions

Before recording the spectrum, verify the following.

(1) the resolution is equal to 0.5 Hz or less by measuring the peak width at half-height using an adequate scale expansion of either (i) the band at δ7.33 ppm or at δ7.51 ppm of the symmetrical multiplet of a 20 per cent v/v solution of *1,2-dichlorobenzene* in *deuterated acetone* or (ii) the band at δ0.00 ppm of a 5 per cent v/v solution of *tetramethylsilane* in *deuterochloroform*.

(2) the signal-to-noise ratio (S/N), measured over the range from δ2 to 5 ppm on the spectrum obtained with a 1 per cent v/v solution of *ethylbenzene* in *deuterochloroform*, is at least 25:1. This ratio is calculated as the mean of five successive determinations from the formula:

$$S/N = 2.5A/H,$$

where A is the amplitude, measured in millimetres, of the largest peak of the methylene quartet of ethylbenzene centred at δ2.65 ppm and H is the peak-to-peak amplitude of the baseline noise measured in millimetres obtained between δ4 and δ5 ppm. The amplitude is measured from a baseline constructed from the centre of the noise on either side of this quartet and at a distance of at least 1 ppm from its centre.

(3) The amplitude of spinning side bands is not more than 2 per cent of the sample peak height in a tube rotating at a speed appropriated for the spectrometer used.

(4) For quantitative measurements verify the repeatability of the integrator responses using a 5 per cent v/v solution of *ethylbenzene* in *deuterochloroform*. Carry out five successive scan of the protons of the phenyl and ethyl groups and determine the mean of the values obtained. None of the individual values differs by more than 2.5 per cent from the mean.

Method

Dissolve the substance being examined as prescribed in the monograph and filter; the solution must be clear. Unless otherwise specified in the monograph, an internal chemical shift standard should be used; for solutions in deuterated organic solvents, 0.5 to 1.0 per cent v/v of *tetramethylsilane* may be added; for solutions in *deuterium oxide*, 0.5 to 1.0 per cent w/v of *sodium tetradeuterodimethylsilapentanoate* may be added. Take the necessary quantity and record the spectrum.

CONTINUOUS WAVE SPECTROMETRY Adjust the spectrometer so that it is operating as closely as possible in the pure absorption mode and use a radio frequency setting that avoids saturation of the signals. Adjust the spectrometer controls so that the height of the strongest peak of the spectrum of the substance being examined reaches almost to full-scale deflection and the peak associated with the internal reference standard registers on the chart at δ0.00 ppm. Record the spectrum over the prescribed spectral width, using a scan speed of not more than 2 Hz per second unless otherwise specified. It is advisable to record an integral spectrum over the same spectral range, using a suitable scan speed.

Directions for quantitative measurements are given in individual monographs.

PULSED SPECTROMETRY Set the spectrometer controls, for example pulse flip angle, pulse amplitude, pulse interval, spectral width, number of data points (resolution) and data acquisition rate, as indicated in the manufacturer's instructions and collect the necessary number of free induction decays. After mathematical transformation of the data by the computer, adjust the phase control in order to obtain as far as possible a pure absorption spectrum and calibrate the spectrum relative to the resonance frequency of the chemical shift internal reference compound. Display the spectrum stored in the computer on a suitable output device and, for quantitative measurements, process the integral according to the facility of the instrument.

APPENDIX 3 CHROMATOGRAPHY AND ELECTROPHORESIS

CHROMATOGRAPHY Chromatography is defined as a procedure by which essential drug principles and other substances encountered in pharmaceutical products are separated by dynamic differential migration process in a system consisting of two or more phases, one of which moves continuously in a given direction and in which the individual substances exhibit different mobilities by reason of differences in adsorption, partition, solubility, vapour pressure, molecular size, or ionic charge density. The individual substances thus obtained can be identified or determined by analytical methods.

The general chromatographic technique requires that a solute undergo distribution between two phases, one of them fixed (stationary phase), the other moving (mobile phase). It is the mobile phase that transfers the solute through the medium until it eventually emerges separated from other solutes that are eluted earlier or later. Generally, the solute is transported through the separation medium by means of a flowing stream of a liquid or a gaseous solvent known as the "eluant". The stationary phase may act through adsorption, as in the case of adsorbants such as activated alumina, silica gel, and ion-exchange resins or it may act by dissolving the solute, thus partitioning the latter between the stationary and mobile phases. In the latter process, a liquid coating held on an inert support serves as the stationary phase. Partitioning is the predominant mechanism of separation in paper chromatography, forms of column chromatography designated as liquid-liquid chromatography, and gas chromatography. In practice, separations frequently result from a combination of adsorption and partitioning effects.

The types of chromatography useful in qualitative and quantitative analyses that are employed in the assays and tests are Paper, Thin-layer, Column, Gas, and High-pressure Liquid Chromatography. Thin-layer chromatography and paper chromatography are ordinarily more useful for purposes of identification, because of their convenience and simplicity. Gas chromatography and high-pressure liquid chromatography require more elaborate apparatus and normally provide high-resolution methods that will identify and quantitate very small amounts of material.

In paper and thin-layer chromatography, the ratio of the distance (this distance being measured to the point of maximum intensity of the spot) traveled on the medium by a given compound to the distance traveled by the front of the mobile phase, from the point of application of the test substance, is designated as the R_f value of the compound. The ratio between the distances traveled by a given compound and a reference substance is the RR_f value.

ELECTROPHORESIS Electrophoresis is a process in which charged species (ions or colloidal particles) are separated based upon differential migration rates in an electrical field. Under the influence of an electrical field, charged particles dissolved or dispersed in an electrolyte solution migrate in the direction of the electrode bearing the opposite polarity. In gel electrophoresis, the movements of the particles are retarded by interactions with the surrounding gel matrix, which acts as a molecular sieve. The opposing interactions of the electrical force and molecular sieving result in differential migration rates according to sizes, shapes and charges of particles. Because of their different physicochemical properties, different macromolecules of a mixture will migrate at different speeds during electrophoresis and will thus be separated into discrete fractions. Electrophoretic separations can be conducted in systems without support phases (e.g., free solution separation in capillary electrophoresis) and in stabilizing media such as thin-layer plates, films or gels.

3.1 THIN-LAYER CHROMATOGRAPHY

Conventional Thin-layer Chromatography

Thin-layer chromatography is used for the rapid separation of compounds by means of a uniform layer of dry, finely powdered material applied to a glass, plastic, or metal sheet or plate, glass plates being most commonly employed. The coated plate can be considered as an "open chromatographic column" and the separations achieved may be based upon adsorption, partition, or a combination of both effects, depending on the particular type of adsorbant, its preparation, and its use with different solvents. Thin-layer chromatography on ion-exchange films can be used for the fractionation of polar compounds.

As R_f values may vary significantly with the experimental conditions, it is always necessary to prepare chromatograms of authentic specimens or reference substances; preferably in varied quantities, alongside the chromatogram of the sample. Positive identification may be effected by observation of 2 spots of identical R_f value and about equal magnitude. A visual comparison of the size of the spots may serve for semi-quantitative estimation. More accurate quantitative measurements can be made by densitometry, fluorescence, and fluorescence quenching, or careful removal of the spots from the plate, followed by elution with a suitable solvent and spectrophotometric measurement. For two-dimensional thin-layer chromatography, the chromatographed plate is turned at a right angle and again chromatographed, usually in another chamber equilibrated with a different solvent system.

APPARATUS The essential apparatus and materials for thin-layer chromatography are as follows:

Flat glass plates of convenient size typically 20 cm × 20 cm.

An aligning tray or a flat surface upon which to align and rest the plates during the application of the adsorbant.

A storage rack to hold the prepared plates during drying and transportation. The rack holding the plates should be kept in a desiccator or be capable of being sealed in order to protect the plates from the environ-

ment after removal from the drying oven.

The adsorbant consists of finely divided adsorbent material, normally 5 to 40 µm in diameter, suitable for chromatography. It can be applied directly to the plate or can be bonded to the plate by means of plaster of Paris (hydrated calcium sulfate) at a ratio of 5 to 15 per cent, or with starch paste or other binders. The former will not yield as hard a surface as will the starch, but it is not affected by strongly oxidizing spray reagents. The adsorbant may contain fluorescing material to aid in the visualization of spots that absorb ultraviolet light.

A spreader, which, when moved over the plate, will apply a uniform layer of adsorbant, 250 to 300 µm thick, over the entire surface of the plate. Other thicknesses might be desirable in some procedures, and an adjustable spreader would be particularly useful in such cases.

A developing chamber of transparent material, usually glass, ground at the top to take a tightly fitting lid, of a size suitable for the plates used.

A template (generally made of plastic) to aid in placing the test spots at definite intervals, to mark distances as needed, and to aid in labelling the plates.

A graduated micropipette capable of delivering 10-µl quantities. Total quantities of sample and standard solutions are specified in the individual monograph.

A reagent sprayer that will emit a fine spray and will not itself be attacked by the reagent.

An ultraviolet light source suitable for observations with short (254 nm) and long (366 nm) ultraviolet wavelengths.

PROCEDURE Clean the plates scrupulously, as by immersion in *chromic acid cleansing mixture*, rinsing them with copious quantities of *water* until the water runs off the plates without leaving any visible water or oily spots, then dry. It is important that the plates be completely free from lint and dust when the adsorbant is applied.

Arrange the plate or plates on the aligning tray, place a 5-cm × 20-cm plate adjacent to the front edge of the first square plate and another 5-cm × 20-cm plate adjacent to the rear edge of the last square, and secure all of the plates so that they will not slip during the application of the adsorbant. Position the spreader on the end plate opposite to the raised end of the aligning tray. Mix 1 part of adsorbant with 2 parts of *water* (or in the ratio suggested by the supplier) by shaking vigorously for about 30 seconds in a glass-stoppered conical flask, and transfer the slurry to the spreader. Usually 30 g of adsorbant and 60 ml of *water* are sufficient for five 20-cm × 20-cm plates. Complete the application of adsorbants using plaster of Paris binder within 2 minutes of addition of the *water*, since thereafter the mixture begins to harden. Draw the spreader smoothly over the plates towards the raised end of the aligning tray, and remove the spreader when it is on the end plate next to the raised end of the aligning tray. (Wash away all traces of adsorbant from the spreader immediately after use). Allow the plates to remain undisturbed for 5 minutes, then transfer the square plates, layer side up, to the storage rack and dry at 105° for 30 minutes. Preferably place the rack at an angle in the drying oven to prevent the condensation of moisture on the back side of the plates in the rack. When the plates are dry, allow them to cool to room temperature, and inspect the uniformity of the distribution and the texture of the adsorbant layer; transmitted light will show uniformity of texture. Store the satisfactory plates over *self-indicating silica gel* in a suitable chamber.

Place two filter-paper wicks, 18 cm in height and as wide as the length of the developing chamber, into the chamber, add about 100 ml of the solvent (sufficient to have a depth of 0.5 to 1 cm at the bottom of the chamber). Seal the cover to the top of the chamber, and allow the system to equilibrate; it is essential that the wicks become completely wet. Alternatively, the chamber may be completely lined with filter paper. In either case, assure that the filter paper dips into the solvent at the bottom of the chamber. Where vapour saturation of the chamber by these methods is undesirable, it is so indicated in the individual monograph.

Apply the sample solution and the standard solution, as directed in the individual monograph, at points about 1.5 cm apart and about 2 cm from the lower edge of the plate (the lower edge is the first part over which the spreader moved in the application of the adsorbant layer), and allow to dry. Avoid physical disturbance of the adsorbant during the spotting procedure (by the pipette or other applicator) or when handling the plates. The template will aid in determining the spot points and the 10-cm to 15-cm distance through which the solvent front should pass.

Mark the sides of the plate 10 to 15 cm above the spot point. Place the plate in the developing chamber. Allow the solvent in the chamber to reach the lower edge of the adsorbant, but do not allow the spot points to be immersed. Put the cover in place, and maintain the system until the solvent ascends to a point 10 to 15 cm above the initial spots, this usually requiring about 15 minutes to 1 hour. Remove and air-dry the plates, and observe first under short-wavelength ultraviolet light (254 nm) and then under long-wavelength ultraviolet light (366 nm). Measure and record the distance of each spot from the point of origin, and indicate for each spot the wavelength under which it was observed. If further directed, spray the spots with the reagent specified, observe, and compare the sample with the standard chromatogram.

Continuous Thin-layer Chromatography

In contrast to conventional thin-layer chromatography, which is carried out in a closed tank, the continuous development or continuous flow technique allows the upper end of the plate to project through a slot in the cover of the developing chamber. When the developing solvent reaches the slot, continuous evaporation occurs, producing a steady flow of solvent over the plate. In conventional thin-layer chromatography, spot migration ceases when the solvent reaches the top of the plate, after which the spots simply enlarge by diffusion.

In the continuous flow process, spot migration continues as long as the plate remains in the tank and the developing solvent is not exhausted.

Development may be continued for several hours after the solvent reaches the top of the plate, to provide adequate migration of the spots. Usually spots of a standard solution, a test solution, and a mixture of equal amounts of test and standard solutions, are initially applied at a standard distance from the base of the plate. Identity of the standard and test substances is confirmed by their migrating equal distances from the origin and by the observation that the two substances applied as a mixture shows no tendency to separate.

A major advantage of continuous development thin-layer chromatography stems from the greater solvent selectivity for solvents of low solvent strength. Solvent strength refers to the property of a developing solvent that causes solutes to migrate, and it is strongly influenced by the polarity of the solvent. Increasing the solvent strength by adding a more polar solvent causes the R_f value to increase. Solvent selectivity refers to the ability of a solvent system to produce different R_f values for closely related substances. In conventional thin-layer chromatography, a solvent system giving an R_f value in the range of 0.3 to 0.7, but with adequate selectivity to permit separation of the substances being examined is usually selected. It is much easier to find solvent systems producing adequate migration than to find those affording adequate selectivity. Solvent systems of lower strength generally exhibit higher selectivity, but are difficult to employ in conventional thin-layer chromatography because they result in very little migration before the solvent reaches the top of the plate. Migration may be increased, however, by repeated drying and redevelopment of the plate or, more conveniently, by providing means for evaporation of solvent at the top of the plate, which results in continuous development. An R_f value cannot be measured in continuous development thin-layer chromatography. Substances may be compared either by their migration distance over a fixed period of time or by comparison with the migration of a standard substance applied to the plate.

APPARATUS Acceptable apparatus and materials for continuous development thin-layer chromatography are the same as those described under Conventional Thin-layer Chromatography, except as follows.

A developing chamber being used consists of a rectangular tank, approximately 23-cm × 23-cm × 9-cm, equipped with a glass solvent trough and a platform about 3.75 cm high to elevate the solvent trough above the base of the tank. The chamber is fitted with a cover having a 21-cm × 6-cm slot in the front edge.

PROCEDURE Apply the standard solution, the test solution and a mixture of equal amounts of the standard solution and the test solution to a line about 2 cm from the base of the plate. Place the plate in the elevated empty solvent trough with the adsorbent on the underside of the leaning plate. The adsorbent rests against a piece of heavy (about 1 mm thick) filter paper measuring 20 cm × 3 cm, folded lengthwise and placed over the front edge of the tank. Place the developing solvent in the trough, set the cover in place, and seal all openings except where the adsorbent contacts the paper wick. The plate extends about 1 cm beyond the top of the tank. After the solvent reaches the top of the tank, allow development to continue for an appropriate time. Then remove and dry the plate, and detect the spots by suitable means.

Identification of Steroids

Use Method I unless otherwise specified.

METHOD I

Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *kieselguhr G* as the coating substance. Impregnate the dry plate by placing it in a chromatographic chamber containing a shallow layer of the specified impregnating solvent, allowing the solvent to ascend to the top, removing the plate and allowing the solvent to evaporate; use within 2 hours, with the flow of the mobile phase in the direction in which impregnation was carried out. Unless otherwise specified, apply separately to the plate, 2 µl of each of the following three solutions in a mixture of 9 volumes of *chloroform* and 1 volume of *methanol*. Solution (A) contains 2.5 mg per ml of the test substance. Solution (B) contains 2.5 mg per ml of the corresponding Reference Substance. Solution (C) is a mixture of equal volumes of solutions (A) and (B). Use the specified mobile phase. After removal of the plate, allow the solvent to evaporate, heat at 120° for 15 minutes and spray the hot plate with *ethanolic sulfuric acid* (*20 per cent*). Heat at 120° for a further 10 minutes, allow to cool and examine in daylight and under ultraviolet light (366 nm). The principal spot in the chromatogram obtained from solution (A) corresponds to that obtained from solution (B). The principal spot in the chromatogram obtained from solution (C) appears as a single, compact spot.

Impregnating solvents

I. A mixture of 1 volume of *formamide* and 9 volumes of *acetone*.

II. A mixture of 1 volume of *propylene glycol* and 9 volumes of *acetone*.

III. A mixture of 1 volume of *liquid paraffin* and 9 volumes of *petroleum ether (boiling range, 40° to 60°)*.

Mobile phases

A. *Chloroform.*

B. A mixture of 75 volumes of *toluene* and 25 volumes of *chloroform.*

C. *Toluene.*

D. A mixture of 80 volumes of *cyclohexane* and 20 volumes of *toluene.*

E. A mixture of equal volumes of *cyclohexane* and *petroleum ether (boiling range, 40° to 60°).*

F. A mixture of 60 volumes of *water* and 40 volumes of *glacial acetic acid*.

G. A mixture of 80 volumes of *hexane* and 20 volumes of *dioxane*.

H. A mixture of 115 volumes of *cyclohexane*, 56 volumes of *chloroform* and 29 volumes of *toluene*.

METHOD II

Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel G* as the coating substance and a mixture of 85 volumes of *ether*, 10 volumes of *toluene* and 5 volumes of *1-butanol* saturated with *water* as the mobile phase. Apply separately to the plate, 2 µl of each of four solutions in a mixture of 9 volumes of *chloroform* and 1 volume of *methanol* containing (A) 2.5 mg per ml of the test substance, (B) 2.5 mg per ml of the corresponding Reference Substance, (C) 1.25 mg per ml each of the test substance and the corresponding Reference Substance, and (D) 1.25 mg per ml each of the test substance and the specified Reference Substance. After removal of the plate, allow it to dry in air and spray with *ethanolic sulfuric acid (20 per cent)*. Heat at 120° for 10 minutes or until spots are produced, allow to cool and examine in daylight and under ultraviolet light (366 nm). The principal spot in the chromatogram obtained from solution (A) is similar in colour in daylight, fluorescence under ultraviolet light (366 nm), position and size to the principal spot in the chromatogram obtained from solution (B) and the chromatogram obtained from solution (C) shows only one spot. The test is not valid unless the chromatogram obtained from solution (D) shows two principal spots that are close to, but separated from, one another.

Related Impurities in Phenothiazines

METHOD

Carry out in subdued light under an atmosphere of nitrogen the method for "Thin-layer Chromatography" (Appendix 3.1), using *silica gel GF254* as the coating substance, but allowing the solvent front to ascend 12 cm above the line of application. Unless otherwise specified, apply separately to the plate, 10 µl of each of two freshly prepared solutions of the test substance in a mixture of 95 volumes of *methanol* and 5 volumes of *diethylamine* containing (A) 20 mg per ml and (B) 100 µg per ml. Use the specified mobile phase. After removal of the plate, allow it to dry in air and examine under ultra-violet light (254 nm). Ignore any spot on the baseline. Unless otherwise specified any secondary spot obtained from solution (A) is not more intense than that obtained from solution (B).

Mobile phase

A. A mixture of 80 volumes of *cyclohexane*, 10 volumes of *acetone* and 10 volumes of *diethylamine*.

B. A mixture of 85 volumes of *hexane*, 10 volumes of *acetone* and 5 volumes of *diethylamine*.

C. A mixture of 15 volumes of *1-butanol* and 3 volumes of 1 M *ammonia*.

Related Foreign Steroids

METHOD I

Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel G* as the coating substance and a mixture of 77 volumes of *dichloromethane*, 15 volumes of *ether*, 8 volumes of *methanol*, and 1.2 volumes of *water* as the mobile phase. Apply separately to the plate, 1 µl of each of three solutions in a mixture of 9 volumes of *chloroform* and 1 volume of *methanol* containing (A) 15 mg per ml of the test substance, (B) 15 mg per ml of the corresponding Reference Substance and (C) 300 µg per ml of each of Prednisolone RS, Prednisone RS and Cortisone Acetate RS. After removal of the plate, allow it to dry in air until the solvents have evaporated, heat at 105° for 10 minutes, cool, and spray with *alkaline tetrazolium blue TS*. The principal spot in the chromatogram obtained from solution (A) corresponds in position, colour and intensity to that obtained from solution (B). Any secondary spot obtained from solution (A) is not more intense than the proximate spot obtained from solution (C).

METHOD II

Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel G* as the coating substance and a mixture of 95 volumes of *1,2-dichloroethane*, 5 volumes of *methanol* and 0.2 volumes of *water* as the mobile phase. Apply separately to the plate, 1 µl of each of three solutions in a mixture of 9 volumes of *chloroform* and 1 volume of *methanol* containing (A) 15 mg per ml of the test substance, (B) 15 mg per ml of the corresponding Reference Substance and (C) 300 µg per ml of each of Prednisone RS, Prednisolone Acetate RS, Cortisone Acetate RS, and Deoxycortone Acetate RS. Complete the procedure described under Method I beginning with "After removal of the plate...".

Related Substances in Sulfonamides

METHOD I

Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel H* as the coating substance and a mixture of 15 volumes of *1-butanol* and 3 volumes of 1 M *ammonia* as the mobile phase. Apply separately to the plate, 10 µl of each of two solutions, in a mixture of 9 volumes of *ethanol* and 1 volume of *strong ammonia solution*, containing (A) 10 mg per ml of the test substance and (B) 50 µg per ml of *sulfanilamide*. After removal of the plate, heat it at 105° for 10 minutes and spray with a 0.1 per cent w/v solution of *4-dimethylaminobenzaldehyde* in *ethanol* containing 1 per cent v/v of *hydrochloric acid*. Any spot in the chromatogram obtained from solution (A), other than the principal spot, is not more intense than that obtained from solution (B).

METHOD II

Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel H* as the coating substance and a mixture of 20 volumes of *chloroform*, 2 volumes of *methanol* and 1 volume of *dimethylformamide* as the mobile phase. Apply separately to the plate, 10 µl of each of two solutions, in a mixture of 9 volumes of *ethanol* and 1 volume of *strong ammonia solution*, containing (A) 2.5 mg per ml of the test substance and (B) 12.5 µg per ml of *sulfanilamide*. After removal of the plate, allow it to dry in air. Spray the plate with a 10 per cent v/v solution of *sulfuric acid* in *ethanol*, heat at 105° for 30 minutes, and immediately expose to nitrous fumes in a closed glass tank for 15 minutes (the nitrous fumes may be generated by adding 7 M *sulfuric acid* dropwise to a solution containing 10 per cent w/v of *sodium nitrite* and 3 per cent w/v of *potassium iodide*). Place the plate in a current of warm air for 15 minutes and spray with a 0.5 per cent w/v solution of N-(*1-naphthyl*)*ethylenediamine dihydrochloride* in *ethanol*. If necessary, allow to dry and repeat the spraying. Any spot in the chromatogram obtained from solution (A), other than the principal spot, is not more intense than that obtained from solution (B).

METHOD III

Carry out the test as described in the "Thin-layer Chromatography" (Appendix 3.1), using *silica gel GF254* as the coating substance and a mixture of 50 volumes of *dioxane*, 40 volumes of *nitromethane*, 5 volumes of *water*, and 3 volumes of 6 M *ammonia* as the mobile phase. Apply separately to the plate, 5 µl of each of the following solutions. For solution (A), dissolve 100 mg of the test substance in 0.5 ml of *strong ammonia solution* and dilute to 5 ml with *methanol*; if the solution is not clear, heat gently until dissolution is complete. Solutions (B) to (D) are solutions in a mixture of 24 volumes of *methanol* and 1 volume of *strong ammonia solution*, containing (B) 4 mg per ml of the test substance, (C) 100 µg per ml of the test substance and (D) 4 mg per ml of the corresponding Reference Substance. After removal of the plate, dry it at 105° and examine under ultraviolet light (254 nm). Any secondary spot in the chromatogram obtained from solution (A) is not more intense than that obtained from solution (C).

3.2 PAPER CHROMATOGRAPHY

In paper chromatography the adsorbant is a sheet of paper of suitable texture and thickness, which may sometimes be impregnated with a liquid phase that is immiscible with the mobile phase.

The concept of R_f value discussed on thin-layer chromatography is applicable to paper chromatography. Because of the nature of the adsorbant it is possible to carry out paper chromatography in either a descending or an ascending mode.

Descending Paper Chromatography

APPARATUS The apparatus is as follows:

A glass chamber of suitable dimensions to accommodate the chromatographic paper used, ground at the top to take a closely fitting glass lid. The lid has a central hole about 1.5 cm in diameter closed by a stopper.

A solvent trough, suspended in the upper part of the chamber, with a device, usually a heavy glass rod, for holding the chromatographic paper. On either side of the trough, parallel to and slightly above its upper edges, are two glass guide rods to support the paper in such a manner that no part of it is in contact with the walls of the chamber.

The chromatographic paper, cut into strips of sufficient length, and of any convenient width between 2.5 cm and the length of the trough. The paper is cut so that the mobile phase runs in the direction of the grain of the paper.

PROCEDURE Place in the bottom of the chromatographic chamber a layer 2 to 3 cm deep of the stationary phase specified in the monograph. Close the chamber, and allow to stand for 24 hours at constant temperature. Maintain the chamber under these conditions throughout the subsequent procedure. Draw a fine pencil line horizontally across the paper at such a distance from one end that, when this end is secured in the solvent trough and the remainder of the paper is hanging freely over the guide rod, the line is a few centimetres below the guide rod and parallel to it. Apply to a spot on the pencil line the volume of the solution specified in the monograph. If the total volume to be applied would produce a spot more than 10 mm in diameter, apply the solution in portions, allowing each to dry before the next application. When more than one chromatogram is to be run on the same strip of paper, space the solutions along the pencil line at points not less than 3 cm apart. Insert the paper in the chamber, close the lid, and allow to stand for 90 minutes. Introduce into the solvent trough, through the hole in the lid, a sufficient quantity of the mobile phase specified in the monograph, close the hole and allow development to proceed for the prescribed distance or time. Remove the paper from the chromatographic chamber and allow to dry in air. The paper should be protected from bright light during the development and drying processes.

Ascending Paper Chromatography

APPARATUS The apparatus is as follows:

A glass chamber of suitable dimensions to accommodate the chromatographic paper used, ground at the top to take a closely fitting glass lid.

A device, in the top of the chamber, to suspend the chromatographic paper and to be capable of being lowered without opening the chamber.

A dish, in the bottom of the chamber, to contain the mobile phase into which the paper may be lowered.

The chromatographic paper, cut into strips of sufficient length, and of any convenient width between 2.5 cm and the length of the trough. The paper is cut so that the mobile phase runs in the direction of the grain of the paper.

PROCEDURE Place in the dish a layer 2 to 3 cm deep of the mobile phase specified in the monograph. If specified in the monograph, pour the stationary phase between the walls of the chamber and the dish. Close the chromatographic chamber and allow to stand for 24 hours at constant temperature. Maintain the chamber at this temperature throughout the subsequent procedure. Apply the test substance to the paper as described in Descending Paper Chromatography. Insert the paper into the chamber, close the lid, and allow to stand for 90 minutes. Lower the paper into the mobile phase specified in the monograph, and allow development to proceed for the prescribed distance or time. Remove the paper from the chamber and allow to dry in air. The paper should be protected from bright light during the development and drying processes.

3.3 COLUMN CHROMATOGRAPHY

Column chromatography offers a wider choice of stationary phases and is useful for the separation of individual compounds, in quantity, from mixtures.

When the stationary phase is a solid, the process is termed adsorption chromatography, while if the stationary phase is liquid, the process is termed partition chromatography. The difference between partition and adsorption chromatography lies in the nature of the forces that determine distribution of the solute between the two phases. In ion-exchange chromatography the stationary phase is an ion-exchange material, usually called ion-exchange resin.

Apparatus

The apparatus required for column chromatographic procedures is simple, consisting only of the chromatographic tube itself and a tamping rod which may be needed to pack a pledget of glass wool or cotton, if needed, in the base of the tube and compress the adsorbant or slurry uniformly within the tube. In some cases a porous glass disc is sealed at the base of the tube in order to support the contents. The tube is cylindrical and is made of glass, unless another material is specified in the individual monograph. A smaller-diameter delivery tube is fused or otherwise attached by a leak-proof joint to the lower end of the main tube. Column dimensions are variable; the dimensions of those commonly used in pharmaceutical analysis range from 10 to 30 mm in uniform inside diameter and 15 to 40 cm in length, exclusive of the delivery tube. The delivery tube, usually 3 to 6 mm in inside diameter, may include a stopcock for accurate control of the flow rate of solvents through the column. The tamping rod, a cylindrical ram firmly attached to a shaft, may be constructed of plastic, glass, stainless steel, or aluminium, unless another material is specified in the individual monograph. The shaft of the rod is substantially smaller in diameter than the column and is not less than 5 cm longer than the effective length of the column. The ram has a diameter about 1 mm smaller than the inside diameter of the column.

Adsorption Chromatography

The adsorbant (such as activated alumina, silicic acid, etc.) as a dry solid or as a slurry is packed into a tube made of glass, quartz, or other material, of suitable dimensions having a restricted out-flow orifice. A solution of the drug in a small amount of solvent is added to the top of the column and allowed to flow into the adsorbant. The drug principles are quantitatively removed from the solution and are adsorbed in a narrow transverse band at the top of the column. As further amounts of solvent are allowed to flow through the column, either by gravity or by application of air pressure, each substance progresses down the column at a characteristic rate resulting in a spatial separation to give what is known as the chromatogram. The rate of movement for a given substance is affected by several variables, including the adsorptive power of the adsorbant, the nature of the solvent, and temperature of the chromatographic system.

If the separated compounds are coloured or if they fluoresce under ultraviolet light, the adsorbant column may be extruded and, by transverse cuts, the appropriate segments may then be isolated. The desired compounds are then extracted from each segment with a suitable solvent. If the compounds are colourless, they may be located by painting or spraying the extruded column with colour-forming reagents.

The "flowing chromatogram" is used quite extensively. With this type, solvents are allowed to flow through the column until the separated drug appears in the effluent solution, known as the "eluate". The drug may be determined in the eluate by titration or by a spectrophotometric or colorimetric method, or the solvent may be evaporated, leaving the drug in more or less pure form. If a second drug principle is involved, it is eluted by continuing the first solvent or by passing a solvent of stronger eluting power through the column.

A modified procedure for adding the mixture to the column is sometimes employed. The drug, in a solid form and, as in the case of a powdered tablet, without separation from the excipients, is mixed with some of the adsorbant and added to the top of a column. The subsequent flow of solvent moves the drug down the column in the usual manner.

Partition Chromatography

In partition chromatography the substances to be separated are partitioned between two immiscible liquids one of which, the immobile phase, is adsorbed on a solid support, thereby presenting a very large surface area to the flowing solvent or mobile phase. The exceedingly high number of successive liquid-liquid contacts allows an efficiency of separation not achieved

in ordinary liquid-liquid extraction.

The solid support is usually polar, and the adsorbed immobile phase more polar than the mobile phase. The solid support that is most widely used is chromatographic siliceous earth having a particle size suitable to permit proper flow of eluant (acid-washed Celite 545 or equivalent is suitable). In reverse-phase partition chromatography the adsorbed immobile phase is less polar than the mobile phase and the solid adsorbant is rendered nonpolar by suitable treatment with a silanizing agent, such as dichlorodimethylsilane, to give silanized chromatographic siliceous earth.

The sample to be chromatographed is usually introduced into the chromatographic system in one of two ways: (a) a solution of the sample in a small volume of the mobile phase is added to the top of the column; or, (b) a solution of the sample in a small volume of the immobile phase is mixed with the solid support and transferred to the column as a layer above a bed of a mixture of immobile phase with adsorbant.

Development and elution are accomplished with flowing solvent as before. The mobile solvent usually is saturated with the immobile solvent before use.

In conventional liquid-liquid partition chromatography, the degree of partition of a given compound between the two liquid phases is expressed by its partition or distribution coefficient. In the case of compounds that dissociate, distribution can be controlled by modifying the pH, dielectric constant, ionic strength, and other properties of the two phases. Selective elution of the components of a mixture can be achieved by successively changing the mobile phase to one that provides a more favourable partition coefficient, or by changing the pH of the immobile phase *in situ* with a mobile phase consisting of a solution of an appropriate acid or base in an organic solvent.

Unless otherwise specified in the individual monograph, assays and tests that employ column partition chromatography are performed according to the following general method.

Solid support Use purified siliceous earth. Use silanized chromatographic siliceous earth for reverse-phase partition chromatography.

Stationary phase Use the solvent or solution specified in the individual monograph. If a mixture of liquids is to be used as the stationary phase, mix them prior to the introduction of the solid support.

Mobile phase Use the solvent or solution specified in the individual monograph. Equilibrate it with water if the stationary phase is an aqueous solution; if the stationary phase is a polar organic fluid, equilibrate with that fluid.

Preparation of chromatographic column Unless otherwise specified in the individual monograph, the chromatographic tube is about 22 mm in inside diameter and 20 to 30 cm in length, without porous glass disc, to which is attached a delivery tube, without stopcock, about 4 mm in inside diameter and about 5 cm in length. Pack a pledget of fine glass wool in the base of the tube. Place the specified volume of stationary phase in a 100- to 250-ml beaker, add the specified amount of solid support, and mix to produce a homogeneous, fluffy mixture. Transfer this mixture to the chromatographic tube, and tamp, using gentle pressure, to obtain a uniform mass. If the specified amount of solid support is more than 3 g, transfer the mixture to the column in portions of approximately 2 g, and tamp each portion.

If the assay or test requires a multi-segment column, with a different stationary phase specified for each segment, tamp after the addition of each segment and add each succeeding segment directly to the previous one.

If a solution of the analyte is incorporated in the stationary phase, complete the quantitative transfer to the chromatographic tube by scrubbing the beaker used for the preparation of the test mixture with a mixture of about 1 g of solid support and several drops of the solvent used to prepare the test solution.

Pack a pledget of fine glass wool above the completed column packing. The mobile phase flows through a properly packed column as a moderate stream or, if reverse-phase chromatography is applied, as a slow trickle.

Procedure Transfer the mobile phase to the column space above the column packing, and allow it to flow through the column under the influence of gravity. Rinse the tip of the chromatographic column with about 1 ml of mobile phase before each change in composition of mobile phase and after completion of the elution. If the analyte is introduced into the column as a solution in the mobile phase, allow it to pass completely into the column packing, then add mobile phase in several small portions, allowing each to drain completely, before adding the bulk of the mobile phase. Where the assay or test requires the use of multiple chromatographic columns mounted in series and the addition of mobile phase in divided portions is specified, allow each portion to drain completely through each column, and rinse the tip of each with mobile phase prior to the addition of each succeeding portion.

Ion-exchange Chromatography

Ion-exchange is defined as the reversible interchange between the ion present in the solution and the counterion of the resinous polymer, modified cellulose, or bonded silica gel support; it may be exemplified for the H^+/Na^+ exchange of a strongly acidic cation exchange resin as:

$$RSO_3H + Na^+ \rightleftharpoons RSO_3Na + H^+$$

and for a Cl^-/OH^- strongly basic anion-exchange resin as:

$$RN(CH_3)_3OH + Cl^- \rightleftharpoons RN(CH_3)_3Cl + OH^-$$

The selection of strong or weak resins, of either type, will largely depend on the pH at which the exchange is to be carried out and on the types of cation or

anion that are to be exchanged. However, the strongly acidic and basic exchange resins will serve in most analytical applications. Their specific capacity may vary from 2 to 5 millimoles per gram (dry basis). In practice, a large (200 to 300 per cent) excess of resin is used over the calculated stoichiometric requirement.

The laws governing the exchange reaction are complete, being in part described by mass action, ionic charge, and activity relationships. The selectivity coefficient is used to indicate the preference of the ion-exchange resin for the uptake of 2 (or more) ions from solution. Generally speaking, the resin will take up divalent (or higher) ions in preference to monovalent ions, and in the case of a choice between ions of the same valence, the resin will take up the heavier ion preferentially.

Treatment of the ion-exchange resin and preparation of the column Usually the ion-exchange resin is immersed in *water* and allowed to swell for 24 hours; it is then packed into a suitable column and, in the case of an anion-exchange resin, converted to the basic form by passing 2 M *sodium hydroxide* through the column at a rate of about 3 ml per minute until the effluent is free of chloride, followed by *carbon dioxide-free water*, to remove alkalinity. In the case of a cation-exchange resin, conversion to the acidic form is achieved by passing 2 M *hydrochloric acid* through the column, followed by *carbon dioxide-free water* until the washings are neutral.

The prepared column is used in a similar manner to that described for adsorption column chromatography except that there is usually no need to monitor the effluent; according to the type of resin chosen and the type of material being determined the volume of effluent detailed in the particular application is collected and titrated with acid or base as appropriate, using a suitable indicator.

After the determination has been completed, the ion-exchange column may be regenerated by washing either with 2 M *sodium hydroxide* for an anion-exchange column, or 2 M *hydrochloric acid* for a cation-exchange column, followed by *water* until a neutral reaction is obtained.

3.4 GAS CHROMATOGRAPHY

Gas chromatography is a method of chromatographic separation in which the mobile phase is a gas (the carrier gas) and the stationary phase is a solid or liquid coated on a suitable solid support contained in a column. On emerging from the column the carrier gas is passed through a suitable detector.

Apparatus

The apparatus consists of an injector, a chromatographic column contained in an oven, a detector and data collection devices. The carrier gas flows through the column at a controlled rate or pressure and then through the detector. The chromatography is carried out either at a constant temperature or according to a given temperature programme.

INJECTORS Sample injection devices range from simple syringes to fully programmable automatic injectors. Direct injections of solutions are the usual mode of injection, unless otherwise prescribed in the monograph. Injection may be carried out either directly at the head of the column using a syringe or an injection valve, or into a vaporization chamber which may be equipped with a stream splitter. The amount of sample that can be injected into a capillary column without overloading is small compared to the amount that can be injected into packed columns and may be less than the smallest amount that can be manipulated satisfactorily by syringe. Capillary columns, therefore, often are used with injectors able to split samples into two fractions, a small one that enters the column and a large one that goes to waste. Such injectors may be used in a splitless mode for analyses of trace or minor components.

Purge and trap injectors are equipped with a sparging device by which volatile compounds in solution are carried into a low-temperature trap. When sparging is complete, trapped compounds are desorbed into the carrier gas by rapid heating of the temperature-programmable trap.

Headspace injectors are equipped with a thermostatically controlled sample heating chamber. Solid or liquid samples in tightly closed containers are heated in the chamber for a fixed period of time allowing the volatile components in the sample to reach an equilibrium between the nongaseous phase and the gaseous or headspace phase.

After this equilibrium has been established, the injector automatically introduces a fixed amount of the headspace in the sample container into the gas chromatograph.

COLUMNS Capillary columns which are usually made of fused silica, are typically 0.2 to 0.53 mm in internal diameter and 5 to 60 m in length. The liquid or stationary phase, which is sometimes chemically bonded to the inner surface, is 0.1 to 1.0 μm thick, although nonpolar stationary phases may be up to 5 μm thick.

Packed columns, made of glass or metal, are 1 to 3 m in length with internal diameters of 2 to 4 mm. Those used for analysis typically are porous polymers or solid supports with liquid phase loadings of about 5 per cent (w/w). High-capacity columns, with liquid phase loadings of about 20 per cent (w/w), are used for large test specimens and for the determination of low molecular weight compounds such as water. The capacity required influences the choice of solid support.

Supports for analysis of polar compounds on low-capacity, low-polarity liquid phase columns must be inert to avoid peak tailing. The reactivity of support materials can be reduced by silanizing prior to coating with liquid phase. Acid-washed, flux-calcined diatomaceous earth is often used for drug analysis. Support materials are available in various mesh sizes, with 80- to

100-mesh and 100- to 120-mesh being most commonly used with 2- to 4-mm columns.

Helium or nitrogen is usually employed as the carrier gas for packed columns, whereas commonly used carrier gases for capillary columns are nitrogen, helium and hydrogen. Retention time and the peak efficiency depend on the carrier gas flow rate; retention time is also directly proportional to column length, while resolution is proportional to the square root of the column length. For packed columns, the carrier gas flow rate is usually expressed in ml per minute at atmospheric pressure and room temperature. It is measured at the detector outlet with a flowmeter while the column is at operating temperature. The linear flow rate through a packed column is inversely proportional to the square of the column diameter for a given flow volume. Flow rates of 60 ml per minute in a 4-mm column and 15 ml per minute in a 2-mm column give identical linear flow rates and thus similar retention times. Unless otherwise specified in the monograph, flow rates for packed columns are about 30 to 60 ml per minute. For capillary columns, linear flow velocity is often used instead of flow rate. This is conveniently determined from the length of the column and the retention time of a dilute methane sample, provided a flame-ionization detector is in use. At high operating temperatures there is sufficient vapour pressure to result in a gradual loss of liquid phase, a process called bleeding.

DETECTORS Flame-ionization detectors are used for most pharmaceutical analyses, with lesser use made of thermal conductivity, electron-capture, nitrogen-phosphorous (alkali flame-ionization), mass spectrometric, Fourier transform infrared spectrophotometric detectors, and others, depending on the purpose of the analysis. For quantitative analyses, detectors must have a wide linear dynamic range: the response must be directly proportional to the amount of compound present in the detector over a wide range of concentrations. Flame-ionization detectors have a wide linear range and are sensitive to most organic compounds. Detector response depends on the structure and concentration of the compound and on the flow rates of the combustion, air, makeup, and carrier gases. Unless otherwise specified in individual monographs, flame-ionization detectors with either helium or nitrogen carrier gas are to be used for packed columns and helium or hydrogen is used for capillary columns.

DATA COLLECTION DEVICES Modern data stations receive the detector output, calculate peak areas and peak heights, and print chromatograms, complete with run parameters and peak data. Chromatographic data may be stored and reprocessed, with integration and other calculation variables being changed as required. Data stations are used also to program the chromatograph, controlling most operational variables and providing for long periods of unattended operation.

Data can also be collected for manual measurement on simple recorders or on integrators whose capabilities range from those providing a printout of peak areas and peak heights calculated and data stored for possible reprocessing.

The design of a particular chromatograph may require modification of the conditions detailed in the monograph. In such a case, the analyst should be satisfied that the modified conditions produce comparable results. If necessary, adjust the flow rate of the carrier gas to improve the quality of the chromatogram or to modify the retention times of the peaks of interest.

Performance

Criteria for assessing the suitability of the system are described in the "Chromatographic Separation Techniques" (Appendix 3.9). The extent to which adjustments of parameters of the chromatographic system can be made to satisfy the criteria of system suitability are also given.

Procedure

Equilibrate the column, the injector and the detector at the temperatures and the gas flow rates specified in the monograph until a stable baseline is achieved. Prepare the test solution(s) and the standard solution(s) as prescribed in the monograph. The solutions must be free from solid particles. Using standard solution determine experimentally suitable instrument settings and volumes of the solutions to be injected to produce an adequate response.

In applications where an internal standard is used, an injection of sample solution containing only the substance being examined should be made to determine whether any peak is present that will interfere with that of the internal standard. If an interfering peak is present, a suitable correction should be made.

Inject the selected volumes of the solutions prescribed in the monograph and record the resulting chromatograms. Repeat the determinations to ensure a consistent response.

For qualitative analysis, the retention time for a peak in the chromatogram obtained for a test specimen is "the same as," or "corresponding to" that obtained for a standard preparation under the conditions specified in the individual monograph.

For quantitative analysis, determine the peak areas or, alternatively, when the symmetry factor is between 0.80 and 1.20, determine the peak heights corresponding to the components of interest. From the values obtained calculate the content of the component or components being determined.

Assays require quantitative comparison of one chromatogram with another, and lack of control of the specimen size injected is a major source of error. Addition of an internal standard to the test specimen minimizes this error. The ratio of peak response of the components of interest to the internal standard is compared from one chromatogram to another. Where the internal standard is chemically similar to the substance being examined, minor variations in column and detector parameters are controlled also. In some cases,

the internal standard may be carried through the assay procedure prior to gas chromatography to control other quantitative aspects of the procedure.

Materials

Supports, stationary phases and internal standards for gas chromatography are stated in the "Materials for Chromatography" (Appendix 1.7).

Solvents and reagents used in the preparation of solutions for examination should be of a quality suitable for use in gas chromatography.

3.5 HIGH-PRESSURE LIQUID CHROMATOGRAPHY

High-pressure liquid chromatography (HPLC), sometimes called high-performance liquid chromatography, is a separation technique based on a solid stationary phase and a liquid mobile phase. Separations are achieved by partition, adsorption, or ion-exchange processes, depending upon the type of stationary phase used. HPLC has distinct advantages over gas chromatography for the analysis of organic compounds. Compounds to be analyzed are dissolved in a suitable solvent, and most separations take place at room temperature. Thus, most drugs, being nonvolatile or thermally unstable compounds, can be chromatographed without decomposition or the necessity of making volatile derivatives. Most pharmaceutical analyses are based on partition chromatography and are completed within 30 minutes.

As in gas chromatography, the elution time of a compound can be described by the capacity factor, k', which depends on the chemical nature of the analyte, the composition and flow rate of the mobile phase, and the composition and surface area of the stationary phase. Column length is an important determinant of resolution. Only compounds having different capacity factors can be separated by HPLC.

Apparatus

A liquid chromatograph consists of a reservoir containing the mobile phase, a pump to force the mobile phase through the system at high pressure, an injector to introduce the sample into the mobile phase, a chromatographic column, a detector, and a data collection device such as a computer, integrator, or recorder.

The mobile phase is pumped under pressure from one or several reservoirs and flows through the column at a constant rate. The composition and flow rate of the mobile phase are stated in the monograph. It is advisable to use as the mobile phase solvent mixtures that have been filtered and de-aerated using a vacuum pump of other suitable means of deaeration that has no effect on the composition of the mixture. Short, smallbore columns containing densely packed particles of stationary phase provide for the rapid exchange of compounds between the mobile and stationary phases. In addition to receiving and reporting detector output, computers are used to control chromatographic settings and operations, thus providing for long periods of unattended operation.

PUMPING SYSTEM HPLC pumping systems deliver metered amounts of mobile phase from the solvent reservoirs to the column through high-pressure tubing and fittings. Modern systems consist of one or more computer-controlled metering pumps that can be programmed to vary the ratio of mobile phase components, as is required for gradient chromatography, or to mix isocratic mobile phases (i.e., mobile phases having a fixed ratio of solvents). However, the proportion of ingredients in premixed isocratic mobile phases can be more accurately controlled than in those delivered by most pumping systems. Operating pressures up to 34,474 kPa (about 5000 psi) or higher, with delivery rates up to about 10 ml per minute are typical. Pumps used for quantitative analysis should be constructed of materials inert to corrosive mobile phase components and be capable of delivering the mobile phase at a constant rate with minimal fluctuations over extended periods of time.

INJECTORS After dissolution in mobile phase or other suitable solution, compounds to be chromatographed are injected into the mobile phase, either manually by a syringe or loop injectors, or automatically by autosamplers. The latter consist of a carousel or rack to hold sample vials with tops that have a pierceable septum or stopper and an injection device to transfer sample from the vials to a loop from which it is loaded into the chromatograph. Some autosamplers can be programmed to control sample volume, the number of injections and loop rinse cycles, the interval between injections, and other operating variables.

A syringe can be used for manual injection of sample through a septum when column head pressures are less than 6897 kPa (about 1000 psi). At higher pressures an injection valve is essential. Some valve systems incorporate a calibrated loop that is filled with the test solution for transfer to the column in the mobile phase. In other systems, the test solution is transferred to a cavity by a syringe and then switched into the mobile phase.

COLUMNS For most pharmaceutical analyses, separation is achieved by partition of compounds in the test solution between the mobile phase and stationary phase. Systems consisting of polar stationary phases and nonpolar mobile phases are described as normal phase, while the opposite arrangement, polar mobile phases and nonpolar stationary phases, is called reverse-phase chromatography. Partition chromatography is almost always used for hydrocarbon-soluble compounds of molecular weight less than 1000. The affinity of a compound for the stationary phase, and thus its retention time on the column, is controlled by making the mobile phase more or less polar. Mobile phase polarity can be varied by the addition of a second, and sometimes a third or even a fourth, component.

Stationary phases for modern, reverse-phase liquid chromatography typically consist of an organic phase chemically bound to silica or other materials. Particles are usually 3 to 10 μm in diameter, but sizes may range up to 50 μm or more for preparative columns. Small particles thinly coated with the organic phase provide for low mass transfer resistance and, hence, rapid transfer of compounds between the stationary and mobile phases. Column polarity depends on the polarity of the bound functional groups, which range from relatively nonpolar octadecyl silane to very polar nitrile groups. Liquid, nonbound stationary phases must be largely immiscible in the mobile phase. Even so, it is usually necessary to presaturate the mobile phase with stationary phase to prevent stripping of the stationary phase from the column. Polymeric stationary phases coated on the support are more durable.

Columns used for analytical separations usually have internal diameters of 2 to 5 mm; larger diameter columns are used for preparative chromatography. Columns may be heated to give more efficient separations, but only rarely are they used at temperatures above 60° because of potential stationary phase degradation or mobile phase volatility. Unless otherwise specified in the individual monograph, columns are used at ambient temperature.

Ion-exchange chromatography is used to separate water-soluble, ionizable compounds of molecular weight less than 1500. The stationary phases are usually synthetic organic resins; cation-exchange resins contain negatively charged active sites and are used to separate basic substances such as amines, while anion-exchange resins have positively charged active sites for separation of compounds with negatively charged groups such as phosphate, sulfonate, or carboxylate groups. Water-soluble ionic or ionizable compounds are attracted to the resins, and differences in affinity bring about the chromatographic separation. The pH of the mobile phase, temperature, ion type, ionic concentration, and organic modifiers affect the equilibrium, and these variables can be adjusted to obtain the desired degree of separation.

In size-exclusion chromatography, columns are packed with a porous stationary phase. Molecules of the compounds being chromatographed are filtered according to size. Those too large to enter the pores pass unretained through the column. Smaller molecules enter the pores and are increasingly retained as molecular size decreases. These columns are typically used to measure aggregation and degradation of large molecules.

DETECTORS Many compendial HPLC methods require the use of spectrophotometric detectors. Such a detector consists of a flow-through cell mounted at the end of the column. A beam of UV radiation passes through the flow cell and into the detector. As compounds elute from the column, they pass through the cell and absorb the radiation, resulting in measurable energy level changes.

Fixed, variable-, and multi-wavelength detectors are widely available. Fixed wavelength detectors operate at a single wavelength, typically 254 nm, emitted by a low-pressure mercury lamp, Variable-wavelength detectors contain a continuous source such as a deuterium or high-pressure xenon lamp, and a monochromator or an interference filter to generate monochromatic radiation at a wavelength selected by the operator. The wavelength accuracy of a variable-wavelength detector equipped with a monochromator should be checked by the procedure recommended by its manufacturer, if the observed wavelengths differ by more than 3 nm from the correct values, recalibration of the instrument is indicated. Modern variable wavelength detectors can be programmed to change wavelength while an analysis is in progress. Multi-wavelength detectors measure absorbance at two or more wavelengths simultaneously. In diode array multi-wavelength detectors, continuous radiation is passed through the sample cell then resolved into its constituent wavelengths, which are individually detected by the photodiode array. These detectors acquire absorbance data over the entire UV-visible range, thus providing the analyst with chromatograms at multiple, selectable wavelengths and spectra of the eluting peaks. Diode array detectors usually have lower-signal-to-noise ratios than fixed or variable wavelength detectors, and thus are less suitable for analysis of compounds present at low concentrations.

Differential refractometer detectors measure the difference between the refractive index of the mobile phase alone and that of the mobile phase containing chromatographed compounds as it emerges from the column. Refractive index detectors are used to detect non-UV absorbing compounds, but they are less sensitive than UV detectors. They are sensitive to small changes in solvent composition, flow rate and temperature so that a reference column may be required to obtain a satisfactory baseline.

Fluorometric detectors are sensitive to compounds that are inherently fluorescent or that can be converted to fluorescent derivatives either by chemical transformation of the compound or by coupling with fluorescent reagents at specific functional groups. If derivatization is required, it can be done prior to chromatographic separation or, alternatively, the reagent can be introduced into mobile phase just prior to its entering the detector.

Potentiometric, voltametric, or polarographic electrochemical detectors are useful for the quantitation of species that can be oxidized or reduced at a working electrode. These detectors are selective, sensitive, and reliable, but require conducting mobile phases free of dissolved oxygen and reducible metal ions. A pulseless pump must be used, and care must be taken to ensure that the pH, ionic strength, and temperature of the mobile phase remain constant. Working electrodes are prone to contamination by reaction products with consequent variable responses.

Electrochemical detectors with carbon-paste electrodes may be used advantageously to measure nano-

gram quantities of easily oxidized compounds, notably phenols and catechols.

New detectors continue to be developed in attempts to overcome the deficiencies of those being used.

DATA COLLECTION DEVICES Modern data stations receive and store detector output and print out chromatograms complete with peak heights, peak areas, sample identification, and method variables. They are also used to program the liquid chromatograph, controlling most variables and providing for long periods of unattended operation.

Data also may be collected on simple recorders for manual measurement or on stand-alone integrators, which range in complexity from those providing a printout of peak areas to those providing chromatograms with peak areas and peak heights calculated and data stored for possible subsequent reprocessing.

Performance

Criteria for assessing the suitability of the system are described in the "Chromatographic Separation Techniques" (Appendix 3.9) The extent to which adjustments of parameters of the chromatographic system can be made to satisfy the criteria of system suitability are also given.

Procedure

The mobile phase composition significantly influences chromatographic performance and the resolution of compounds in the mixture being chromatographed. For accurate quantitative work, high-purity reagents and "HPLC grade" organic solvents must be used. Water of suitable quality should have low conductivity and low UV absorption, appropriate to the intended use.

Reagents used with special types of detectors (e.g., electrochemical, mass spectrometer) may require the establishment of additional tolerances for potential interfering species. Composition has a much greater effect than temperature on the capacity factor, k'.

In partition chromatography, the partition coefficient, and hence the separation, can be changed by addition of another component to the mobile phase. In ion-exchange chromatography, pH and ionic strength, as well as changes in the composition of the mobile phase, affect capacity factors. The technique of continuously changing the solvent composition during the chromatographic run is called gradient elution or solvent programming. It is sometimes used to chromatograph complex mixtures of components differing greatly in their capacity factors. Detectors that are sensitive to change in solvent composition, such as the differential refractometer, are more difficult to use with the gradient elution technique.

The detector must have a broad linear dynamic range, and compounds to be measured must be resolved from any interfering substances. The linear dynamic range of a compound is the range over which the detector signal response is directly proportional to the amount of the compound. For maximum flexibility in quantitative work, this range should be about three orders of magnitude. HPLC systems are calibrated by plotting peak responses in comparison with known concentrations of a reference standard, using either an external or an internal standardization procedure.

Reliable quantitative results are obtained by external calibration if automatic injectors or autosamplers are used. This method involves direct comparison of the peak responses obtained by separately chromatographing the test and reference standard solutions. If syringe injection, which is irreproducible at the high pressures involved, must be used, better quantitative results are obtained by the internal calibration procedure where a known amount of a noninterfering compound, the internal standard, is added to the test and reference standard solutions, and the ratios of peak responses of drug and internal standard are compared.

3.6 SIZE-EXCLUSION CHROMATOGRAPHY

Size-exclusion chromatography is a chromatographic technique which separates molecules in solution according to their size. With organic mobile phases, the technique is known as gel-permeation chromatography and with aqueous mobile phases, the term gel-filtration chromatography has been used. The sample is introduced into a column, which is filled with a gel or a porous particle packing material and is carried by the mobile phase through the column. The size separation takes place by repeated exchange of the solute molecules between the solvent of the mobile phase and the same solvent in the stationary liquid phase within the pores of the packing material. The pore-size range of the packing material determines the molecular-size range within which separation can occur.

Molecules small enough to penetrate all the pore spaces elute at the total permeation volume, V_T. On the other hand, molecules apparently larger than the maximum pore size of the packing material migrate along the column only through the spaces between the particles of the packing material without being retained and elute at the exclusion volume, V_O (void volume). Separation according to molecular size occurs between the exclusion volume and the total permeation volume, useful separation usually occurring in the first two-thirds of this range.

Apparatus

COLUMN If necessary, the column is temperature-controlled. It is packed with a separation material that is capable of fractionation in the appropriate range of molecular sizes and through which the eluent is passed at a constant rate. One end of the column is usually fitted with a suitable device for applying the sample, such as a flow adaptor, a syringe through a septum or an injection valve, and it may also be connected to a suitable pump for controlling the flow of the eluent. Alternatively, the sample may be applied directly to the drained bed surface, or, where the sample is denser than

the eluent, it may be layered beneath the eluent. The packing material may be a soft support such as a swollen gel or a rigid support composed of a material such as glass, silica, or a solvent-compatible, cross-linked organic polymer. Rigid supports usually require pressurized systems giving faster separations. The mobile phase is chosen according to sample type, separation medium, and method of detection.

DETECTOR The outlet of the column is usually connected to a suitable detector fitted with an automatic recorder that enables the monitoring of the relative concentrations of separated components of the sample. Detectors are usually based on photometric, refractometric, or luminescent properties. An automatic fraction collector may be attached, if necessary.

Performance

Criteria for assessing the suitability of the system are described in the "Chromatographic Separation Techniques" (Appendix 3.9). The extent to which adjustments of parameters of the chromatographic system can be made to satisfy the criteria of system suitability are also given.

Procedure

Before carrying out the separation, the packing material is treated and the column is packed, as described in the individual monograph or according to the manufacturer's instructions. The elution characteristics of a compound in a particular column may be described by the distribution coefficient, K_D, which is calculated by the formula:

$$(V_I - V_O)/(V_T - V_O),$$

in which V_O, V_T, and V_I are the retention volumes for the non-retained component, the component that has full access to all the pores in the support, and the compound under test, respectively. Each retention volume is measured from the time of application to the time of the peak maximum.

DETERMINATION OF RELATIVE COMPONENT COMPOSITION OF MIXTURE Carry out the separation as directed in the individual monograph. Monitor the elution of the components continuously, and measure the corresponding peak areas. If all the components under test exhibit equivalent responses to the physicochemical property being monitored (for example, if they exhibit corresponding absorptivities), calculate the relative amount of each component by dividing the respective peak area by the sum of the peak areas of all the components under test. If the responses to the property used for the detection of the components under test are not equivalent, calculate the content either from calibration curves obtained from the calibration procedure specified in the individual monograph or by any other means stated in the individual monograph.

DETERMINATION OF MOLECULAR WEIGHTS Size-exclusion chromatography is used to determine molecular weights of components under test by comparison to calibration standards specified in the individual monograph. Plot the retention volumes of the calibration standards versus the logarithm of their molecular weights. Draw the line that best fits the plotted points within the exclusion and total permeation limits for the particular separation medium. From the calibration curve, molecular weights of components under test are estimated. This calibration is valid only for the particular macromolecular solute-solvent system used under the specified experimental conditions.

DETERMINATION OF MOLECULAR WEIGHT DISTRIBUTION OF POLYMERS The material used for calibration and the methods for determination of the distribution of molecular weights of polymers are specified in the individual monograph. However, sample comparison is valid only for results obtained under identical experimental conditions.

3.7 ELECTROPHORESIS

Electrophoresis is a physical method of analysis permitting the separation of compounds that are capable of acquiring an electrical charge in a conducting electrolyte. In this medium the ionized particles move more or less rapidly under the influence of an electrical field.

The electrophoretic mobility is the rate of migration of the substance measured in cm/s under the influence of a potential gradient of 1 V/cm, and is expressed in $cm^2V^{-1}s^{-1}$.

The measurement of electrophoretic mobility is significant only where experimental conditions have been precisely defined. This mobility depends on the characteristics of the substance, its nature, size, form, and electrical charge. It also depends on the composition of the conducting liquid, its nature, concentration, pH, the presence of additional solvents, and viscosity. The direction of migration depends on the sign of the electrical charge of the particle as it moves towards the electrode of opposite sign.

According to the methods used, the eletrophoretic mobility is either measured directly or compared with that of a reference substance.

Moving Boundary (Free-flow) Electrophoresis

This technique, used exclusively for the determination of the mobility, is particularly suitable for substances of high molecular weight with poor diffusion properties.

The boundaries are usually measured both before and after the application of an electrical field by a physical method, such as refractometry or conductometry. The concentration of the substance in the conducting liquid, the characteristics of the latter and the details of the procedure, including quantitative evaluation of the fractions, are specified in the monographs.

Zone Electrophoresis (Electrophoresis Using a Supporting Medium)

This method uses only small sample sizes. The nature of the supporting medium (for example, paper, cellulose acetate, starch gel, agar gel, polyacrylamide gel, mixed gel) introduces additional factors influencing the mobility. The rate of migration depends on the mobility of the particles and also on the electro-endosmotic current (in the case of carriers with polar properties), on the currents due to evaporation (caused by heat generated through the Joule effect), and on the gradient of the electrical field.

In practice, the mobility of the electrophoretic zones and their signs are ignored; the zones are located by experience or by comparison with those given by a reference substance treated in the same way.

After separation of the constituents, the position of colourless substances may be determined by treating the electrophoretogram with a reagent that will convert them to coloured or fluorescent derivatives. For quantitative purposes, the spot (zone) may be carefully separated, the substance eluted with a suitable solvent and then determined by a sufficiently sensitive method, such as spectrophotometric measurement, either directly or after a chemical reaction. In another quantitative procedure after conversion to a coloured derivative, the zone intensity can be measured with the aid of a scanning densitometer.

Apparatus

The apparatus for electrophoresis consists essentially of the following components.

(1) An appropriate power source supplying a constant, direct current and provided with means for indicating and controlling either the output voltage or the current consumption as appropriate; additional circuitry may be incorporated to stabilize the output.

(2) An electrophoretic assembly with an appropriate support-carrying device.

For electrophoresis using paper, agar gel, agarose gel, agarose-starch gel, or cellulose acetate as the electrophoretic support, the assembly consists of a tank with a close-fitting lid made of glass or other suitable material. The tank should be fitted with suitable safety devices to ensure that electrical supply is disconnected when the lid is removed. Two double troughs provided with a central lengthwise partition are inserted in the tank, one at each end; alternatively the troughs may be integral parts of the tank. One platinum electrode is laid along the bottom of one compartment of each double trough and the electrodes are connected through insulated leads, sealed through the walls of the tank, to external insulated cables which are connected to the power source. The troughs are filled with sufficient of the specified eletrolyte solution to ensure that the electrodes are fully immersed. Contact between the inner and outer compartment of each double trough is made either by means of "bridges" of electrophoresis paper or by perforating the central partition with several holes or by any other suitable method.

For agar-gel, agarose-gel and agarose-starch-gel electrophoresis the tank is designed to allow ventilation that will prevent the condensation of moisture or the drying of the layer of the solid medium. For polyacrylamide-gel electrophoresis the assembly consists of two buffer reservoirs made of poly (methylmethacrylate) or similar material, each fitted with a platinum or graphite electrode. The upper reservoir is mounted vertically above the lower and its height is adjustable. It has a number of rubber holders in its base situated equidistant from the electrode. The electrodes are connected by insulated cables to the power source so that the cathode is in the upper reservoir and the anode in the lower.

(3) An electrophoretic support.

For paper and cellulose acetate electrophoresis the electrophoretic support is in the form of strips held between the troughs on a uniform surface composed of inert plastic or glass contact points, spaced so as to minimize capillary diffusion of the electrolyte solution.

For agar-gel, agarose-gel and agarose-starch-gel electrophoresis the electrophoretic support is in the form of a gel, 6 mm thick, spread on a glass which in the case of agar-gel electrophoresis is placed on a hollow metal plate through which a cooling liquid may be circulated and the upper surface of which is machined to allow intimate contact with the glass plate.

The electrophoretic support is connected either directly (paper and cellulose acetate electrophoresis) or indirectly by means of wicks of suitable material (agar-gel, agarose-gel and agarose-starch-gel electrophoresis) to the compartment of each double trough that does not contain the electrode.

For polyacrylamide-gel electrophoresis the electrophoretic support is in the form of a gel contained in clean glass tubes, 7.5 cm long and 0.5 cm in internal diameter.

(4) A measuring device or means of detection.

Methods

Different methods in zone electrophoresis are described below.

PAPER ELECTROPHORESIS

Fill the troughs of the tank with the electrolyte solution specified in the monograph.

Apply separately to points along the line of application (drawn about 13 cm from one end of the electrophoresis paper), 1 cm from the edge of the paper and not less than 2.5 cm apart, the volumes of solutions prepared as described in the monograph.

Allow the spots to dry and then place the end of the paper nearer the line of application in the appropriate compartment of the anode trough and the other end in the appropriate compartment of the cathode trough. Wet the paper with the electrolyte solution by a suitable method (for example using a brush, starting from the ends of the paper and working towards the line of application). Do not wet the strip that includes the applied samples. Close the lid, allow the electrolyte solution to diffuse across the line of application, if

necessary cover the apparatus so as to exclude light, connect the cables to the power source and switch on the current. Adjust the voltage to about 20 V per cm of paper between the troughs and allow electrophoresis to proceed for the time indicated or until the marker substances have moved to the specified distances. Switch off the current, remove the paper, dry, if necessary in the dark, in a current of air, and examine under ultraviolet light (254 nm).

CELLULOSE ACETATE ELECTROPHORESIS

Use Method I unless otherwise directed.

Method I Fill the trough of the apparatus with the electrolyte solution specified in the monograph. Immerse cellulose acetate foil of suitable dimensions for 5 minutes in the same solution and press the strips dry between filter paper. Apply separately to the foil at points 1 cm from the anode edge and 2.5 cm apart 1 µl of each of the solutions prescribed in the monograph. Adjust the voltage to that specified in the monograph and allow electrophoresis to proceed for the specified time. Press the strips dry and immerse in a solution prepared by dissolving 1 g of *potassium hexacyanoferrate (III)* in 50 ml of *water* and adding 2 ml of a saturated solution of *iron(III) chloride*. Wash with a 5 per cent v/v solution of *phosphoric acid* until the background is as pale as possible and finally wash with *water*. Examine the electrophoretogram.

Method II Fill the troughs of the apparatus with *mixed barbital buffer pH 8.6*. Using 10 separate strips of cellulose acetate for each solution prescribed in the monograph, apply either 2.5 µl of solution as a 10-mm band or, if narrower strips are used, 0.25 µl of solution per mm of strip width. Apply a suitable electric field such that the most rapid band migrates at least 30 mm. Stain the strips with a 0.5 per cent w/v solution of *naphthalene black 12B* in a mixture of 90 volumes of *methanol* and 10 volumes of 5 M *acetic acid* for 5 minutes and then decolorize with a mixture of 90 volumes of *methanol* and 10 volumes of *acetic acid* so that the background is just free of colour. Wash the strips with a mixture of 81 volumes of *methanol* and 19 volumes of 5 M *acetic acid* until the background is as transparent as possible. Measure the absorbance of the bands at 600 nm in an instrument having a linear response over the range of at least 0 to 3 (Appendix 2.2). Calculate the result as the mean of the measurements of each of the ten strips.

AGAR-GEL ELECTROPHORESIS

Method I Place a suitable metal or plastic frame on the glass plate, seal its inner edge to the plate with a small quantity of liquefied Medium A. Place the plate on a level surface and pour on sufficient liquefied Medium A previously inoculated with 1 per cent v/v of the Inoculum of Test Organism A to produce a layer 1.2 to 1.6 mm thick. Allow the medium to set, remove the frame and bore at least 32 holes, about 1 mm in diameter, in the medium in such a manner that the solutions may be applied either in the form of a Latin Square or in a Randomized Block Design and that they are adequately spaced to allow the separation of the components.

Place 5-µl quantities of each of the four solutions specified in the monograph in the holes in accordance with the chosen design. Transfer the prepared plate to the electrophoresis apparatus and place in each trough the same volume of Liquid Medium A, ensuring that the plate is level. Using wicks composed of a double layer of absorbent lint and moistened with Liquid Medium A, connect the contents of the appropriate compartment of each trough with the solid medium on the prepared plate; for the latter, the wicks should extend 2 cm across the solid medium and be pressed gently into contact with it. Close the tank and apply a potential between the electrodes to produce a voltage of 15 to 20 V per cm of the length of the layer of Medium A preferably using a stabilized voltage source. During electrophoresis circulate water or another suitable cooling liquid through the metal plate to prevent the temperature from rising above 15°. In atmospheres of high humidity, condensation of moisture may occur on the surface of the layer of Medium A if the ventilation of the box and cooling are not adequately controlled.

Allow electrophoresis to proceed until separation of the substance to be determined has been attained. Disconnect the electrodes, remove the glass plate, cover it, and incubate at 30° to 35° for 18 hours, taking precautions to ensure that the layer of medium does not become dry. Measure the diameter, at right angles to the direction of migration, of the zones of inhibition produced by the reference solutions and any corresponding zones produced by the substance being examined. Calculate the result as a percentage of the substance being examined.

Method II Carry out Method I with the following modifications.

Use Medium B, Liquid Medium B and Inoculum of Test Organism B in place of Medium A, Liquid Medium A and Inoculum of Test Organism A, respectively.

Apply a potential between the electrodes to produce a voltage of 25 to 30 V per cm of the length of the layer of Medium B. Allow electrophoresis to proceed for 2 hours.

AGAROSE-GEL ELECTROPHORESIS

Use a glass plate of suitable dimensions over the surface of which is deposited a firmly adhering layer of uniform thickness of a gel prepared from Agarose for Electrophoesis. Use Electrolyte Solution I to equilibrate the agarose. Apply separately to the gel, 2 to 3 µl of the solutions prepared as described in the monograph. Close the tank, connect the electrodes to the power supply and allow electrophoresis to proceed at a current of 1 to 2 mA per cm of gel width at a potential of 300 V for about 10 minutes. Disconnect the electrodes, remove the glass plate and stain the gel using a 0.1 per cent w/v solution of Toluidine Blue removing the excess by washing. Evaluate the gels as prescribed in the monograph.

AGAROSE-STARCH-GEL ELECTROPHORESIS

Use a glass plate of suitable dimensions. Place a suitable metal or plastic frame on the glass plate, seal its inner edge to the plate with a small quantity of liquefied Medium C, place the plate on a level surface, and pour on sufficient liquefied Medium C to produce a layer about 1 mm thick. Allow the medium to set, remove the frame and bore two holes 1 mm in diameter in the surface of the medium. The holes should be 5 cm apart and 5 cm from one end of the plate.

Place 5-µl portions of each of the solutions specified in the monograph in the holes, pour into each compartment of the tank a sufficient volume of Electrolyte Solution II, and place the plate, gel uppermost, in the tank, ensuring that the plate is level. Using wicks composed of a double layer of absorbent lint and moistened with Electrolyte Solution II, connect the contents of the appropriate compartment of each trough with the solid medium on the prepared plate so that the cathode is connected to the end of the plate at which the holes are located; the wicks should extend 2 cm across the solid medium and be pressed gently into contact with it. Close the tank and apply a potential between the electrodes to produce a voltage of 20 V per cm of the layer of medium, preferably using a stabilized voltage source. Allow electrophoresis to proceed for 30 minutes, disconnect the electrodes and remove the glass plate. Using the same procedure as described above, cover the gel with a layer of liquefied Medium A previously inoculated with 1 per cent v/v of Inoculum of Test Organism A to produce a layer 1.2 to 1.6 mm thick. Incubate at 30° to 35° for 18 hours and measure the zones of inhibition produced.

POLYACRYLAMIDE ROD GEL ELECTROPHORESIS

In polyacrylamide rod gel electrophoresis, the stationary phase is a gel which is prepared from a mixture of acrylamide and *N,N'*-methylenebisacrylamide. Rod gels are prepared in tubes 7.5 cm long and 0.5 cm in internal diameter, one solution being applied to each rod.

Apparatus The platinum electrode is recommended.

Procedure The solutions should usually be degassed before polymerization and the gels used immediately after preparation. Prepare the gel mixture as prescribed and pour into suitable glass tubes, stoppered at the bottom, to an equal height in each tube and to about 1 cm from the top, taking care to ensure that no air bubbles are trapped in the tubes. Cover the gel mixture with a layer of *water* to exclude air and allow to set. Gel formation usually takes about 30 minutes and is complete when a sharp interface appears between the gel and the water layer. Remove the water layer. Fill the lower reservoir with the prescribed buffer solution and remove the stoppers from the tubes. Fit the tubes into the holders of the upper reservoir and adjust so that the bottom of the tubes are immersed in the buffer solution in the lower reservoir. Carefully fill the tubes with the prescribed buffer solution. Prepare the test and reference solutions containing the prescribed marker dye and make them dense by dissolving in them *sucrose*, for example. Apply the solutions to the surface of a gel using a different tube for each solution. Add the same buffer to the upper reservoir. Connect the electrodes to the power supply and allow electrophoresis to proceed at the prescribed temperature and using the prescribed constant voltage or current. Switch off the power supply when the marker dye has migrated almost into the lower reservoir. Immediately remove each tube from the apparatus and extrude the gel. Locate the position of the bands in the electrophoretogram as prescribed.

SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Polyacrylamide gel electrophoresis is used for the qualitative characterization of proteins in biological preparations, for control of purity and quantitative determinations.

Analytical gel electrophoresis is an appropriate method with which to identify and to assess the homogeneity of proteins in pharmaceutical preparations. The method is routinely used for the estimation of protein subunit molecular masses and for determining the subunit compositions of purified proteins. Ready-to-use gels and reagents are widely available on the market and can be used instead of those described in this text, provided that they give equivalent results and that they meet the validity requirements given below under Validation of the test.

Characteristics of polyacrylamide gels The sieving properties of polyacrylamide gels are established by the three-dimensional network of fibres and pores which is formed as the bifunctional bisacrylamide cross-links adjacent polyacrylamide chains. Polymerization is catalyzed by a free radical-generating system composed of *ammonium persulfate* and *tetramethylethylenediamine*. As the acrylamide concentration of a gel increases, its effective pore size decreases. The effective pore size of a gel is operationally defined by its sieving properties; that is, by the resistance it imparts to the migration of macromolecules. There are limits on the acrylamide concentrations that can be used. At high acrylamide concentrations, gels break much more easily and are difficult to handle. As the pore size of a gel decreases, the migration rate of a protein through the gel decreases. By adjusting the pore size of a gel, through manipulating the acrylamide concentration, the resolution of the method can be optimized for a given protein product. Thus, a given gel is physically characterized by its respective composition in acrylamide and bisacrylamide. In addition to the composition of the gel, the state of the protein is an important component to the electrophoretic mobility. In the case of proteins, the electrophoretic mobility is dependent on the pK value of the charged groups and the size of the molecule. It is influenced by the type, concentration and

pH of the buffer, by the temperature and the field strength as well as by the nature of the support material.

Denaturing polyacrylamide gel electrophoresis The method cited as an example is limited to the analysis of monomeric polypeptides with a mass range of 14,000 to 100,000 daltons[1]. It is possible to extend this mass range by various techniques (e.g., gradient gels, particular buffer system) but those techniques are not discussed in this appendix.

Denaturing polyacrylamide gel electrophoresis using sodium dodecyl sulfate (SDS-PAGE) is the most common mode of electrophoresis used in assessing the pharmaceutical quality of protein products and will be the focus of the example method. Typically, analytical electrophoresis of proteins is carried out in polyacrylamide gels under conditions that ensure dissociation of the proteins into their individual polypeptide subunits and that minimize aggregation. Most commonly, the strongly anionic detergent *sodium dodecyl sulfate* (SDS) is used in combination with heat to dissociate the proteins before they are loaded on the gel. The denatured polypeptides bind to SDS, become negatively charged and exhibit a consistent charge-to-mass ratio regardless of protein type. Because the amount of SDS bound is almost always proportional to the molecular mass of the polypeptide and is independent of its sequence, SDS-polypeptide complexes migrate through polyacrylamide gels with mobilities dependent on the size of the polypeptide.

The electrophoretic mobilities of the resultant detergent-polypeptide complexes all assume the same functional relationship to their molecular masses. Migration of SDS complexes is towards the anode in a predictable manner, with low-molecular-mass complexes migrating faster than larger ones. The molecular mass of a protein can therefore be estimated from its relative mobility in calibrated SDS-PAGE and the occurrence of a single band in such a gel is a criterion of purity.

Modifications to the polypeptide backbone, such as *N*- or *O*-linked glycosylation, however, have a significant impact on the apparent molecular mass of a protein since SDS does not bind to a carbohydrate moiety in a manner similar to a polypeptide. Thus, a consistent charge-to-mass ratio is not maintained. The apparent molecular mass of proteins having undergone post-translational modifications is not a true reflection of the mass of the polypeptide chain.

REDUCING CONDITIONS Polypeptide subunits and three-dimensional structure is often maintained in proteins by the presence of disulfide bonds. A goal of SDS-PAGE analysis under reducing conditions is to disrupt this structure by reducing disulfide bonds. Complete denaturation and dissociation of proteins by treatment with *2-mercaptoethanol* or *dithiothreitol* (DTT) will result in unfolding of the polypeptide backbone and subsequent complexation with SDS. In these conditions, the molecular mass of the polypeptide subunits can be calculated by linear regression in the presence of suitable molecular-mass standards.

NON-REDUCING CONDITIONS For some analyses, complete dissociation of the protein into subunit peptides is not desirable. In the absence of treatment with reducing agents such as *2-mercaptoethanol* or *DTT*, disulfide covalent bonds remain intact, preserving the oligomeric form of the protein. Oligomeric SDS-protein complexes migrate more slowly than their SDS-polypeptide subunits. In addition, non-reduced proteins may not be completely saturated with SDS and, hence, may not bind the detergent in a constant mass ratio. This makes molecular-mass determinations of these molecules by SDS-PAGE less straightforward than analyses of fully denatured polypeptides, since it is necessary that both standards and unknown proteins be in similar configurations for valid comparisons. However, the staining of a single band in such a gel is a criterion of purity.

Characteristics of discontinuous buffer system gel electrophoresis The most popular electrophoretic method for the characterization of complex mixtures of proteins involves the use of a discontinuous buffer system consisting of two contiguous, but distinct gels: a resolving or separating (lower) gel and a stacking (upper) gel. The two gels are cast with different porosities, pH, and ionic strengths. In addition, different mobile ions are used in the gel and electrode buffers. The buffer discontinuity acts to concentrate large volume samples in the stacking gel, resulting in improved resolution. When power is applied, a voltage drop develops across the sample solution which drives the proteins into the stacking gel. Glycinate ions from the electrode buffer follow the proteins into the stacking gel. A moving boundary region is rapidly formed with the highly mobile chloride ions in the front and the relatively slow glycinate ions in the rear. A localized high-voltage gradient forms between the leading and trailing ion fronts, causing the SDS-protein complexes to form into a thin zone (stack) and migrate between the chloride and glycinate phases. Within broad limits, regardless of the height of the applied sample, all SDS-proteins condense into a very narrow region and enter the resolving gel as a well-defined, thin zone of high protein density. The large-pore stacking gel does not retard the migration of most proteins and serves mainly as an anticonvective medium. At the interface of the stacking and resolving gels, the proteins experience a sharp increase in retardation due to the restrictive pore size of the resolving gel. Once in the resolving gel, proteins continue to be slowed by the sieving of the matrix. The glycinate ions overtake the proteins, which then move in a space of uniform pH formed by the tris-(hydroxymethyl) aminomethane and glycine. Molecular sieving causes the SDS-polypeptide complexes to separate on the basis of their molecular masses.

[1]One dalton is equivalent to 1.65×10^{-24} g.

Preparing vertical discontinuous buffer SDS polyacrylamide gels

ASSEMBLING OF THE GEL MOULDING CASSETTE Clean the two glass plates (size: e.g., 10 cm × 8 cm), the polytetrafluoroethylene comb, the two spacers and the silicone rubber tubing (diameter: e.g., 0.6 mm × 35 cm) with mild detergent and rinse extensively with *water*. Dry all the items with a paper towel or tissue. Lubricate the spacers and the tubing with non-silicone grease. Apply the spacers along each of the two short sides of the glass plate 2 mm away from the edges and 2 mm away from the long side corresponding to the bottom of the gel. Begin to lay the tubing on the glass plate by using one spacer as a guide. Carefully twist the tubing at the bottom of the spacer and follow the long side of the glass plate. While holding the tubing with one finger along the long side twist again the tubing and lay it on the second short side of the glass plate, using the spacer as a guide. Place the second glass plate in perfect alignment and hold the mould together by hand pressure. Apply two clamps on each of the two short sides of the mould. Carefully apply four clamps on the longer side of the gel mould thus forming the bottom of the gel mould. Verify that the tubing is running along the edge of the glass plates and has not been extruded while placing the clamps. The gel mould is now ready for pouring the gel.

PREPARATION OF THE GEL In a discontinuous buffer SDS polyacrylamide gel, it is recommendesd to pour the resolving gel, let the gel set, and then pour the stacking gel since the composition of the two gels in acrylamide-bisacrylamide, buffer and pH are different.

Preparation of the resolving gel In a conical flask, prepare the appropriate volume of solution containing the desired concentration of *acrylamide* for the resolving gel, using the values given in Table 1. Mix the components in the order shown. Where appropriate, before adding the ammonium persulfate solution and the teramethylethylenediamine (TEMED), filter the solution if necessary under vacuum through a cellulose acetate membrane (pore diameter: 0.45 µm); keep the solution under vacuum by swirling the filtration unit until no more bubbles are formed in the solution. Add appropriate amounts of *ammonium persulfate solution* and TEMED as indicated in Table 1, swirl and pour immediately into the gap between the two glass plates of the mould. Leave sufficient space for the stacking gel (the length of the teeth of the comb plus 1 cm). Using a tapered glass pipette, carefully overlay the solution with water-saturated 2-methyl-1-propanol. Leave the gel in a vertical position at room temperature to allow polymerization.

Preparation of the stacking gel After polymerization is complete (about 30 minutes), pour off 2-*methyl-1-propanol* and wash the top of the gel several times with *water* to remove the 2-methyl-1-propanol overlay and any unpolymerized acrylamide. Drain as much fluid as possible from the top of the gel, and remove any remaining water with the edge of a paper towel. In a conical flask, prepare the appropriate volume of solution containing the desired concentration of *acrylamide*, using the values given in Table 2. Mix the components in the order shown. Where appropriate, before adding the ammonium persulfate solution and the TEMED, filter the solution if necessary under vacuum through a cellulose acetate membrane (pore diameter: 0.45 µm); keep the solution under vacuum by swirling the filtration unit until no more bubbles are formed in the solution.

Add appropriate amounts of *ammonium persulfate solution* and TEMED as indicated in Table 2, swirl and pour immediately into the gap between the two glass plates of the mould directly onto the surface of the polymerized resolving gel. Immediately insert a clean polytetrafluoroethylene comb into the stacking gel solution, being careful to avoid trapping air bubbles. Add more stacking gel solution to fill the spaces of the comb completely. Leave the gel in a vertical position and allow to polymerize at room temperature.

MOUNTING THE GEL IN THE ELECTROPHORESIS ELECTROPHORESIS APPARATUS AND ELECTROPHORETIC SEPARATION After polymerization is complete (about 30 minutes), remove the polytetrafluoroethylene comb carefully. Rinse the wells immediately with *water* or with the SDS-PAGE running buffer to remove any unpolymerized acrylamide. If necessary, straighten the teeth of the stacking gel with a blunt hypodermic needle attached to a syringe. Remove the clamps on one short side, carefully pull out the tubing and replace the clamps. Proceed similarly on the other short side. Remove the tubing from the bottom part of the gel. Mount the gel in the electrophoresis apparatus. Add the electrophoresis buffers to the top and bottom reservoirs. Remove any bubbles that become trapped at the bottom of the gel between the glass plates. This is best done with a bent hypodermic needle attached to a syringe. Never prerun the gel before loading the samples, since this will destroy the discontinuity of the buffer systems. Before loading the sample, carefully rinse the slot with SDS-PAGE running buffer. Prepare the test and reference solutions in the recommended sample buffer and treat as specified in the individual monograph. Apply the appropriate volume of each solution to the stacking gel wells. Start the electrophoresis using the conditions recommended by the manufacturer of the equipment. Manufacturers of SDS-PAGE equipment may provide gels of different surface area and thickness. Electrophoresis running time and current/voltage may need to vary as described by the manufacturer of the apparatus in order to achieve optimum separation. Check that the dye front is moving into the resolving gel. When the dye is reaching the bottom of the gel, stop the electrophoresis. Remove the gel assembly from the apparatus and separate the glass plates. Remove the spacers, cut off and discard the stacking gel and immediately proceed with staining.

Detection of proteins in gels Coomassie staining is the most common protein staining method with a

Table 1 Preparation of Resolving Gel

Solution Components	Component Volumes (ml) per Gel Mould Volume of							
	5 ml	10 ml	15 ml	20 ml	25 ml	30 ml	40 ml	50 ml
6 per cent acrylamide								
Water	2.6	5.3	7.9	10.6	13.2	15.9	21.2	26.5
Acrylamide solution	1.0	2.0	3.0	4.0	5.0	6.0	8.0	10.0
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
100 g/l SDS *	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
100 g/l APS**	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED†	0.004	0.008	0.012	0.016	0.02	0.024	0.032	0.04
8 per cent acrylamide								
Water	2.3	4.6	6.9	9.3	11.5	13.9	18.5	23.2
Acrylamide solution	1.3	2.7	4.0	5.3	6.7	8.0	10.7	13.3
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
100 g/l SDS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
100 g/l APS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.003	0.006	0.009	0.012	0.015	0.018	0.024	0.03
10 per cent acrylamide								
Water	1.9	4.0	5.9	7.9	9.9	11.9	15.9	19.8
Acrylamide solution	1.7	3.3	5.0	6.7	8.3	10.0	13.3	16.7
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
100 g/l SDS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
100 g/l APS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
12 per cent acrylamide								
Water	1.6	3.3	4.9	6.6	8.2	9.9	13.2	16.5
Acrylamide solution	2.0	4.0	6.0	8.0	10.0	12.0	16.0	20.0
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
100 g/l SDS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
100 g/l APS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
14 per cent acrylamide								
Water	1.4	2.7	3.9	5.3	6.6	8.0	10.6	13.8
Acrylamide solution	2.3	4.6	7.0	9.3	11.6	13.9	18.6	23.2
1.5 M Tris (pH 8.8)	1.2	2.5	3.6	5.0	6.3	7.5	10.0	12.5
100 g/l SDS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
100 g/l APS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
15 per cent acrylamide								
Water	1.1	2.3	3.4	4.6	5.7	6.9	9.2	11.5
Acrylamide solution	2.5	5.0	7.5	10.0	12.5	15.0	20.0	25.0
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
100 g/l SDS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
100 g/l APS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02

*a 100 g/l solution of *sodium dodecyl sulfate*.

**a 100 g/l solution of *ammonium persulfate*. Ammonium persulfate provides the free radicals that drive polymerization of acrylamide and bisacrylamide. Since ammonium persulfate solution decomposes slowly, fresh solutions must be prepared weekly.

†*tetramethylethylenediamine*.

Table 2 Preparation of Stacking Gel

Solution Components	Component Volumes (ml) per Gel Mould Volume of							
	1 ml	2 ml	3 ml	4 ml	5 ml	6 ml	8 ml	10 ml
Water	0.68	1.4	2.1	2.7	3.4	4.1	5.5	6.8
Acrylamide solution	0.17	0.33	0.5	0.67	0.83	1.0	1.3	1.7
1.0 M Tris (pH 6.8)	0.13	0.25	0.38	0.5	0.63	0.75	1.0	1.25
100 g/l SDS*	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
100 g/l APS**	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
TEMED†	0.001	0.002	0.003	0.004	0.005	0.006	0.008	0.01

*a 100 g/l solution of *sodium dodecyl sulfate*.

**a 100 g/l solution of *ammonium persulfate*. Ammonium persulfate provides the free radicals that drive polymerization of acrylamide and bisacrylamide. Since ammonium persulfate solution decomposes slowly, fresh solutions must be prepared weekly.

†*tetramethylethylenediamine*.

detection level of the order of 1 to 10 μg of protein per band. Silver staining is the most sensitive method for staining proteins in gels and a band containing 10 to 100 ng can be detected.

All of the steps in gel staining are done at room temperature with gentle shaking (e.g., on an orbital shaker platform) in any convenient container. Gloves must be worn when staining gels, since fingerprints will stain.

COOMASSIE STAINING Immerse the gel in a large excess of Coomassie staining solution and allow to stand for at least 1 hour. Remove the staining solution.

Destain the gel with a large excess of Destaining solution. Change the destaining solution several times, until the stained protein bands are clearly distinguishable on a clear background. The more thoroughly the gel is destained, the smaller is the amount of protein that can be detected by the method. Destaining can be speeded up by including a few grams of anion-exchange resin or a small sponge in the Destaining solution. (**Note** The acid-alcohol solutions used in this procedure do not completely fix proteins in the gel.) This can lead to losses of some low-molecular-mass proteins during the staining and destaining of thin gels. Permanent fixation is obtainable by allowing the gel to stand in a mixture of 1 volume of *trichloroacetic acid*, 4 volumes of *methanol* and 5 volumes of *water* for 1 hour before it is immersed in the Coomassie staining solution.

SILVER STAINING Immerse the gel in a large excess of fixing solution and allow to stand for 1 hour. Remove the fixing solution, add fresh fixing solution and incubate either for at least 1 hour or overnight, if convenient. Discard the fixing solution and wash the gel in a large excess of *water* for 1 hour. Soak the gel for 15 minutes in a 1 per cent v/v solution of *glutaraldehyde*. Wash the gel twice for 15 minutes in a large excess of *water*. Soak the gel in fresh Silver staining solution for 15 minutes, in darkness. Wash the gel three times for 5 minutes in a large excess of *water*. Immerse the gel for about 1 minute in Developing solution until satisfactory staining has been obtained. Stop the development by incubation in the Blocking solution for 15 minutes. Rinse the gel with *water*.

Drying of stained SDS polyacrylamide gels Depending on the staining method used, gels are treated in a slightly different way. For Coomassie staining, after the destaining step, allow the gel to stand in a 10 per cent w/v solution of *glycerol* for at least 2 hours (overnight incubation is possible). For silver staining, add to the final rinsing a step of 5 minutes in a 2 per cent w/v solution of *glycerol*. Immerse two sheets of porous cellulose film in *water* and incubate for 5 to 10 minutes. Place one of the sheets on a drying frame. Carefully lift the gel and place it on the cellulose film. Remove any trapped air bubbles and pour a few millilitres of *water* around the edges of the gel. Place the second sheet on top and remove any trapped air bubbles. Complete the assembly of the drying frame. Place in an oven or leave at room temperature until dry.

Molecular-mass determination Molecular masses of proteins are determined by comparison of their mobilities with those of several marker proteins of known molecular weight. Mixtures of proteins with precisely known molecular masses blended for uniform staining are available for calibrating gels. They are obtainable in various molecular mass ranges. Concentrated stock solutions of proteins of known molecular mass are diluted in the appropriate sample buffer and loaded on the same gel as the protein sample to be studied. Immediately after the gel has been run, the position of the bromophenol blue tracking dye is marked to identify the leading edge of the electrophoretic ion front. This can be done by cutting notches in the edges of the gel or by inserting a needle soaked in India ink into the gel at the dye front. After staining, measure the migration distances of each protein band (markers and unknowns) from the top of the resolving gel. Divide the migration distance of each protein by the distance travelled by the tracking dye. The normalized migration distances so obtained are called the relative mobilities of the proteins (relative to the dye front) and conventionally denoted as R_f. Construct a plot of the logarithm of the relative molecular masses (M_r) of the protein standards as a function of the R_f values. Note that the graphs are slightly sigmoid. Unknown molecular masses can be estimated by linear regression analysis or interpolation from the curves of

log M_r against R_f as long as the values obtained for the unknown samples are positioned along the linear part of the graph.

Validation of the test The test is not valid unless the proteins of the molecular mass marker are distributed along 80 per cent of the length of the gel and over the required separation range (e.g., the range covering the product and its dimer or the product and its related impurities) the separation obtained for the relevant protein bands shows a linear relationship between the logarithm of the molecular mass and the R_f. Additional validation requirements with respect to the solution under test may be specified in individual monographs.

Quantification of impurities Where the impurity limit is specified in the individual monograph, a reference solution corresponding to that level of impurity should be prepared by diluting the test solution. For example, where the limit is 5 per cent, a reference solution would be a 1:20 dilution of the test solution. No impurity (any band other than the main band) in the electropherogram obtained with the test solution may be more intense than the main band obtained with the reference solution.

Under validated conditions impurities may be quantified by normalization to the main band using an integrating densitometer. In this case, the responses must be validated for linearity.

SAFETY PRECAUTIONS

Voltages used in electrophoresis can readily deliver a lethal shock. The hazard is increased by the use of aqueous buffer solutions and the possibility of working in damp environments.

The equipment, with the possible exception of the power supply, should be enclosed in either a grounded metal case or a case made of insulating material. The case should have an interlock that de-energizes the power supply when the case is opened, after which reactivation should be prevented until activation of a reset switch is carried out.

High-voltage cables from the power supply to the apparatus should preferably be a type in which a braided metal shield completely encloses the insulated central conductor, and the shield should be grounded. The base of the apparatus should be grounded metal or contain a grounded metal rim which is constructed in such a way that any leakage of electrolyte will produce a short which will de-energize the power supply before the electrolyte can flow beyond the protective enclosure.

If the power supply contains capacitors as part of a filter circuit, it should also contain a bleeder resistor to ensure discharge of the capacitors before the protective case is opened. A shorting bar that is activated by opening the case may be considered as an added precaution.

Because of the potential hazard associated with electrophoresis, laboratory personnel should be completely familiar with electrophoresis equipment before using it.

REAGENTS

Acrylamide solution Prepare a solution containing 290 g of *acrylamide* and 10 g of *methylenebisacrylamide* per litre of *water* and filter.

Agarose for electrophoresis Use electrophoretic grade of commerce.

Blocking solution A 10 per cent v/v solution of *acetic acid*.

Coomassie staining solution A 0.125 per cent w/v solution of *acid blue 83* in a mixture of 1 volume of *glacial acetic acid*, 4 volumes of *methanol* and 5 volumes of *water*.

Destaining solution A mixture of 1 volume of *glacial acetic acid*, 4 volumes of *methanol* and 5 volumes of *water*.

Developing solution

Formaldehyde Solution	0.30	ml
Citric Acid	0.025	g
Water sufficient to produce	500.0	ml

Electrolyte solution I Mix 50 ml of *glacial acetic acid* and 800 ml of *water,* adjust the pH to 3.0 with *lithium hydroxide* and dilute to 1000 ml with *water*.

Electrolyte solution II Dissolve 1.015 g of *dipotassium hydrogenphosphate* and 340 mg of *potassium dihydrogenphosphate* in sufficient *water* to produce 1000 ml.

Electrophoresis paper Suitable filter paper (Whatman 3 MM or equivalent is suitable) that has been washed chromatographically for 16 hours with a mixture of 2 volumes of *acetone* and 1 volume of *water*. After drying, cut the paper into strips of appropriate size.

Fixing solution To 250 ml of *methanol* and 0.27 ml of *formaldehyde solution* and dilute with sufficient *water* to produce 500 ml.

Inoculum of test organism A Grow *Bacillus subtilis* (ATCC 11774, NCTC 8236) at a temperature of 37° to 39° for 7 days on the surface of Medium A to which has been added 0.001 per cent w/v of *manganese(II) sulfate*. Using sterile *water*, wash off the growth, which consists mainly of spores, and dilute to give a suitable suspension; the degree of dilution should be determined experimentally. A suitable suspension usually contains between 10^7 and 10^8 spores per ml. The suspension may be stored for long periods at a temperature not exceeding 4°.

Inoculum of test organism B Prepare as for Inoculum of test organism A but using Medium B in place of Medium A.

Medium A

Dried peptone	6.0	g
Pancreatic digest of casein	4.0	g
Beef extract	1.5	g
Yeast extract	3.0	g
Dextrose monohydrate	1.0	g

Agar	15.0	g
Water sufficient to produce	1000	ml

Sterilize under pressure at 121° for 15 minutes. Immediately before use adjust to pH 6.5 by the addition of 0.1 M *hydrochloric acid*.

Liquid medium A Prepare as described for Medium A using the same ingredients but omitting the agar.

Medium B

Dried peptone	3.0	g
Pancreatic digest of casein	2.0	g
Beef extract	0.75	g
Yeast extract	1.5	g
Agar	10.0	g
Water sufficient to produce	1000	ml

Sterilize under pressure at 121° for 15 minutes. Immediately before use adjust to pH 6.5 by the addition of 0.1 M *hydrochloric acid*.

Liquid medium B Prepare as described for Medium B, using the same ingredients but omitting the agar.

Medium C

Agarose for electrophoresis	10.0	g
Hydrolyzed starch	10.0	g
Electrolyte solution II sufficient to produce	1000	ml

Add the Agarose for Electrophoresis and Hydrolyzed Starch to the electrolyte solution and heat in saturated steam at 121° until they are dissolved. Maintain the molten gel at a temperature of 50° until required for use.

Pretreatment solution Mix together 250 ml of *methanol*, 250 ml of *water* and 0.1 ml of *formaldehyde solution*.

Sample application buffer

Tris(hydroxymethyl)methylamine	1.5	g
1 M Hydrochloric acid	12.0	ml
Urea	96.0	g
Water sufficient to produce	200.0	ml

Sample buffer

Tris(hydroxymethyl)methylamine	0.76	g
Glycerol	5.0	ml
Sodium dodecyl sulfate	1.0	g
Bromophenol blue	0.1	g
2-Mercaptoethanol	1.0	ml
Water	25.0	ml

Mix together and adjust the pH to 6.8 with 6 M *hydrochloric acid* and dilute to 50.0 ml with *water*.

Silver staining solution Mix 40 ml of 1 M *sodium hydroxide* with 3 ml of *strong ammonia solution*, add 8 ml of a 20 per cent w/v solution of *silver nitrate*, and dilute with sufficient *water* to produce 200 ml.

Solution A

Tris(hydroxymethyl)methylamine	36.6	g
N,N,N′,N′-Tetramethylethylenediamine	0.23	ml
1 M Hydrochloric acid	48.0	ml
Water sufficient to produce	100.0	ml

Solution B

Acrylamide	30.0	g
N,N′-Methylenebisacrylamide	0.735	g
Water sufficient to produce	100.0	ml

Solution C Dissolve 6.0 g of *tris(hydroxymethyl)-methylamine* in 70 ml of *water*. Adjust the pH to 8.8 with 6 M *hydrochloric acid* and dilute with sufficient *water* to produce 100.0 ml.

Solution D Dissolve 6.0 g of *tris(hydroxymethyl)-methylamine* in 70 ml of *water*. Adjust the pH to 6.8 with 6 M *hydrochloric acid* and dilute with sufficient *water* to produce 100.0 ml.

Starch, hydrolyzed Use electrophoretic grade of commerce.

1.0 M Tris (pH 6.8) Dissolve 60.6 g of *tris(hydroxymethyl)aminomethane* in 400 ml of *water*, adjust the pH with *hydrochloric acid* and dilute to 500 ml with *water*.

1.5 M Tris (pH 8.8) Dissolve 90.8 g of *tris(hydroxymethyl)methylamine* in 400 ml of *water*, adjust the pH with *hydrochloric acid* and dilute to 500 ml with *water*.

Toluidine blue (Toluidine blue O) $C_{15}H_{16}ClN_3S$ = 305.83

DESCRIPTION Dark green powder.

SOLUBILITY Soluble in *water*; slightly soluble in *ethanol*.

3.8 CAPILLARY ELECTROPHORESIS

Electrophoresis refers to the migration of charged electrical species when dissolved or suspended in an electrolyte through which an electric current is passed. Cations migrate towards the negatively charged electrode (cathode), while anions are attracted towards the positively charged electrode (anode). Neutral particles are met attracted towards either electrode.

The use of capillaries as a migration channel in electrophoresis has enabled analysts to perform electrophoretic separations on an instrumental level comparable to that of high-performance liquid chromatography (HPLC), albeit with some distinct operational differences, advantage, and disadvantages relative to HPLC. This method of analysis is commonly known as capillary electrophoresis (CE). The migration velocity of an analyte under an electric field of intensity, E, is determined by the electrophoretic mobility of the analyte and the electro-osmotic mobility of the buffer inside the capillary. The electrophoretic mobility of a solute (μ_{ep}) depends on the characteristics of the solute (electric charge, molecular size and shape) and those of the buffer in which the migration takes place (type and ionic strength of the electrolyte, pH, viscosity and additives). The electrophoretic velocity (ν_{ep}) of a solute, assuming a spherical shape, is given by the equation:

$$v_{ep} = \mu_{ep} \times E = \left(\frac{q}{6\pi\eta r}\right) \times \left(\frac{V}{L}\right),$$

where q = effective charge of the solute,
η = viscosity of the electrolyte solution,
r = Stoke's radius of the solute,
V = applied voltage, and
L = total length of the capillary.

When an electric field is applied through the capillary filled with buffer, a flow of solvent is generated inside the capillary, called electro-osmotic flow. The velocity of the electro-osmotic flow depends on the electro-osmotic mobility (μ_{eo}) which in turn depends on the charge density on the capillary internal wall and the buffer characteristics. The electro-osmotic velocity (v_{eo}) is given by the equation:

$$v_{eo} = \mu_{eo} \times E = \left(\frac{\varepsilon\zeta}{\eta}\right) \times \left(\frac{V}{L}\right),$$

where ε = dielectric constant of the buffer, and
ζ = zeta potential of the capillary surface.

The velocity of the solute (v) is given by the equation:

$$v = v_{ep} + v_{eo}$$

The electrophoretic mobility of the analyte and the electro-osmotic mobility may act in the same direction or in opposite directions, depending on the charge of the solute. In normal capillary electrophoresis, anions will migrate in the opposite direction to the electro-osmotic flow and their velocities will be smaller than the electro-osmotic velocity. Cations will migrate in the same direction as the electro-osmotic flow and their velocities will be greater than the electro-osmotic velocity. Under conditions in which there is a fast electro-osmotic velocity with respect to the electrophoretic velocity of the solutes, both cations and anions can be separated in the same run.

The time (t) taken by the solute to migrate the distance (l) from the injection end of the capillary to the detection point (capillary effective length) is given by the equation:

$$t = \frac{l}{v_{ep} + v_{eo}} = \frac{l \times L}{(\mu_{ep} + \mu_{eo}) \times V}$$

In general, uncoated fused-silica capillaries above pH 3 have negative charge due to ionized silanol groups in the inner wall. Consequently, the electro-osmotic flow is from anode to cathode. The electro-osmotic flow must remain constant from run to run if good reproducibility is to be obtained in the migration velocity of the solutes. For some applications, it may be necessary to reduce or suppress the electro-osmotic flow by modifying the inner wall of the capillary or by changing the concentration, composition and/or pH of the buffer solution.

After the introduction of the sample into the capillary, each analyte ion of the sample migrates within the background electrolyte as an independent zone, according to its electrophoretic mobility. Zone dispersion, that is the spreading of each solute band, results from different phenomena. Under ideal conditions the sole contribution to the solute-zone broadening is molecular diffusion of the solute along the capillary (longitudinal diffusion). In this ideal case the efficiency of the zone, expressed as the number of theoretical plates (N), is given by the equation:

$$N = \frac{(\mu_{ep} + \mu_{eo}) \times V \times l}{2 \times D \times L},$$

where D = molecular diffusion coefficient of the solute in the buffer.

In practice, other phenomena such as heat dissipation, sample adsorption onto the capillary wall, mismatched conductivity between sample and buffer, length of the injection plug, detector cell size, and unlevelled buffer reservoirs can also significantly contribute to band dispersion. Separation between 2 bands (expressed as the resolution, R_s) can be obtained by modifying the electrophoretic mobility of the analytes, the electro-osmotic mobility induced in the capillary and by increasing the efficiency for the band of each analyte, according to the equation:

$$R_s = \frac{\sqrt{N}(\mu_{epb} - \mu_{epa})}{4(\bar{\mu}_{ep} + \mu_{eo})},$$

where μ_{epa} and μ_{epb} = electrophoretic mobilities of the 2 analytes separated,
$\bar{\mu}_{ep}$ = mean electrophoretic mobility of the 2 analytes, and
$\bar{\mu}_{ep} = 1/2\ (\mu_{epa} + \mu_{epb})$

Apparatus

An apparatus for capillary electrophoresis is composed of:

(a) a high-voltage, controllable direct-current power supply;

(b) 2 buffer reservoirs, held at the same level, containing the prescribed anodic and cathodic solutions;

(c) 2 electrode assemblies (the cathode and the an ode), immersed in the buffer reservoirs and connected to the power supply;

(d) a separation capillary (usually made of fusedsilica) which, when used with some specific types of detectors, has an optical viewing window aligned with the detector. The ends of the capillary are placed in the buffer reservoirs. The capillary is filled with the solution prescribed in the monograph;

(e) a suitable injection system;

(f) a detector able to monitor the amount of substances of interest passing through a segment of the separation capillary at a given time; it is usually based on absorption spectrophotometry (UV and visible) or fluorimetry, but conductimetric, amperometric or mass spectrometric detection can be useful for specific appli cations; indirect detection is an alternative method used to detect non-UV-absorbing and non-fluorescent com-

pounds;

(g) a thermostatic system able to maintain a constant temperature inside the capillary is recommended to obtain a good separation reproducibility; and

(h) a recorder and a suitable integrator or a computer.

The definition of the injection process and its automation are critical for precise quantitative analysis. Modes of injection include gravity, pressure or vacuum injection and electrokinetic injection. The amount of each sample component introduced electrokinetically depends on its electrophoretic mobility, leading to possible discrimination using this injection mode. Use the capillary, the buffer solutions, the preconditioning method, the sample solution and the migration conditions prescribed in the monograph of the considered substance. The employed electrolytic solution is filtered to remove particles and degassed to avoid bubble formation that could interfere with the detection system or interrupt the electrical contact in the capillary during the separation run. A rigorous rinsing procedure should be developed for each analytical method to achieve reproducible migration times of the solutes.

Capillary Zone Electrophoresis

In capillary zone electrophoresis, analytes are separated in a capillary containing only buffer without any anticonvective medium. With this technique, separation takes place because the different components of the sample migrate as discrete bands with different velocities. The velocity of each band depends on the electro-phoretic mobility of the solute and the electro-osmotic flow in the capillary (see General Principles). Coated capillaries can be used to increase the separation capacity of those substances adsorbing on fused-silica surfaces. Using this mode of capillary electrophoresis, the analysis of both small ($M_r < 2000$) and large molecules ($2000 < M_r < 100{,}000$) can be accomplished. Due to the high efficiency achieved in capillary zone electrophoresis, separation of molecules having only minute differences in their charge-to-mass ratio can be effected. This separation mode also allows the separation of chiral compounds by addition of chiral selectors to the separation buffer.

Optimization of the separation is a complex process where several separation parameters can play a major role. The main factors to be considered in the development of separations are instrumental and electrolytic solution parameters.

INSTRUMENTAL PARAMETERS

Voltage A Joule heating plot is useful in optimizing the applied voltage and capillary temperature. Separation time is inversely proportional to applied voltage. However, an increase in the voltage used can cause excessive heat production, giving rise to temperature and, as a result thereof, viscosity gradients in the buffer inside the capillary. This effect causes band broadening and decreases resolution.

Polarity Electrode polarity can be normal (anode at the inlet and cathode at the outlet) and the electro-osmotic flow will move toward the cathode. If the electrode polarity is reversed, the electro-osmotic flow is away from the outlet and only charged analytes with electrophoretic mobilities greater than the electro-osmotic flow will pass to the outlet.

Temperature The main effect of temperature is observed on buffer viscosity and electrical conductivity, and therefore on migration velocity. In some cases, an increase in capillary temperature can cause a conformational change in proteins, modifying their migration time and the efficiency of the separation.

Capillary The dimensions of the capillary (length and internal diameter) contribute to analysis time, efficiency of separations and load capacity. Increasing both effective length and total length can decrease the electric fields (working at constant voltage) which increases migration time. For a given buffer and eslectric field, heat dissipation, and hence sample bandbroadening, depend on the internal diameter of the capillary. The latter also affects the detection limit, depending on the sample volume injected and the detection system employed. Since the adsorption of the sample components on the capillary wall limits efficiency, methods to avoid these interactions should be considered in the development of a separation method. In the specific case of proteins, several strategies have been devised to avoid adsorption on the capillary wall. Some of these strategies (use of extreme pH and adsorption of positively charged buffer additives) only require modification of the buffer composition to prevent protein adsorption. In other strategies, the internal wall of the capillary is coated with a polymer, covalently bonded to the silica, that prevents interaction between the proteins and the negatively charged silica surface. For this purpose, ready-to-use capillaries with coatings consisting of neutral-hydrophilic, cationic and anionic polymers are available.

ELECTROLYTIC SOLUTION PARAMETERS

Buffer type and concentration Suitable buffers for capillary electrophoresis have an appropriate buffer capacity in the pH range of choice and low mobility to minimize current generation. Matching buffer-ion mobility to solute mobility, whenever possible, is important for minimizing band distortion. The type of sample solvent used is also important to achieve on column sample focusing, which increases separation efficiency and improves detection. An increase in buffer concentration (for a given pH) decreases electro-osmotic flow and solute velocity.

Buffer pH The pH of the buffer can affect separation by modifying the charge of the analyte or additives, and by changing the electro-osmotic flow. In protein and peptide separation, changing the pH of the buffer from above to below the isoelectric point (pI) changes the net charge of the solute from negative to positive.

An increase in the buffer pH generally increases the electro-osmotic flow.

Organic solvents Organic modifiers (methanol, acetonitrile, etc.) may be added to the aqueous buffer to increase the solubility of the solute or other additives and/or to affect the degree of ionization of the sample components. The addition of these organic modifiers to the buffer generally causes a decrease in the electro-osmotic flow.

Additives for chiral separations For the separation of optical isomers, a chiral selector is added to the separation buffer. The most commonly used chiral selectors are cyclodextrins, but crown ethers, polysaccha-rides and proteins may also be used. Since chiral recognition is governed by the different interactions between the chiral selector and each of the enantiomers, the resolution achieved for the chiral compounds depends largely on the type of chiral selector used. In this regard, for the development of a given separation it may be useful to test cyclodextrins having a different cavity size (α-, β-, or γ-cyclodextrin) or modified cyclodextrins with neutral (methyl, ethyl, hydroxyalkyl, etc.) or ionizable (aminomethyl, carboxymethyl, sulfobutyl ether, etc.) groups. When using modified cyclodextrins, batch-to-batch variations in the degree of substitution of the cyclodextrins must be taken into account since it will influence the selectivity. Other factors controlling the resolution in chiral separations are concentration of chiral selector, composition and pH of the buffer and temperature. The use of organic additives, such as methanol or urea, can also modify the resolution achieved.

Capillary Gel Electrophoresis

In capillary gel electrophoresis, separation takes place inside a capillary filled with a gel that acts as a molecular sieve. Moleculess with similar charge-to-mass ratios are separated according to molecular size since smaller molecules move more freely through the network of the gel and therefore migrate faster than larger molecules. Different biological macromolecules (for example, proteins and DNA fragments), which often have similar charge-to-mass ratios, can thus be separated according to their molecular mass by capillary gel electrophoresis.

CHARACTERISTICS OF GELS Two types of gels are used in capillary electrophoresis: permanently coated gels and dynamically coated gels. Permanently coated gels, such as cross-linked polyacrylamide, are prepared inside the capillary by polymerization of the monomers. They are usually bonded to the fused-silica wall and cannot be removed without destroying the capillary. If the gels are used for protein analysis under reducing conditions, the separation buffer usually contains sodium dodecyl sulfate and the samples are denatured by heating in a mixture of sodium dodecyl sulfate and 2-mercaptoethanol or dithiothreitol before injection. When non-reducing conditions are used (for example, analysis of an intact antibody), 2-mercaptoethanol and dithiothreitol are not used. Separation in cross-linked gels can be optimized by modifying the separation buffer (as indicated in the capillary zone electrophoresis section) and controlling the gel porosity during the gel preparation. For cross-linked polyacrylamide gels, the porosity can be modified by changing the concentration of acrylamide and/or the proportion of cross-linker. As a rule, a decrease in the porosity of the gel leads to a decrease in the mobility of the solutes. Due to the rigidity of these gels, only electrokinetic injection can be used. Dynamically coated gels are hydrophilic polymers, such as linear polyacrylamide, cellulose derivatives, dextran, etc., which can be dissolved in aqueous separation buffers giving rise to a separation medium that also acts as a molecular sieve. These separation media are easier to prepare than cross-linked polymers. They can be prepared in a vial and filled by pressure in a wall-coated capillary (with no electro-osmotic flow). Replacing the gel before every injection generally improves the separation reproducibility. The porosity of the gels can be increased by using polymers of higher molecular mass (at a given polymer concentration) or by decreasing the polymer concentration (for a given polymer molecular mass). A reduction in the gel porosity leads to a decrease in the mobility of the solute for the same buffer. Since the dissolution of these polymers in the buffer gives low viscosity solutions, both hydrodynamic and electrokinetic injection techniques can be used.

Capillary Isoelectric Focusing

In isoelectric focusing, the molecules migrate under the influence of the electric field, so long as they are charged, in a pH gradient generated by ampholytes having pI values in a wide range (poly-aminocarboxylic acids), dissolved in the separation buffer. The three basic steps of isoelectric focusing are loading, focusing and mobilization.

LOADING STEP Two methods may be employed:

(a) loading in one step: the sample is mixed with ampholytes and introduced into the capillary either by pressure or vacuum;

(b) sequential loading: a leading buffer, then the ampholytes, then the sample mixed with ampholytes, again ampholytes alone and finally the terminating buffer are introduced into the capillary. The volume of the sample must be small enough not to modify the pH gradient.

FOCUSING STEP When the voltage is applied, ampholytes migrate towards the cathode or the anode, according to their net charge, thus creating a pH gradient from anode (lower pH) to cathode (higher pH). During this step the components to be separated migrate until they reach a pH corresponding to their isoelectric point (pI) and the current drops to very low values.

MOBILIZATION STEP If mobilization is required for detection, use one of the following methods.

(a) in the first method, mobilization is accom-

plished during the focusing step under the effect of the electro-osmotic flow; the electro-osmotic flow must be small enough to allow the focusing of the components;

(b) in the second method, mobilization is accomplished by applying positive pressure after the focusing step;

(c) in the third method, mobilization is achieved after the focusing step by adding salts to the cathode reservoir or the anode reservoir (depending on the direction chosen for mobilization) in order to alter the pH in the capillary when the voltage is applied. As the pH is changed, the proteins and ampholytes are mobilized in the direction of the reservoir which contains the added salts and pass the detector.

The separation achieved, expressed as ΔpI, depends on the pH gradient (dpH/dx), the number of ampholytes having different pI values, the molecular diffusion coefficient (D), the intensity of the electric field (E) and the variation of the electrophoretic mobility of the analyte with the pH ($-d\mu/dpH$):

$$\Delta pI = 3 \times \sqrt{\frac{D(dpH/dx)}{E(-d\mu/dpH)}}$$

OPTIMIZATION The main parameters to be considered in the development of separations are:

Voltage Capillary isoelectric focusing utilizes very high electric fields, 300 V/cm to 1000 V/cm in the focusing step.

Capillary The electro-osmotic flow must be reduced or suppressed depending on the mobilization strategy (see above). Coated capillaries tend to reduce the electro-osmotic flow.

Solutions The anode buffer reservoir is filled with a solution with a pH lower than the pI of the most acidic ampholyte and the cathode reservoir is filled with a solution with a pH higher than the pI of the most basic ampholyte. Phosphoric acid for the anode and sodium hydroxide for the cathode are frequently used.

Addition of a polymer, such as methylcellulose, in the ampholyte solution tends to suppress convective forces (if any) and electro-osmotic flow by increasing the viscosity. Commercial ampholytes are available covering many pH ranges and may be mixed if necessary to obtain an expanded pH range. Broad pH ranges are used to estimate the isoelectric point where as narrower ranges are employed to improve accuracy. Calibration can be done by correlating migration time with isoelectric point for a series of protein markers.

During the focusing step precipitation of proteins at their isoelectric point can be prevented, if necessary, using buffer additives such as glycerol, surfactants, urea or zwitterionic buffers. However, depending on the concentration, urea denatures proteins.

Micellar Electrokinetic Chromatography (MEKC)

In micellar electrokinetic chromatography, separation takes place in an electrolyte solution which contains a surfactant at a concentration above the critical micellar concentration (*cmc*). The solute molecules are distributed between the aqueous buffer and the pseudo-stationary phase composed of micelles, according to the partition coefficient of the solute. The technique can therefore be considered as a hybrid of electrophoresis and chromatography. It is a technique that can be used for the separation of both neutral and charged solutes, maintaining the efficiency, speed and instrumental suitability of capillary electrophoresis. One of the most widely used surfactants in MEKC is the anionic surfactant sodium dodecyl sulfate, although other surfactants, for example cationic surfactants such as cetyltrimethylammonium salts, are also used.

The separation mechanism is as follows. At neutral and alkaline pH, a strong electro-osmotic flow is generated and moves the separation buffer ions in the direction of the cathode. If sodium dodecyl sulfate is employed as the surfactant, the electrophoretic migration of the anionic micelle is in the opposite direction, towards the anode. As a result, the overall micelle migration velocity is slowed down compared to the bulk flow of the electrolytic solution. In the case of neutral solutes, since the analyte can partition between the micelle and the aqueous buffer, and has no electrophoretic mobility, the analyte migration velocity will depend only on the partition coefficient between the micelle and the aqueous buffer. In the electropherogram, the peaks corresponding to each uncharged solute are always between that of the electro-osmotic flow marker and that of the micelle (the time elapsed between these two peaks is called the separation window). For electrically charged solutes, the migration velocity depends on both the partition coefficient of the solute between the micelle and the aqueous buffer, and on the electrophoretic mobility of the solute in the absence of micelle.

Since the mechanism in MEKC of neutral and weakly ionized solutes is essentially chromatographic, migration of the solute and resolution can be rationalized in terms of the retention factor of the solute (k), also referred to as mass distribution ratio (D_m), which is the ratio of the number of moles of solute in the micelle to those in the mobile phase. For a neutral compound, k is given by:

$$k = \frac{t_R - t_0}{t_0 \times \left(1 - \frac{t_R}{t_{mc}}\right)} = K \times \frac{V_s}{V_M},$$

where t_R = migration time of the solute,
t_0 = analysis time of an unretained solute (determined by injecting an electro-osmotic flow marker which does not enter the micelle, for instance methanol),
t_{mc} = micelle migration time (measured by injecting a micelle marker, such as Sudan III, which migrates while continuously associated in the micelle),

K = partition coefficient of the solute,
V_S = volume of the micellar phase, and
V_M = volume of the mobile phase.

Likewise, the resolution between 2 closely migrating solutes (R_S) is given by the equation:

$$R_s = \frac{\sqrt{N}}{4} \times \frac{\alpha - 1}{\alpha} \times \frac{k_b}{k_b + 1} \times \frac{1 - \left(\frac{t_0}{t_{mc}}\right)}{1 + k_a \times \left(\frac{t_0}{t_{mc}}\right)},$$

where N = number of theoretical plates for one of the solutes,
α = selectivity, and
k_a and k_b = retention factors for both solutes, respectively ($k_b > k_a$).

Similar, but not identical, equations give k and R_S values for electrically charged solutes.

The main parameters to be considered in the development of separations by MEKC are instrumental and electrolytic solution parameters.

INSTRUMENTAL PARAMETERS

Voltage Separation time is inversely proportional to applied voltage. However, an increase in voltage can cause excessive heat production that gives rise to temperature gradients and viscosity gradients of the buffer in the cross-section of the capillary. This effect can be significant with high conductivity buffers such as those containing micelles. Poor heat dissipation causes band broadening and decreases resolution.

Temperature Variations in capillary temperature affect the partition coefficient of the solute between the buffer and the micelles, the critical micellar concentration and the viscosity of the buffer. These parameters contribute to the migration time of the solutes. The use of a good cooling system improves the reproducibility of the migration time for the solutes.

Capillary As in capillary zone electrophoresis, the dimensions of the capillary (length and internal diameter) contribute to analysis time and efficiency of separations. Increasing both effective length and total length can decrease the electric fields (working at constant voltage), increase migration time and improve the separation efficiency. The internal diameter controls heat dissipation (for a given buffer and electric field) and consequently the sample band broadening.

ELECTROLYTIC SOLUTION PARAMETERS

Surfactant type and concentration The type of surfactant, in the same way as the stationary phase in chromatography, affects the resolution since it modifies separation selectivity. Also, the log k of a neutral compound increases linearly with the concentration of surfactant in the mobile phase. Since resolution in MEKC reaches a maximum when k approaches the value of $\sqrt{t_{mc}/t_o}$, modifying the concentration of surfactant in the mobile phase changes the resolution obtained.

Buffer pH Although pH does not modify the partition coefficient of non-ionized solutes, it can modify the electro-osmotic flow in uncoated capillaries. A decrease in the buffer pH decreases the electro-osmotic flow and therefore increases the resolution of the neutral solutes in MEKC, resulting in a longer analysis time.

Organic solvents To improve MEKC separation of hydrophobic compounds, organic modifiers (methanol, propanol, acetonitrile, etc.) can be added to the electrolytic solution. The addition of these modifiers usually decreases migration time and the selectivity of the separation. Since the addition of organic modifiers affects the critical micellar concentration, a given surfactant concentration can be used only within a certain percentage of organic modifier before the micellization is inhibited or adversely affected, resulting in the absence of micelles and, therefore, in the absence of partition. The dissociation of micelles in the presence of a high content of organic solvent does not always mean that the separation will no longer be possible; in some cases the hydrophobic interaction between the ionic surfactant monomer and the neutral solutes forms solvophobic complexes that can be separated electrophoretically.

Additives for chiral separations For the separation of enantiomers using MEKC, a chiral selector is included in the micellar system, either covalently bound to the surfactant or added to the micellar separation electrolyte. Micelles that have a moiety with chiral discrimination properties include salts of *N*-dodecanoyl-L-amino acids, bile salts, etc. Chiral resolution can also be achieved using chiral discriminators, such as cyclodextrins, added to the electrolytic solutions which contain micellised achiral surfactants.

Other additives Several strategies can be carried out to modify selectivity, by adding chemicals to the buffer. The addition of several types of cyclodextrins to the buffer can also be used to reduce the interaction of hydrophobic solutes with the micelle, thus increasing the selectivity for this type of compound.

The addition of substances able to modify solutemicelle interactions by adsorption on the latter, is used to improve the selectivity of the separations in MEKC. These additives may be a second surfactant (ionic or non-ionic) which gives rise to mixed micelles or metallic cations which dissolve in the micelle and form co-ordination complexes with the solutes.

Quantification

Peak areas must be divided by the corresponding migration time to give the corrected area in order to:

(a) compensate for the shift in migration time from run to run, thus reducing the variation of the response,

(b) compensate for the different responses of sample constituents with different migration times.

Where an internal standard is used, verify that no peak of the substance to be examined is masked by that of the internal standard.

CALCULATIONS From the values obtained, calculate the content of the component or components being examined. When prescribed, the percentage content of one or more components of the sample to be examined is calculated by determining the corrected area(s) of the peak(s) as a percentage of the total of the corrected areas of all peaks, excluding those due to solvents or any added reagents (normalization procedure). The use of an automatic integration system (integrator or data acquisition and processing system) is recommended.

System Suitability

In order to check the behaviour of the capillary electrophoresis system, system suitability parameters are used. The choice of these parameters depends on the mode of capillary electrophoresis used. They are: retention factor (k) (only for micellar electrokinetic chromatography), apparent number of theoretical plates (N), symmetry factor (A_s) and resolution (R_s). In previous sections, the theoretical expressions for N and R_s have been described, but more practical equations that allow these parameters to be calculated from the electrophero-grams are given below.

APPARENT NUMBER OF THEORETICAL PLATES The apparent number of theoretical plates (N) may be calculated using the equation:

$$N = 5.54(t/W_{h/2})^2,$$

where t = migration time or distance along the baseline from the point of injection to the perpendicular dropped from the maximum of the peak corresponding to the component, and
$W_{h/2}$ = width of the peak at half-height.

RESOLUTION The resolution (R) between peaks of similar height of two components may be calculated using the equation:

$$R = \frac{2(t_2 - t_1)}{1.70(W_{1,h/2} + W_{2,h/2})}, \quad t_2 > t_1,$$

where t_1 and t_2 = migration times or distances along the baseline from the point of injection to the perpendiculars dropped from the maxima of two adjacent peaks, and
$W_{1,h/2}$ and $W_{2,h/2}$ = peak widths at half-height.

When appropriate, the resolution may be calculated by measuring the height of the valley (H_v) between 2 partly resolved peaks in a standard preparation and the height of the smaller peak (H_p) and calculating the peak-to-valley ratio:

$$\frac{p}{v} = \frac{H_p}{H_v}$$

SYMMETRY FACTOR OR TAILING FACTOR The symmetry factor (T) of a peak may be calculated using the equation:

$$T = \frac{W_{0.05}}{2f},$$

where $W_{0.05}$ = width of the peak at one-twentieth of the peak height, and
f = distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at one-twentieth of the peak height.

Tests for area repeatability (standard deviation of areas or of the area/migration-time ratio) and for migration time repeatability (standard deviation of migration time) are introduced as suitability parameters. Migration time repeatability provides a test for the suitability of the capillary washing procedures. An alternative practice to avoid the lack of repeatability of the migration time is to use migration time relative to an internal standard. A test for the verification of the signal-to-noise ratio for a standard preparation (or the determination of the limit of quantification) may also be useful for the determination of related substances.

SIGNAL-TO-NOISE RATIO The detection limit and quantification limit correspond to signal-to-noise ratios of 3 and 10 respectively. The signal-to-noise ratio (S/N) is calculated using the expression:

$$\frac{S}{N} = \frac{2H}{h},$$

where H = height of the peak corresponding to the component concerned, in the electro pherogram obtained with the prescribed reference solution, measured from the maximum of the peak to the extrapolated baseline of the signal observed over a distance equal to twenty times the width at half-height, and
h = range of the background in an electropherogram obtained after injection of a blank, observed over a distance equal to twenty times the width at the half-height of the peak in the electropherogram obtained with the prescribed reference solution and, if possible, situated equally around the place where this peak would be found.

3.9 CHROMATOGRAPHIC SEPARATION TECHNIQUES

Interpretation of Chromatograms

Figure 1 represents a typical chromatographic separation of two substances, 1 and 2, where t_1 and t_2 are the respective retention times. h, $h/2$, and $W_{h/2}$ are the height, the half-height, and the width at half-height, respectively, for peak 1. W_1 and W_2 are the respective widths of peaks 1 and 2 at the baseline. Air peaks are a feature of gas chromatograms and correspond to the solvent front in liquid chromatography.

Chromatographic retention times are characteristic of the compounds they represent but are not unique. Coincidence of retention times of a test and a reference substance can be used as a feature in construction of an identity profile but is insufficient on its own to establish identity. Absolute retention times of a given compound vary from one chromatogram to the next.

Because in most procedures there is no need to identify an unretained peak, comparisons are normally made in terms of relative retention times, R_r:

$$R_r = \frac{t_2}{t_1},$$

where t_2, and t_1 are retention times, measured from the point of injection, of the test and reference substances, respectively, determined under identical experimental conditions on the same column.

Other procedures may identify the peak position using the relative retention, r:

$$r = \frac{t_2 - t_M}{t_1 - t_M},$$

where t_M is the retention time of a non-retained marker, which needs to be defined in the procedure.

The number of theoretical plates, N, is a measure of column efficiency. For Gaussian peaks, it is calculated by the equation:

$$N = 16\left(\frac{t}{W}\right)^2,$$

where t is the retention time of the substance and W is the width of the peak at its base, obtained by extrapolating the relatively straight sides of the peak to the baseline. The value of N depends on the substance being chromatographed as well as the operating conditions such as mobile phase or carrier gas flow rates and temperature, the quality of the packing, the uniformity of the packing within the column and, for capillary columns, the thickness of the stationary phase film, and the internal diameter and length of the column.

The separation of two components in a mixture, the resolution, R, is determined by the equation:

$$R = \frac{2(t_2 - t_1)}{W_2 + W_1},$$

in which t_2 and t_1 are the retention times of the two components, and W_2 and W_1 are the corresponding widths at the bases of the peaks obtained by extrapolating the relatively straight sides of the peaks to the baseline.

Where electronic integrators are used, it may be convenient to determine the resolution, R, by the equation:

$$R = \frac{2(t_2 - t_1)}{1.70(W_{1,h/2} + W_{2,h/2})},$$

and to determine the number of theoretical plates, N, by the equation:

$$N = 5.54(t/W_{2,h/2})^2,$$

where $W_{h/2}$ is the peak width at half-height, obtained directly by electronic integrators. However, in the event of dispute, only equations based on peak width at baseline are to be used.

Peak areas and peak heights are usually proportional to the quantity of compound eluting. These are commonly measured by electronic integrators but may be determined by more classical approaches. Peak areas are generally used but may be less accurate if peak interference occurs. For manual measurements, the chart should be run faster than usual, or a comparator should be used to measure the width at half-height and the width at the base of the peak, to minimize error in these measurements.

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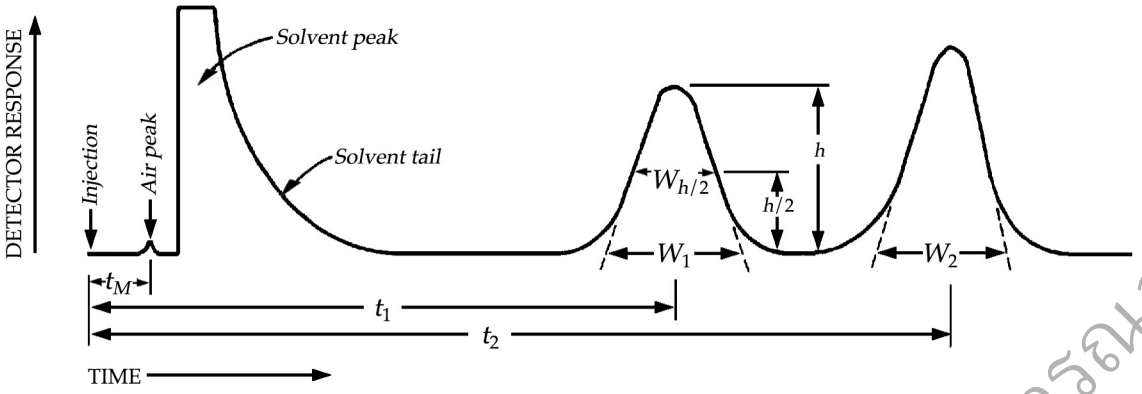

Fig. 1 Chromatographic Separation of Two Substances

For accurate quantitative work, the components to be measured should be separated from any interfering components. Peak tailing and fronting and the measurement of peaks on solvent tails are to be avoided.

Chromatographic purity tests for drug raw materials are sometimes based on the determination of peaks due to impurities, expressed as a percentage of the area due to the drug peak. It is preferable, however, to compare impurity peaks to the chromatogram of a standard at a similar concentration. The standard may be the drug itself at a level corresponding to, for example, 0.5 per cent impurity, or in the case of toxic or signal impurities, a standard of the impurity itself.

System Suitability

System suitability tests are an integral part of gas and liquid chromatographic methods. They are used to verify that the detection sensitivity, resolution, and reproducibility of the chromatographic system are adequate for the analysis to be done. The tests are based on the concept that the equipment electronics, analytical operations, and samples to be analyzed constitute an integral system that can be evaluated as such.

The detection sensitivity is a measure used to ensure the suitability of a given chromatographic procedure for the complete detection of the impurities in the Chromatographic purity or Related compounds tests by injecting a volume of a quantitation limit solution equal to that of the Test solution. Unless otherwise specified in the individual monograph, the quantitation limit solution may be prepared by dissolving the Drug Reference Substance in the same solvent as that used for the Test solution at a 0.05 per cent concentration level relative to the amount of drug substance in the Test solution for drug substances, and a 0.1 per cent level relative to the amount of drug substance in the Test solution for drug products. The signal-to-noise ratio for the drug substance peak obtained with the quantitation limit solution should be not less than 10.

The resolution, *R*, (**Note** All terms and symbols are defined in the Glossary of Symbols) is a function of column efficiency, *N*, and is specified to ensure that closely eluting compounds are resolved from each other, to establish the general resolving power of the system, and to ensure that internal standards are resolved from the drug. Column efficiency may be specified also as a system suitability requirement, especially if there is only one peak of interest in the chromatogram; however, it is a less reliable means to ensure resolution than direct measurement. Column efficiency is a measure of peak sharpness, which is important for the detection of trace components.

Replicate injections of a standard preparation used in the assay or other standard solution are compared to ascertain whether requirements for precision are met. Unless otherwise specified in the individual monograph, data from five replicate injections of the analyte are used to calculate the relative standard deviation, *RSD*, if the requirement is 2.0 per cent or less; data from six replicate injections are used if the relative standard deviation requirement is more than 2.0 per cent.

The symmetry factor (or tailing factor), *T*, a measure of peak symmetry, is unity for perfectly symmetrical peaks and its value increases as tailing becomes more pronounced (Fig. 2). In some cases, values less than unity may be observed. As peak asymmetry increases, integration and hence precision, becomes less reliable.

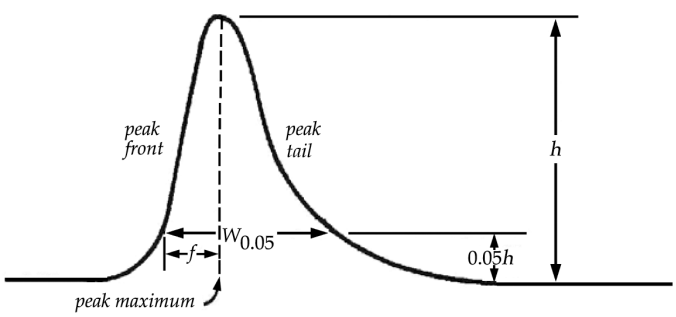

Fig. 2 Asymmetrical Chromatographic Peak

These tests are performed by collecting data from replicate injections of standard or other solutions as specified in the individual monograph. The specification of definitive parameters in a monograph does not

preclude the use of other suitable operating conditions. Adjustments of operating conditions to meet system suitability requirements may be necessary.

Unless otherwise directed in the monograph, system suitability parameters are determined from the analyte peak.

Relative retention times may be provided in monographs for informational purposes only, to aid in peak identification. There are no acceptance criteria applied to relative retention times.

To ascertain the effectiveness of the final operating system, it should be subjected to suitability testing. Replicate injections of the standard preparation required to demonstrate adequate system precision may be made before the injection of samples or may be interspersed among sample injections. System suitability must be demonstrated throughout the run by injection of an appropriate control preparation at appropriate intervals. The control preparation can be a standard preparation or a solution containing a known amount of analyte and any additional materials useful in the control of the analytical system, such as excipients or impurities. Whenever there is a significant change in equipment or in a critical reagent, suitability testing should be performed before the injection of samples. No sample analysis is acceptable unless the requirement of system suitability have been met. Sample analyses obtained while the system fails system suitability requirements are unacceptable.

Adjustment of Chromatographic Conditions

The extent to which the various parameters of a chromatographic test may be adjusted to satisfy the system suitability criteria without fundamentally modifying the methods are listed below for information. The chromato-graphic conditions described have been validated during the elaboration of the monograph. The system suitability tests are included to ensure the separation required for satisfactory performance of the test or assay. Nonetheless, since the stationary phases are described in a general way and there is such a variety available commercially, with differences in chromatographic behaviour, some adjustments of the chromatographic conditions may be necessary to achieve the prescribed system suitability requirements. With reverse-phase liquid chromatographic methods, in particular, adjustment of the various parameters will not always result in satisfactory chromatography. In that case, it may be necessary to replace the column with another of the same type (e.g., octadecylsilyl silica gel) which exhibits the desired chromatographic behaviour.

For critical parameters the adjustments are defined clearly in the monograph to ensure the system suitability.

Multiple adjustments which may have a cumulative effect in the performance of the system are to be avoided.

Thin-layer Chromatography and Paper Chromatography

COMPOSITION OF THE MOBILE PHASE; pH OF THE AQUEOUS COMPONENT OF THE MOBILE PHASE; CONCENTRATION OF SALTS The adjustments can be made as described under High-pressure liquid chromatography.

APPLICATION VOLUME The application volume can be adjusted to 10 to 20 per cent of the prescribed volume if using fine particle size plates (2 to 10 μm).

MIGRATION DISTANCE The migration distance of the solvent front is to be not less than 50 mm or 30 mm on high-performance plates.

High-pressure Liquid Chromatography

COMPOSITION OF THE MOBILE PHASE The following adjustment limits apply to minor components of the mobile phase (specified at 50 per cent or less). The amount(s) of these component(s) can be adjusted by ±30 per cent relative. However, the change in any component cannot exceed ±10 per cent absolute (i.e., in relation to the total mobile phase), nor can the final concentration of any component be reduced to zero. Adjustment can be made to one minor component in a ternary mixture. Examples of adjustments are given below.

Specified ratio of 50:50 Thirty per cent of 50 is 15 per cent absolute, but this exceeds the maximum permitted change of ±10 per cent absolute in either component. Therefore, the mobile phase ratio may be adjusted only within the range of 40:60 to 60:40.

Specified ratio of 2:98 Thirty per cent of 2 is 0.6 per cent absolute. Therefore, the maximum allowed justment is within the range of 1.4:98.6 to 2.6:97.4.

Specified ratio of 60:35:5 For the second component, 30 per cent of 35 is 10.5 per cent absolute, which exceeds the maximum permitted change of ±10 per cent absolute in any component. Therefore, the second component may be adjusted only within the range of 25 per cent to 45 per cent absolute. For the third component, 30 per cent of 5 is 1.5 per cent absolute. In all cases, a sufficient quantity of the first component is used to give a total of 100 per cent. Therefore, mixture ranges of 50:45:5 to 70:25:5 or 58.5: 35:6.5 to 61.5:35:3.5 would meet the requirement.

pH OF THE AQUEOUS COMPONENT OF THE MOBILE PHASE The pH of the aqueous buffer used in the preparation of the mobile phase can be adjusted to within ±0.2 units of the value or range specified, or ±1.0 units when neutral substances are to be examined.

CONCENTRATION OF SALTS The concentration of the salts used in the preparation of the aqueous buffer used in the mobile phase can be adjusted to within ±10 per cent, provided the permitted pH variation is met.

DETECTOR WAVELENGTH No adjustment permitted.

STATIONARY PHASE
— column length: ±70 per cent,
— column internal diameter: ±25 per cent,
— particle size: maximal reduction of 50 per cent, no increase permitted.

FLOW RATE The flow rate can be adjusted by as much as ±50 per cent.

COLUMN TEMPERATURE The column temperature can be adjusted by as much as ±10°. Column thermostating is recommended to improve control and reproducibility of retention time.

INJECTION VOLUME The injection volume can be reduced as far as is consistent with accepted precision and detection limits.

Gas Chromatography

STATIONARY PHASE
— column length: ±70 per cent,
— column internal diameter: ±50 per cent,
— particle size: maximal reduction of 50 per cent, no increase permitted,
— film thickness: –50 per cent to +100 per cent.

FLOW RATE The flow rate can be adjusted by as much as ±50 per cent.

OVEN TEMPERATURE The oven temperature can be adjusted by as much as ±10 per cent.

OVEN TEMPERATURE PROGRAM Adjustment of temperatures is permitted as stated above. For the times specified for the temperature to be maintained or for the temperature to be changed from one value to another, an adjustment of up to ±20 per cent is permitted.

INJECTION VOLUME The injection volume can be reduced as far as is consistent with accepted precision and detection limits.

Glossary of Symbols

To promote uniformity of interpretation, the following symbols and definitions are employed where applicable in presenting formulas in the individual monographs. Where a different symbol or definition is used in an individual monograph, the monograph text takes precedence. (**Note** Where the terms *W* and *t* both appear in the same equation, they must be expressed in the same units.)

f distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 5 per cent of the peak height from the baseline.

k' capacity factor,

$$k' = \frac{\text{amount of substance in stationary phase}}{\text{amount of substance in mobile phase}}$$

$$k' = \frac{\text{time spent by substance in stationary phase}}{\text{time spent by substance in mobile phase}} = \frac{t}{t_M} - 1$$

N number of theoretical plates in a chromatographic column,

$$N = 16\left(\frac{t}{W}\right)^2 \text{ or } N = 5.54\left(\frac{t}{W_{h/2}}\right)^2$$

r relative retention,

$$r = \frac{t_2 - t_M}{t_1 - t_M}$$

R resolution between two chromatographic peaks,

$$R = \frac{2(t_2 - t_1)}{W_1 + W_2} \text{ or } R = \frac{2(t_2 - t_1)}{1.70(W_{1,h/2} + W_{2,h/2})}$$

R_r relative retention time,

$$R_r = \frac{t_2}{t_1}$$

RSD (%) relative standard deviation in percentage,

$$\text{RSD}(\%) = \frac{100}{\overline{X}}\left[\frac{\sum_{i=1}^{n}\left(X_i - \overline{X}\right)^2}{n-1}\right]^{1/2}$$

where X_i is an individual measurement in a set of n measurements and $\overline{X}$ is the arithmetic mean of the set.

T symmetry factor (or tailing factor),

$$T = \frac{W_{0.05}}{2f}$$

t retention time measured from time of injection to time of elution of peak maximum.

t_M retention time of non-retained component, air with thermal conductivity detection.

V retention volume,

$$V = \text{flow rate} \times t$$

V_O retention volume of non-retained component (void volume of the column)

W width of peak measured by extrapolating the relatively straight sides to the baseline.

$W_{h/2}$ width of peak at half height.

$W_{0.05}$ width of peak at 5 per cent height.

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APPENDIX 4 PHYSICAL TESTS

4.1 CLARITY OF SOLUTION

Use Method I unless otherwise directed.

Method I

Into separate matched, flat-bottomed tubes of colourless, transparent, neutral glass, 15 to 20 mm in diameter, place sufficient of the test solution and of a suitable reference suspension, freshly prepared as specified below, such that the tubes are filled to a depth of 40 mm, accurately measured. Five minutes after preparation of the reference suspension, compare the contents of the tubes against a black background by viewing under diffused light down the vertical axes of the tubes. The diffusion of the light must be such that reference suspension I can be readily distinguished from water and from reference suspension II.

Standard of opalescence Dissolve 1.0 g of *hydrazine sulfate* in sufficient *water* to produce 100.0 ml, and allow to stand for 4 to 6 hours. To 25.0 ml of this solution add a solution containing 2.5 g of *methenamine* in 25.0 ml of *water*, mix well and allow to stand for 24 hours. This suspension is stable for 2 months, provided that it is stored in a glass container free from surface defects.

To prepare the standard of opalescence, dilute 15.0 ml of the suspension to 1000.0 ml with *water*. This suspension must be used within 24 hours of preparation.

Reference suspensions Reference suspensions I to IV should be prepared as indicated in Table 1. Each suspension should be mixed well and shaken before use.

Table 1

	Reference Suspension			
	I	II	III	IV
Standard of opalescence (ml)	5.0	10.0	30.0	50.0
Water (ml)	95.0	90.0	70.0	50.0

Expression of clarity and degree of opalescence

A solution is termed *clear* if its opalescence is not more pronounced than that of reference suspension I.

A solution is termed *slightly opalescent* if its opalescence is more pronounced than that of reference suspension I, but not more pronounced than that of reference suspension II.

A solution is termed *opalescent* if its opalescence is more pronounced than that of reference suspension II, but not more pronounced than that of reference suspension III.

A solution is termed *very opalescent* if its opalescence is more pronounced than that of reference suspension III, but not more pronounced than that of reference suspension IV.

Method II

Use Procedure A or B as directed.

Procedure A Into separate matched, flat-bottomed tubes of colourless, transparent, neutral glass, 12 mm in internal diameter, place 2.0 ml of the solution being examined and 2.0 ml of a suitable reference solution, freshly prepared as specified below. Five minutes after preparation of the reference solution, compare the contents of the tubes, in darkness, by passing laterally through the tubes a beam of light from an electric lamp giving a luminosity of 1000 lux at a distance of 1 m.

Procedure B Into separate matched, flat-bottomed tubes of colourless, transparent, neutral glass, 16 mm in internal diameter, place 10.0 ml of the solution being examined and 10.0 ml of a suitable reference solution, freshly prepared as specified below. Five minutes after preparation of the reference solution, compare the contents of the tubes against a black background by viewing under diffused light down the vertical axes of the tubes.

Expression of clarity and degree of opalescence

A solution is termed *clear* if its appearance is the same as that of water, or of solvent used, or of the mixture of reagents in the absence of the test substance.

A solution is termed *very slightly opalescent* if its opalescence is not more pronounced than that of reference solution A_1 or B_1.

A solution is termed *slightly opalescent* if its opalescence is more pronounced than that of reference solution A_1 or B_1, but not more pronounced than that of reference solution A_2 or B_2.

A solution is termed *opalescent* if its opalescence is more pronounced than that of reference solution A_2 or B_2, but not more pronounced than that of reference solution A_3 or B_3.

A solution is termed *very opalescent* if its opalescence is more pronounced than that of reference solution A_3 or B_3, but not more pronounced than that of reference solution A_4 or B_4.

Reagents

STANDARD CHLORIDE SOLUTION I Dilute 1.0 ml of 0.2 M *sodium chloride* with sufficient *water* to produce 100.0 ml.

STANDARD CHLORIDE SOLUTION II Dilute 20.0 ml of *standard chloride solution I* with sufficient *water* to produce 100.0 ml.

STANDARD CHLORIDE SOLUTION III Dilute 1.0 ml of *standard chloride solution I* with sufficient *water* to produce 100.0 ml.

REFERENCE SOLUTIONS Reference solutions A_1 to A_4 and B_1 to B_4 should be prepared without vigorous shaking, as indicated in Table 2.

Table 2

	Reference Solution (ml)							
	A_1	B_1	A_2	B_2	A_3	B_3	A_4	B_4
Standard chloride solution III	0.5	2.5	7.5	37.5	-	-	-	-
Standard chloride solution II	-	-	-	-	1.5	7.5	2.5	12.5
2 M nitric acid	10	50	10	50	10	50	10	50
Water	7.5	37.5	0.5	2.5	6.5	32.5	5.5	27.5
0.1 M *silver nitrate*	2.0	10	2.0	10	2.0	10	2.0	10

4.2 COLOUR OF SOLUTION

The examination of the colour of solution is carried out by comparing the test solution prepared as specified in the monograph with a matching fluid indicated in the monograph. The composition of the matching fluid is selected depending on the hue and intensity of the colour of the test solution corresponding to the limits permitted in the specifications.

Method

Unless otherwise specified in the monograph, carry out the comparison in flat-bottomed tubes of transparent glass that are matched as closely as possible in internal diameter and in all other respects. The colour of the test solution is not more intense than that of the matching fluid when viewed down the vertical axis of the tubes in diffused light against a white background.

Colorimetric Solutions (CS)

COBALT(II) CHLORIDE CS Dissolve about 65 g of *cobalt (II) chloride* ($CoCl_2.6H_2O$) in enough of a mixture of 25 ml of *hydrochloric acid* and 975 ml of *water* to make 1000 ml. Pipette 5 ml of this solution into a 250-ml iodine flask, and add 5 ml of *hydrogen peroxide TS (10 volumes)* and 10 ml of a 30 per cent w/v solution of *sodium hydroxide*. Boil gently for 10 minutes, allow to cool and add 60 ml of 1 M *sulfuric acid* and 2 g of *potassium iodide*. Close the flask and dissolve the precipitate by shaking gently. Titrate the liberated iodine with 0.1 M *sodium thiosulfate VS*, using *starch TS*, added towards the end of the titration, as indicator. The end-point is reached when the solution turns pink. Perform a blank determination with the same quantities of the same reagents, and make any necessary correction. Each ml of 0.1 M *sodium thiosulfate VS* is equivalent to 23.79 mg of $CoCl_2.6H_2O$. Adjust the final volume of the solution by adding enough of the hydrochloric acid and water mixture so that each ml contains 59.5 mg of $CoCl_2.6H_2O$.

COPPER(II) SULFATE CS Dissolve about 65 g of *copper (II) sulfate* ($CuSO_4.5H_2O$) in enough of a mixture of 25 ml of *hydrochloric acid* and 975 ml of *water* to make 1000 ml. Pipette 10 ml of this solution into a 250-ml iodine flask, add 40 ml of *water*, 4 ml of *acetic acid*, 5 ml of *hydrochloric acid*, and 3 g of *potassium iodide*. Titrate the liberated iodine with 0.1 M *sodium thiosulfate VS*, using *starch TS*, added towards the end of the titration, as indicator. The end-point is reached when the solution shows a slight pale brown colour. Perform a blank determination with the same quantities of the same reagents, and make any necessary correction. Each ml of 0.1 M *sodium thiosulfate VS* is equivalent to 24.97 mg of $CuSO_4.5H_2O$. Adjust the final volume of the solution by adding enough of the hydrochloric acid and water mixture so that each ml contains 62.4 mg of $CuSO_4.5H_2O$.

IRON(III) CHLORIDE CS Dissolve about 55 g of *iron(III) chloride* ($FeCl_3.6H_2O$) in enough of a mixture of 25 ml of *hydrochloric acid* and 975 ml of *water* to make 1000 ml. Pipette 10 ml of this solution into a 250-ml iodine flask, add 15 ml of *water*, 5 ml of *hydrochloric acid* and 3 g of *potassium iodide*. Close the flask, allow to stand protected from light for 15 minutes and add 100 ml of *water*. Titrate the liberated iodine with 0.1 M *sodium thiosulfate VS*, using *starch TS*, added towards the end of the titration, as indicator. The end-point is reached when the solution turns colourless. Perform a blank determination with the same quantities of the same reagents, and make any necessary correction. Each ml of 0.1 M *sodium thiosulfate VS* is equivalent to 27.03 mg of $FeCl_3.6H_2O$. Adjust the final volume of the solution by adding enough of the hydrochloric acid and water mixture so that each ml contains 45.0 mg of $FeCl_3.6H_2O$.

Matching Fluids

To serve as standards of comparison, twenty matching fluids, each designated by a letter of the alphabet, are provided, the composition of each being as indicated in the accompanying table. To prepare the matching fluid required, pipette the prescribed volumes of the colorimetric solutions and water into one of the matching tubes, and mix the solution in the tube. Make the comparison as directed in the individual monograph, under the viewing conditions previously described. The matching fluids, or other combinations of the colorimetric solutions, may be used in very low concentrations to measure deviation from colourlessness.

Matching Fluids

Matching Fluid	Parts of Cobalt(II) Chloride Colorimetric Solution (ml)	Parts of Iron(III) Chloride Colorimetric Solution (ml)	Parts of Copper(II) Sulfate Colorimetric Solution (ml)	Parts of water (ml)
A	0.1	0.4	0.1	4.4
B	0.3	0.9	0.3	3.5
C	0.1	0.6	0.1	4.2
D	0.3	0.6	0.4	3.7
E	0.4	1.2	0.3	3.1
F	0.3	1.2	0.0	3.5
G	0.5	1.2	0.2	3.1
H	0.2	1.5	0.0	3.3
I	0.4	2.2	0.1	2.3
J	0.4	3.5	0.1	1.0
K	0.5	4.5	0.0	0.0
L	0.8	3.8	0.1	0.3
M	0.1	2.0	0.1	2.8
N	0.0	4.9	0.1	0.0
O	0.1	4.8	0.1	0.0
P	0.2	0.4	0.1	4.3
Q	0.2	0.3	0.1	4.4
R	0.3	0.4	0.2	4.1
S	0.2	0.1	0.0	4.7
T	0.5	0.5	0.4	3.6

4.3 DETERMINATION OF MELTING RANGE AND MELTING TEMPERATURE

For Pharmacopoeial purposes, the melting range of a substance is defined as the range between the temperature at which the substance begins to collapse or form droplets on the wall of a capillary tube and the temperature at which it is completely melted as shown by the disappearance of the solid phase; the latter temperature is also considered to be the melting temperature, except as defined otherwise for Classes II and III below. Any apparatus or method capable of equal accuracy may be used. The apparatus should be calibrated with appropriate substances of known melting point.[1]

Five procedures for the determination of melting range and melting temperature are given herein, varying in accordance with the nature of the substance. When no class is designated in the monograph, use the procedure for Class Ia. The procedure known as the mixed-melting point determination, whereby the melting range of a solid under test is compared with that of an intimate mixture of equal parts of the solid and an authentic sample of it, may be used as a confirmatory identification test. Agreement of the observations on the original and the mixture constitutes reliable evidence of chemical identity.

[1]Suitable substances are issued by the World Health Organization Collaborating Centre for Chemical Reference Substances, Apotekens Centrallaboratorium, Box 3045, 17103 SOLNA 3, Sweden.

Apparatus I

An example of a suitable melting range Apparatus I consists of a glass container for a bath of transparent fluid, a suitable stirring device, an accurately standardized thermometer, and a controlled source of heat. The thermometers should cover the range of –10° to +360°, the length of one degree on the scale being not less than 0.8 mm. Each thermometer should preferably be of the mercury-in-glass, solid-stem type with a cylindrical bulb and made of approved thermometric glass suitable for the range covered, and should have a safety chamber. The bath fluid is selected with a view to the temperature required, but liquid paraffin is used generally and certain liquid silicones are well adapted to the higher temperature ranges. The fluid is deep enough to permit immersion of the thermometer to its specified immersion depth so that the bulb is still about 2 cm above the bottom of the bath. The heat may be supplied electrically or by an open flame. The capillary tube is about 10 cm long and 0.8 to 1.2 mm in internal diameter with walls, 0.2 to 0.3 mm in thickness.

The thermometer is selected for the desired accuracy and range of temperature.

The procedures for Classes I and Ia are applied to those substances readily reduced to fine powders.

Apparatus II

An instrument may be used in the procedures for Classes I, Ia, and Ib. An example of a suitable melting range Apparatus II consists of a block of metal that may be heated at a controlled rate, its temperature being

monitored by a sensor. The block accommodates the capillary tube containing the test substance and permits monitoring of the melting process, typically by means of a beam of light and a detector. The detector signal may be processed by a microcomputer to determine and display the melting point or range, or the detector signal may be plotted to allow visual estimation of the melting point or range.

Procedure for Class I

Apparatus I—Reduce the substance under test to a very fine powder. Unless otherwise directed, render it anhydrous when it contains water of hydration by drying at the temperature specified in the monograph. When the substance contains no water of hydration, dry it as directed for Loss on drying in the individual monograph; or if no Loss on drying test is specified, dry it over a suitable desiccant for not less than 16 hours.

Charge a capillary glass tube, one end of which is sealed, with sufficient quantity of the dry powder to form a column in the bottom of the tube 2.5 to 3.5 mm high when packed down as closely as possible by moderate tapping on a solid surface.

Heat the bath until the temperature is about 30° below the expected melting point. Remove the thermometer, and quickly attach the capillary tube to the thermometer by wetting both with a drop of the liquid of the bath, and adjust its height so that the material in the capillary is level with the thermometer bulb. Replace the thermometer, and continue the heating, with constant stirring sufficiently to cause the temperature to rise at a rate of about 3° per minute. When the temperature is about 3° below the lowest figure of the melting range for the substance under test, reduce the heating so that the temperature rises at a rate of about 1° to 2° per minute. Continue heating until melting is complete.

The temperature at which the column of the substance under test is observed to collapse definitely against the side of the tube at any point is defined as the beginning of melting and the temperature at which the test substance becomes liquid throughout is defined as the end of melting or the "melting temperature". The two temperatures fall within the limits of the melting range.

Apparatus II—Prepare the substance under test and charge the capillary tube as directed for *Class I, Apparatus I*. Operate the apparatus according to the manufacturer's instructions: Heat the block until the temperature is about 30° below the expected melting point. Insert the capillary tube into the heating block, and continue heating at a rate of temperature increase of about 1° to 2° per minute until melting is complete.

The temperature at which the detector signal first leaves its initial value is defined as the beginning of melting, and the temperature at which the detector signal reaches its final value is defined as the end of melting, or the melting point. The two temperatures fall within the limits of the melting range.

In the event of dispute, only the melting range or temperature obtained as directed for *Class I, Apparatus I*, is definitive.

Procedure for Class Ia

Apparatus I—Prepare the substance under test and charge the capillary as directed for *Class I*.

Heat the bath until the temperature is about 10° below expected melting point and is rising at a rate of 1°±0.5° per minute. Insert the capillary as directed under *Class I* when the temperature is about 5° below the lowest figure of the melting range for the substance under test and continue heating until melting is complete. Record the melting range as directed for *Class I, Apparatus I*.

Procedure for Class Ib

Apparatus I—Place the substance under test in a closed container and cool to 10°, or lower, for at least 2 hours. Without previous powdering, charge the cooled material into the capillary tube as directed for *Class I*, immediately place the charged tube in a vacuum desiccator and dry at a pressure not exceeding 2.7 kPa (about 20 Torr) for 3 hours. Immediately upon removal from the desiccator, fire-seal the open end of the tube, and as soon as practicable proceed with the determination of the melting range as follows: Heat the bath until a temperature is 10°±1° below the lowest figure of the melting range for the substance under test, introduce the charged tube, and heat at a rate of rise of 3°±0.5° per minute until melting is complete. Record the melting range as directed for *Class I, Apparatus I*.

If the particle size of the material is too large for the capillary, pre-cool the test substance as directed above, then with as little pressure as possible gently crush the particles to fit the capillary, and immediately charge the tube.

Procedure for Class II

This procedure is applied to such substances as fats, fatty acids, paraffins or waxes, that are insoluble in water and not readily reduced to powder. Carefully melt the material under test at as low a temperature as possible, and draw it into a capillary tube, which is left open at both ends, to a height of about 10 mm. Cool the charged tube at 10°, or lower, for 24 hours, or in contact with ice for at least 2 hours. Then attach the tube to the thermometer by a suitable means, adjust it in a water-bath so that the upper edge of the material is 10 mm below the water level, and heat as directed for *Class I* except, within 5° of the expected melting temperature, to regulate the rate of rise of temperature to 0.5° to 1.0° per minute. The temperature at which the material is observed to rise in the capillary tube is the melting temperature.

Procedure for Class III

This procedure is applied to petrolatums. Melt a quantity of the substance under test slowly, while

stirring, until it reaches a temperature of 90° to 92°. Remove the source of the heat and allow the molten substance to cool to a temperature of 8° to 10° above the expected melting temperature. Chill the bulb of a suitable thermometer to 5°, wipe it dry, and while still cold, dip into the molten substance so that approximately the lower half of the bulb is submerged. Withdraw it immediately, and hold vertically away from the heat until the wax surface dulls. Then dip for 5 minutes into a water-bath having a temperature not higher than 16°.

Fix the thermometer securely in a test-tube so that the lower end is 1.5 cm above the bottom of the test-tube. Suspend the test-tube in a water-bath adjusted to about 16°, and raise the temperature of the bath at a rate of 2° per minute to 30°. Then change to a rate of 1° per minute, and note the temperature at which the first drop of melted substance leaves the thermometer. Repeat the determination twice on a freshly melted portion of the substance under test. If the variation of three determinations is less than 1°, take the average of the three as the melting temperature. If the variation of three determinations is more than 1°, make two additional determinations and take the average of the five.

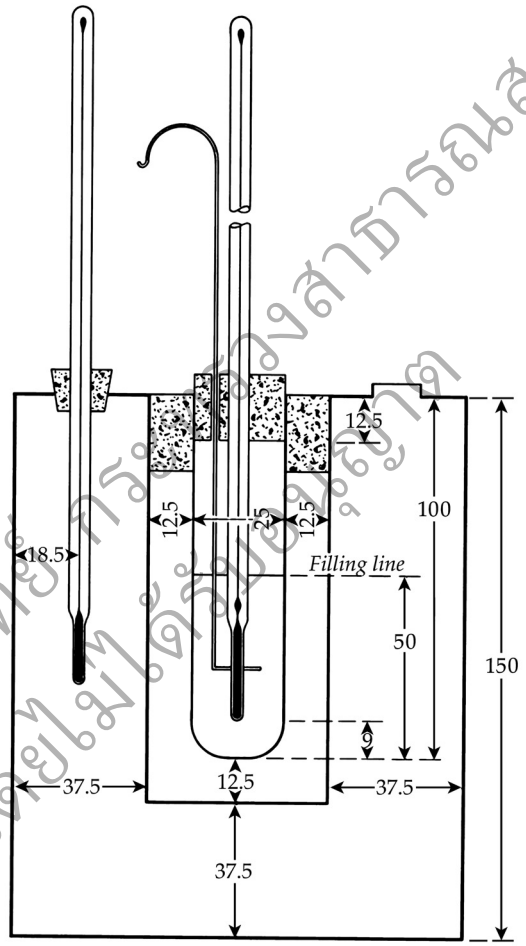
4.4 DETERMINATION OF FREEZING TEMPERATURE

The temperature at which a substance passes from the liquid to the solid state upon cooling is a useful index to purity if heat is liberated when the solidification takes place, provided that any impurities present dissolve in the liquid only, and not in the solid. Pure substances have a well-defined freezing point, but mixtures generally freeze over a range of temperatures. For many mixtures, the freezing temperature, as determined by strict adherence to the following empirical methods, is a useful index of purity. The method for determining freezing temperature set forth here is applicable to substances that melt between –20° and 150°, the range of the thermometer used in the bath. The freezing temperature is the maximum point (or lacking a maximum, the point of inflection) in the temperature-time curve.

Apparatus

Assemble an apparatus similar to that illustrated, in which the container for the substance is a 25-mm × 100-mm test-tube. This is provided with a suitable, short-range thermometer suspended in the center, and a wire stirrer, about 300 mm long, bent at its lower end into a horizontal loop around the thermometer. (Use a thermometer having a range not exceeding 30°, graduated in 0.1° divisions, and calibrated for, but not used at, 76-mm immersion. Other temperature-measuring devices may be used if they are validated for this procedure. Dimensions should be within ±20 per cent of those given in the illustration.)

The sample container is supported, by means of a cork, in a suitable water-tight cylinder about 50 mm in internal diameter and 110 mm in length. The cylinder, in turn, is supported in a suitable bath sufficient to provide not less than a 37-mm layer surrounding the sides and bottom of the cylinder. The outside bath is provided with a suitable thermometer.



Apparatus for Determination of Freezing Temperature
Dimension in mm

Procedure

Use a thermometer having a range not exceeding 30°, graduated in 0.1° divisions, and calibrated for, but not used at, 76-mm immersion. Melt the substance, if a solid, at a temperature not exceeding 20° above its expected freezing temperature, and pour it into the test-tube to a height of 50 to 57 mm. Assemble the apparatus with the bulb of the test-tube thermometer immersed halfway between the top and bottom of the sample in the test-tube. Fill the bath to about 12 mm from the top of the tube with suitable fluid at a temperature of 4° to 5° below the expected freezing temperature.

In case the substance is a liquid at room temperature, carry out the determination using a bath temperature about 15° below the expected freezing temperature.

When the test sample has cooled to about 5° above its expected freezing temperature, adjust the bath to a temperature 7° to 8° below the expected freezing temperature. Stir the sample continuously during the

remainder of the test by moving the loop up and down between the top and bottom of the sample, at a regular rate of 20 complete cycles per minute.

Solidification frequently may be induced by rubbing the inner walls of the test-tube with the thermometer, or by introducing a crystal of the substance being examined. Pronounced supercooling may cause deviation from the normal pattern of temperature changes. If the latter occurs, repeat the test, introducing small particles of the material under test in solid form at 1° intervals as the temperature approaches the expected freezing temperature.

Record the reading of the test-tube thermometer every 30 seconds. Continue stirring only so long as the temperature is gradually falling, stopping when the temperature becomes constant or starts to rise slightly. Continue recording the temperature in the test-tube every 30 seconds for at least 3 minutes after the temperature again begins to fall after remaining constant.

The average of not less than four consecutive readings that lie within a range of 0.2° constitutes the freezing temperature. These readings lie about a point of inflection or a maximum, in the temperature-time curve, that occurs after the temperature becomes constant or starts to rise and before it again begins to fall. The average to the nearest 0.1° is the freezing temperature.

4.5 DETERMINATION OF BOILING RANGE (DISTILLATION RANGE)

The boiling range is the temperature interval, corrected for a pressure of 101.3 kPa (760 Torr), within which a liquid, or a specified fraction of a liquid, distils in the following conditions.

Apparatus

The apparatus (Fig. 1) consists of a distillation flask (A), a straight tube condenser (B) which fits onto the side arm of the flask and a plain-bend adaptor (C) attached to the end of the condenser. The lower end of the condenser may, alternatively, be bent to replace the adaptor. A thermometer is inserted in the neck of the flask so that the upper end of the mercury reservoir is 5 mm lower than the junction of the lower wall of the lateral tube. The thermometer is graduated at 0.2°-intervals and the scale covers a range of about 50°. During the determination, the flask, including its neck, is protected from draughts by a suitable screen.

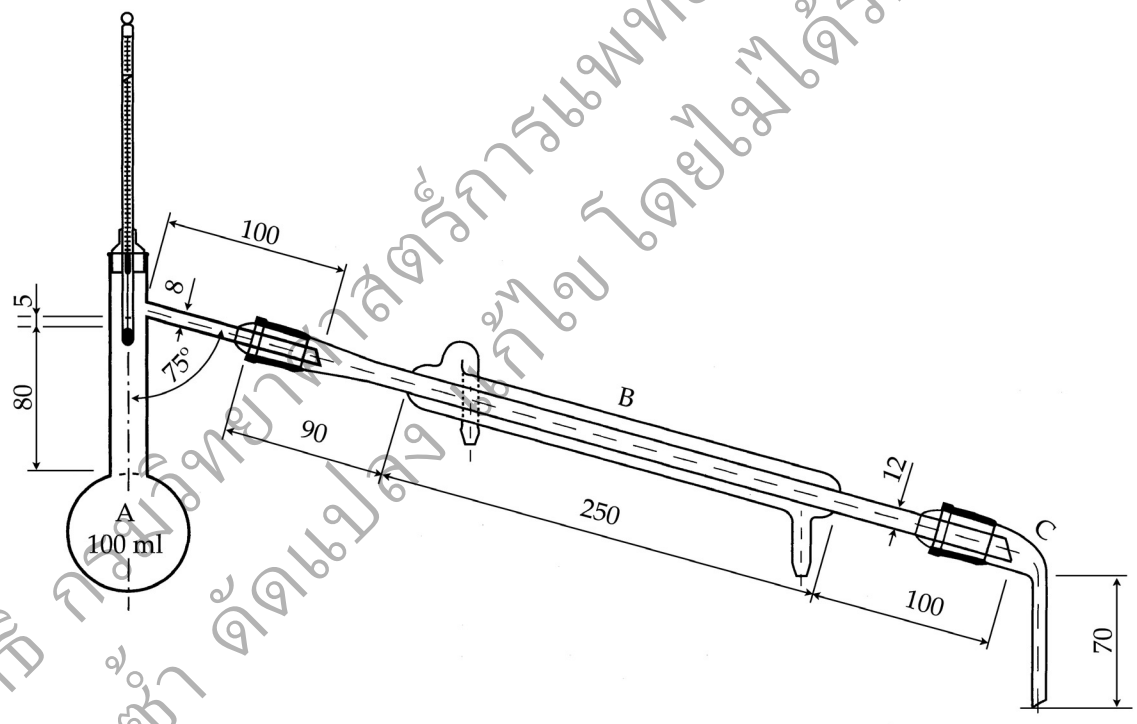


Fig. 1 Apparatus for the Determination of Boiling Range
Dimensions in mm

Method

Place in the flask (A) 50.0 ml of the liquid to be examined and a few pieces of porous material. Collect the distillate in a 50-ml cylinder graduated in 1 ml. Cooling by circulating water is essential for liquids distilling below 150°. Heat the flask so that boiling is rapidly achieved and note the temperature at which the first drop of distillate falls into the cylinder. Adjust the heating to give a regular rate of distillation of 2 to 3 ml per minute and note the temperature when the whole or the prescribed fraction of the liquid, measured at 20°, has distilled.

Correct the observed temperatures for barometric pressure by means of the formula:

$$t_1 = t_2 + k\,(101.3 - b),$$

where t_1 = the corrected temperature,
t_2 = the observed temperature,

k = the correction factor taken from Table 1 unless the factor is given, and
b = the barometric pressure, expressed in kilopascals, during the distillation.

Table 1 Temperature Correction in Relation to the Pressure

Boiling Temperature	Correction Factor k
Up to 100°	0.30
Above 100° up to 140°	0.34
Above 140° up to 190°	0.38
Above 190° up to 240°	0.41
Above 240°	0.45

4.6 DETERMINATION OF BOILING TEMPERATURE

The boiling temperature is the corrected temperature at which the vapour pressure of a liquid is equal to 101.3 kPa (760 Torr).

Apparatus

The apparatus is that used for the "Determination of Boiling Range" (Appendix 4.5) with the exception that the thermometer is inserted in the neck of the flask so that the lower end of the mercury reservoir is level with the lower end of the neck of the distillation flask and that the flask is placed on a plate of isolating material pierced by a hole 35 mm in diameter.

Method

Place in the flask (A) 20 ml of the liquid to be examined and a few pieces of porous material. Heat the flask so that boiling is rapidly achieved and record the temperature at which liquid runs from the side-arm into the condenser. Correct the observed temperature for barometric pressure by means of the formula:

$$t_1 = t_2 + k\,(101.3 - b),$$

where t_1 = the corrected temperature,
t_2 = the observed temperature,
k = the correction factor taken from Table 1 under the "Determination of Boiling Range" (Appendix 4.5), and
b = the barometric pressure, expressed in kilopascals, at the time of the determination.

4.7 DETERMINATION OF REFRACTIVE INDEX

The refractive index (n) of a substance is the ratio of the velocity of light in a vacuum to its velocity in the substance. It varies with the wavelength of the light (λ) used in its measurement and with the temperature (t). It is therefore necessary to specify these conditions (n_λ^t). In practice it is usually convenient to measure the refraction with respect to air and the substance, rather than with respect to a vacuum and the substance, since, for Pharmacopoeial purposes, this has no significant influence on the observed values.

The refractive index may also be defined as the ratio of the sine of the angle of incidence to the sine of the angle of refraction.

The measurement of the refractive index is employed for Pharmacopoeial purposes mainly to establish the identity of liquid substances. It may also be used to test the purity of such substances.

Refractive indices are usually stated in terms of the wavelength of the sodium D line (589.3 nm) and at a temperature of 25° (n_D^{25}) unless otherwise specified in the individual monograph.

The accuracy of the measurement should be related to the requirements of the monograph. For Pharmacopoeial purposes it is usually adequate to express the refractive index to three decimal places.

Apparatus

Commercial refractometers are normally constructed for use with white light but are calibrated to give the refractive index in terms of the sodium D wavelength (589.3 nm).

The optical parts of the apparatus should be kept brilliantly clean. The working surfaces of prisms should be free from scratches.

Subject to the directions given above, the manufacturer's instructions relating to a suitable light source should be followed.

The instrument should be calibrated against a standard provided by the manufacturer; the temperature control of the liquid being examined and the cleanliness of the prism should be checked frequently by determining the refractive index of distilled water, which is 1.3330 at 20° and 1.3325 at 25°.

4.8 DETERMINATION OF OPTICAL ROTATION AND SPECIFIC ROTATION

Many drugs, in pure state or in solution, possess the inherent property of rotating the plane of incident polarized light; this property is called optical activity. The measurement of optical activity is used for Pharmacopoeial purposes mainly to establish the identity of the substance. It may also be employed to test the purity of the substance (absence of optically non-active foreign substances) and as an assay procedure.

Optical rotation The optical rotation is the angle through which the plane of polarization is rotated when polarized light passes through a layer of a liquid. Substances are described as dextrorotatory or levorotatory according to whether the plane of polarization is rotated clockwise or counter-clockwise, respectively, as determined by viewing towards the light source. Dextrorotation is designated (+) and levorotation is designated (–).

The optical rotation, unless otherwise specified, is measured at the wavelength of the sodium D line (589.3 nm) at 25° on a layer 1 dm thick.

Specific rotation (specific optical rotation) The specific rotation of a liquid substance is the angle of rotation, usually measured at the wavelength of the

sodium D line at 25°, unless otherwise specified, calculated with reference to a layer 1 dm thick, and divided by the specific gravity (relative density) measured at the same temperature.

The specific rotation of a solid substance is the angle of rotation, usually measured at the wavelength of sodium D line at 25°, unless otherwise specified, and calculated with reference to a layer 1 dm thick of a solution containing 1 g of the substance per ml.

$$\text{For liquids, } [\alpha]_D^{25} = \frac{\alpha}{ld};$$

$$\text{For solid, } [\alpha]_D^{25} = \frac{100\alpha}{lc} = \frac{100\alpha}{ldp},$$

where $[\alpha]_D^{25}$ = specific rotation determined at 25° using sodium D line,
α = angle of rotation in degrees read at 25°,
l = length in dm of the polarimeter tube,
c = number of g of substance contained in 100 ml of solution,
d = specific gravity of the liquid or solution at 25°, and
p = number of g of substance contained in 100 g of solution.

Apparatus

Optical rotation is measured with a polarimeter. Generally, a polarimeter accurate to 0.05° of angular rotation, and capable of being read with the same precision, suffices for Pharmacopoeial purposes; in some cases, a polarimeter accurate to 0.01° of angular rotation, and read with comparable precision, may be required.

Polarimeters for visual measurement: commercial instruments are normally constructed for use with a sodium or mercury vapour lamp or xenon.

Photoelectric polarimeters: where it is directed in the individual monograph to determine the optical rotation photoelectrically, use a photoelectric polarimeter capable of an accuracy of at least 0.01°.

Measurement of Optical Rotation

The accuracy and precision of optical rotatory measurements will be increased if they are carried out with due regard for the following general considerations.

Optical elements of the instrument must be brilliantly clean and in exact alignment. The match point should lie close to the normal zero mark. The light source should be rigidly set and well aligned with respect to the optical bench. It should be supplemented by a filtering system capable of transmitting light of a sufficiently monochromatic nature. Precision polarimeters generally are designed to accommodate interchangeable discs to isolate the D line from sodium light or the 546.1-nm line from the mercury spectrum. With polarimeters not thus designed, cells containing suitably coloured liquids may be employed as filters.

Observations should be accurate and reproducible to the extent that differences between replicates, or between observed and true values of rotation (the latter value having been established by calibration of the polarimeter scale with suitable standards), shall not exceed one-fourth of the range given in the individual monograph for the rotation of the substance being tested.

Polarimeter tubes should be filled in such a way as to avoid creating or leaving air bubbles that interfere with the passage of the beam of light. Interference from bubbles is minimized with tubes in which the bore is expanded at one end. However, with tubes of uniform bore, such as semimicro or micro tubes, care is required for proper filling.

In closing tubes having removable end-plates fitted with gaskets and caps, the latter should be tightened only enough to ensure a leak-proof seal between the end-plate and the body of the tube. Excessive pressure on the end-plate may set up strains that result in interference with the measurement. In determining the optical rotation of a substance of low rotatory power, it is desirable to loosen the caps and tighten them again between successive readings in the measurement of both the rotation and the zero point. Differences arising from end-plate strain thus generally will be revealed, and appropriate adjustments to eliminate the cause may be made.

Procedure

Determine the zero point of the polarimeter with the tube empty and closed for liquid substances and filled with the specified solvent for solutions of solid substances.

Where the substance is a solid, accurately weigh a suitable portion and transfer to a volumetric flask by means of *water*, or other solvent if specified in the monograph, reserving a portion of the solvent for the blank determination. Add enough solvent to bring the meniscus close to but still below the mark, and adjust the flask contents to 25° by suspending the flask in a constant-temperature bath. Add solvent to the mark, and mix. Transfer the solution to the polarimeter tube, preferably within 30 minutes from the time the substance was dissolved, taking care to standardize the elapsed time in the case of substances known to undergo racemization or mutarotation. During the elapsed time interval, maintain the solution at a temperature of 25°.

Where the substance is a liquid, adjust its temperature to 25°, and transfer directly to the polarimeter tube.

When a polarimeter is used for visual measurement, make at least 6 readings, both on blank and sample, at 25°, of the observed rotation. Take half the readings in a clockwise and the other half in a counter-clockwise direction. The zero correction is the average of the blank readings, and is subtracted from the average observed rotation. It is necessary in this calculation to use the observed signs of rotation, whether positive or negative, to give the corrected observed rotation.

When a photoelectric polarimeter is used, a smaller number of readings are required, depending on the type of instrument.

4.9 DETERMINATION OF WEIGHT PER MILLILITRE, SPECIFIC GRAVITY AND RELATIVE DENSITY

Weight per Millilitre

The weight per millilitre of a liquid is the weight in g of 1 ml of a liquid when weighed in air at 20°, unless otherwise specified in the monograph.

The weight per millilitre is determined by dividing the weight in air, expressed in g, of the quantity of liquid that fills a pycnometer at the specified temperature by the capacity, expressed in ml, of the pycnometer at the same temperature. The capacity of the pycnometer is ascertained from the weight in air, expressed in g, of the quantity of water required to fill the pycnometer at that temperature. The weight of a litre of water at specified temperatures when weighed against brass weights in air of density 0.0012 g per ml is given in the following table. Ordinary deviations in the density of air from the above value, here taken as the mean, do not affect the result of a determination in the significant figures prescribed for Pharmacopoeial substances.

Temperature(°)	Weight of a Litre of Water (g)
20	997.18
25	996.02
30	994.62

Specific Gravity

Unless otherwise stated in the individual monograph, the specific gravity determination is applicable only to liquids, and, unless otherwise stated, is based on the ratio of the weight of a liquid in air at 25° to that of an equal volume of *water* at the same temperature. Where a temperature is specified in the individual monograph, the specific gravity is the ratio of the weight of liquid in air at the specified temperature to that of an equal volume of *water* at the same temperature. When the substance is a solid at 25°, determine the specific gravity of the melted material at the temperature directed in the individual monograph, and refer to *water* at 25°.

Unless otherwise stated in the individual monograph, the density is defined as the mass of a unit volume of the substance at 25°, expressed in kilograms per cubic metre or grams per cubic centimetre (1 $kg.m^{-3} = 10^{-3}$ $g.cm^{-3}$).

Unless otherwise directed in the individual monograph, use Method I.

Method I Select a scrupulously clean, dry pycnometer that previously has been calibrated by determining its weight and the weight of recently boiled *water* contained in it at 25°. Adjust the temperature of the liquid to about 20°, and fill the pycnometer with it. Adjust the temperature of the filled pycnometer to 25°, remove any excess of the liquid, and weigh. When the monograph specifies a temperature different from 25°, filled pycnometers must be brought to the temperature of the balance before they are weighed. Subtract the tare weight from the filled weight.

The specific gravity of the liquid is the quotient obtained by dividing the weight of the liquid contained in the pycnometer by the weight of water contained in it, both determined at 25° unless otherwise directed in the individual monograph.

Method II The procedure includes the use of the Oscillating transducer density meter. The apparatus consists of the following:

— a U-shaped tube, usually of borosilicate glass, which contains the liquid to be examined;

— a magneto-electrical or piezo-electrical excitation system that causes the tube to oscillate as a cantilever oscillator at a characteristic frequency depending on the density of the liquid to be examined;

— a means of measuring the oscillation period (T), which may be converted by the apparatus to give a direct reading of density or used to calculate density by using the constants A and B described below; and

— a means to measure and/or control the temperature of the oscillating transducer containing the liquid to be tested.

The oscillation period is a function of the spring constant (c) and the mass of the system:

$$T^2 = \left(\frac{M}{c} + \frac{\rho \times V}{c} \right) \times 4\pi^2$$

where ρ is the density of the liquid to be tested, M is the mass of the tube, and V is the volume of the filled tube.

Introduction of two constants, $A = C/(4\pi^2 \times V)$ and $B = M/V$, leads to the classical equation for the oscillating transducer:

$$\rho = A \times T^2 - B$$

The specific gravity of the liquid is given by the expression:

$$\rho_L / \rho_W,$$

where ρ_L and ρ_W are the densities of the liquid and water, respectively, both determined at 25°, unless otherwise directed in the individual monograph.

CALIBRATION The constants A and B are determined by operating the instrument with the U-tube filled with two different samples of known density (e.g., degassed *water* and air). Perform the control measurements daily, using degassed *water*: the results displayed for the control measurement using degassed *water* do not deviate from the reference value ($\rho_{25} = 0.997043$ $g.cm^{-3}$) by more than its specified error. Precision is a function of the repeatability and stability of the oscillator frequency. Density meters are able to achieve measurements with an error on the order of 1×10^{-3} $g.cm^{-3}$ to 1×10^{-5} $g.cm^{-3}$ and a repeatability of 1×10^{-4} $g.cm^{-3}$ to 1×10^{-6} $g.cm^{-3}$. For example, an instrument specified to $\pm 1 \times 10^{-4}$ $g.cm^{-3}$ must display 0.9970 ± 0.0001 $g.cm^{-3}$ in order to be suitable for further measurement, otherwise a readjustment is necessary. Calibration with certified reference materials should be carried out regularly.

PROCEDURE Using the manufacturer's instructions, perform the measurements using the same procedure as for Calibration. If necessary, equilibrate the liquid to be examined at 25° before introduction into the tube to avoid the formation of bubbles and to reduce the time required for measurement. Factors affecting accuracy include the following:

— temperature uniformity throughout the tube,
— nonlinearity over a range of density,
— parasitic resonant effects, and
— viscosity, if the oscillating transducer density meters used do not provide automatic compensation of sample viscosity influence.

Relative Density

The relative density $d_{t_2}^{t_1}$ of a substance is the ratio of the mass of a certain volume of a substance at temperature t_1 to the mass of an equal volume of water at temperature t_2. Unless otherwise indicated, the relative density d_{20}^{20} is used. Relative density is also commonly expressed as d_4^{20}

Density ρ_{20}, defined as the mass of a unit volume of the substance at 20°, may also be used, expressed in kilograms per cubic metre or grams per cubic centimetre ($1\ kg.m^{-3} = 10^{-3}\ g.cm^{-3}$). These quantities are related by the following equations where density is expressed in grams per cubic centimetre:

$$\rho_{20} = 0.998203 \times d_{20}^{20} \quad \text{or} \quad d_{20}^{20} = 1.00180 \times \rho_{20}$$

$$\rho_{20} = 0.999972 \times d_4^{20} \quad \text{or} \quad d_4^{20} = 1.00003 \times \rho_{20}$$

$$d_4^{20} = 0.998230 \times d_{20}^{20}$$

Relative density or density is measured with the precision to the number of decimals prescribed in the monograph using a density bottle (solids or liquids), a hydrostatic balance (solids), a hydrometer (liquids) or a digital density meter with an oscillating transducer (liquids and gases). When the determination is made by weighing, the buoyancy of air is disregarded, which may introduce an error of 1 unit in the third decimal place. When using a density meter, the buoyancy of air has no influence.

Proceed as directed under "Specific Gravity, Method II."

4.10 DETERMINATION OF VISCOSITY

Viscosity is a property of liquids that is closely related to the resistance to flow. It is defined in terms of the force required to move one plane surface continuously past another under specified steady-state conditions when the space between is filled by the liquid in question. In other words it is the shear stress divided by the rate of shear strain. The unit[1] is the pascal second (Pa.s), viscosities commonly encountered represent fractions of the pascal second, so that the millipascal second (mPa.s) proves to be the more convenient unit. The specifying of temperature is important because viscosity changes with temperature; in general, viscosity decreases as temperature is raised. While on the absolute scale viscosity is measured in pascal second or millipascal second, for convenience, the kinematic scale, in which the units are square centimetres per second ($cm^2.s^{-1}$) and square millimetres per second ($mm^2.s^{-1}$), commonly is used. To obtain the kinematic viscosity from the absolute viscosity, the latter is divided by the density of the liquid at the same temperature, i.e. kinematic viscosity = (absolute viscosity)/(density). The sizes of the units are such that viscosities in the ordinary ranges are conveniently expressed in square millimetres per second. The approximate viscosity in square millimetres per second at room temperature of ether is 0.2; of water, 1; of kerosene, 2.5; of liquid paraffin, 20 to 70; and of honey, 10,000.

Absolute viscosity can be measured directly if accurate dimensions of the measuring instruments are known, but it is more common practice to calibrate the instrument with a liquid of known viscosity and to determine the viscosity of the unknown fluid by comparison with that of the known.

Many substances, such as the gums employed in pharmacy, have variable viscosity, and most of them are less resistant to flow at higher flow rates. In such cases, a given set of conditions is selected for measurement, and the measurement obtained is considered to be an apparent viscosity. Since a change in the conditions of measurement would yield a different value for the apparent viscosity of such substances, the instrument dimensions and conditions for measurement must be closely adhered to by the operator.

[1]In the centimetre-gram-second (CGS) system, the unit of absolute viscosity is the poise or the centipoise and the unit of kinematic viscosity is the stoke or the centistoke.

Unit Equivalence

SI Unit	CGS Unit
10^{-1} Pa.s	= 1 poise
1 mPa.s	= 1 centipoise
1 $cm^2.s^{-1}$	= 1 stoke
1 $mm^2.s^{-1}$	= 1 centistoke

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Method I

APPARATUS The apparatus consists of a glass U-tube viscometer (Fig. 1) made of clear borosilicate glass and constructed in accordance with the dimensions shown in the figure and in Table 1; the monograph states the size of a viscometer to be used.

PROCEDURE Fill the viscometer with the test liquid through tube L to slightly above the mark G, using a long pipette to minimize wetting the tube above the mark. Place the tube vertically in a water-bath and when it has attained the specified temperature, adjust the volume of the liquid so that the bottom of the meniscus settles at the mark G. Suck or blow the liquid to a point about 5 mm above the mark E. After releasing pressure or suction, measure the time taken for the bottom of the meniscus to fall from the top edge of mark E to the top edge of mark F.

Calculate, as required, either the kinematic viscosity (ν) in square millimetres per second ($mm^2.s^{-1}$) from the equation:

$$\nu = Kt,$$

or the absolute viscosity (η) in pascal seconds (Pa.s) from the equation:

$$\eta = K\rho t,$$

where t = time in seconds for the meniscus to fall from E to F, and
ρ = mass per volume ($g.cm^{-3}$).

The constant (K) of the instrument is determined using the appropriate reference liquid for viscometers.

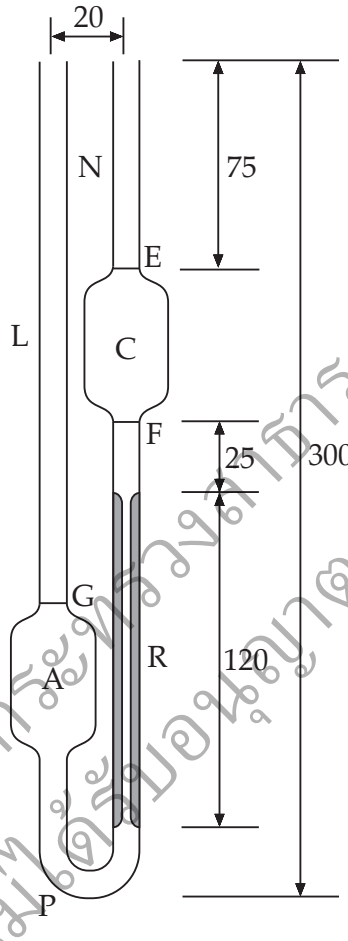

Fig. 1 U-Tube Viscometer
Dimensions in mm unless otherwise stated; tolerances are ±10 per cent or ±10 mm, whichever is the less.

Table 1 U-Tube Viscometer-Dimensions

Size No.	Nominal Viscometer Constant $mm^2.s^{-2}$	Kinematic Viscosity Range $mm^2.s^{-1}$	Inside Diameter of Tube R mm (±2%)	Outside Diameter of Tubes*		Volume of Bulb C ml (±5%)	Vertical Distance F to G mm	Outside Diameter of Bulbs A and C mm
				L and P mm	N mm			
A**	0.003	0.9 to 3	0.50	8 to 9	6 to 7	5.0	91±4	21 to 23
B	0.01	2 to 10	0.71	8 to 9	6 to 7	5.0	87±4	21 to 23
C	0.03	6 to 30	0.88	8 to 9	6 to 7	5.0	83±4	21 to 23
D	0.1	20 to 100	1.40	9 to 10	7 to 8	10.0	78±4	25 to 27
E	0.3	60 to 300	2.00	9 to 10	7 to 8	10.0	73±4	25 to 27
F	1.0	200 to 1,000	2.50	9 to 10	7 to 8	10.0	70±4	25 to 27
G	3.0	600 to 3,000	4.00	10 to 11	9 to 10	20.0	60±3	32 to 35
H	10.0	2,000 to 10,000	6.10	10 to 11	9 to 10	20.0	50±3	32 to 35

*Use 1 to 1.25 mm wall tubing for L, N, and P.

**300 s minimum flow times; 200 s minimum flow time for all other sizes.

Method II

APPARATUS The apparatus consists of a glass suspended-level viscometer (Fig. 2) made of clear borosilicate glass and constructed in accordance with the dimensions shown in Table 2; the size to be used is specified in the monograph.

PROCEDURE Fill the viscometer through tube L with a sufficient quantity of the test liquid to fill bulb A but ensuring that the level of liquid in bulb B is below the exit to ventilation tube M. After the tube has been placed vertically in the bath and has attained the specified temperature, close tube M and apply suction to tube N until the liquid reaches a level about 8 mm above mark E. Hold the liquid at this level by closing tube N and open tube M. When the liquid is clear of the capillary end of tube N and the lower end of tube M, open tube N. Measure the time taken for the bottom of the meniscus to fall from the top edge of mark E to the top edge of mark F.

If the end of tube M becomes blocked by the liquid at any time while the flow time is being measured, the determination must be repeated.

Calculate the kinematic viscosity (ν) in square millimetres per second ($mm^2.s^{-1}$) from the equation:

$$\nu = Kt,$$

where t = time in seconds for the meniscus to fall from E to F.

The constant (K) of the instrument is determined using the appropriate reference liquid for viscometers.

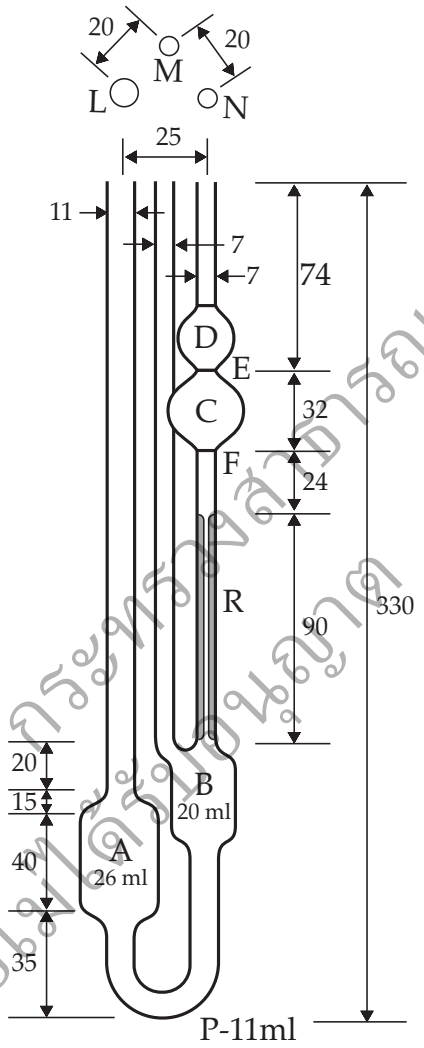

Fig. 2 Suspended-level Viscometer
Dimensions in mm unless otherwise stated; tolerances are ±10 per cent or ±10 mm, whichever is the less.

Table 2 Suspended-level Viscometer-Dimensions

Size No.	Nominal Viscometer Constant $mm^2.s^{-2}$	Kinematic Viscosity Range $mm^2.s^{-1}$	Inside Diameter of Tube R mm (±2%)	Volume of Bulb C ml (±5%)	Inside Diameter of Tube N mm
1*	0.01	3.5 to 10	0.64	5.6	2.8 to 3.2
1A	0.03	6 to 30	0.84	5.6	2.8 to 3.2
2	0.1	20 to 100	1.15	5.6	2.8 to 3.2
2A	0.3	60 to 300	1.51	5.6	2.8 to 3.2
3	1.0	200 to 1,000	2.06	5.6	3.7 to 4.3
3A	3.0	600 to 3,000	2.74	5.6	4.6 to 5.4
4	10	2,000 to 10,000	3.70	5.6	4.6 to 5.4
4A	30	6,000 to 30,000	4.97	5.6	5.6 to 6.4
5	100	20,000 to 100,000	6.76	5.6	6.8 to 7.5

*350 s minimum flow time; 200 s minimum flow time for all other sizes.

Method III

APPARATUS The apparatus consists of a falling-sphere viscometer. It comprises the following parts:

(a) A bath constructed of clear glass or having clear glass windows which allow the observation of all the graduations on the fall tube. It is provided with a cover bared to accommodate the thermometer, a tube to hold the balls to be used in the test, a stirring device, and the fall tube. The bath is filled to at least 30 mm above the topmost graduation of the fall tube, and the temperature in the bath is maintained between 19.9° and 20.1°.

(b) A fall tube (see Fig. 3) of nominal internal diameter 25 mm and between 317 and 323 mm long, made of clear glass. The tube is etched with six fine marks at the distances given in the figure, measured from a line 30 mm from the closed end. The marks encircle the tube at right-angles to the axis and no interval shall be in error by more than 0.5 mm. The numbers are etched below the lines. The tube is fitted with a bored plug of inert non-absorbent material carrying a glass delivery tube with a funnel top, or with a glass insert adjusted so that the lower end of the delivery tube is 5 mm above the topmost graduation mark; an internal diameter of 2.5 mm is suitable.

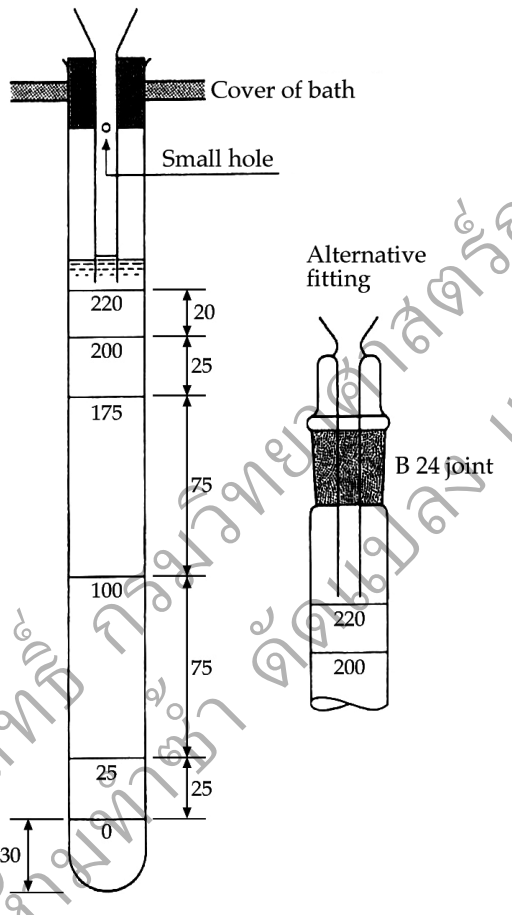

Fig. 3 Falling-sphere Viscometer
Dimensions in mm

(c) A suitable thermometer of the "total immersion" type.

(d) A sphere of steel or other suitable material 1.59 mm in diameter. Steel spheres should be kept in liquid paraffin or other suitable materials to prevent rusting.

PROCEDURE Fill the fall tube with the test liquid to about 10 mm above the 220-mm mark, place vertically in the bath, and allow to stand for air bubbles to clear and for temperature equilibrium to be attained. Clean the sphere, immerse it in a portion of the test liquid maintained at a temperature, between 19.9° and 20.1°, and when it is at this temperature, introduce it, without wiping, into the delivery tube. Observe the time for the lowest part of the sphere to pass through the planes of the tops of the 175-mm mark and the 25-mm mark, using a telescope or other suitable device to avoid errors due to parallax. The average of three readings concordant to within 0.5 per cent is taken as the time of fall.

Calculate the kinematic viscosity (ν) in square millimetres per second ($mm^2.s^{-1}$) from the equation:

$$\nu = \frac{d^2 g(\delta - \rho)0.867}{0.18 v \rho},$$

where d = the diameter of the sphere in centimetres,
δ = density of the sphere in grams per millilitre,
ρ = density of the test liquid in grams per millilitre,
v = velocity of fall in centimetres per second, and
g = local acceleration due to gravity in centimetres per second.

Method IV (for non-newtonian systems)

Operate a rotating cylinder viscometer in accordance with the manufacturer's instructions. The temperature and the angular velocity or shear rate, at which the dermination should be carried out, are indicated in the monograph.

Calculate the absolute viscosity (η) in pascal seconds (Pa.s) from the equation:

$$\eta = KL/\omega,$$

where L = the angular momentum in newton metres, and
ω = the angular speed in radians per second.

The constant (K) of the instrument is determined using the appropriate reference liquid for viscometers, or by reference to tables supplied by the instrument manufacturers giving the constants in relation to the surface area of the cylinders used and their speed of rotation.

4.11 DETERMINATION OF pH

The pH value of a solution is determined potentiometrically by means of a glass electrode, a reference electrode and a pH meter.

All solutions of substances being examined and the standard buffer solutions must be prepared using *carbon dioxide-free water.*

Preparation of Standard Buffer Solutions

Potassium Tetraoxalate Standard Buffer Solution (0.05 M): Dissolve 12.61 g of *potassium tetraoxalate* ($C_4H_3KO_8.2H_2O$) in *water*, and dilute to 1000.0 ml with the same solvent.

Potassium Hydrogentartrate Standard Buffer Solution (saturated at 25°): Shake an excess of *potassium hydrogentartrate* ($C_4H_5KO_6$) vigorously with *water* at 25°. Filter or decant. Prepare immediately before use.

Potassium Dihydrogencitrate Standard Buffer Solution (0.05 M): Dissolve 11.41 g of *potassium dihydrogencitrate* ($C_6H_7KO_7$) in *water* and dilute to 1000.0 ml with the same solvent. Prepare immediately before use.

Potassium Hydrogenphthalate Standard Buffer Solution (0.05 M): Dissolve 10.13 g of *potassium hydrogenphthalate* ($C_8H_5KO_4$), dried at 110° to 135°, in *water* and dilute to 1000.0 ml with the same solvent.

Phosphate Standard Buffer Solution pH 6.8 (*potassium dihydrogenphosphate* 0.025 M + *anhydrous disodium hydrogenphosphate* 0.025 M): Dissolve 3.39 g of *potassium dihydrogenphosphate* (KH_2PO_4) and 3.53 g of *anhydrous disodium hydrogenphosphate* (Na_2HPO_4), both dried for 2 hours at 110° to 130°, in *water* and dilute to 1000.0 ml with the same solvent.

Phosphate Standard Buffer Solution pH 7.4 (*potassium dihydrogenphosphate* 0.0087 M + *anhydrous disodium hydrogenphosphate* 0.0303 M): Dissolve 1.18 g of *potassium dihydrogenphosphate* (KH_2PO_4) and 4.30 g of *anhydrous disodium hydrogenphosphate* (Na_2HPO_4), both dried for 2 hours at 110° to 130°, in *water* and dilute to 1000.0 ml with the same solvent.

Sodium Tetraborate Standard Buffer Solution (0.01 M): Dissolve 3.80 g of *sodium tetraborate* ($Na_2B_4O_7.10H_2O$) in *water* and dilute to 1000.0 ml with the same solvent. Store protected from the carbon dioxide of the air.

Sodium Carbonate Standard Buffer Solution (*anhydrous sodium carbonate* 0.025 M + *sodium hydrogen carbonate* 0.025 M): Dissolve 2.64 g of *anhydrous sodium carbonate* (Na_2CO_3) and 2.09 g of *sodium hydrogencarbonate* ($NaHCO_3$) in *water* and dilute to 1000.0 ml with the same solvent.

Calcium Hydroxide Standard Buffer Solution (saturated at 25°). Shake an excess of *calcium hydroxide* with *carbondioxide-free water* and decant at 25°. Store protected from atmospheric carbon dioxide.

Calibration of pH Meter

Unless otherwise prescribed in the monograph, all measurements are made at the same temperature (at 25°±2°). The table shows the variation of pH with respect to temperature of a number of standard buffer solutions used for calibration. For the temperature correction, when necessary, follow the manufacturer's instructions.

To standardize the pH meter, select two Buffer Solutions for Standardization whose difference in pH does not exceed 4 units and such that the expected pH of the material under test falls between them. Fill the cell with one of the Buffer Solutions for Standardization at the temperature at which the test material is to be measured. Set the "temperature" control at the temperature of the solution, and adjust the calibration control to make the observed pH value identical with that tabulated. Rinse the electrodes and cell with several portions of the second Buffer Solutions for Standardization, then fill the cell with it, at the same temperature as the material to be measured. The pH of the second buffer solution is within ±0.07 pH unit of the tabulated value. If a larger deviation is noted, examine the electrodes and, if they are faulty, replace them. Adjust the "slope" or "temperature" control to make the observed pH value identical with that tabulated. Repeat the standardization until both Buffer Solutions for Standardization give observed pH value within 0.02 pH unit of the tabulated value without further adjustment of the controls. When the system is functioning satisfactorily, rinse the electrodes and cell several times with a few portions of the test material, fill the cell with the test material, and read the pH value. Use *carbon dioxide-free water* for solution or dilution of test material in pH determinations. In all pH measurements, allow a sufficient time for stabilization.

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pH of Standard Buffer Solutions at Various Temperatures

Temperature (°)	Potassium Tetraoxalate Standard Buffer Solution	Potassium Hydrogen-tartrate Standard Buffer Solution	Potassium Dihydrogen-citrate Standard Buffer Solution	Potassium Hydrogen-phthalate Standard Buffer Solution	Phosphate Standard Buffer Solution pH 6.8	Phosphate Standard Buffer Solution pH 7.4	Sodium Tetraborate Standard Buffer Solution	Sodium Carbonate Standard Buffer Solution	Calcium Hydroxide Standard Buffer Solution
15	1.67		3.80	4.00	6.90	7.45	9.28	10.12	12.81
20	1.68		3.79	4.00	6.88	7.43	9.23	10.06	12.63
25	1.68	3.56	3.78	4.01	6.87	7.41	9.18	10.01	12.45
30	1.68	3.55	3.77	4.02	6.85	7.40	9.14	9.97	12.29
35	1.69	3.55	3.76	4.02	6.84	7.39	9.10	9.93	12.13
$\frac{\Delta pH^*}{\Delta t}$	+0.001	–0.0014	–0.0022	+0.0012	–0.0028	–0.0028	–0.0082	–0.0096	–0.034

*pH variation per degree Celsius.

Method

Immerse the electrodes in the solution to be examined and measure the pH at the same temperature as for the standard solutions. At the end of a set of measurements, take a reading of the solution used to standardize the meter and electrodes. If the difference between this reading and the original value is more than 0.05, the set of measurements must be repeated.

When measuring pH values above 10.0, ensure that the glass electrode is suitable for use under alkaline conditions and apply any necessary correction.

4.12 DETERMINATION OF WATER

Karl Fischer Method

Principle The titrimetric determination of water is based upon the quantitative reaction of water with an anhydrous solution of sulfur dioxide and iodine dissolved in pyridine and methanol. The test sample may be titrated with Karl Fischer reagent directly or the analysis may be carried out by a residual titration procedure. In the residual titration, excess Karl Fischer reagent is added to the test sample, sufficient time is allowed for the reaction to reach completion, and the unconsumed Karl Fischer reagent is titrated with a standard solution of water in methanol. The residual titration procedure is applicable generally and avoids the difficulties that may be encountered in the direct titration of substances from which the bound water is released slowly.

The stoichiometry of the reaction is not exact, and the reproducibility of a determination depends upon such factors as the relative concentrations of the Karl Fischer reagent ingredients, the nature of the inert solvent used to dissolve the test sample, and the technique used in the particular determination. Therefore, an empirically standardized technique is used in order to achieve the desired accuracy. Precision in the method is governed largely by the extent to which atmospheric moisture is excluded from the system. The titration of water is usually carried out with the use of *anhydrous methanol* as the solvent for the test sample; however, other suitable solvents may be used for special or unusual test sample.

Apparatus Use a closed system consisting of a suitable titration vessel, previously dried at 105° and cooled in a desiccator, fitted with two platinum electrodes, a nitrogen inlet tube, a stopper which accommodates the burette tip, a vent tube protected by a suitable desiccant, and a magnetic stirrer, connected to a suitable electro-metric apparatus. This apparatus embodies a simple electrical circuit which serves to pass 5 to 10 μA of direct current between a pair of platinum electrodes immersed in the solution to be titrated. At the end-point of the titration a slight excess of the reagent increases the flow of current to between 50 and 150 μA for 30 seconds or longer, depending upon the solution being titrated.

Reagent

KARL FISCHER REAGENT Dissolve 125 g of *iodine* in 170 ml of *pyridine*, add 670 ml of *anhydrous methanol*, and cool. To 100 ml of *pyridine* kept in an ice-bath, add liquid anhydrous *sulfur dioxide* until the volume reaches 200 ml. Slowly add this solution to the cooled iodine mixture, and shake well to dissolve the iodine. Allow the solution to stand for 24 hours before use. Preserve the reagent under refrigeration in a suitably sealed, glass-stoppered bottle, protected from light and from moisture in the air. This methanolic reagent is somewhat unstable and requires frequent standardization. A more stable reagent may be prepared by substituting 2-*methoxyethanol* for *anhydrous methanol*. A commercially available, stabilized solution of Karl Fischer reagent may be used.

METHANOL SOLUTION Add sufficient *water* (usually 2 ml per litre) to anhydrous methanol so that each ml of the resulting methanol solution is equivalent to about 0.5 ml of Karl Fischer reagent.

Standardization of the reagent

WATER EQUIVALENCE OF KARL FISCHER REAGENT (*F*)

Standardize the Karl Fischer reagent no longer than 1 hour before use by one of the following methods.

Place enough *anhydrous methanol* in the titration vessel to cover the electrodes, and add sufficient Karl Fischer reagent to give the characteristic end-point colour, or 100±50 μA of direct current at about 200 mV of applied potential.

For determination of trace amounts of water (less than 1 per cent), *sodium tartrate* may be used as a convenient water reference substance.

Quickly add 120 to 300 mg of *sodium tartrate* ($C_4H_4Na_2O_6.2H_2O$), accurately weighed by difference, and titrate to the end-point. Calculate the water equivalence of the Karl Fischer reagent as follows:

$$F = 2(18.02/230.08)(W/Vr),$$

where F = water equivalence of the Karl Fischer reagent in terms of mg of water per ml,
18.02 = molecular weight of water,
W = weight of *sodium tartrate* in mg,
230.08 = molecular weight of *sodium tartrate*, and
Vr = ml of Karl Fischer reagent consumed in the second titration.

For the precise determination of significant amounts of water (more than 1 per cent), use *water* obtained by distillation as the reference substance.

Quickly add between 25 and 250 mg of *water*, accurately weighed by difference, from a weighing pipette or from a pre-calibrated syringe or micropipette. Titrate to the end-point. Calculate the water equivalence of Karl Fischer reagent as follows:

$$F = W/Vr,$$

where F = water equivalence of the Karl Fischer reagent in terms of mg of water per ml,
W = weight of water in mg, and
Vr = ml of Karl Fischer reagent consumed in the second titration.

KARL FISCHER REAGENT EQUIVALENCE OF METHANOL SOLUTION (*f*)

Titrate a known volume of Karl Fischer reagent with methanol solution until the end-point is reached. Calculate the millilitres of Karl Fischer reagent equivalent to each ml of methanol solution as follows:

$$f = Vr/Vm,$$

where f = ml of Karl Fischer reagent equivalent to each ml of methanol solution,
Vr = ml of Karl Fischer reagent used, and
Vm = ml of methanol solution used.

Sample preparation For Pharmacopoeial articles, unless otherwise specified in the individual monograph, use an accurately weighed or measured amount of the specimen under test estimated to contain 10 to 250 mg of water.

TABLETS AND CAPSULES Tablets, use accurately weighed powder from not less than 4 tablets ground to a fine powder in an atmosphere of about 10 per cent relative humidity.

Capsules, use an accurately weighed portion of the mixed contents of not less than 4 capsules.

OINTMENTS AND OILS Use an accurately weighed portion of about 1 to 2 g.

AEROSOLS WITH PROPELLANT Store it in a suitable freezing unit having a temperature of not higher than 0° for at least 2 hours. After removing from the freezer, immediately open the container, and test 10.0 ml of the well-mixed sample. In titrating the sample, determine the end-point at a temperature of 10° or higher.

HYGROSCOPIC POWDERS Accurately weigh the immediate container. Using a suitable dry hypodermic needle and syringe, inject an appropriate volume of *anhydrous methanol*, accurately measured, into the container and shake to dissolve the contents. Using the same syringe, remove the solution from the container and transfer it to a titration vessel containing 20 ml of a mixture of *carbon tetrachloride*, *chloroform* and *anhydrous methanol* (2:2:1), previously titrated to the end-point as directed under Titration procedure. Repeat the procedure with a second portion of *anhydrous methanol*, accurately measured, add this washing to the titration vessel, and immediately titrate. Determine the volume, in ml, of Karl Fischer reagent equivalent to a portion of *anhydrous methanol* of the same total volume as that used to dissolve the sample and to wash the container and syringe, as directed under Titration procedure. Subtract this value from the volume, in ml, of Karl Fischer reagent obtained in the titration of the sample under test. Dry the container and its closure at 100° for 3 hours, allow to cool in a desiccator, and weigh. Determine the weight of the sample tested from the difference in weight from the initial weight of the container.

Titration procedure Determine the water by Method I, unless otherwise specified in the individual monograph. In the case of a colourless solution that is titrated directly, the end-point may be observed visually as a change in colour from canary-yellow to amber. The reverse is observed in the case of a test sample that is titrated residually. More commonly, however, the end-point is determined electrometrically.

METHOD I: DIRECT TITRATION Add 20 ml of *anhydrous methanol* to the titration vessel, and titrate with Karl Fischer reagent to the electrometric or visual end-point to consume any moisture that may be present. (Disregard the volume consumed, since it does not enter into the calculations.) Quickly add an accurately weighed or measured amount of the sample under test, mix, and again titrate with Karl Fischer reagent to the electrometric or visual end-point.

METHOD II: RESIDUAL TITRATION Where the individual monograph specifies that the water content is to be determined by Method II, the residual titration procedure, transfer 20 ml of *anhydrous methanol* to the titration vessel, and titrate with Karl Fischer reagent to the electrometric or visual end-point. Quickly add an accurately weighed or measured amount of the sample under test, mix, and add an accurately measured excess of Karl Fischer reagent. Allow sufficient time for the reaction to reach completion, and titrate the unconsumed Karl Fischer reagent with standardized methanol solution to the electrometric or visual end-point.

Calculation

Calculate the percentage of water in the sample as follows:

If titration Method I is used:

Percentage of water in weighed samples
$= (Vr \times F \times 100)/Ws$

Percentage of water in aerosols
$= Vr \times F/(\text{ml of sample used} \times 10)$

Percentage of water in hygroscopic powders
$= [(Vr - Vb) \times F \times 100]/Ws$

If titration Method II is used:

Percentage of water in weighed samples
$= [(Vr - Vm \times f) \times F \times 100]/Ws$

Percentage of water in aerosols
$= [(Vr - Vm \times f) \times F]/(\text{ml of sample used} \times 10)$

Percentage of water in hygroscopic powders
$= [(Vr - Vm \times f - Vb) \times F \times 100]/Ws$

Where f = ml of Karl Fischer reagent equivalent to each ml of methanol solution determined as directed in Karl Fischer reagent equivalence of methanol solution,

F = water equivalence of the Karl Fischer reagent determined as directed in Water Equivalence of Karl Fischer reagent of this section,

Vb = ml of Karl Fischer reagent equivalent to the *anhydrous methanol* used as a sample solvent,

Vm = ml of methanol solution used,

Vr = ml of Karl Fischer reagent used, and

Ws = weight of the sample in mg.

Coulometric Titration

Principle The Karl Fischer reaction is used in the coulometric determination of water. Iodine, however, is not added in the form of a volumetric solution but is produced in an iodide-containing solution by anodic oxidation. The reaction cell usually consists of a large anode compartment and a small cathode compartment that are separated by a diaphragm. Other suitable types of reaction cells (e.g., without diaphragms) may also be used. Each compartment has a platinum electrode that conducts current through the cell. Iodine, which is produced at the anode electrode, immediately reacts with water present in the compartment. When all the water has been consumed, an excess of iodine occurs, which usually is detected electrometrically, thus indicating the end-point. Moisture is eliminated from the system by pre-electrolysis. Changing the Karl Fischer reagent after each determination is not necessary since individual determinations can be carried out in succession in the same reagent solution. A requirement for this method is that each component of the test sample is compatible with the other components, and no side reactions take place. Samples are usually transferred into the vessel as solutions by means of injection through a septum. Gases can be introduced into the cell by means of a suitable gas inlet tube. Precision in the method is predominantly governed by the extent to which atmospheric moisture is excluded from the system; thus, the introduction of solids into the cell is not recommended, unless elaborate precautions are taken, such as working in a glove-box in an atmosphere of dry inert gas. Control of the system may be monitored by measuring the amount of baseline drift. This method is particularly suited to chemically inert substances like hydrocarbons, alcohols, and ethers. In comparison with the volumetric Karl Fischer titration, coulometry is a micromethod.

Apparatus Any commercially available apparatus consisting of an absolutely tight system fitted with the necessary electrodes and a magnetic stirrer is appropriate. The instrument's microprocessor controls the analytical procedure and displays the results. Calibration of the instrument is not necessary, as the current consumed can be measured absolutely.

Reagent See Reagent under Karl Fischer Method.

Sample preparation Where the sample is a soluble solid, dissolve an appropriate quantity, accurately weighed, in *anhydrous methanol* or other suitable solvents. Liquids may be used as such or as accurately prepared solutions in appropriate anhydrous solvents.

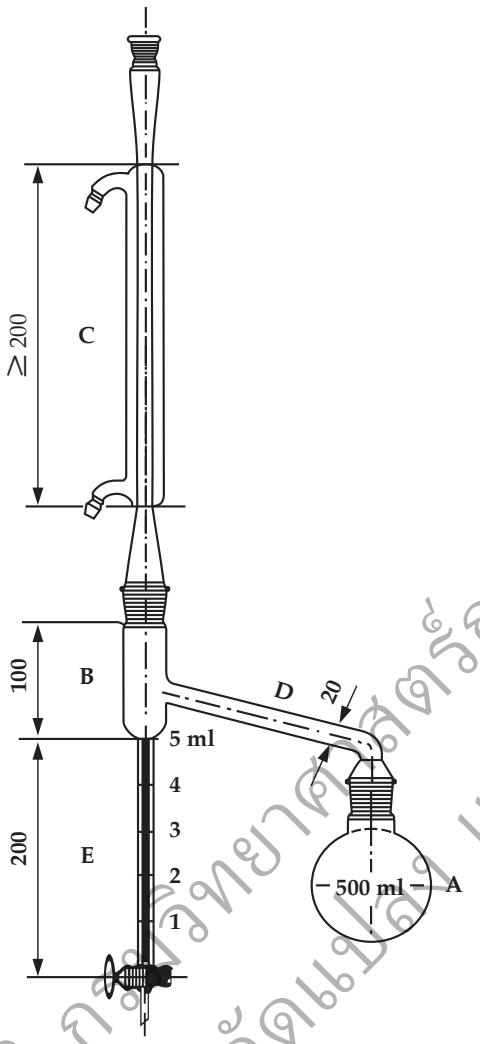
Where the sample is an insoluble solid, the water may be extracted using a suitable anhydrous solvent from which an appropriate quantity, accurately weighed, may be injected into the anolyte solution. Alternatively an evaporation technique may be used in which water is released and evaporated by heating the specimen in a tube in a stream of dry inert gas, this gas being then passed into the cell.

Procedure Using a dry syringe, quickly inject sample preparation, accurately measured and estimated to contain 0.5 to 5 mg of water, or as recommended by the instrument manufacturer into the anolyte, mix, and perform the coulometric titration to the electrometric end-point. Read the water content of sample preparation directly from the instrument's display, and calculate the percentage that is present in the substance. Perform a blank determination, and make any necessary corrections.

Calculation One mole of iodine corresponds to 1 mole of water, a quantity of electricity of 10.71 C corresponds to 1 mg of water.

Azeotropic Distillation Method

Apparatus The apparatus (see figure) consists of a glass flask (A) connected by a tube (D) to a cylindrical tube (B) fitted with a graduated receiving tube (E) and a reflux condenser (C). The receiving tube (E) is graduated in 0.1-ml subdivisions so that the error of reading is not more than 0.05 ml. The source of heat is preferably an electric heater with rheostat control or an oil-bath. The upper portion of the flask and the connection tube may be insulated with asbestos.



Apparatus for Determination of Water by the Azeotropic Distillation Method
Dimensions in mm

Method Clean the receiving tube and the condenser of the apparatus by a suitable method, thoroughly rinse with *water*, and dry.

Introduce 200 ml of *toluene* and about 2 ml of *water* into the dry flask. Distil for about 2 hours, allow to cool to room temperature and read the water volume to an accuracy of 0.05 ml. Place in the flask a quantity of the substance, weighed to the nearest centigram, expected to give about 2 to 3 ml of water. If the substance is of a pasty character, weigh it in a boat of metal foil. Add a few pieces of porous material and heat the flask gently for 15 minutes. When the toluene begins to boil, distil at the rate of 2 drops per second until most of the water has distilled over, and then increase the rate of distillation to about 4 drops per second. When the water has all distilled over, rinse the inside of the condenser tube with *toluene*. Continue the distillation for 5 minutes, remove the heat, allow the receiving tube to cool to room temperature, and dislodge any droplets of water which adhere to the walls of the receiving tube. When the water and toluene have completely separated, read the volume of water and calculate the percentage present in the substance using the expression:

$$\frac{100(n' - n)}{p},$$

where p = the weight in g of the substance to be examined,
n = the volume in ml of water obtained in the first distillation, and
n' = the total volume in ml of water obtained in the two distillations.

4.13 DETERMINATION OF INSOLUBLE MATTER

Dissolve the quantity of the sample specified in the monograph in 100 ml of *water*, heat to boiling unless otherwise directed, in a covered beaker, and warm on a water-bath for 1 hour. Filter the hot solution through a filtering crucible with an asbestos mat or a crucible with a sponge platinum mat or a sintered-glass crucible of fine porosity or a porous porcelain crucible, which has been tared. Wash the beaker and the filter thoroughly with hot *water*, dry at 105° to constant weight, cool in a desiccator, and weigh.

4.14 CRYSTALLINITY

Use the method specified in the individual monograph.

Method I

To prepare the sample for examination, mount a few particles in *liquid paraffin* on a clean glass slide. Examine the sample by means of a polarizing microscope. The particles reveal the phenomena of birefringence and extinction positions on revolving the microscope stage.

Method II

To prepare the sample for examination, mount a few particles in *liquid paraffin* on a clean glass slide, add 1 drop of *ethanol*, and allow to react for about 30 seconds. Then proceed as directed in Method I.

4.15 LOSS ON DRYING

The procedure set forth in this section determines the amount of volatile matter of any kind that is driven off under the conditions specified. For substances appearing to contain water as the only volatile constituent, the procedure given in "Determination of Water" (Appendix 4.12), is appropriate, and is specified in the individual monograph.

Unless otherwise directed in the monograph, conduct the determination on 1 to 2 g of the substance (2 to 5 g in case of crude drugs), previously mixed and accurately weighed. If the test substance is in the form of large crystals, reduce the particle size to about 2 mm by quickly crushing. Tare a glass-stoppered, shallow weighing bottle that has been dried for 30 minutes under the same conditions to be employed in the determination. Put the test substance in the bottle, replace the cover, and accurately weigh the bottle and the contents. By gentle, sidewise shaking distribute the test substance as evenly as practicable to a depth of about 5 mm generally, and not over 10 mm in the case of bulky materials. Place the loaded bottle in the drying chamber, removing the stopper and leaving it also in the chamber. Dry the test substance at the temperature and for the time specified in the monograph. The temperature of heating is within the range of ±2° of the stated figure in the monograph. Upon opening the chamber, close the bottle promptly and allow it to come to room temperature in a desiccator before weighing.

If the substance melts at a lower temperature than that specified for the determination of Loss on drying, maintain the bottle with its contents for 1 to 2 hours at a temperature 5° to 10° below the melting temperature, then dry at the specified temperature.

Where the sample under test is Capsules, use a portion of the mixed contents of not less than 4 capsules.

Where the sample under test is Tablets, use powder from not less than 4 tablets ground to a fine powder.

Where the individual monograph directs that loss on drying be determined by thermogravimetric analysis, a sensitive electrobalance is to be used.

Where drying in vacuum over a desiccant is directed in the individual monograph, a vacuum desiccator or a vacuum drying pistol, or other suitable vacuum drying apparatus, is to be used.

Where drying in a desiccator is specified, exercise particular care to ensure that the desiccant is kept fully effective by frequent replacement.

Where drying in a capillary-stoppered bottle in vacuum is directed in the individual monograph, use a bottle or tube fitted with a stopper having a 225±25 μm diameter capillary, and maintain the heating chamber at a pressure not exceeding 0.7 kPa (about 5 Torr). At the end of the heating period, admit dry air to the heating chamber, remove the bottle, and with the capillary stopper still in place allow it to cool in a desiccator before weighing.

4.16 LOSS ON IGNITION

This procedure is provided for the purpose of determining the percentage of test material that is volatilized and driven off under the conditions specified. The procedure, as generally applied, is nondestructive to the substance under test; however, the substance may be converted to another form such as an anhydride.

Perform the test on finely powdered material, and break up lumps, if necessary, with the aid of a mortar and pestle before weighing the sample. Weigh the sample to be tested without further treatment, unless a pre-liminary drying at a lower temperature, or other special pretreatment, is specified in the individual monograph. Unless other equipment is designated in the individual monograph, conduct the ignition in a suitable muffle furnace or oven that is capable of maintaining a temperature within 25° of that required for the test, and use a suitable crucible, complete with cover, previously ignited for 1 hour to a low red heat or at the temperature specified for the test, cooled in a desiccator, and accurately weighed.

Unless otherwise directed in the individual monograph, transfer to the tared crucible an accurately weighed quantity, in g, of the substance to be tested, about equal to that calculated by the formula 10/L, in which L is the limit (or the mean value of the limits) for Loss on ignition, in percentage. Ignite the loaded uncovered crucible, and cover to a low red heat or at the specified temperature (±25°) and for the period of time designated in the individual monograph. Ignite for successive 1-hour periods where ignition to constant weight is indicated. Upon completion of each ignition, cover the crucible, and allow it to cool in a desiccator to room temperature before weighing.

4.18 X-RAY DIFFRACTION

Every crystal form of a compound produces its own characteristic X-ray diffraction pattern. These diffraction patterns can be derived either from a single crystal or from a powdered sample (containing numerous crystals) of the material. The spacings between and the relative intensities of the diffracted maxima can be used for qualitative and quantitative analysis of crystalline materials. Powder diffraction techniques are most commonly employed for routine identification and the determination of relative purity of crystalline materials. Small amounts of impurity, however, are not normally detectable by X-ray diffraction method, and for quantitative measurements it is necessary to prepare the sample carefully to avoid preferred orientation effects.

The powder methods provide an advantage over other means of analysis in that they are usually nondestructive in nature (sample preparation is usually limited to grinding to ensure a randomly oriented sample, and deleterious effects of X-rays on solid pharmaceutical compounds are not commonly encountered).

The principal use of single-crystal diffraction data is for the determination of molecular weights and analysis of crystal structures at the atomic level. However, diffraction established for a single crystal can be used to support a specific powder pattern as being truly representative of a single phase.

Solids A solid substance can be classified as being crystalline, noncrystalline, or a mixture of the two forms. In crystalline materials, the molecular or atomic species are ordered in a three-dimensional array, called a lattice, within the solid particles. This ordering of molecular components is lacking in noncrystalline material. Noncrystalline solids sometimes are referred to as glasses or amorphous solids when repetitive order is nonexistent in all three dimensions. It is also possible for order to exist in only one or two dimensions, resulting in mesomorphic phases (liquid crystals). Although crystalline materials are usually considered to have well-defined visible external morphologies (their habits), this is not a necessity for X-ray diffraction analysis.

The relatively random arrangement of molecules in noncrystalline substances makes them poor coherent scatterers of X-rays, resulting in broad, diffuse maxima in diffraction patterns. Their X-ray patterns are quite distinguishable from crystalline sample, which give sharply defined diffraction patterns.

Many compounds are capable of crystallizing in more than one type of crystal lattice. At any particular temperature and pressure, only one crystalline form (polymorph) is thermodynamically stable. Since the rate of phase transformation of a metastable polymorph to the stable one can be quite slow, it is not uncommon to find several polymorphs of crystalline pharmaceutical compounds existing under normal handling conditions.

In addition to exhibiting polymorphism, many compounds form crystalline solvates in which the solvent molecule is an integral part of the crystal structure. Just as every polymorph has its own characteristic X-ray patsterns, so does every solvate. Sometimes the differences in the diffraction patterns of different polymorphs are relatively minor, and must be very carefully evaluated before a definitive conclusion is reached. In some instances, these polymorphs and/or solvate show varying dissolution rates. Therefore, on the time scale of pharmaceutical bioavailability, different total amounts of drug are dissolved, resulting in potential bioinequivalence of the several forms of the drug.

Fundamental principles A collimated beam of monochromatic X-rays is diffracted in various directions when it impinges upon a rotating crystal or randomly oriented powdered crystal. The crystal acts as a three-dimensional diffraction grating to this radiation. This phenomenon is described by Bragg's law, which states that diffraction (constructive interference) can occur only when waves that are scattered from different regions of the crystal, in a specific direction, travel distances differing by integral numbers (n) of the wavelength (λ). Under such circumstances, the waves are in phase. This condition is described by the Bragg's equation:

$$\frac{n\lambda}{2 \sin \theta} = d_{khl},$$

in which d_{hkl} denotes the interplanar spacings and θ is the angle of diffraction.

A family of planes in space can be indexed by three whole numbers, usually referred to as Miller indices. These indices are the reciprocals, reduced to smallest integers, of the intercepts that a plane makes along the axes corresponding to three nonparallel edges of the unit cell (basic crystallographic unit). The unit cell dimensions are given by the lengths of the spacings along the three axes, a, b, c, and the angles between them, α, β and γ. The interplanar spacing for a specific set of parallel planes hkl is denoted by d_{hkl}. Each such family of planes may show higher orders of diffraction where the d values for the related families of planes nh, nk, nl are diminished by the factor $1/n$ (n being an integer: 2, 3, 4, etc.). Every set of planes throughout a crystal has a corresponding Bragg diffraction angle associated with it (for a specific λ).

The amplitude of a diffracted X-ray beam from any set of planes is dependent upon the following atomic properties of the crystal: (1) position of each atom in the unit cell, (2) the respective atomic scattering factors, and (3) the individual thermal motions. Other factors that directly influence the intensities of the diffracted beam are: (1) the intensity and wavelength of the incident radiation, (2) the volume of crystalline sample, (3) the absorption of the X-radiation by the sample, and (4) the experimental arrangement utilized to record the intensity data. Thus, the experimental conditions are especially important for measurement of diffraction intensities.

Only a limited number of Bragg planes are in a position to diffract when monochromatized X-rays pass through a single crystal. Techniques of recording the intensities of all of the possible diffracting hkl planes involve motion of the single crystal and the recording media. Recording of these data is accomplished by photographic techniques (film) or with radiation detectors.

A beam passing through a very large number of small, randomly oriented crystals produces continuous cones of diffracted rays from each set of lattice planes. Each cone corresponds to the diffraction from various planes having a similar interplanar spacing. The intensities of these Bragg reflections are recorded by either film or radiation detectors. The Bragg angle can be measured easily from a film, but the advent of radiation detectors has made possible the construction of diffractometers that read this angle directly. The intensities and d spacings are more conveniently determined with powder diffractometers employing radiation detectors than by film methods. Microphotometers are frequently used for precise intensity measurements of films.

An example of powder pattern for a solid phase of magaldrate is shown in the accompanying figure. This diffraction pattern was derived from a powder diffractometer equipped with a Geiger-Müller detector; nickel-filtered Cu $K\alpha$ radiation was used.

Radiation The principal radiation sources utilized for X-ray diffraction are vacuum tubes utilizing copper, molybdenum, iron, and chromium as anodes; copper X-rays are employed most commonly for organic substances. For each of these radiations there is an element that will filter off the $K\beta$ radiation and permit the $K\alpha$ radiation to pass (nickel is used, in the case of copper radiation). In this manner the radiation is practically monochromatized. The choice of radiation to be used depends upon the absorption characteristics of the material and possible fluorescence by atoms present in the sample.

Caution Care must be taken in the use of such radiation. Those not familiar with the use of X-ray equipment should seek expert advice. Improper use can result in harmful effects to the operator.

Test preparation In an attempt to improve randomness in the orientation of crystallites (and, for film techniques, to avoid a grainy pattern), the sample may be ground in a mortar to a fine powder. Grinding pressure has been known to induce phase transformations; therefore, it is advisable to check the diffraction pattern of the unground sample.

In general, the shapes of many crystalline particles tend to give a sample that exhibits some degree of preferred orientation in the sample holder. This is especially evident for needle-like or plate-like crystals where size reduction yields finer needles or platelets. Preferred orientation in the sample influences the relative intensities of various reflections.

Several specialized handling techniques may be employed to minimize preferred orientation, but further reduction of particle size is often the best approach.

Where very accurate measurement of the Bragg angles is necessary, a small amount of an internal standard can be mixed into the sample. This enables the film or recorder tracing to be calibrated. If comparisons to literature values (including compendial limits) of d are being made, calibrate the diffractometer. NIST[1] standards are available covering to a d-value of 0.998 nm. Tetradecanol[2] may be used (d is 3.963 nm) for larger spacing. The absorption of the radiation by any sample is determined by the number and kinds of atoms through which the X-ray beam passes. An organic matrix usually absorbs less of the diffracted radiation than does an inorganic matrix. Therefore, it is important in quantitative studies that standard curves relating amount of material to the intensity of certain d spacings for that substance be determined in a matrix similar to that in which the substance will be analyzed.

In quantitative analyses of materials, a known amount of standard usually is added to a weighed amount of sample to be analyzed. This enables the amount of the substance to be determined relative to the amount of standard added. The standard used should have approximately the same density as the sample and similar absorption characteristics. More important, its diffraction pattern should not overlap to any extent with that of the material to be analyzed. Under these conditions a linear relationship between line intensity and concentration exists. In favourable cases, amounts of crystalline materials as small as 10 per cent may be determined in solid matrices.

Identification of crystalline materials can be accomplished by comparison of X-ray powder diffraction patterns obtained for known[3] materials with those of the unknown. The intensity ratio (ratio of the peak intensity of a particular d spacing to the intensity of the strongest maxima in the diffraction pattern) and the d spacing are used in the comparison. If a reference material (e.g., Reference Substance) is available, it is preferable to generate a primary reference pattern on the same equipment used for running the unknown sample, and under the same conditions. For most organic crystals, it is appropriate to record the diffraction pattern to include values for 2θ that range from as near zero degree as possible to 40 degrees. Agreement between sample and reference should be within the calibrated precision of the diffractometer for diffraction angle (2θ values should typically be reproducible to ±0.10 or 0.20 degrees), while relative intensities between sample and reference may vary up to 20 per cent. For other types of samples (e.g., inorganic salts), it may be necessary to extend the 2θ region scanned to well beyond 40 degrees. It is generally sufficient to scan past the ten strongest reflection identified in the Powder Diffraction File[3].

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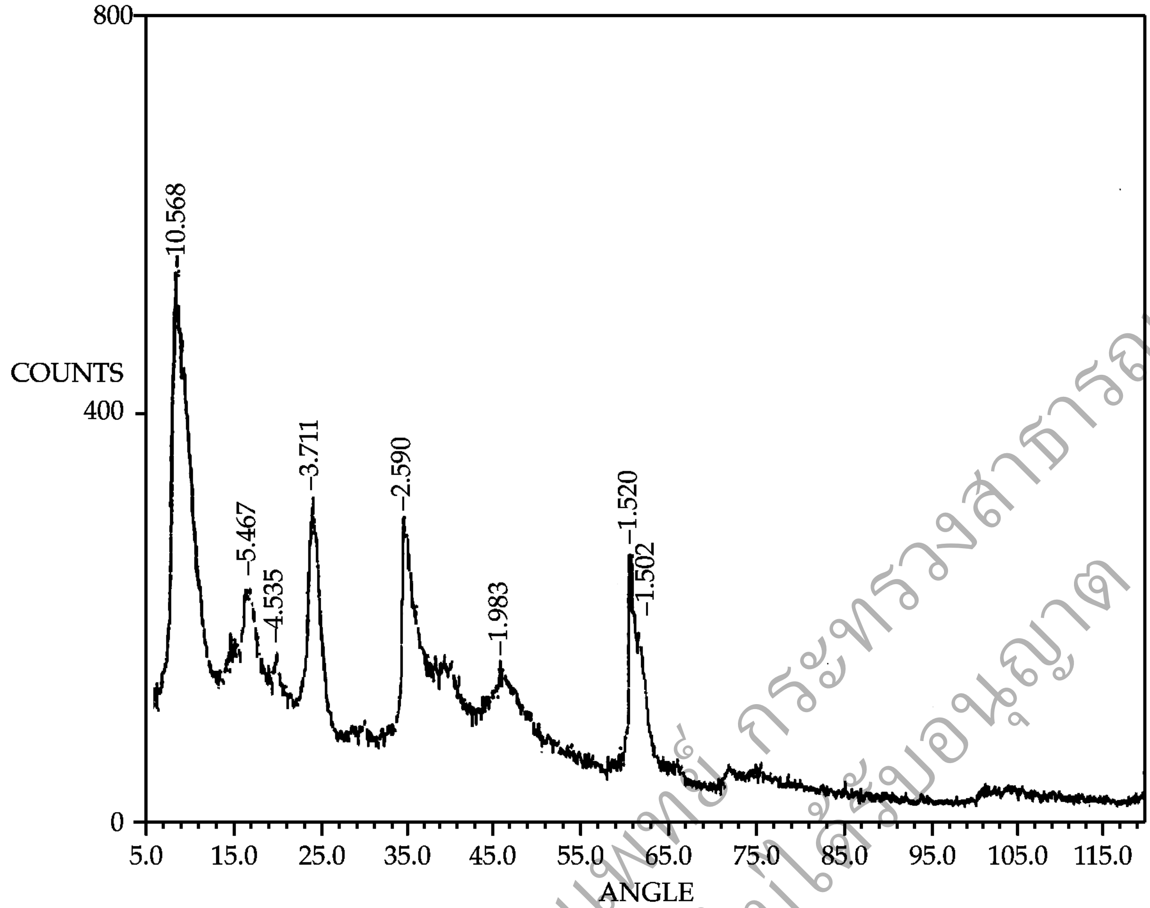
[1]U.S. National Institute of Standards and Technology.

[2]G. W. Brindley and G. Brown, eds., *Crystal Structures of Clay Minerals and their X-ray Identification*, Mineralogical Society Monograph No. 5, London, 1980, pp. 318 ff.

[3]The International Centre for Diffraction Data, Newtown Square Corporate Campus, 12 Campus Boulevard, Newtown Square, PA 19073, maintains a file on more than 60,000 crystalline materials, both organic and inorganic, suitable for such comparisons.



Typical Powder Pattern for a Solid Phase of Magaldrate[1]

4.21 DELIVERABLE VOLUME

The following tests are designed to provide assurance that oral solutions and suspensions will, when transferred from the original container, deliver the volume of dosage form that is declared on the label of the article. These tests are applicable to products labelled to contain not more than 250 ml, whether supplied as liquid preparations or liquid preparations that are constituted from solids upon the addition of a designated volume of a specific diluent. They are not required for an article packaged in single-unit containers when the monograph includes "Uniformity of Dosage Units" (Appendix 4.28).

For the determination of deliverable volume, select not fewer than 30 containers, and proceed as follows for the dosage form designated.

ORAL SOLUTIONS, ORAL SUSPENSIONS AND SYRUPS Mix the contents of 10 containers individually.

POWDERS THAT ARE LABELLED TO STATE THE VOLUME OF ORAL SOLUTION OR ORAL SUSPENSION THAT RESULTS WHEN THE POWDER IS CONSTITUTED WITH THE VOLUME OF DILUENT STATED IN THE LABELLING Constitute 10 containers with the volume of diluent stated in the labelling accurately measured and mix.

[1]*Thai Pharmacopoeia* Vol. I Part 2, Bangkok, Department of Medical Sciences, Ministry of Public Health, 1993, p. 1075.

PROCEDURE

SINGLE-UNIT CONTAINERS Gently pour the contents of each container into a separate dry graduated cylinder of a rated capacity not exceeding two and a half times the volume to be measured and calibrated "to contain", being careful to avoid the formation of bubbles and allowing them to drain for a period not to exceed 5 seconds unless otherwise specified in the monograph. When free from air bubbles, measure the volume of each mixture. Alternatively, in the case of products of low volume, it can be computed by discharging into a suitable tared container (allowing drainage for not more than 5 seconds), by determining the weight by difference and by computing the volume after determining the apparent density. The average volume of solution, suspension, or syrup obtained from the 10 containers is not less than 100 per cent and the volume of no container is less than 95 per cent and more than 110 per cent of the volume declared in the labelling. If A, the average volume is less than 100 per cent of that declared in the labelling, but the volume of no container is less than 95 per cent of the labelled amount, or B, the volume of not more than one container is less than 95 per cent, but is not less than 90 per cent of the labelled volume, perform the test on 20 additional containers. The average volume of solution, suspension, or syrup obtained from the containers is not less than 100 per cent of the volume declared in the labelling; and the volume of

ลิขสิทธิ์ กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข
ห้ามทำซ้ำ ดัดแปลง แก้ไข โดยไม่ได้รับอนุญาต

solution, suspension, or syrup obtained from not more than one of the 30 containers is less than 95 per cent, but not less than 90 per cent of that declared in the labelling; and is more than 110 per cent, but not more than 115 per cent of that declared on the labelling.

MULTIPLE-UNIT CONTAINERS Gently pour the contents of each container into a separate dry graduated cylinder of a rated capacity not exceeding two and a half times the volume to be measured, and calibrated "to contain", being careful to avoid the formation of bubbles and allowing them to drain for a period not to exceed 30 minutes unless otherwise specified in the monograph. When free from air bubbles, measure the volume of each mixture. The average volume of solution, suspension, or syrup obtained from the 10 containers is not less than 100 per cent and the volume of no container is less than 95 per cent of the volume declared in the labelling. If A, the average volume is less than 100 per cent of that declared in the labelling, but the volume of no container is less than 95 per cent of the labelled amount, or B, the volume of not more than one container is less than 95 per cent, but is not less than 90 per cent of the labelled volume, perform the test on 20 additional containers. The average volume of solution, suspension, or syrup obtained from the containers is not less than 100 per cent of the volume declared in the labelling; and the volume of solution, suspension, or syrup obtained from not more than one of the 30 containers is less than 95 per cent, but not less than 90 per cent of that declared in the labelling.

4.22 DISINTEGRATION TEST FOR SUPPOSITORIES AND PESSARIES

This test is provided to determine compliance with the limits on Disintegration stated in the individual monographs except where the label states that the suppositories or pessaries are intended for modified release or for prolonged local action. The test for disintegration of suppositories and pessaries determines whether suppositories and pessaries disintegrate or soften within a prescribed time when placed in an immersion fluid under the prescribed experimental conditions.

Apparatus

(a) A transparent sleeve of glass or suitable plastic, of height 60 mm with an internal diameter of 52 mm and an appropriate wall thickness (Fig. 1).

(b) A metal device consisting of two stainless metal discs, each of which contains 39 holes, each 4 mm in diameter, being distributed as indicated in Fig. 1. The diameter of the discs is closely similar to the internal diameter of the sleeve. The discs are separated by a distance of about 30 mm. The metal device is attached to the outer sleeve by means of three equally-spaced hooks.

For vaginal tablets use with the hook-end downwards as indicated in Fig. 2.

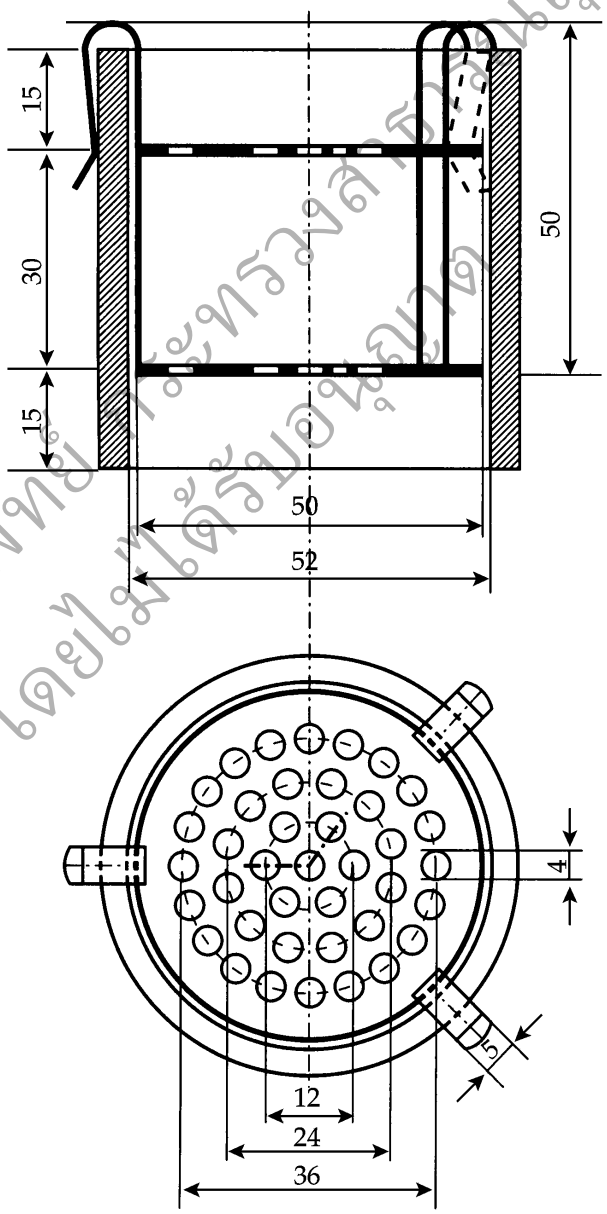


Fig. 1 Apparatus for the Disintegration of Suppositories and Pessaries
Dimensions in mm

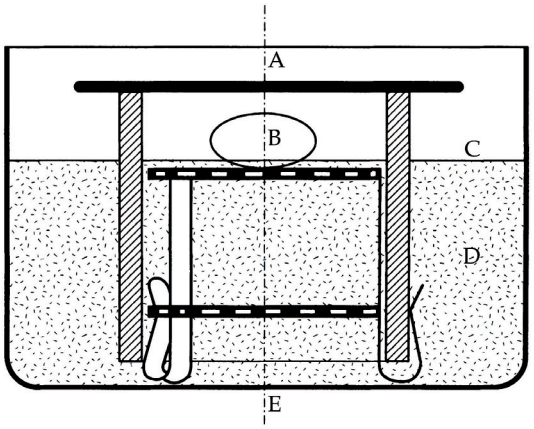


Fig. 2 Apparatus for the Disintegration of Vaginal Tablets

A - Glass plate
B - Vaginal tablet
C - Water surface
D - Water
E - Dish, Beaker

Procedure

MOULDED SUPPOSITORIES Place a suppository on the lower perforated disc of the metal device and then insert the device into the cylinder and attach this to the sleeve. Repeat the operation with a further two suppositories and metal devices and sleeves. Unless otherwise stated in the individual monograph, place each piece of apparatus in a vessel containing at least 4 litres[1] of water at 36.5°±0.5° and fitted with a slow stirrer and a means of holding the top of the apparatus 90 mm below the surface of the water. After each 10 minutes invert each apparatus without removing it from the liquid.

Disintegration is complete when the moulded suppository

(a) is completely dissolved or

(b) has dispersed into its component parts, which may collect on the surface (melted fatty substances), sink to the bottom (insoluble powders) or dissolve or

(c) has become soft, which may involve an appreciable change in shape, without necessarily separating into its components, and the mass has no solid core offering resistance to pressure with a glass rod.

Disintegration occurs in not more than 30 minutes for fat-based suppositories and in not more than 60 minutes for water-soluble suppositories, unless otherwise stated in the individual monograph.

RECTAL CAPSULES Carry out the procedure described under Moulded Suppositories. Disintegration is complete when the gelatin shell ruptures, allowing release of the contents.

Disintegration occurs in not more than 30 minutes.

MOULDED PESSARIES Carry out the procedure and use the criteria for disintegration described under Moulded Suppositories.

[1]All three pieces of apparatus may alternatively be placed together in a vessel containing at least 12 litres.

Disintegration occurs in not more than 1 hour unless otherwise stated in the individual monograph.

VAGINAL CAPSULES Carry out the procedure described under Moulded Suppositories and use the criterion for disintegration described under Rectal Capsules.

Disintegration occurs in not more than 30 minutes.

VAGINAL TABLETS Place the apparatus in a vessel of suitable diameter containing water at 36.5°±0.5°. Adjust the level of the liquid by the gradual addition of water at 36.5°±0.5° until the perforations in the metal disc are just covered by a uniform layer of water. Place one vaginal tablet on the upper perforated disc and cover the apparatus with a glass plate to maintain appropriate conditions of humidity. Repeat the operation with a further two vaginal tablets.

Disintegration is complete when

(a) there is no residue on the perforated plate or

(b) if a residue remains, it consists only of a soft or frothy mass having no solid core offering resistance to pressure with a glass rod.

Disintegration occurs in not more than 30 minutes, unless otherwise stated in the individual monograph.

4.23 DISINTEGRATION TEST FOR TABLETS AND CAPSULES

This test is provided to determine compliance with the limits on Disintegration stated in the individual monographs except where the label states that the tablets or capsules are intended for use as troches, or are to be chewed, or are designed as modified-release dosage forms (Dissolution Test, Appendix 4.24). Determine the type of units under test from the labelling and from observation, and apply the appropriate procedure to six or more dosage units.

For the purposes of this test, disintegration does not imply complete solution of the unit or even of its active constituent. Complete disintegration is defined as that state in which no residue, except fragments of undissolved tablet coating or of capsule shell, remains on the screen of the test apparatus or adheres to the lower surface of the disc if a disc has been used; if any other residue remains, it consists of a soft mass having no palpably firm core.

Apparatus

(a) A rigid basket-rack assembly supporting six open-ended cylindrical transparent tubes 77.5±2.5 mm long, approximately 21.85±1.15 mm in internal diameter and with a wall thickness of about 1.9±0.9 mm (see Figure).

(b) The tubes are held in a vertical position by two plates, each 90±2 mm in diameter and 6.75±1.75 mm in thickness, with six holes, each 24±2 mm in diameter, equidistant from the center of the plate and equally spaced from one another. Attached to the under surface of the lower plate is a woven stainless steel wire cloth, which has a plain square weave with 2.0±0.2 mm apertures and with a wire diameter of 0.615±0.045 mm.

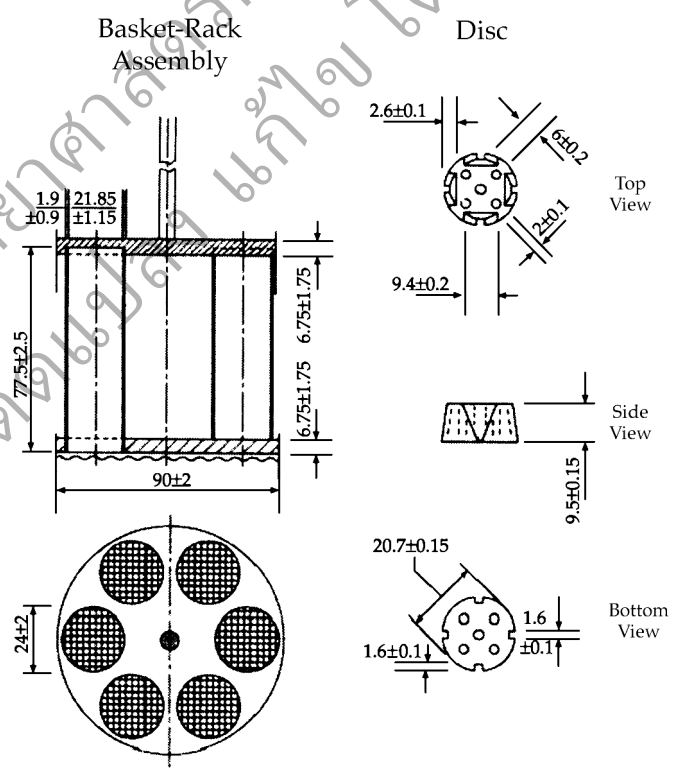
(c) The parts of the apparatus are assembled and rigidly held by means of three bolts passing through the two plates. A suitable means is provided to suspend the basket-rack assembly from the raising and lowering device using a point on its axis. The device for raising and lowering the basket in the immersion fluid is set at a constant frequency rate between 29 and 32 cycles per minute through a distance of 55±2 mm.

(d) The apparatus consists of a basket-rack assembly, a 1000-ml, low-form beaker, 149±11 mm in height and having an inside diameter of 106±9 mm for the immersion fluid, a thermostatic arrangement for heating the fluid between 37°±2°. The volume of the fluid in the vessel is such that at the highest point of the upward stroke the wire mesh remains at least 15 mm below the surface of the fluid and descends to not less than 25 mm from the bottom of the vessel on the downward stroke. At no time should the top of the basket-rack assembly become submerged. The time required for the upward stroke is equal to the time required for the downward stroke, and the change in stroke direction is a smooth transition, rather than an abrupt reversal of motion. The basket-rack assembly moves vertically along its axis. There is no appreciable horizontal motion or movement of the axis from the vertical.

The design of the basket-rack assembly may be varied provided that the specifications for the tubes and wire mesh are maintained.

(e) A suitable device maintains the temperature of the liquid at 37°±2°, unless otherwise specified.

(f) The use of discs is permitted only where specified or allowed in the monograph. If specified in the individual monograph, each tube is provided with a cylindrical disc 9.5±0.15 mm thick and 20.7±0.15 mm in diameter. The disc is made of a suitable transparent plastic material having a specific gravity of between 1.18 and 1.20. Five parallel 2±0.1-mm holes extend between the ends of the cylinder. One of the holes is centered on the cylindrical axis. The other holes are centered 6±0.2 mm from the axis on imaginary lines perpendicular to the axis and parallel to each other. Four identical trapezoidal-shaped planes are cut into the wall of the cylinder, nearly perpendicular to the ends of the cylinder. The trapezoidal shape is symmetrical; its parallel sides coincide with the ends of the cylinder and are parallel to an imaginary line connecting the centers of two adjacent holes 6 mm from the cylindrical axis. The parallel side of the trapezoid on the bottom of the cylinder has a length of 1.6±0.1 mm, and its bottom edges lie at a depth of 1.6±0.1 mm from the cylinder's circumference. The parallel side of the trapezoid on the top of the cylinder has a length of 9.4±0.2 mm, and its center lies at a depth of 2.6±0.1 mm from the cylinder's circumference. All surfaces of the disc are smooth. If the use of discs is specified in the individual monograph, add a disc to each tube, and operate the apparatus as directed under Procedure. The discs conform to dimensions (see Figure).



Apparatus for the Disintegration Test of Tablets and Capsules
Dimensions in mm

Procedure

Select six units and proceed as follows for the dosage form designated.

UNCOATED TABLETS Introduce one tablet in each of the six tubes of the basket and, if prescribed, add a disc. Operate the apparatus, using *water* or the specified medium as the immersion fluid, maintained at 37°±2°. At the end of the 15-minute time limit unless otherwise specified in the monograph, lift the basket from the fluid, and observe the tablets: all of the tablets have disintegrated completely. If one or two tablets fail to disintegrate completely, repeat the test on 12 additional tablets. The requirement is met if not less than 16 of the total of 18 tablets tested are disintegrated.

SOLUBLE TABLETS AND DISPERSIBLE TABLETS Introduce one tablet into each of the six tubes and operate the apparatus, using *water* at 20°±1° as the immersion fluid. The tablets disintegrate within 3 minutes unless otherwise stated in the individual monograph.

EFFERVESCENT TABLETS Place one tablet in a 50-ml beaker containing 200 ml of *water* at 20°±5°; numerous gas bubbles are evolved. When the evolution of gas around the tablet or its fragments has ceased the tablet shall have disintegrated, being either dissolved or dispersed in the water so that no agglomerates of particles remain. Repeat the operation on a further five tablets. The tablets comply with the test if each of the six tablets used in the test disintegrates in the manner prescribed within 5 minutes, unless otherwise stated in the individual monograph.

BUCCAL TABLETS Apply the test for Uncoated Tablets. After 4 hours, lift the basket from the fluid, and observe the tablets: all of the tablets have disintegrated. If one or two tablets fail to disintegrate completely, repeat the test on 12 additional tablets: not less than 16 of the total of 18 tablets tested disintegrate completely.

SUBLINGUAL TABLETS Apply the test for Uncoated Tablets. At the end of the time limit specified in the individual monograph: all of the tablets have disintegrated. If one or two tablets fail to disintegrate completely, repeat the test on 12 additional tablets: not less than 16 of the total of 18 tablets tested disintegrate completely.

COATED TABLETS Introduce one tablet into each of the six tubes, add disc to each tube, and operate the apparatus, using *water* as the immersion fluid. Unless otherwise stated in the individual monograph film-coated tablets disintegrate within 30 minutes and other coated tablets disintegrate within 60 minutes. For coated tablets other than film-coated tablets, if any of the tablets have not disintegrated, repeat the test on a further six tablets, replacing the water in beaker with 0.1 M *hydrochloric acid*. The tablets comply with the test if all six tablets have disintegrated in the acid medium.

For coated tablets including film-coated tablets, if the tablets fail to comply because of adherence of the tablets to the disc, repeat the test on a further six tablets omitting the discs. The tablets comply with the test if all six tablets have disintegrated.

DELAYED-RELEASE TABLETS (ENTERIC-COATED TABLETS) Introduce one tablet in each of the six tubes of the basket and, if the tablet has a soluble external sugar coating, immerse the basket in water at room temperature for 5 minutes. Then operate the apparatus using *simulated gastric fluid TS* maintained at 37°±2° as the immersion fluid. After 2 hours of operation in *simulated gastric fluid TS*, lift the basket from the fluid, and observe the tablets: the tablets show no evidence of disintegration, cracking, or softening. Operate the apparatus, using *simulated intestinal fluid TS* maintained at 37°±2° as the immersion fluid, for 60 minutes unless otherwise specified in the monograph. Lift the basket from the fluid, and observe the tablets: all of the tablets disintegrate completely. If one or two tablets fail to disintegrate completely, repeat the test on 12 additional tablets: not less than 16 of the total of 18 tablets tested disintegrate completely.

HARD CAPSULES Apply the test for Uncoated Tablets. Attach a removable wire cloth, which has a plain square weave with 2±0.2-mm mesh apertures and with a wire diameter of 0.615±0.045 mm, as described under Apparatus, to the surface of the upper plate of the basket-rack assembly. Observe the capsules within 30 minutes unless otherwise specified in the individual monograph: all of the capsules have disintegrated except for fragments from the capsule shell.

If one or two capsules fail to disintegrate completely, repeat the test on 12 additional capsules: not less than 16 of the total of 18 capsules tested disintegrate completely.

SOFT CAPSULES Proceed as directed under Hard Capsules.

DELAYED-RELEASE CAPSULES (ENTERIC CAPSULES) Introduce one capsule into each of the six tubes and unless otherwise specified in the individual monograph operate the apparatus for 2 hours[1], using 0.1 M *hydrochloric acid* as the immersion fluid. No capsule shows signs of disintegration or of rupture permitting the escape of the contents. Change the immersion fluid to *mixed phosphate buffer pH 6.8* and operate the apparatus for a further 60 minutes. The capsules pass the test if all six have disintegrated.

[1]The time of resistance to the acid medium varies according to the formulation of the preparation being examined. It is normally 3 hours but even with authorized deviations it is not less than 1 hour.

4.24 DISSOLUTION TEST

This test is provided to determine compliance with the dissolution requirements where stated in the individual monograph for dosage forms administered orally. In this appendix, a dosage unit is defined as one tablet or one capsule or the amount specified. Of the types of apparatus described herein, use the one specified in the individual monograph. Where the label states that a dosage form is enteric-coated, and where a dissolution or disintegration test that does not specifically state that it is to be applied to delayed-release dosage forms is included in the individual monograph, the procedure and interpretation given for Delayed-Release Dosage Forms is applied unless otherwise specified in the individual monograph. For hard or soft gelatin capsules and gelatin-coated tablets that do not conform to the dissolution specification, repeat the test as follows. Where water or a medium with a pH of less than 6.8 is specified as the medium in the individual monograph, the same medium specified may be used with the addition of purified pepsin that results in an activity of 750,000 Units or less per 1000 ml. For media with a pH of 6.8 or more pancreatin can be added to produce not more than 1750 Units of protease activity per 1000 ml.

APPARATUS

Apparatus 1 (Basket apparatus, Fig. 1) The assembly consists of the following: a covered vessel (C) made of borosilicate glass or other inert, transparent material; a motor; a metallic drive shaft (A); and a cylindrical basket (B). The vessel is partially immersed in a suitable water-bath of any convenient size that permits holding the temperature inside the vessel at 37°±0.5° during the test and keeping the bath fluid in constant, smooth motion. No part of the assembly, including the environment in which the assembly is placed, contributes significant motion, agitation, or vibration beyond that due to the smoothly rotating stirring element. Apparatus that permits observation of the specimen and stirring element during the test is preferable. The vessel is cylindrical, with a hemispherical bottom and with one of the following dimensions and capacities: for a nominal capacity of 1000 ml, the height is 185±25 mm and its inside diameter is 102±4 mm; for a nominal capacity of 2000 ml, the height is 290±10 mm and its inside diameter is 102±4 mm; and for a nominal capacity of 4000 ml, the height is 290±10 mm and its inside diameter is 150±5 mm. Its sides are flanged at the top. A fitted cover may be used to retard evaporation[1]. The shaft is positioned so that its axis is not more than 2 mm at any point from the vertical axis of the vessel and rotates smoothly and without significant wobble. A speed-regulating device is used that allows the shaft rotation speed to be selected and maintained at the rate specified in the individual monograph, within ±4 per cent.

[1]If a cover is used it provides sufficient openings to allow ready insertion of the thermometer and withdrawal of specimens.

The basket consists of two components. The top part is of solid metal with a vent and is attached to the shaft. It is fitted with three spring clips or other suitable means that allow removal of the lower part for introduction of the dosage unit and that firmly hold the lower part of the basket concentric with the axis of the vessel during rotation. The shaft and basket components are made of stainless steel of suitable quality. The lower detachable part of the basket is made of welded-seam, stainless steel cloth formed into a cylinder with a narrow rim of sheet metal around the top and bottom; unless otherwise prescribed, the cloth has a wire thickness of 0.28±0.03 mm in diameter with wire openings of 0.40±0.04 mm. A basket having a gold coating 2.5 µm (0.0001 inch) thick may be used with acidic media. The dosage unit is placed in a dry basket at the beginning of each test. The distance between the inside bottom of the vessel and the basket is maintained at 25±2 mm during the test.

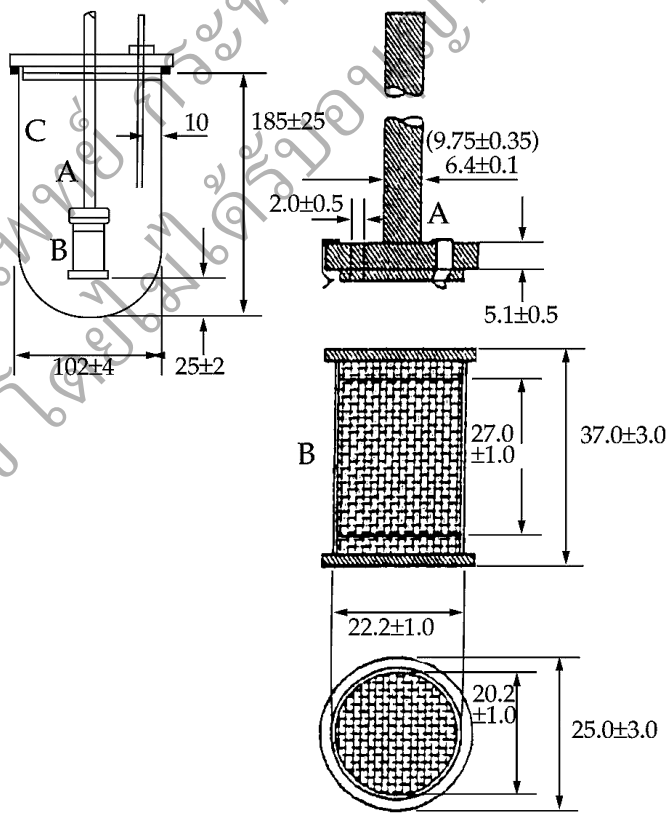


Fig. 1 Basket Apparatus
Dimensions in mm

Apparatus 2 (Paddle apparatus, Fig. 2) Use the assembly from Apparatus 1, except that a paddle (D) formed from a blade and a shaft is used as the stirring element. The shaft is positioned so that its axis is not more than 2 mm at any point from the vertical axis of the vessel, and rotates smoothly without significant wobble. The vertical centre line of the blade passes through the axis of the shaft so that the bottom of the blade is flush with the bottom of the shaft. The paddle conforms to the specifications shown in the figure. The distance of 25±2 mm between the blade and the inside bottom of the vessel is maintained during the test. The metallic or

suitably inert, rigid blade and shaft comprise a single entity. A suitable two-part detachable design may be used provided the assembly remains firmly engaged during the test. The paddle blade and shaft may be coated with a suitable coating so as to make them inert. The dosage unit is allowed to sink to the bottom of the vessel before rotation of the blade is started. A small, loose piece of nonreactive material such as not more than a few turns of wire helix, may be attached to dosage units that would otherwise float. An alternative sinker device is shown in Fig. 2a. Other validated sinker devices may be used.

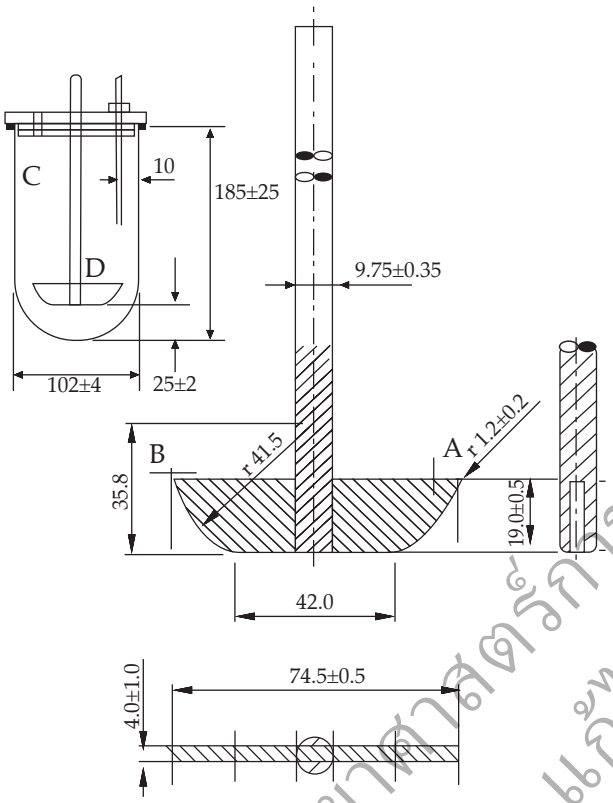
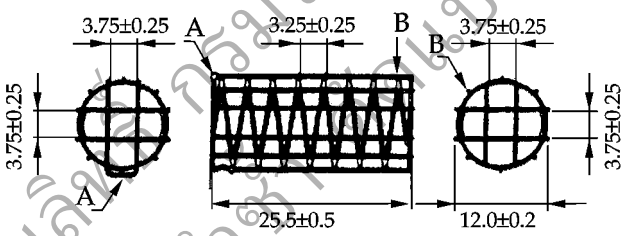


Fig. 2 Paddle Apparatus
Dimensions in mm



A: acid-resistant wire clasp
B: acid-resistant wire support

Fig. 2a Alternative Sinker
Dimensions in mm

Apparatus 3 (Flow-through cell apparatus, Fig. 3) The assembly consists of a reservoir and a pump for the dissolution medium, a flow-through cell and a water-bath that maintains the dissolution medium at 37°±0.5°. The cell size is specified in the individual monograph.

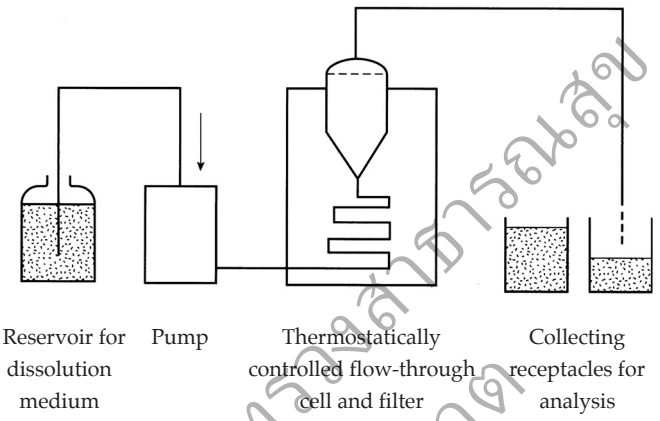
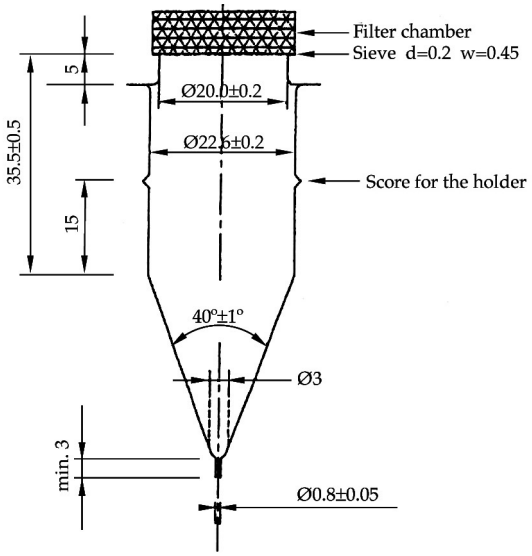
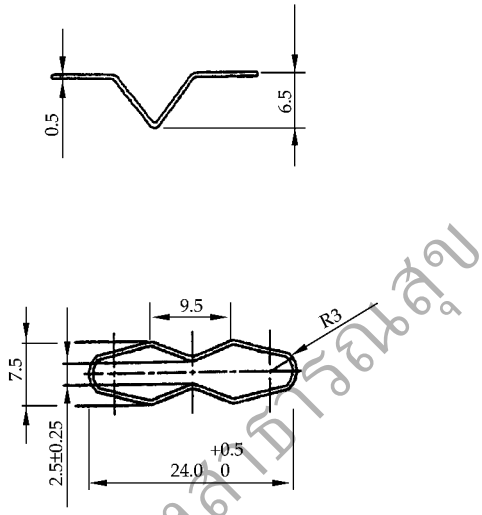


Fig. 3 Flow-through Cell Apparatus

The pump forces the dissolution medium upwards through the flow-through cell. The pump has a delivery range between 240 and 960 ml per hour, with standard flow rates of 4, 8, and 16 ml per minute. It must be volumetric to deliver a constant flow (±5 per cent of the nominal flow rate) independent of flow resistance in the filter device; the flow profile is sinusoidal with a pulsation of 120±10 pulses per minute. The flow-through cell (Figs. 3a and 3b), of transparent and inert material, is mounted vertically with a filter system (specified in the individual monograph) that prevents escape of undissolved particles from the top of the cell; standard cell diameters are 12 and 22.6 mm; the bottom cone is usually filled with small glass beads of the cell; standard cell diameters are 12 and 22.6 mm; the bottom cone is usually filled with small glass beads of about 1-mm diameter with one bead of about 5 mm positioned at the apex to protect the fluid entry tube; a tablet holder (Figs. 3a and 3b) is available for positioning of special dosage forms such as inlay tablets. The cell is immersed in a water-bath and the temperature is maintained at 37°±0.5°. The apparatus uses a clamp mechanism and two O-rings for the fixation of the cell assembly. The pump is separated from the dissolution unit in order to shield the latter against any vibrations originating from the pump. The position of the pump should not be on a level higher than the reservoir flasks. Tube connections are as short as possible. Use suitably inert tubing, such as polytef, with about 1.6-mm inner diameter and chemically inert flanged-end connections.

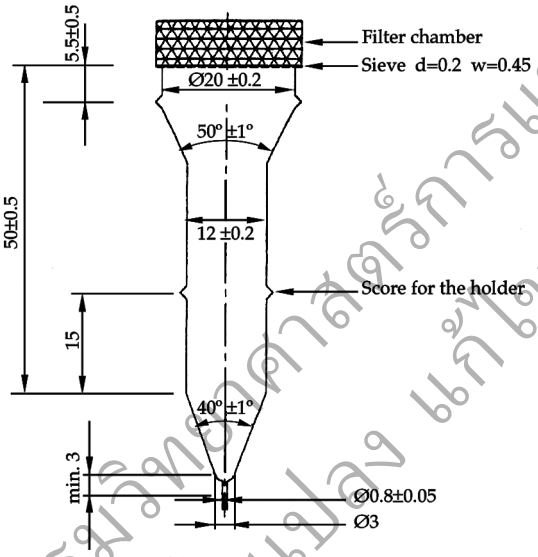


Large Cell for Tablets and Capsules

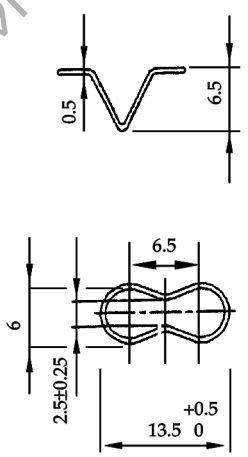


Tablet Holder for the Large Cell

Fig. 3a Flow-through Cell (Large Cell)
Dimensions in mm



Small Cell for Tablets and Capsules



Tablet Holder for the Small Cell

Fig. 3b Flow-through Cell (Small Cell)
Dimensions in mm

Apparatus suitability The determination of suitability of the apparatus to perform dissolution testing must include conformance to the dimensions and tolerances of the apparatus as given above. In addition, critical test parameters that have to be monitored periodically during use include volume and temperature of the dissolution medium, rotation speed (Apparatus 1 and Apparatus 2), and flow rate of medium (Apparatus 3).

Determine the acceptable performance of the dissolution test apparatus periodically.

PROCEDURE

Immediate-Release Dosage Forms (Capsules, Uncoated Tablets and Plain Coated Tablets)

Apparatuses 1 and 2 Place the stated volume of the dissolution medium (±1 per cent) in the vessel of the apparatus specified in the individual monograph, assemble the apparatus, equilibrate the dissolution medium to 37°±0.5°, and remove the thermometer. Place one tablet or one capsule in the apparatus, taking care to exclude air bubbles from the surface of the dosage-form unit, and immediately operate the appara-

ลิขสิทธิ์ กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข
ห้ามดัดแปลง แก้ไข โดยไม่ได้รับอนุญาต

tus at the rate specified in the individual monograph. Within the time interval specified, or at each of the times stated, withdraw a sample from a zone midway between the surface of the dissolution medium and the top of the rotating basket or blade, not less than 1 cm from the vessel wall. (**Note** Where multiple sampling times are specified, replace the aliquots withdrawn for analysis with equal volumes of fresh dissolution medium at 37° or, where it can be shown that replacement of the medium is not necessary, correct for the volume change in the calculation. Keep the vessel covered for the duration of the test, and verify the temperature of the mixture under test at suitable times.) Perform the analysis as directed in the individual monograph. (**Note** Test specimens are filtered immediately upon sampling unless filtration is demonstrated to be unnecessary. Use an inert filter that does not cause adsorption of the active ingredient or contain extractable substances that would interfere with the analysis.) Repeat the test with additional dosage-form units.

If automated equipment is used for sampling or the apparatus is otherwise modified, verification that the modified apparatus will produce results equivalent to those obtained with the standard apparatus described in this appendix is necessary.

Dissolution medium Use the solvent specified in the individual monograph. The volume specified refers to measurements made between 20° and 25°. If the dissolution medium is a buffered solution, adjust the solution so that its pH is within 0.05 unit of the pH specified in the individual monograph. (**Note** Dissolved gases can cause bubbles to form which may change the results of the test. In such cases, dissolved gases should be removed prior to testing.[1])

Time Where a single time specification is given, the test may be concluded in a shorter period if the requirement for minimum amount dissolved is met. If two or more times are specified, sample are to be withdrawn only at the stated times, within a tolerance of ±2 per cent.

Procedure for a Pooled Sample for Immediate-Release Dosage Forms

Use this procedure where Procedure for a pooled sample is specified in the individual monograph. Proceed as directed under Procedure for Immediate-Release Dosage Forms. Combine equal volumes of the filtered solutions of the six or twelve individual samples withdrawn, and use the pooled sample as the test solution. Determine the average amount of the active ingredient dissolved in the pooled sample.

Where capsule shells interfere with the analysis, remove the contents of not less than 6 capsules as completely as possible, and dissolve the empty capsule shells in the specified volume of dissolution medium. Perform the analysis as directed in the individual monograph. Make any necessary correction. Correction factors greater than 25 per cent of the labelled content are unacceptable.

Dissolution medium and Time Proceed as directed for Immediate-Release Dosage Forms under Apparatus 1 and Apparatus 2.

Apparatus 3 Place the glass beads into the cell specified in the monograph and one dosage-form unit on top of the beads or, if specified in the monograph, on a tablet holder. Assemble the filter head and fix the parts together by means of a suitable clamping device. Introduce by the pump the dissolution medium warmed to 37°±0.5° through the bottom of the cell to obtain the flow rate specified in the individual monograph and measured with an accuracy of 5 per cent. Collect the eluate by fractions at each of the times stated. Perform the analysis as directed in the individual monograph. Repeat the test with additional dosage form units.

Extended-Release Dosage Forms

Use the apparatus specified in the individual monograph.

Apparatus 1, Apparatus 2, Apparatus 3, Apparatus suitability, Dissolution medium and **Procedure** Proceed as directed under Immediate-Release Dosage Forms.

Time The test-time point, generally three, are expressed in hours. Samples are to be withdrawn within a tolerance of ±2 per cent of the stated time.

Delayed-Release (Enteric-Coated) Dosage forms

Use Method A or Method B and Apparatus 1 or 2 specified in the individual monograph. All test times stated are to be observed within a tolerance of ±2 per cent, unless otherwise specified.

METHOD A:

Procedure (unless otherwise directed in the individual monograph)

ACID STAGE Place 750 ml of 0.1 M *hydrochloric acid* in the vessel and assemble the apparatus. Allow the medium to equilibrate to a temperature of 37°±0.5°. Place one tablet or one capsule in the apparatus, cover the vessel, and operate the apparatus for 2 hours at the rate specified in the monograph.

After 2 hours of operation in 0.1 M *hydrochloric acid*, withdraw an aliquot of the fluid, and proceed immediately as directed under Buffer stage.

Perform an analysis of the aliquot using the Procedure specified in the test for Dissolution in the individual monograph.

BUFFER STAGE (**Note** Complete the operations of adding the buffer, and adjusting the pH within 5 minutes.) With the apparatus operating at the rate specified

[1]One method of deaeration is as follows: heat the medium, while stirring gently, to about 41°, immediately filter under vacuum using a filter having a porosity of 0.45 µm or less, with vigorous stirring, and continue stirring under vacuum for about 5 minutes. Other validated deaeration techniques for removal of dissolved gases may be used.

in the monograph, add to the fluid in the vessel 250 ml of 0.20 M *trisodium phosphate* that has been equilibrated to 37°±0.5°. Adjust, if necessary, with 2 M *hydrochloric acid* or 2 M *sodium hydroxide* to a pH of 6.8±0.05. Continue to operate the apparatus for 45 minutes, or for the time specified in the individual monograph. At the end of the time period, withdraw an aliquot of the fluid, and perform the analysis using the Procedure specified in the test for Dissolution in the individual monograph. The test may be concluded in a shorter time period than that specified for the Buffer stage if the requirement for minimum amount dissolved is met at an earlier time.

METHOD B:

Procedure (unless otherwise directed in the individual monograph)

ACID STAGE Place 1000 ml of 0.1 M *hydrochloric acid* in the vessel, and assemble the apparatus. Allow the medium to equilibrate to a temperature of 37°±0.5°. Place one tablet or one capsule in the apparatus, cover the vessel, and operate the apparatus for 2 hours at the rate specified in the individual monograph. After 2 hours of operation in 0.1 M *hydrochloric acid*, withdraw an aliquot of the fluid and proceed immediately as directed under Buffer stage.

Perform an analysis of the aliquot using the Procedure specified in the test for Dissolution in the individual monograph.

BUFFER STAGE (**Note** For this stage of the procedure, use buffer that previously has been equilibrated to a temperature of 37°±0.5°). Drain the acid from the vessel, and add to the vessel 1000 ml of pH 6.8 phosphate buffer, prepared by mixing 0.1 M *hydrochloric acid* with 0.2 M *trisodium phosphate* (3:1) and adjusting, if necessary, with 2 M *hydrochloric acid* or 2 M *sodium hydroxide* to a pH of 6.8±0.05. (**Note** This may be accomplished also by removing from the apparatus the vessel containing the acid and transferring the dosage unit to the vessel containing the buffer.) Continue to operate the apparatus for 45 minutes, or for the time specified in the individual monograph. At the end of the time period, withdraw an aliquot of the fluid, and perform the analysis using the Procedure specified in the test for Dissolution in the individual monograph. The test may be concluded in a shorter time period than that specified for the Buffer stage if the requirement for minimum amount dissolved is met at an earlier time.

INTERPRETATION

Immediate-Release Dosage Forms (Capsules, Uncoated Tablets and Plain Coated Tablets)

Unit Sample Unless otherwise specified in the individual monograph, the requirements are met if the quantities of active ingredient dissolved from the dosage units tested conform to the accompanying Acceptance Table 1 for Unit Sample. Continue testing through the three stages unless the results conform at either S_1 or S_2. The quantity, Q, is the amount of dissolved active ingredient specified in the individual monograph, expressed as a percentage of the labelled content of dosage unit; the 5 per cent, 15 per cent and 25 per cent values in the Acceptance Table 1 are percentages of the labelled content so that these values and Q are in the same terms.

Acceptance Table 1 for Unit Sample

Stage	Number Tested	Acceptance Criteria
S_1	6	Each unit is not less than Q + 5 per cent.
S_2	6	Average of 12 units (S_1 + S_2) is equal to or greater than Q, and no unit is less than Q – 15 per cent.
S_3	12	Average of 24 units (S_1 + S_2 + S_3) is equal to or greater than Q, not more than 2 units are less than Q – 15 per cent, and no unit is less than Q – 25 per cent.

Pooled Sample Unless otherwise specified in the individual monograph, the requirements are met if the quantities of active ingredient dissolved from the pooled sample conform to the accompanying Acceptance Table 1 for a Pooled Sample. Continue testing through the three stages unless the results conform at either S_1 or S_2. The quantity, Q, is the amount of dissolved active ingredient specified in the individual monograph, expressed as a percentage of the labelled content.

Acceptance Table 1 for a Pooled Sample

Stage	Number Tested	Acceptance Criteria
S_1	6	Average amount dissolved is not less than Q + 10 per cent.
S_2	6	Average amount dissolved (S_1 + S_2) is equal to or greater than Q + 5 per cent.
S_3	12	Average amount dissolved (S_1 + S_2 + S_3) is equal to or greater than Q.

Extended-Release Dosage Forms

Unless otherwise specified in the individual monograph, the requirements are met if the quantities of active ingredient dissolved from the units tested conform to Acceptance Table 2. Continue testing through the three levels unless the results conform at either L_1 or L_2. Limits on the amounts of active ingredient dissolved are expressed in terms of the percentage of labelled content. The limits embrace each value of Q_i, the amount dissolved at each specified fractional dosing interval. Where more than one range is specified in the individual monograph, the acceptance criteria apply individually to each range.

Acceptance Table 2

Level	Number Tested	Acceptance Criteria
L_1	6	No individual value lies outside each the stated ranges and no individual value is less than the stated amount at the final test time.
L_2	6	The average value of the 12 units ($L_1 + L_2$) lies within each of the stated ranges and is not less than the stated amount at the final test time; none is more than 10 per cent of labelled content outside each of the stated ranges; and none is more than 10 per cent of labelled content below the stated amount at the final test time.
L_3	12	The average value of the 24 unit ($L_1 + L_2 + L_3$) lies within each of the stated ranges, and is not less than the stated amount at the final test time; not more than 2 of the 24 units are more than 10 per cent of labelled content outside each of the stated ranges; not more than 2 of the 24 units are more than 10 per cent of labelled content below the stated amount at the final test time; and none of units is more than 20 per cent of labelled content outside each of the stated ranges or more than 20 per cent of labelled content below the stated amount at the final test time.

Delayed-Release (Enteric-Coated) Dosage Forms

ACID STAGE Unless otherwise specified in the individual monograph, the requirements of this portion of the test are met if the quantities, based on the percentage of the labeled content, of active ingredient dissolved from the units tested conform to Acceptance Table 3. Continue testing through all levels unless the results of both acid and buffer stages conform at an earlier level.

Acceptance Table 3

Level	Number Tested	Acceptance Criteria
A_1	6	No individual value exceeds 10 per cent dissolved.
A_2	6	Average of the 12 units ($A_1 + A_2$) is not more than 10 per cent dissolved, and no individual unit is greater than 25 per cent dissolved.
A_3	12	Average of the 24 units ($A_1 + A_2 + A_3$) is not more than 10 per cent dissolved, and no individual unit is greater than 25 per cent dissolved.

BUFFER STAGE Unless otherwise specified in the individual monograph, the requirements are met if the quantities of active ingredient dissolved from the units tested conform to Acceptance Table 4. Continue testing through the three levels unless the results of both stages conform at an earlier level. The value of *Q* in Acceptance Table 4 is 75 per cent dissolved unless otherwise specified in the individual monograph. The quantity, *Q*, specified in the individual monograph, is the total amount of active ingredient dissolved in both the acid and buffer stages, expressed as a percentage of the labelled content. The 5 per cent, 15 per cent and 25 per cent values in Acceptance Table 4 are percentages of the labelled content so that these values and *Q* are in the same terms.

Acceptance Table 4

Level	Number	Tested Acceptance Criteria
B_1	6	Each unit is not less than *Q* + 5 per cent.
B_2	6	Average of 12 units ($B_1 + B_2$) is equal to or greater than *Q*, and no unit is less than *Q* – 15 per cent.
B_3	12	Average of 24 units ($B_1 + B_2 + B_3$) is equal to or greater than *Q*, not more than 2 units are less than *Q* – 15 per cent, and no unit is less than *Q* – 25 per cent.

4.26 MINIMUM FILL

The following tests and specifications apply to articles such as creams, gels, jellies, lotions, ointments, pastes, powders, and aerosols, including pressurized and nonpressurized topical sprays and pressurized metered-dose inhalers, that are packaged in containers in which the labelled content is not more than 150 g or 150 ml.

Procedure for Dosage Forms Other than Aerosols

For containers labelled by weight, select a sample of 10 filled containers, and remove any labelling that might be altered in weight during the removal of the container contents. Thoroughly cleanse and dry the outside of the containers by a suitable means, and weigh individually. Quantitatively remove the contents from each container, cutting the latter open and washing with a suitable solvent, if necessary, taking care to retain the closure and other parts of each container. Dry, and again weigh each empty container together with its corresponding parts. The difference between the two weights is the net weight of the contents of the container.

For containers labelled by volume, pour the contents of 10 containers into 10 suitable graduated cylinders, and allow to drain completely. Record the volume of the contents of each of the 10 containers. The average net content of the 10 containers is not less than the labelled amount, and the net content of any single container is not less than 90 per cent of the labelled

amount where the labelled amount is 60 g or 60 ml or less, or not less than 95 per cent of the labelled amount where the labelled amount is more than 60 g or 60 ml but not more than 150 g or 150 ml. If this requirement is not met, determine the content of 20 additional containers. The average content of the 30 containers is not less than the labelled amount and the net content of not more than 1 of the 30 containers is less than 90 per cent of the labelled amount where the labelled amount is 60 g or 60 ml or less, or less than 95 per cent of the labelled amount where the labelled amount is more than 60 g or 60 ml but not more than 150 g or 150 ml.

Procedure for Aerosols

Select a sample of 10 filled containers, and remove any labelling that might be altered in weight during the removal of the container contents. Thoroughly cleanse and dry the outsides of the containers by suitable means, and weigh individually. Remove the contents from each container by employing any safe technique (e.g., chill to reduce the internal pressure, remove the valve, and pour). Remove any residual contents with suitable solvents and then rinse with a few portions of *methanol*. Retain as a unit the container, the valve, and all associated parts, and heat them at 100° for 5 minutes. Cool, and again weigh each of the containers together with its corresponding parts. The difference between the original weight and the weight of the empty aerosol container is the net fill weight. Determine the net fill weight for each container tested. The requirements are met if the net weight of the contents of the 10 containers is not less than the labelled amount.

4.27 PARTICULATE MATTER IN INJECTIONS

Particulate Contamination: Subvisible Particles

Particulate matter consists of mobile, randomly-sourced, extraneous substances, other than gas bubbles, that cannot be quantitated by chemical analysis due to the small amount of material that it represents and to its heterogeneous composition. Injectable solutions, including solutions constituted from sterile solids intended for parenteral use, are essentially free from particulate matter that can be observed on visual inspection. The tests described herein are physical tests performed for the purpose of enumerating subvisible extraneous particles within specific size ranges.

Microscopic and light obscuration procedures for the determination of particulate matter are given herein. This appendix provides a test approach in two stages. The injection is first tested by the light obscuration procedure (stage 1). If it fails to meet the prescribed limits, it must pass the microscopic procedure (stage 2) with its own set of test limits. Where for technical reasons the injection cannot be tested by light obscuration, microscopic testing may be used exclusively. Documentation demonstrating that the light obscuration procedure is incapable of testing the injection or produces invalid results is required in each case. It is expected that most articles will meet the requirements on the basis of the light obscuration test alone; however, it may be necessary to test some articles by the light obscuration test followed by the microscopic test to reach a conclusion on conformance to requirements.

All large-volume injections for single-dose infusion and those small-volume injections for which the monographs specify such requirements are subject to the particulate matter limits set forth for the test being applied, unless otherwise specified in the individual monograph. Excluded from the requirements of this appendix are injections intended solely for intramuscular and subcutaneous administration.

Not all injection formulations can be examined for particles by one or both of these tests. Any product that is not a pure solution having a clarity and a viscosity approximating those of water may provide erroneous data when analyzed by the light obscuration counting method. Such materials may be analyzed by the microscopic method. Emulsions, colloids and liposomal preparations are examples. Similarly, products that produce air or gas bubbles when drawn into the sensor, such as bicarbonate-buffered formulations, may also require microscopic testing. Refer to the specific monographs when a question of test applicability occurs. Higher limits are appropriate for certain articles and will be specified in the individual monographs.

In some instances, the viscosity of a material to be tested may be sufficiently high so as to preclude its analysis by either test method. In this event, a quantitative dilution with an appropriate diluent may be made to decrease viscosity, as necessary, to allow the analysis to be performed.

In the tests described below for large-volume and small-volume injections, the results obtained in examining a discrete unit or group of units for particulate matter cannot be extrapolated with certainty to other units that remain untested. Thus, statistically sound sampling plans based upon known operational factors must be developed if valid inferences are to be drawn from observed data to characterize the level of particulate matter in a large group of units. Sampling plans should be based on consideration of product volume, numbers of particles historically found to be present in comparison to limits, particle size distribution of particles present, and variability of particle counts between units.

Light obscuration particle count test Use a suitable apparatus based on the principle of light blockage which allows an automatic determination of the size of particles and the number of particles according to size.

The apparatus is calibrated using suitable certified reference materials consisting of dispersions of spherical particles of known sizes between 10 μm and 25 μm. These standard particles are dispersed in *particle-free water*. Care must be taken to avoid aggregation of particles during dispersion.

GENERAL PRECAUTIONS The test is carried out under conditions limiting particulate contamination, preferably in a laminar-flow cabinet.

Very carefully wash the glassware and filtration equipment used, except for the membrane filters, with a warm detergent solution and rinse with abundant amounts of water to remove all traces of detergent. Immediately before use, rinse the equipment from top to bottom, outside and then inside, with *particle-free water*.

Take care not to introduce air bubbles into the preparation to be examined, especially when fractions of the preparation are being transferred to the container in which the determination is to be carried out.

In order to check that the environment is suitable for the test, that the glassware is properly cleaned and that the water to be used is particle-free, the following test is carried out: determine the particulate contamination of five samples of *particle-free water*, each of 5 ml, according to the procedure described below. If the number of particles of 10 µm or more size exceeds 25 for the combined 25 ml, the precautions taken for the test are not sufficient.

The preparatory steps must be repeated until the environment, glassware and water are suitable for the test.

PROCEDURE Mix the contents of the sample by slowly inverting the container 20 times successively. If necessary, cautiously remove the sealing closure. Clean the outer surfaces of the container opening using a jet of *particle-free water* and remove the closure, avoiding any contamination of the contents. Eliminate gas bubbles by appropriate measures such as allowing to stand for 2 minutes or sonicating.

For large-volume parenterals, single units are tested. For small-volume parenterals less than 25 ml in volume, the contents of 10 or more units are combined in a cleaned container to obtain a volume of not less than 25 ml; where justified and authorized, the test solution may be prepared by mixing the contents of a suitable number of vials and diluting to 25 ml with *particle-free water* or with an appropriate solvent without contamination of particles when *particle-free water* is not suitable. Small-volume parenterals having a volume of 25 ml or more may be tested individually.

Powders for parenteral use are reconstituted with *particle-free water* or with an appropriate solvent without contamination of particles when *particle-free water* is not suitable.

The number of test specimens must be adequate to provide a statistically sound assessment. For large-volume parenterals or for small-volume parenterals having a volume of 25 ml or more, less than 10 units may be tested, based on an appropriate sampling plan.

Remove 3 portions, each of not less than 5 ml, and count the number of particles equal to or more than 10 µm and 25 µm. Disregard the result obtained for the first portion, and calculate the mean number of particles for the preparation to be examined.

INTERPRETATION The preparation meets the requirements of the test if the calculated number of particles present in each discrete unit tested or in each pooled sample tested does not exceed the appropriate value listed in Table 1. If the average number of particles exceeds the limit, test the article by Microscopic Particle Count Test.

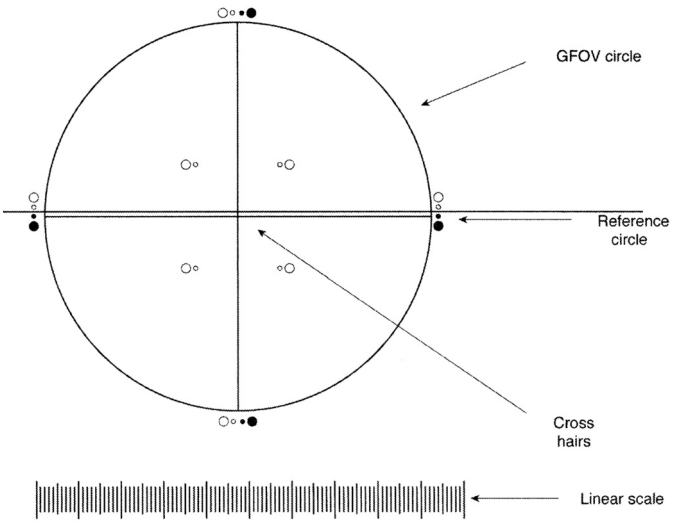
Table 1 Maximum Limit of Number of Particles by Light Obscuration Test

Injections	Particle Size	
	≥10 µm	≥25 µm
Small-Volume	6000 per container	600 per container
Large-Volume	25 per ml	3 per ml

Microscopic particle count test Use a suitable binocular microscope, filter assembly for retaining particulate contamination and membrane filter for examination.

The microscope is equipped with an ocular micrometer calibrated with an objective micrometer, a mechanical stage capable of holding and traversing the entire filtration area of the membrane filter, two suitable illuminators to provide episcopic illumination in addition to oblique illumination, and is adjusted to 100±10 magnifications.

The ocular micrometer is a circular diameter graticule (see Figure) and consists of a large circle divided by crosshairs into quadrants, transparent and black reference circles 10 µm and 25 µm in diameter at 100 magnifications, and a linear scale graduated in 10 µm increments. It is calibrated using a stage micrometer that is certified by either a domestic or international standard institution. A relative error of the linear scale of the graticule within ±2 per cent is acceptable. The large circle is designated the graticule field of view (GFOV).



Circular Diameter Graticule

Two illuminators are required. One is an episcopic brightfield illuminator internal to the microscope, and the other is an external, focusable auxiliary illuminator

adjustable to give reflected oblique illumination at an angle of 10° to 20°.

The filter assembly for retaining particulate contamination consists of a filter holder made of glass or other suitable material, and is equipped with a vacuum source and a suitable membrane filter.

The membrane filter is of suitable size, black or dark grey in colour, non-gridded or gridded, and 1.0 µm or finer in nominal pore size.

GENERAL PRECAUTIONS The test is carried out under conditions limiting particulate contamination, preferably in a laminar-flow cabinet.

Very carefully wash the glassware and filter assembly used, except for the membrane filter, with a warm detergent solution and rinse with abundant amounts of water to remove all traces of detergent. Immediately before use, rinse both sides of the membrane filter and the equipment from top to bottom, outside and then inside, with *particle-free water*.

In order to check that the environment is suitable for the test, that the glassware and the membrane filter are properly cleaned and that the water to be used is particle-free, the following test is carried out: determine the particulate contamination of a 50 ml volume of *particle-free water* according to the procedure described below. If more than 20 particles 10 µm or larger in size or if more than 5 particles 25 µm or larger in size are present within the filtration area, the precautions taken for the test are not sufficient.

The preparatory steps must be repeated until the environment, glassware, membrane filter and water are suitable for the test.

PROCEDURE Mix the contents of the samples by slowly inverting the container 20 times successively. If necessary, cautiously remove the sealing closure. Clean the outer surfaces of the container opening using a jet of *particle-free water* and remove the closure, avoiding any contamination of the contents.

For large-volume parenterals, single units are tested. For small-volume parenterals less than 25 ml in volume, the contents of 10 or more units are combined in a cleaned container; where justified and authorized, the test solution may be prepared by mixing the contents of a suitable number of vials and diluting to 25 ml with *particle-free water* or with an appropriate solvent without contamination of particles when *particle-free water* is not suitable. Small-volume parenterals having a volume of 25 ml or more may be tested individually. Powders for parenteral use are constituted with *particle-free water* or with an appropriate solvent without contamination of particles when *particle-free water* is not suitable.

The number of test specimens must be adequate to provide a statistically sound assessment. For large-volume parenterals or for small-volume parenterals having a volume of 25 ml or more, less than 10 units may be tested, based on a appropriate sampling plan.

Wet the inside of the filter holder fitted with the membrane filter with several millilitres of *particle-free water*. Transfer to the filtration funnel the total volume of a solution pool or of a single unit, and apply vacuum. If needed, add stepwise a portion of the solution until the entire volume is filtered. After the last addition of solution, begin rinsing the inner walls of the filter holder by using a jet of *particle-free water*. Maintain the vacuum until the surface of the membrane filter is free from liquid. Place the filter in a Petri dish and allow the filter to air-dry with the cover slightly ajar. After the filter has been dried, place the Petri dish on the stage of the microscope, scan the entire membrane filter under the reflected light from the illuminating device, and count the number of particles that are equal to or greater than 10 µm and the number of particles that are equal to or greater than 25 µm. Alternatively, partial filter count and determination of the total filter count by calculation is allowed. Calculate the mean number of particles for the preparation to be examined.

The particle sizing process with the use of the circular diameter graticule is carried out by transforming mentally the image of each particle into a circle and then comparing it to the 10 µm and 25 µm graticule reference circles. Thereby the particles are not moved from their initial locations within the graticule field of view and are not superimposed on the reference circles for comparison. The inner diameter of the transparent graticule reference circles is used to size white and transparent particles, while dark particles are sized by using the outer diameter of the black opaque graticule reference circles.

In performing the microscopic particle count test do not attempt to size or enumerate amorphous, semi-liquid, or otherwise morphologically indistinct materials that have the appearance of a stain or discoloration on the membrane filter. These materials show little or no surface relief and present a gelatinous or film-like appearance. In such cases the interpretation of enumeration may be aided by testing a sample of the solution by the light obscuration particle count test.

INTERPRETATION The preparations meets the requirements of the test if the number of particles present (actual or calculated) in each discrete unit tested or in each pooled sample tested does not exceed the appropriate value listed in Table 2.

Table 2 Maximum Limit of Number of Particles by Microscopic Particle Count Test

Injections	Particle Size	
	≥10 µm	≥25 µm
Small-Volume	3000 per container	300 per container
Large-Volume	12 per ml	2 per ml

Particulate Contamination: Visible Particles

Particulate contamination of injections and infusions consists of extraneous, mobile undissolved particles, other than gas bubbles, unintentionally present in the solutions. The test is intended to provide a simple procedure for the visual assessment of the quality of parenteral solutions as regards visible particles. Other validated methods may be used.

APPARATUS The apparatus consists of a viewing station comprising: a matt black panel of appropriate size held in a vertical position, a non-glare white panel of appropriate size held in a vertical position next to the black panel, an adjustable lampholder fitted with a suitable, shaded, white-light source and with a suitable light diffuser (a viewing illuminator containing two 13 W fluorescent tubes, each 525 mm in length, is suitable). The intensity of illumination at the viewing point is maintained between 2000 lux and 3750 lux, although higher values are preferable for coloured glass and plastic containers.

PROCEDURE Remove any adherent labels from the container, wash and dry the outside. Gently swirl or invert the container, ensuring that air bubbles are not introduced, and observe for about 5 seconds in front of the white panel. Repeat the procedure in front of the black panel. Record the presence of any particles.

4.28 UNIFORMITY OF DOSAGE UNITS

Dosage units are defined as dosage forms containing a single dose or a part of a dose of drug substance in each unit. (**Note** In this appendix, unit and dosage unit are synonymous.) To ensure the consistency of dosage units, each unit in a batch should have a drug substance content within a narrow range around the label claim. The term "uniformity of dosage units" is defined as the degree of uniformity in the amount of the drug substance among dosage units. Therefore, the requirements of this appendix apply to each drug substance being comprised in dosage units containing one or more drug substances, unless otherwise specified in the individual monograph.

The uniformity of dosage units can be demonstrated by either of two methods, Content Uniformity or Weight Variation (Table 1). The test for Content Uniformity is based on the assay of the individual content of drug substance(s) in a number of individual dosage units to determine whether the individual content is within the limits set. The method of Content Uniformity may be applied in all cases. The test for Content Uniformity is required for those dosage forms described below:

(1) coated tablets, other than film-coated tablets containing 25 mg or more of a drug substance that comprises 25 per cent or more (by weight) of one tablet;

(2) transdermal systems;

(3) suspensions or emulsions or gels in unit-dose containers or in soft capsules that are intended for systemic administration only (not for those drug products that are intended for external, cutaneous administration);

(4) inhalations (other than solutions for inhalation packaged in glass or plastic ampoules and intended for use in nebulizers) packaged in premetered dosage units. For inhalers and premetered dosage units labelled for use with a named inhalation device;

(5) solids (including sterile solids) that are packaged in single-unit containers and that contain active or inactive added substances, except that the test for Weight Variation may be applied in the special situations stated in (b) and (c) below; and

(6) suppositories.

The test for Weight Variation is applicable for the following dosage forms:

(a) solutions for inhalation that are packaged in glass or plastic ampoules and intended for use in nebulizers, and oral solutions packaged in unit-dose containers and into soft capsules;

(b) solids (including sterile solids) that are packaged in single-unit containers and contain no added substances, whether active or inactive;

(c) solids (including sterile solids) that are packaged in single-unit containers, with or without added substances, whether active or inactive, that have been prepared from true solutions and freeze-dried in the final containers and are labelled to indicate this method of preparation; and

(d) hard capsules, uncoated tablets, or film-coated tablets, containing 25 mg or more of a drug substance comprising 25 per cent or more, by weight, of the dosage unit or, in the case of hard capsules, the capsule contents, except that uniformity of other drug substances present in lesser proportions is demonstrated by meeting the requirements of Content Uniformity.

The test for Content Uniformity is required for all dosage forms not meeting the above conditions for the test for Weight Variation. Alternatively, products listed in item (d) above that do not meet the 25 mg/25 per cent threshold limit may be tested for uniformity of dosage units by Weight Variation instead of the test for Content Uniformity if the concentration relative standard deviation (RSD) of the drug substance in the final dosage units is not more than 2 per cent based on process validation data and development data. The concentration RSD is the RSD of the concentration per dosage unit (w/w or w/v), where concentration per dosage unit equals the assay result per dosage unit divided by the individual dosage unit weight. See the RSD formula in Table 2.

Content Uniformity

Select not less than 30 units and proceed as follows for the dosage form designated. Where the amount of drug substance in a single dosage unit differs from that required in the Assay, adjust the degree of dilution of the solutions and/or the volume of aliquots so that the concentration of the drug substances in the final solution is of the same order as that obtained in the Assay procedure; or, in the case of a titrimetric assay, use a titrant of a different concentration, if necessary, so that an adequate volume of titrant is required. If any such modifications are made in the Assay procedure set forth in the individual monograph, make the appropriate corresponding changes in the calculation formula and titration factor.

Table 1 Application of Content Uniformity (CU) and Weight Variation (WV) Tests for Dosage Forms

Dosage Form	Type	Subtype	Dose and Ratio of Drug Substance	
			≥25 mg and ≥25 per cent	<25 mg or <25 per cent
Tablets	Uncoated		WV	CU
	Coated	Film	WV	CU
		Others	CU	CU
Capsules	Hard		WV	CU
	Soft	Suspension, emulsion, or gel	CU	CU
		Solutions	WV	WV
Solids in single-unit containers	Single component		WV	WV
	Multiple components	Solution freeze-dried in final container	WV	WV
		Others	CU	CU
Suspension, emulsion, or gel for systemic use only, packaged in single-unit containers			CU	CU
Solutions for inhalation packaged in glass or plastic ampoules and intended for use in nebulizers, and oral solutions packaged in unit-dose containers and into soft capsules			WV	WV
Inhalations (other than solutions for inhalation packaged in glass or plastic ampoules and intended for use in nebulizers) packaged in premetered dosage units			CU	CU
Transdermal systems			CU	CU
Suppositories			CU	CU
Others			CU	CU

Where a special procedure is specified in the test for Content Uniformity in the individual monograph, make any necessary correction of the results obtained as follows.

(1) Prepare a composite sample of a sufficient number of dosage units to provide the amount of specimen called for in the Assay in the individual monograph plus the amount required for the special procedure in the monograph by finely powdering tablets or mixing the contents of capsules or oral solutions, suspensions, emulsions, gels, or solids in single-unit containers to obtain a homogeneous mixture. If a homogeneous mixture cannot be obtained in this manner, use suitable solvents or other procedures to prepare a solution containing all of the drug substance, and use appropriate aliquot portions of this solution for the specified procedures.

(2) Assay separate, accurately measured portions of the composite sample of capsules or tablets or suspensions or inhalations or solids in single-unit containers, both (a) as directed in the Assay, and (b) using the special procedure for Content Uniformity in the monograph.

(3) Calculate the weight of drug substance equivalent to one average dosage unit, by (a) using the results obtained by the assay procedure, and by (b) using the results obtained by the special procedure in the monograph.

(4) Calculate the correction factor, F, by the formula:

$$F = W/P$$

in which W is the weight of drug substance equivalent to one average dosage unit obtained by the Assay procedure, and P is the weight of drug substance

Table 2 Variables Used in the Calculation of Uniformity of Dosage Units

Variable	Definition	Conditions	Value
$\overline{X}$	Mean of individual contents (x_1, x_2, ..., x_n), expressed as a percentage of the label claim		
x_1, x_2, ..., x_n	Individual contents of the units tested, expressed as a percentage of the label claim		
n	Sample size (number of units in a sample)		
k	Acceptability constant	If $n = 10$, then $k =$	2.4
		If $n = 30$, then $k =$	2.0
s	Sample standard deviation		$\left[\frac{\sum_{i=1}^{n}(x_i - \overline{X})^2}{n-1}\right]^{\frac{1}{2}}$
RSD	Relative standard deviation (the sample standard deviation expressed as a percentage of the mean)		$\frac{100s}{\overline{X}}$
M (case 1) to be applied when $T \le 101.5$ per cent	Reference value	If 98.5 per cent $\le \overline{X} \le 101.5$ per cent, then	$M = \overline{X}$ ($AV = ks$)
		If $\overline{X} < 98.5$ per cent, then	$M = 98.5$ per cent ($AV = 98.5 - \overline{X} + ks$)
		If $\overline{X} > 101.5$ per cent, then	$M = 101.5$ per cent ($AV = \overline{X} - 101.5 + ks$)
M (case 2) to be applied when $T > 101.5$ per cent	Reference value	If 98.5 per cent $\le \overline{X} \le T$, then	$M = \overline{X}$ ($AV = ks$)
		If $\overline{X} < 98.5$ per cent, then	$M = 98.5$ per cent ($AV = 98.5 - \overline{X} + ks$)
		If $\overline{X} > T$, then	$M = T$ per cent ($AV = \overline{X} - T + ks$)
Acceptance value (AV)			general formula: $\lvert M - \overline{X} \rvert + ks$ (Calculations are specified above for the different cases.)

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Table 2 Variables Used in the Calculation of Uniformity of Dosage Units (continued)

Variable	Definition	Conditions	Value
L1	Maximum allowed acceptance value		*L1* = 15.0 unless otherwise specified in the individual monograph
L2	Maximum allowed range for deviation of each dosage unit tested from the calculated value of *M*	On the low side, no dosage unit result can be less than $(1 - L2 \times 0.01)M$, while on the high side no dosage unit result can be more than $(1 + L2 \times 0.01)M$. (This is based on an *L2* value of 25.0)	*L2* = 25.0 unless otherwise specified in the individual monograph
T	Target test sample amount at time of manufacture. For purposes of this Pharmacopoeia, unless otherwise specified in the individual monograph, *T* is 100.0 per cent, and for manufacturing purposes, *T* is the manufacturer's approved target test amount value at the time of manufacture.		

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equivalent to one average dosage unit obtained by the special procedure. If

$$\frac{100\,|\,W - P\,|}{W}$$

is more than 10, the use of a correction factor is not valid.

(5) The correction factor is to be applied only if F is not less than 1.030 nor more than 1.100, or not less than 0.900 nor more than 0.970. If F is between 0.970 and 1.030, no correction is required.

(6) If F lies between 1.030 and 1.100, or between 0.900 and 0.970, calculate the weight of drug substance in each dosage unit by multiplying each of the weights found using the special procedure by F.

UNCOATED, COATED, OR MOLDED TABLETS, CAPSULES, ORAL SOLUTIONS IN SINGLE-UNIT CONTAINERS, ORAL SUSPENSIONS OR ORAL EMULSIONS OR ORAL GELS IN SINGLE-UNIT CONTAINERS, AND SOLIDS (INCLUDING STERILE SOLIDS) IN SINGLE-UNIT CONTAINERS Assay 10 units individually as directed in the Assay in the individual monograph, unless otherwise specified in the procedure for Content Uniformity in the individual monograph. Calculate the acceptance value as directed below.

For oral solutions, oral suspensions, oral emulsions, or oral gels in single-unit containers, conduct the Assay on the amount of well-mixed material that drains from an individual container in not more than 5 seconds, or for highly viscous products, conduct the Assay on the amount of well-mixed material that is obtained by quantitatively removing the contents from an individual container, and express the results as the delivered dose.

Calculation of acceptance value Calculate the acceptance value by the formula:

$$|\,M - \overline{X}\,| + ks$$

in which the terms are as defined in Table 2.

SUPPOSITORIES, TRANSDERMAL SYSTEMS, AND INHALATIONS PACKAGED IN PREMETERED DOSAGE UNITS (**Note** Acceptance value calculations are not required for these dosage forms.) Assay 10 units individually as directed in the Assay in the individual monograph, unless otherwise specified in the procedure for Content Uniformity.

Weight Variation

Carry out the Assay on a representative sample of the batch as directed in the individual monograph. The result of the Assay, obtained as directed in the individual monograph, is designated as result A, expressed as a percentage of the label claim as described under Calculation of Acceptance Value. Assume that the concentration (weight of drug substance per weight of dosage unit) is uniform. Select not less than 30 dosage units, and proceed as follows for the dosage form designated. (**Note** Specimens other than these test units may be drawn from the same batch for assay determinations.)

UNCOATED OR FILM-COATED TABLETS Accurately weigh 10 tablets individually. Calculate the drug substance content, expressed as a percentage of the label claim, of each tablet from the weight of the individual tablet and the result of the Assay. Calculate the acceptance value.

HARD CAPSULES Accurately weigh 10 capsules individually, taking care to preserve the identity of each capsule. Remove the contents of each capsule by a suitable means. Accurately weigh the emptied shells individually, and calculate for each capsule the net weight of its contents by subtracting the weight of the shell from the respective gross weight. Calculate the drug substance content, expressed as a percentage of the label claim, of each capsule from the net weight of the individual capsule content and the result of the Assay. Calculate the acceptance value.

SOFT CAPSULES Accurately weigh 10 intact capsules individually to obtain their gross weights, taking care to preserve the identity of each capsule. Then cut open the capsules by means of a suitable clean, dry cutting instrument such as scissors or a sharp open blade, and remove the contents by washing with a suitable solvent. Allow the occluded solvent to evaporate from the shells at room temperature over a period of about 30 minutes, taking precautions to avoid uptake or loss of moisture. Weigh the individual shells, and calculate the net contents. Calculate the drug substance content, expressed as a percentage of the label claim, in each capsule from the net weight of product removed from the individual capsules and the result of the Assay. Calculate the acceptance value.

SOLIDS (INCLUDING STERILE SOLIDS) IN SINGLE-UNIT CONTAINERS Proceed as directed for Hard Capsules, treating each unit as described therein. Calculate the acceptance value.

ORAL SOLUTIONS PACKAGED IN SINGLE-UNIT CONTAINERS Accurately weigh the amount of liquid that drains in not more than 5 seconds from each of 10 individual containers. If necessary, compute the equivalent volume after determining the density. Calculate the drug substance content, expressed as a percentage of the label claim, in the liquid drained from each unit from the net weight of the individual container content and the result of the Assay. Calculate the acceptance value.

Calculation of acceptance value Calculate the acceptance value as described under Content Uniformity, except that the individual contents of the units are replaced with the individual estimated contents defined below.

$x_1, x_2, \ldots, x_n$ = individual estimated contents of the units tested, where $xi = wi \times A / \overline{W}$,
$w_1, w_2, \ldots, w_n$ = individual weights of the units tested,
A = content of drug substance (per cent of label claim) determined as described in the Assay, and

$\overline{W}$ = mean of individual weights (w_1, w_2, ..., w_n).

SOLUTIONS FOR INHALATION PACKAGED IN GLASS OR PLASTIC AMPOULES AND INTENDED FOR USE IN NEBULIZERS (**Note** Acceptance value calculations are not required for these dosage forms.) Accurately weigh 10 containers individually, taking care to preserve the identity of each container. Remove the contents of each container by a suitable means. Accurately weigh the emptied containers individually, and calculate for each container the net weight of its contents by subtracting the weight of the container from the respective gross weight. From the results of the Assay, obtained as directed in the individual monograph, calculate the drug substance content, expressed as a percentage of the label claim, in each of the containers.

Criteria

Apply the following criteria, unless otherwise specified in the individual monograph.

UNCOATED, COATED, OR MOLDED TABLETS, CAPSULES, ORAL SOLUTIONS IN SINGLE-UNIT CONTAINERS, ORAL SUSPENSIONS OR ORAL EMULSIONS OR ORAL GELS IN SINGLE-UNIT CONTAINERS, AND SOLIDS (INCLUDING STERILE SOLIDS) IN SINGLE-UNIT CONTAINERS The requirements for dosage uniformity are met if the acceptance value of the first 10 dosage units is less than or equal to *L1*. If the acceptance value is more than *L1*, test the next 20 units and calculate the acceptance value. The requirements are met if the final acceptance value of the 30 dosage units is less than or equal to *L1*, and no individual content of any dosage unit is less than $(1 - L2 \times 0.01)M$ nor more than $(1 + L2 \times 0.01)M$ as specified in the Calculation of Acceptance Value under Content Uniformity or under Weight Variation. Unless otherwise specified in the individual monograph, *L1* is 15.0 and *L2* is 25.0.

SUPPOSITORIES

Limit A (if the average of the limits specified in the potency definition in the individual monograph is 100.0 per cent or less) Unless otherwise specified in the individual monograph, the requirements for dosage uniformity are met if the amount of the drug substance in each of the 10 dosage units as determined from the method of Content Uniformity lies within the range of 85.0 per cent to 115.0 per cent of the label claim, and the RSD is less than or equal to 6.0 per cent.

If one unit is outside the range of 85.0 per cent to 115.0 per cent of the label claim, and no unit is outside the range of 75.0 per cent to 125.0 per cent of the label claim, or if the RSD is more than 6.0 per cent, or if both conditions prevail, test 20 additional units. The requirements are met if not more than one unit of the 30 is outside the range of 85.0 per cent to 115.0 per cent of the label claim, and no unit is outside the range of 75.0 per cent to 125.0 per cent of the label claim and the RSD of the 30 dosage units does not exceed 7.8 per cent.

Limit B (if the average of the limits specified in the potency definition in the individual monograph is more than 100.0 per cent)

(1) If the average value of the dosage units tested is 100.0 per cent or less, the requirements are as in Limit A.

(2) If the average value of the dosage units tested is greater than or equal to the average of the limits specified in the potency definition in the individual monograph, the requirements are as specified under Limit A, except that the words "label claim" are replaced by the words "label claim multiplied by the average of the limits specified in the potency definition in the monograph divided by 100".

(3) If the average value of the dosage units tested is between 100 per cent and the average of the limits specified in the potency definition in the individual monograph, the requirements are as specified under Limit A, except that the words "label claim" are replaced by the words "label claim multiplied by the average value of the dosage units tested (expressed as a per cent of label claim) divided by 100".

TRANSDERMAL SYSTEMS AND INHALATIONS PACKAGED IN PREMETERED DOSAGE UNITS

Limit A (if the average of the limits specified in the potency definition in the individual monograph is 100.0 per cent or less) Unless otherwise specified in the individual monograph, the requirements for dosage uniformity are met if the amount of the drug substance in not fewer than 9 of the 10 dosage units as determined from the method of Content Uniformity (or, in the case of solutions for inhalation packaged in glass or plastic ampoules and intended for use in nebulizers, from either the method of Content Uniformity or the Weight Variation) lies within the range of 85.0 per cent to 115.0 per cent of label claim, and no unit is outside the range of 75.0 per cent to 125.0 per cent of the label claim, and the RSD of the 10 dosage units is less than or equal to 6.0 per cent.

If two or three dosage units are outside the range of 85.0 per cent to 115.0 per cent of the label claim, but not outside the range of 75.0 per cent to 125.0 per cent of the label claim, or if the RSD is more than 6.0 per cent or if both conditions prevail, test 20 additional units. The requirements are met if not more than 3 units of the 30 are outside the range of 85.0 per cent to 115.0 per cent of the label claim and no unit is outside the range of 75.0 per cent to 125.0 per cent of the label claim, and the RSD of the 30 dosage units does not exceed 7.8 per cent.

Limit B (if the average of the limits specified in the potency definition in the individual monograph is more than 100.0 per cent)

(1) If the average value of the dosage units tested is 100.0 per cent or less, the requirements are as in Limit A.

(2) If the average value of the dosage units tested is more than or equal to the average of the limits specified in the potency definition in the individual monograph, the requirements are as specified under Limit A, except

that the words "label claim" are replaced by the words "label claim multiplied by the average of the limits specified in the potency definition in the monograph divided by 100".

(3) If the average value of the dosage units tested is between 100 per cent and the average of the limits specified in the potency definition in the individual monograph, the requirements are as specified under Limit A, except that the words "label claim" are replaced by the words "label claim multiplied by the average value of the dosage units tested (expressed as a percentage of label claim) divided by 100".

4.30 FRIABILITY OF UNCOATED TABLETS

This test is intended to determine, under defined conditions, the friability of uncoated tablets, the phenomenon whereby tablet surfaces are damaged and/or show evidence of lamination or breakage when subjected to mechanical shock or attrition.

Apparatus

Use a drum with an internal diameter between 283 and 291 mm and a depth between 36 and 40 mm, made of a transparent synthetic polymer with polished internal surfaces and not subject to static build-up (Fig. 1). One side of the drum is removable. The tablets are tumbled at each turn of the drum by a curved projection with an inside radius between 75.5 and 85.5 mm that extends from the middle of the drum to the outer wall. The outer diameter of the central ring is between 24.5-25.5 mm. The drum is attached to the horizontal axis of a device that rotates at 25±1 rpm. Thus, at each turn the tablets roll or slide and fall onto the drum wall or onto each other.

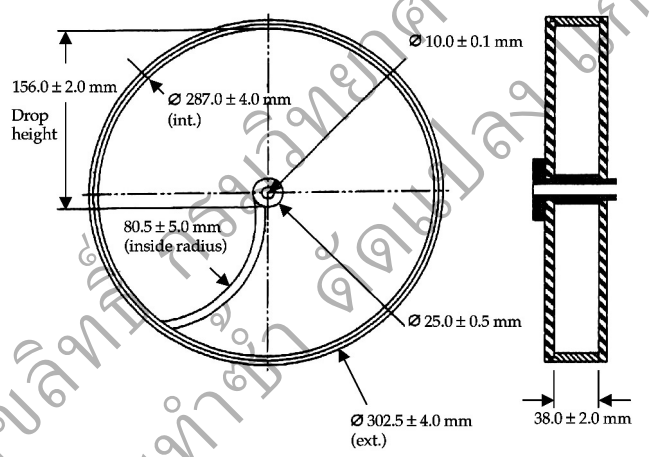


Fig.1 Tablet Friability Apparatus

Procedure

For tablets weighing up to 0.65 g each, take a sample of 20 tablets; for tablets weighing more than 0.65 g each, take 10 tablets. Place the tablets on a *No. 1000 sieve* and remove any loose dust with the aid of air pressure or a soft brush. Accurately weigh the tablet sample and place the tablets in the drum. Rotate the drum 100 times and remove the tablets. Remove any loose dust from the tablets as before. If no tablets are cracked, split or broken, weigh the tablets to the nearest milligram.

In general, the test is run once. If the results are doubtful or if the weight loss is more than 1 per cent, repeat the test twice and determine the mean of the three tests. A maximum loss of 1 per cent of the weight of the tablets tested is considered to be acceptable for most products.

For tablets having a diameter of 13 mm or more, problems of reproducibility may be encountered due to frequent irregular tumbling. In such cases, adjust the drum so that the tablets may fall freely and do not bind together when lying next to each other, adjusting the drum, so that the axis forms a 10° angle with the base, is usually satisfactory.

Effervescent tablets and chewable tablets may have different specifications as far as friability is concerned. In the case of hygroscopic tablets, an appropriate humidity-controlled environment is required for testing.

Drums with dual scooping projections, or an apparatus with more than one drum, for the running of multiple samples at one time, are also permitted.

Expression of the Results

The friability is expressed as the loss of mass and it is calculated as percentage of the initial mass. Indicate the number of tablets used.

4.31 RESISTANCE TO CRUSHING OF TABLETS

This test is intended to determine, under defined conditions, the resistance to crushing of tablets or hardness measured by the force needed to disrupt them by crushing.

Apparatus

The apparatus consists of two jaws facing each other, one of which moves towards the other. The flat surfaces of the jaws are perpendicular to the direction of movement. The crushing surfaces of the jaws are flat and larger than the zone of contact with the tablet. The apparatus is calibrated using a system with a precision of 1 newton.

Procedure

Place the tablet between the jaws, taking into account, where applicable, the shape, the break-mark and the inscription; for each measurement orient the tablet in the same way with respect to the direction of application of the force. Carry out the measurement on 10 tablets. Taking care that all fragments of the tablets have been removed before each determination.

This procedure does not apply when fully automated equipment is used.

Expression of the Results

Express the results as the mean, minimum and maximum values of the forces measured, all expressed in newtons.

Indicate the type of apparatus and, where applicable, the orientation of the tablets.

4.32 APPARENT VOLUME

This test for apparent volume is intended to determine under defined conditions the apparent volumes, before and after settling, the ability to settle and the apparent densities of divided solids (for example, powders, granules).

Apparatus

The apparatus is specified in Fig. 1 and consists of the following components:

(a) A settling apparatus capable of producing in 1 minute 250±15 taps from a height of 3±0.2 mm. The support for the graduated cylinder, with its holder, has a mass of 450±5 g.

(b) A 250-ml graduated cylinder (2-ml intervals) with a mass of 220±40 g.

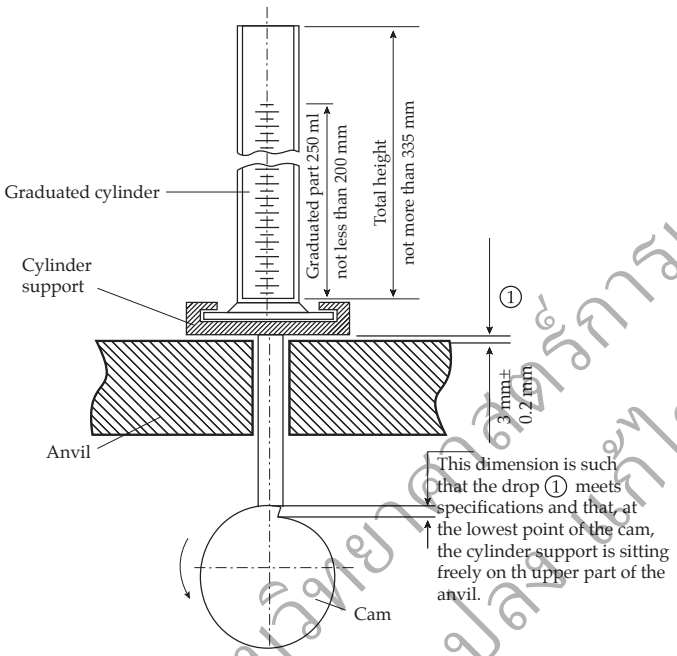


Fig. 1 Apparent Volume Apparatus

Procedure

Into the dry cylinder, introduce without compacting 100.0 g (*W*) of the substance being examined. If this is not possible, select a test sample with an apparent volume between 50 and 250 ml and specify the weight in the expression of results. Secure the cylinder in its holder. Read the unsettled apparent volume, V_0, to the nearest millilitre. Carry out 10, 500 and 1250 taps and read the corresponding volumes V_{10}, V_{500} and V_{1250}, to the nearest millilitre. If the difference between V_{500} and V_{1250} is more than 2 ml, carry out another 1250 taps.

Calculation

(a) Apparent volumes:

– apparent volume before settling or bulk volume: V_0 ml.

– apparent volume after settling or settled volume: V_{1250} or V_{2500}.

(b) Ability to settle: difference V_{10} ml – V_{500} ml.

(c) Apparent densities:

The apparent densities are expressed as follows:

– apparent density before settling or density of bulk product: W/V_0, in g per ml (bulk or poured density).

– apparent density after settling or density of settled product: W/V_{1250} or W/V_{2500}, in g per ml (tapped density).

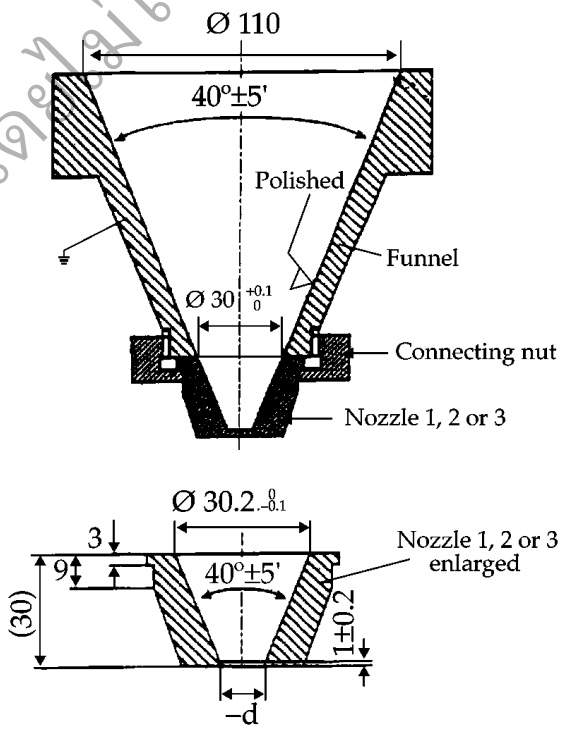
4.33 FLOWABILITY

This test flowability is intended to determine the ability of divided solids (for example, powders and granules) to flow vertically under defined conditions.

Apparatus

According to the flow properties of the material to be tested, funnels with or without stem, with different angles and orifice diameters are used. The funnel is maintained upright by a suitable device. The assembly must be protected from vibrations.

The dimensions and tolerances of typical pieces of apparatus are specified in Figs. 1 and 2.



Nozzle	Diameter (*d*) of the Outflow Opening (mm)
1	10±0.01
2	15±0.01
3	25±0.01

Fig. 1 Flow Funnel and Nozzle
Dimensions in mm

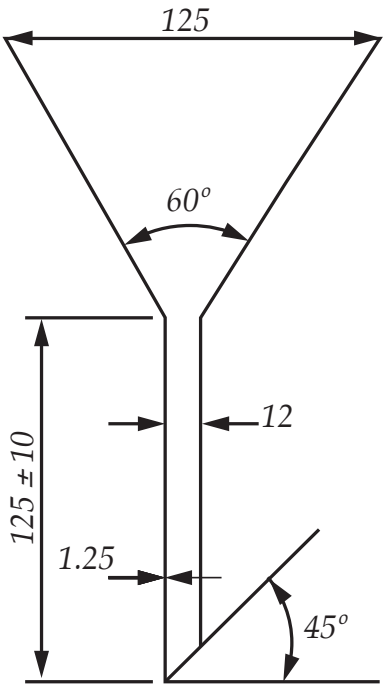


Fig. 2 Funnel with Stem
Dimensions in mm

Procedure

Into a dry funnel, the bottom opening of which has been blocked by suitable means, introduce without compacting a test sample weighed with 0.5 per cent accuracy. The amount of the sample depends on the "Apparent Volume" (Appendix 4.32), and the apparatus used. Unblock the bottom opening of the funnel and measure the time needed for the entire sample to flow out of the funnel. Carry out three determinations.

Calculation

The flowability is expressed in seconds and tenths of seconds, related to 100 g of sample.

The results depend on the storage conditions of the material to be tested.

The results can be expressed as (1) the mean of the determinations, if none of the individual values deviates from the mean value by more than 10 per cent; (2) as a range, if the individual values deviate from the mean value by more than 10 per cent; (3) as a plot of the weight against the flow time; (4) as and infinite time, if the entire sample fails to flow through.

4.34 POWDER FLOW

The widespread use of powders in the pharmaceutical industry has generated a variety of methods for characterizing powder flow. Not surprisingly, scores of references appear in the pharmaceutical literature, attempting to correlate the various measures of powder flow to manufacturing properties. The development of such a variety of test methods was inevitable; powder behavior is multifaceted and thus complicates the effort to characterize powder flow.

The purpose of this appendix is to review the methods for characterizing powder flow that have appeared most frequently in the pharmaceutical literature. In addition, while it is clear that no single and simple test method can adequately characterize the flow properties of pharmaceutical powders, this chapter proposes the standardization of test methods that may be valuable during pharmaceutical development.

Four commonly reported methods for testing powder flow are:

- angle of repose,
- compressibility index or Hausner ratio,
- flow rate through an orifice, and
- shear cell.

In addition, numerous variations of each of these basic methods are available. Given the number of test methods and variations, standardizing the test methodology, where possible, would be advantageous.

With this goal in mind, the most frequently used methods are discussed below. Important experimental considerations are identified and recommendations are made regarding standardization of the methods. In general, any method of measuring powder flow must be practical, useful, reproducible and sensitive, and must yield meaningful results. It bears repeating that no simple powder flow method will adequately or completely characterize the wide range of flow properties experienced in the pharmaceutical industry. An appropriate strategy may well be the use of multiple standardized test methods to characterize the various aspects of powder flow as needed by the pharmaceutical scientist.

Angle of Repose

The angle of repose has been used in several branches of science to characterize the flow properties of solids. Angle of repose is a characteristic related to interparticulate friction, or resistance to movement between particles. Angle of repose test results are reported to be very dependent upon the method used. Experimental difficulties arise due to segregation of material and consolidation or aeration of the powder as the cone is formed. Despite its difficulties, the method continues to be used in the pharmaceutical industry, and a number of examples demonstrating its value in predicting manufacturing problems appear in the literature.

The angle of repose is the constant, three-dimensional angle (relative to the horizontal base) assumed by a cone-like pile of material formed by any of several different methods, described briefly below.

BASIC METHODS FOR ANGLE OF REPOSE A variety of angle of repose test methods are described in the literature. The most common methods for determining the static angle of repose can be classified based on 2 important experimental variables:

- the height of the "funnel" through which the powder passes may be fixed relative to the base, or the height may be varied as the pile forms;

– the base upon which the pile forms may be of fixed diameter or the diameter of the powder cone may be allowed to vary as the pile forms.

VARIATIONS IN ANGLE OF REPOSE METHODS Variations of the above methods have also been used to some extent in the pharmaceutical literature:

– *drained angle of repose* is determined by allowing an excess quantity of material positioned above a fixed diameter base to "drain" from the container. Formation of a cone of powder on the fixed diameter base allows determination of the drained angle of repose;

– *dynamic angle of repose* is determined by filling a cylinder (with a clear, flat cover on one end) and rotating it at a specified speed. The dynamic angle of repose is the angle (relative to the horizontal) formed by the flowing powder. The internal angle of kinetic friction is defined by the plane separating those particles sliding down the top layer of the powder and those particles that are rotating with the drum (with roughened surface).

GENERAL SCALE OF FLOWABILITY FOR ANGLE OF REPOSE While there is some variation in the qualitative description of powder flow using the angle of repose, much of the pharmaceutical literature appears to be consistent with the classification by Carr (Table 1). There are examples in the literature of formulations with an angle of repose in the range of 40 to 50 degrees that manufactured satisfactorily. When the angle of repose exceeds 50 degrees, the flow is rarely acceptable for manufacturing purposes.

Table 1 Flow Properties and Corresponding Angles of Repose*

Flow Property	Angle of Repose (degrees)
Excellent	25-30
Good	31-35
Fair (aid not needed)	36-40
Passable (may hang up)	41-45
Poor (must agitate, vibrate)	46-55
Very poor	56-65
Very, very poor	>66

*R.L. Carr, Evaluating flow properties of solids, *Chem Eng.*, 1965, *72*, 163-168.

EXPERIMENTAL CONSIDERATIONS FOR ANGLE OF REPOSE Angle of repose is not an intrinsic property of the powder, that is to say, it is very much dependent upon the method used to form the cone of powder. On this subject, the existing literature raises these important considerations:

– the peak of the cone of powder can be distorted by the impact of powder from above. By carefully building the powder cone, the distortion caused by impact can be minimized;

– the nature of the base upon which the powder cone is formed influences the angle of repose. It is recommended that the powder cone be formed on a "common base", which can be achieved by forming the cone of powder on a layer of powder. This can be done by using a base of fixed diameter with a protruding outer edge to retain a layer of powder upon which the cone is formed.

RECOMMENDED PROCEDURE FOR ANGLE OF REPOSE Form the angle of repose on a fixed base with a retaining lip to retain a layer of powder on the base. The base must be free of vibration. Vary the height of the funnel to carefully build up a symmetrical cone of powder. Care must be taken to prevent vibration as the funnel is moved. The funnel height is maintained at approximately 2 to 4 cm from the top of the powder pile as it is being formed in order to minimize the impact of falling powder on the tip of the cone. If a symmetrical cone of powder cannot be successfully or reproducibly prepared, this method is not appropriate. Determine the angle of repose by measuring the height of the cone of powder and calculating the angle of repose, α, from the following equation:

$$\tan(\alpha) = \frac{\text{height}}{0.5 \times \text{base}}$$

Compressibility Index and Hausner Ratio

In recent years the compressibility index and the closely related Hausner ratio have become the simple, fast, and popular methods of predicting powder flow characteristics. The compressibility index has been proposed as an indirect measure of bulk density, size and shape, surface area, moisture content, and cohesiveness of materials, because all of these can influence the observed compressibility index. The compressibility index and the Hausner ratio are determined by measuring both the bulk volume and tapped volume of a powder.

BASIC METHODS FOR COMPRESSIBILITY INDEX AND HAUSNER RATIO While there are some variations in the method of determining the compressibility index and Hausner ratio, the basic procedure is to measure the unsettled apparent volume, (V_0), and the final tapped volume, (V_f), of the powder after tapping the material until no further volume changes occur. The compressibility index and the Hausner ratio are calculated as follows:

$$\text{compressibility index} = 100 \times \frac{V_0 - V_f}{V_0}$$

$$\text{Hausner ratio} = \frac{V_0}{V_f}$$

Alternatively, the compressibility index and Hausner ratio may be calculated using measured values of bulk density (ρ_{bulk}) and tapped density (ρ_{tapped}) as follows:

$$\text{compressibility index} = 100 \times \frac{\rho_{tapped} - \rho_{bulk}}{\rho_{tapped}}$$

$$\text{Hausner ratio} = \frac{\rho_{tapped}}{\rho_{bulk}}$$

In a variation of these methods, the rate of consolidation is sometimes measured rather than, or in addi-

tion to, the change in volume that occurs on tapping. For the compressibility index and the Hausner ratio, the generally accepted scale of flow ability is given in Table 2.

EXPERIMENTAL CONSIDERATIONS FOR THE COMPRESSIBILITY INDEX AND HAUSNER RATIO Compressibility index and Hausner ratio are not intrinsic properties of the powder, that is to say, they are dependent upon the methodology used. The existing literature points out several important considerations affecting the determination of the unsettled apparent volume, V_0, of the final tapped volume, V_f, of the bulk density, ρ_{bulk}, and of the tapped density, ρ_{tapped}:

– the diameter of the cylinder used,
– the number of times the powder is tapped to achieve the tapped density,
– the mass of material used in the test, and
– rotation of the sample during tapping.

RECOMMENDED PROCEDURE FOR COMPRESSIBILITY INDEX AND HAUSNER RATIO Use a 250 ml volumetric cylinder with a test sample weight of 100 g. Smaller amounts and volumes may be used, but variations in the method must be described with the results. An average of three determinations is recommended.

Flow Through an Orifice

The flow rate of a material depends upon many factors, some of which are particle-related and some related to the process. Monitoring the rate of flow of material through an orifice has been proposed as a better measure of powder flowability. Of particular significance is the utility of monitoring flow continuously, since pulsating flow patterns have been observed even for free-flowing materials. Changes in flow rate as the container empties can also be observed. Empirical equations relating flow rate to the diameter of the opening, particle size, and particle density have been determined. However, determining the flow rate through an orifice is useful only with free-flowing materials.

The flow rate through an orifice is generally measured as the mass per time flowing from any of a number of types of containers (cylinders, funnels, hoppers). Measurement of the flow rate can be in discrete increments or continuous.

BASIC METHODS FOR FLOW THROUGH AN ORIFICE There are a variety of methods described in the literature. The most common for determining the flow rate through an orifice can be classified based on three important experimental variables:

– the type of container used to contain the powder. Common containers are cylinders, funnels, and hoppers from production equipment;
– the size and shape of the orifice used. The orifice diameter and shape are critical factors in determining powder flow rate;
– the method of measuring powder flow rate. Flow rate can be measured continuously using an electronic balance with some sort of recording device (strip chart recorder, computer). It can also be measured in discrete samples (for example, the time it takes for 100 g of powder to pass through the orifice to the nearest tenth of a second or the amount of powder passing through the orifice in 10 to the nearest tenth of a gram).

VARIATIONS IN METHODS FOR FLOW THROUGH AN ORIFICE Either mass flow rate or volume flow rate can be determined. Mass flow rate is the easier of the methods, but it biases the results in favour of high-density materials. Since die fill is volumetric, determining volume flow rate may be preferable. A vibrator is occasionally attached to facilitate flow from the container, however, this appears to complicate interpretation of results. A moving orifice device has been proposed to more closely simulate rotary press conditions. The minimum diameter orifice through which powder flows can also be identified.

GENERAL SCALE OF FLOWABILITY FOR FLOW THROUGH AN ORIFICE No general scale is available because flow rate is critically dependent on the method used to measure it. Comparison between published results is difficult.

EXPERIMENTAL CONSIDERATIONS FOR FLOW THROUGH AN ORIFICE Flow rate through an orifice is not an intrinsic property of the powder. It is very much dependent upon the methodology used. The existing literature points out several important considerations affecting these methods:

– the diameter and shape of the orifice,
– the type of container material (metal, glass, plastic),
– the diameter and height of the powder bed.

Table 2 Scale of Flowability*

Compressibility Index (per cent)	Flow Character	Hausner Ratio
1-10	Excellent	1.00-1.11
11-15	Good	1.12-1.18
16-20	Fair	1.19-1.25
21-25	Passable	1.26-1.34
26-31	Poor	1.35-1.45
32-37	Very poor	1.46-1.59
>38	Very, very poor	>1.60

*R.L. Carr, "Evaluating flow properties of solids", *Chem Eng.*, *72*, 1965, pp. 163-168.

RECOMMENDED PROCEDURE FOR FLOW THROUGH AN ORIFICE Flow rate through an orifice can be used only for materials that have some capacity to flow. It is not useful for cohesive materials. Provided that the height of the powder bed (the 'head' of powder) is much more than the diameter of the orifice, the flow rate is virtually independent of the powder head. It is advisable to use a cylinder as the container, because the walls of the container must have little effect on flow. This configuration results in flow rate being determined by the movement of powder over powder, rather than powder along the wall of the container. Powder flow rate often increases when the height of the powder column is less than twice the diameter of the column. The orifice must be circular and the cylinder must be free of vibration. General guidelines for dimensions of the cylinder are as follows:

– diameter of the opening more than 6 times the diameter of the particles,

– diameter of the cylinder more than twice the diameter of the opening.

Use of a hopper as the container may be appropriate and representative of flow in a production situation. It is not advisable to use a funnel, particularly one with a stem, because flow rate will be determined by the size and length of the stem as well as the friction between the stem and the powder. A truncated cone may be appropriate, but flow will be influenced by the powder-wall friction coefficient, thus, selection of an appropriate construction material is important.

For the opening in the cylinder, use a flat-faced bottom plate with the option to vary orifice diameter to provide maximum flexibility and better ensure a powder-over-powder flow pattern. Rate measurement can be either discrete or continuous. Continuous measurement using an electronic balance can more effectively detect momentary flow rate variations.

Shear Cell Methods

In an effort to put powder flow studies and hopper design on a more fundamental basis, a variety of powder shear testers and methods that permit more through and precisely defined assessment of powder flow properties have been developed. Shear cell methodology has been used extensively in the study of pharmaceutical materials. From these methods, a wide variety of parameters can be obtained, including the yield loci representing the shear stress-shear strain relationship, the angle of internal friction, the unconfined yield strength, the tensile strength, and a variety of derived parameters such as the flow factor and other flowability indices. Because of the ability to control experimental parameters more precisely, flow properties can also be determined as a function of consolidation load, time, and other environmental conditions. These methods have been successfully used to determine critical hopper and bin parameters.

BASIC METHODS FOR SHEAR CELL One type of shear cell is the cylindrical shear cell which is split horizontally, forming a shear plane between the lower stationary base and the upper moveable portion of the shear cell ring. After powder bed consolidation in the shear cell (using a well-defined procedure), the force necessary to shear the powder bed by moving the upper ring is determined. Annular shear cell designs offer some advantages over the cylindrical shear cell design, including the need for less material. A disadvantage, however, is that because of its design, the powder bed is not sheared as uniformly because material on the outside of the annulus is sheared more than material in the inner region. A third type of shear cell (plate-type) consists of a thin sandwich of powder between a lower stationary rough surface and an upper rough surface that is moveable.

All of the shear cell methods have their advantages and disadvantages, but a detailed review is beyond the scope of this chapter. As with the other methods for characterizing powder flow, many variations are described in the literature. A significant advantage of shear cell methodology in general is a greater degree of experimental control. The methodology generally is rather time-consuming and requires significant amounts of material and a well-trained operator.

RECOMMENDATIONS FOR SHEAR CELL The many existing shear cell configurations and test methods provide a wealth of data and can be used very effectively to characterize powder flow. They are also helpful in the design of equipment such as hoppers and bins. Because of the diversity of available equipment and experimental procedures, no specific recommendations regarding methodology are presented in this appendix. It is recommended that the results of powder flow characterization using shear cell methodology include a complete description of equipment and methodology used.

4.35 OSMOLALITY

Osmolality is a practical means of giving an overall measure of the contribution of the various solutes present in a solution to the osmotic pressure of the solution.

An acceptable approximation for the osmolality (ξ_m) of a given aqueous solution is given by:

$$\xi_m = vm\Phi$$

where m = molality of the solution, that is the number of moles of solute per kilogram of solvent,

Φ = molal osmotic coefficient which takes account of the interactions between ions of opposite charge in the solution. It is dependent on the value of m. As the complexity of solutions increases, Φ becomes difficult to measure.

If the solute is not ionized, v is 1; otherwise, v is the total number of ions already present or formed by

solvolysis from one molecule of solute.

The unit of osmolality is osmole per kilogram (Osmol kg^{-1}), but the submultiple milliosmole per kilogram (mOsmol kg^{-1}) is usually used.

Unless otherwise prescribed, osmolality is determined by measurement of the depression of freezing point.

The following relationship exists between the osmolality and the depression of freezing point ΔT:

$$\xi_m = \frac{\Delta T}{1.86} \times 1000 \text{ mOsmol/kg}$$

Apparatus

The apparatus (osmometer) consists of:

(a) a means of cooling the container used for the measurement,

(b) a system for measuring temperature consisting of a resistor sensitive to a temperature (thermistor), with an appropriate current or potential difference measurement device that may be graduated in temperature depression or directly in osmolality,

(c) a means of mixing the sample is usually included.

Method

Prepare reference solutions as described in Table 1, as required. Determine the zero of the apparatus using *distilled water*. Calibrate the apparatus using the reference solutions: introduce 50 to 250 µl of the solutions into the measurement cell and start the cooling system. Usually, the mixing device is programmed to operate at a temperature below that expected through cryoscopic depression to prevent supercooling. A suitable device indicates attainment of equilibrium. Before each measurement, rinse the measurement cell with the solution being examined.

Carry out the same operations with the test sample. Read directly the osmolality or calculate it from the measured depression of freezing point. The test is not valid unless the value found is within two values of the calibration scale.

Table 1 Reference Solution for Osmometer Calibration

Sodium Chloride (g/kg of water)	Real Osmolality (mOsmol/kg)	Ideal Osmolality (mOsmol/kg)	Molal Osmotic Coefficient, Φ	Cryoscopic Depression (°C)
3.087	100	105.67	0.9463	0.186
6.260	200	214.20	0.9337	0.372
9.463	300	323.83	0.9264	0.558
12.684	400	434.07	0.9215	0.744
15.916	500	544.66	0.9180	0.930
19.147	600	655.24	0.9157	1.116
22.380	700	765.86	0.9140	1.302

4.36 TOTAL ORGANIC CARBON

Total Organic Carbon (TOC) determination is an indirect measure of organic substances present in water for pharmaceutical use. TOC determination can also be used to monitor the performance of various operations in the preparation of medicines.

A variety of acceptable methods is available for determining TOC. Rather than prescribing a given method to be used, this appendix describes the procedures used to qualify the chosen method and the interpretation of results in limit tests. A standard solution is analyzed at suitable intervals, depending on the frequency of measurements; the solution is prepared with a substance that is expected to be easily oxidizable (for example, sucrose) at a concentration adjusted to give an instrument response corresponding to the TOC limit to be measured. The suitability of the system is determined by analysis of a solution prepared with a substance expected to be oxidizable with difficulty (for example, 1,4-benzoquinone).

The various types of apparatus used to measure TOC in water for pharmaceutical use have in common the objective of completely oxidizing the organic molecules in the sample water to produce carbon dioxide followed by measurement of the amount of carbon dioxide produced, the result being used to calculate the carbon concentration in the water.

The apparatus used must discriminate between organic and inorganic carbon, the latter being present as carbonate. The discrimination may be effected either by measuring the inorganic carbon and subtracting it from the total carbon, or by purging inorganic carbon from the sample before oxidization. Purging may also entrain organic molecules, but such purgeable organic carbon is present in negligible quantities in water for pharmaceutical use.

Apparatus Use a calibrated instrument installed either online or off-line. Verify the system suitability at suitable intervals as described below. The apparatus must have a limit of detection specified by the manufacturer of 0.05 mg or less of carbon per litre.

Reagent water Use Purified Water complying with the following specifications:

– conductivity: not more than 1.0 µS/cm at 25°,
– total organic carbon: not more than 0.1 mg/l.

Depending on the type of apparatus used, the content of heavy metals and copper may be critical. The manufacturer's instructions should be followed.

Glassware preparation Use glassware that has been scrupulously cleaned by a method that will remove organic matter. Use the reagent water for the final rinse of glassware.

Standard solution Dissolve *sucrose*, previously dried at 105° for 3 hours in the reagent water to obtain a solution containing 1.19 mg of sucrose per litre (0.50 mg of carbon per litre).

Test solution Using all due care to avoid contamination, collect water to be tested in a tightly closed container leaving minimal head-space. Examine the water with minimum delay to reduce contamination from the container and its closure.

System suitability solution Dissolve *1,4-Benzoquinone* in the reagent water to obtain a solution having a concentration of 0.75 mg of 1,4-benzoquinone per litre (0.50 mg of carbon per litre).

Reagent water control Use the reagent water obtained at the same time as that used to prepare the standard solution and the system suitability solution.

Other control solutions Prepare suitable blank solutions or other solutions needed for establishing the baseline or for calibration adjustments following the manufacturer's instructions; run the appropriate blanks to zero the instrument.

System suitability Test the reagent water control in the apparatus, and record the response, r_w. Repeat the test using the standard solution, and record the response, r_S. Calculate the corrected standard solution response, which is also the limit response, by subtracting the reagent water control response from the response of the standard solution. The theoretical limit of 0.50 mg of carbon per litre is equal to the corrected standard solution response, $r_S - r_w$. Test the system suitability solution in the apparatus, and record the response, r_{ss}. Calculate the corrected system suitability solution response by subtracting the reagent water control response from the response of the system suitability solution, $r_{ss} - r_w$. Calculate the response efficiency for the System suitability solution by the formula:

$$\frac{r_{ss} - r_w}{r_S - r_w} \times 100$$

The system is suitable if the response efficiency is not less than 85 per cent and not more than 115 per cent of the theoretical response.

Procedure Run the test solution and record the response (r_U). The test solution complies with the test if r_U is not more than $r_S - r_w$.

The method can also be applied using on-line instrumentation that has been adequately calibrated and shown to have acceptable system suitability. The location of instrumentation must be chosen to ensure that the responses are representative of the water used.

4.37 WATER CONDUCTIVITY

Electrical conductivity in water is a measure of the ion-facilitated electron flow passing through it. Water molecules dissociate into ions as a function of pH and temperature and result in a very predictable conductivity. Some gases, most notably carbon dioxide, readily dissolve in water and interact to form ions, which predictably affect conductivity as well as pH. For the purpose of this discussion, these ions and their resulting conductivity can be considered intrinsic to the water.

Water conductivity is also affected by the presence of extraneous ions. The extraneous ions used in modeling the conductivity specifications described below are the chloride and sodium ions. The conductivity of the ubiquitous chloride ion and the ammonium ion represent a major portion of the allowed water impurity level. A balancing quantity of cations, such as sodium ions, is included in this allowed impurity level to maintain electroneutrality. Extraneous ions such as these may have significant impact on the water's chemical purity and suitability for use in pharmaceutical applications.

Water conductivity must be measured accurately using calibrated instrumentation. On-line conductivity testing provides real-time measurements and opportunities for real-time process control, decision, and intervention. Precaution should be taken while collecting water samples for off-line conductivity measurements. The sample may be affected by the sampling method, the sampling container, and environmental factors such as ambient carbon dioxide concentration and organic vapours.

Apparatus

CONDUCTIVITY CELL

– Electrodes of a suitable material.

– The conductivity cell constant must be within ±2 per cent of the given value.

– The cell constant can be verified directly by using a solution of known or traceable conductivity, or indirectly by comparing the instrument reading taken with the conductivity sensor in question to readings from a conductivity sensor of known or traceable cell constant.

CONDUCTIVITY METER

– Meter calibration is accomplished by replacing the conductivity sensor with a traceable precision resistors (accurate to ±0.1 per cent of the stated value, traceable to the official standard) or an equivalently accurate adjustable resistance device, such as a Wheatstone Bridge, to give a predicted instrument response. Each scale on the meter may require separate calibration prior to use.

– The frequency of recalibration is a function of instrument design, degree of use, etc. However, because some multiple-scale instruments have a single

calibration adjustment, recalibration may be required between each use of a different scale.

– The instrument accuracy must be ±0.1 µS/cm, excluding the conductivity sensor cell constant accuracy.

SYSTEM VERIFICATION

Periodic verification of the entire equipment shall be performed. This could be done by comparing the conductivity/resistivity values displayed by the measuring equipment with those of an external calibrated conductivity-measuring device. The two nontemperature-compensated conductivity or resistivity values must be equivalent to within ±20 per cent of each other, or at a difference that is acceptable on the basis of product water criticality and/or the water conductivity ranges in which the measurements are taken. The two conductivity sensors should be positioned close enough together to measure the same water sample in the same environmental conditions.

A similar verification performed in temperature-compensated mode could be performed to ensure an appropriate accuracy of the equipment when such a mode is used for trending or other purposes.

CONDUCTIVITY TEMPERATURE COMPENSATION

Many instruments automatically correct the actual reading to display the value that theoretically would be observed at the nominal temperature of 25°. This is typically done using a temperature sensor embedded in the conductivity sensor and an algorithm in the instrument's circuitry. This temperature compensation algorithm may not be accurate. Conductivity values used in this method are nontemperature-compensated measurements.

– Temperature measurement is required for the performance of the Stage 1 test. It may be made using the temperature sensor embedded in the conductivity cell sensor. An external temperature sensor positioned near the conductivity sensor is also acceptable. Accuracy of the temperature measurement must be ±2°.

Procedure

FOR BULK WATER

Stage 1

Stage 1 is intended for on-line measurement or may be performed off-line in a sufficient amount of water to a suitable container.

1. Determine the temperature of the water and the conductivity of the water using a nontemperature-compensated conductivity reading.

2. Using the Stage 1 temperature and conductivity requirements table, find the temperature value that is not greater than the measured temperature, i.e. the next lower temperature. The corresponding conductivity value on this table is the limit. (**Note** Do not interpolate.)

3. If the measured conductivity is not greater than the table value, the water meets the requirements of the test for conductivity. If the conductivity is greater than the table value, proceed with Stage 2.

Stage 1 Temperature and Conductivity Requirements (for Nontemperature-compensated Conductivity Measurements Only)

Temperature	Conductivity Requirement (µS/cm)
0	0.6
5	0.8
10	0.9
15	1.0
20	1.1
25	1.3
30	1.4
35	1.5
40	1.7
45	1.8
50	1.9
55	2.1
60	2.2
65	2.4
70	2.5
75	2.7
80	2.7
85	2.7
90	2.7
95	2.9
100	3.1

Stage 2

4. Transfer a sufficient amount of water (100 ml or more) to a suitable container, and stir the test sample. Adjust the temperature, if necessary, and, while maintaining it at 25°±1°, begin vigorously agitating the test sample while periodically observing the conductivity. When the change in conductivity (due to uptake of atmospheric carbon dioxide) is less than a net of 0.1 µS/cm per 5 minutes, note the conductivity.

5. If the conductivity is not more than 2.1 µS/cm, the water meets the requirements of the test for conductivity. If the conductivity is more than 2.1 µS/cm, proceed with Stage 3.

Stage 3

6. Perform this test within approximately 5 minutes of the conductivity determination in Step 5, while maintaining the sample temperature at 25°±1°. Add a recently prepared saturated solution of *potassium chloride* to the same test sample (0.3 ml per 100 ml of the test sample), and determine the pH to the nearest 0.1 pH unit, as directed under "Determination of pH" (Appendix 4.11).

7. Referring to the Stage 3 pH and Conductivity Requirements table, determine the conductivity limit at the measured pH value. If the measured conductivity in Step 4 is not more than the conductivity requirements for the pH determined in Step 6, the water meets the requirements of the test for conductivity. If either the

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measured conductivity is greater than this value or the pH is outside the range of 5.0 to 7.0, the water does not meet the requirements of the test for conductivity.

Stage 3 pH and Conductivity Requirements (for Atmosphere- and Temperature-equilibrated Samples Only)

pH	Conductivity Requirement (μS/cm)
5.0	4.7
5.1	4.1
5.2	3.6
5.3	3.3
5.4	3.0
5.5	2.8
5.6	2.6
5.7	2.5
5.8	2.4
5.9	2.4
6.0	2.4
6.1	2.4
6.2	2.5
6.3	2.4
6.4	2.3
6.5	2.2
6.6	2.1
6.7	2.6
6.8	3.1
6.9	3.8
7.0	4.6

FOR STERILE WATER

Transfer a sufficient amount of water to a suitable container, and stir the test sample. Adjust the temperature, if necessary, and, while maintaining it at 25°±1°, begin vigorously agitating the test sample while periodically observing the conductivity. When the change in conductivity (due to uptake of ambient carbon dioxide) is less than a net of 0.1 μS/cm per 5 minutes, note the conductivity.

For containers with a nominal volume of 10 ml or less, if the conductivity is not more than 25 μS/cm, the water meets the requirements. For containers with a nominal volume more than 10 ml, if the conductivity is not more than 5 μS/cm, the water meets the requirements.

4.38 ANALYTICAL FILTRATION BY FILTER PAPER

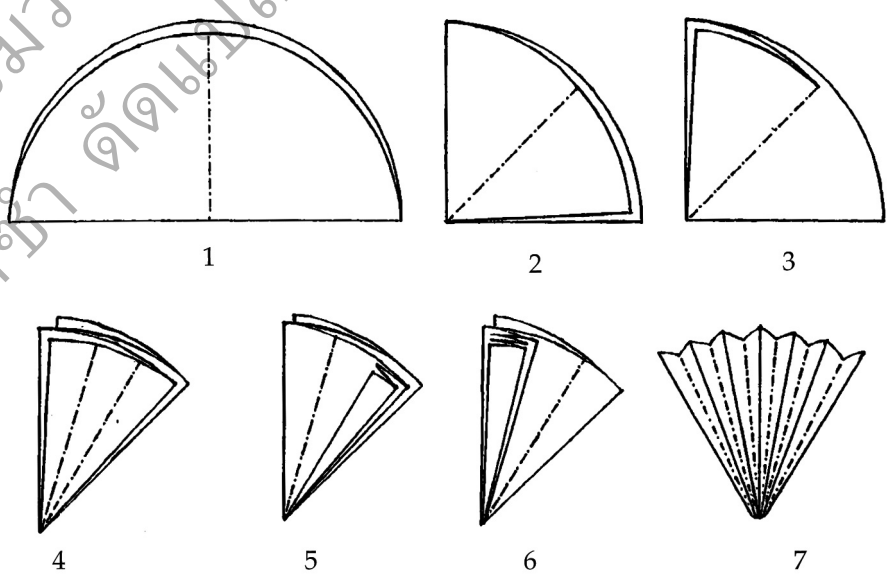
The following considerations are provided as a guide when filtration is utilized in the assay.

Only high quality filter paper should be used to assure maximum filtering efficacy. For analytical work, pleated filter paper folded in a special way or prepleated filter paper should be used to increase filtering speed and prevent tearing at the apex.

The steps in folding a pleated filter paper are illustrated in the figure below.

When the filter paper has been accurately pleated and creases emphasized by pressure, it is then carefully opened and placed in a dry funnel. Pour the assay preparation into the dry filter (in case the solution-volume has been adjusted). The stream should be delivered upon one of the sides. The first appropriate millilitres of the filtrate should be discarded in order to eliminate insofar as possible contamination of the assay preparation by free fibres associated with most filter paper and to maintain the concentration of the assay preparation.

In folding a filter paper, care should be observed not to extend the creases entirely to the apex, but to end them at a distance of about half an inch from it, because the point at which all the creases converge would be thereby so weakened that the weight of the assay preparation may rupture the filter paper.



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APPENDIX 5 CHEMICAL TESTS

5.1 GENERAL IDENTIFICATION TESTS

Acetates

A. Acetates, when warmed with a 50 per cent v/v solution of *sulfuric acid*, yield acetic acid, which is recognizable by its odour.

B. Acetates, when warmed with *sulfuric acid* and a small quantity of *ethanol*, yield ethyl acetate, which is recognizable by its odour.

C. Neutral solutions of acetates produce a red-brown colour with *iron(III) chloride TS* and a red-brown precipitate when boiled. The precipitate dissolves and the colour of the solution changes to yellow by the addition of *hydrochloric acid*.

Acetyl Groups

In a test-tube (about 180 mm × 18 mm) place about 15 mg of the test substance or the prescribed quantity and 3 drops of *phosphoric acid*. Close the tube with a stopper through which passes a small test-tube (about 100 mm × 10 mm) containing water to act as a condenser. On the outside of the smaller tube hang a drop of a 5 per cent w/v solution of *lanthanum nitrate*.

Place the apparatus in a water-bath for 5 minutes and remove the smaller tube. Mix the drop with 1 drop of 0.01 M *iodine* on a tile. Add at the edge 1 drop of 2 M *ammonia*. After 1 to 2 minutes a blue colour develops at the junction of the two drops which intensifies and persists for a short time.

For substances hydrolyzable only with difficulty, heat the mixture slowly to boiling point over an open flame instead of using a water-bath.

Alkaloids

Dissolve a few mg of alkaloids, or the prescribed quantity, in 5 ml of *water*, add *dilute hydrochloric acid* until an acid reaction occurs. Then add 1 ml of *acetic potassium iodobismuthate TS*: an orange or orange-red precipitate is produced immediately.

Aluminium Salts

A. Solutions of aluminium salts yield with *ammonia TS* a gelatinous, white precipitate which does not dissolve upon the addition of excess *ammonia TS*. *Sodium hydroxide TS* or *sodium sulfide TS* produces the same precipitate which dissolves in an excess of either of these reagents.

B. To a solution of aluminium salts, add *ammonia TS* until a faint precipitate is produced, and then add 5 drops of a freshly prepared 0.05 per cent w/v solution of *quinalizarin* in a 1 per cent w/v solution of *sodium hydroxide*. Heat to boiling, cool, and acidify with an excess of 5 M *acetic acid*: a reddish violet colour is produced.

Amines, Primary Aromatic

Acidify the prescribed solution with 2 M *hydrochloric acid*, or dissolve 100 mg in 2 ml of 2 M *hydrochloric acid*, and add 4 drops of *sodium nitrite TS*. After 1 to 2 minutes add the solution to 1 ml of 2-*naphthol TS*: an intense orange or red colour, usually together with a precipitate of the same colour, is produced.

Amino Acids

To 5 ml of a 0.1 per cent w/v solution of the test substance add 1 ml of *ninhydrin TS*. After heating for 3 minutes, add 20 ml of *water* and allow to stand for 15 minutes: a blue-purple colour develops.

Ammonium Salts

Ammonium salts are decomposed by the addition of an excess of *sodium hydroxide TS*, with the evolution of ammonia, recognizable by its odour and by its alkaline effect upon moistened *red litmus paper* exposed to the vapour. Warming the solution accelerates the decomposition.

Antimony Compounds

Dissolve with gentle heating 10 mg of the substance being examined in a solution of 500 mg of *potassium sodium tartrate* in 10 ml of *water* and allow to cool. To 2 ml of this solution or to 2 ml of the prescribed solution add *sodium sulfide TS1* dropwise: an orange-red precipitate is produced which dissolves on addition of 2 M *sodium hydroxide*.

Arsenic Compounds

Heat 5 ml of the prescribed solution on a water-bath with 5 ml of *hypophosphorous TS*: a brown precipitate is produced.

Barbiturates, Non-nitrogen Substituted

Dissolve 5 mg of the substance being examined in 3 ml of *methanol*, add 2 drops of a solution containing 10 per cent w/v of *cobalt(II) nitrate* and 10 per cent w/v of *calcium chloride*, mix and add, with shaking, 2 drops of 2 M *sodium hydroxide*: a violet-blue colour and precipitate are produced.

Barium

With 1 M *sulfuric acid*, solutions of barium salts yield a white precipitate. This precipitate is insoluble in *hydrochloric acid* and in *nitric acid*. Barium salts impart a yellowish green colour to a nonluminous flame that appears blue when viewed through green glass.

Benzoates

A. To 1 ml of a 10 per cent w/v neutral solution of benzoates add 0.5 ml of *iron(III) chloride TS*: a dull yellow precipitate, soluble in *ether*, is produced.

B. Moisten 200 mg of benzoates, treated if necessary as prescribed, with 4 to 6 drops of *sulfuric acid* and gently warm the bottom of the tube: a white sublimate is deposited on the inner walls of the tube.

C. Dissolve 500 mg of benzoates in 10 ml of *water*, or use 10 ml of the prescribed solution and add 0.5 ml of *hydrochloric acid*: the precipitate obtained, after crystallization from *water* and drying in vacuum, melts between 120° and 124° (Appendix 4.3).

Bicarbonates

A. Solutions of bicarbonates, on boiling, liberate carbon dioxide which produces a white precipitate in *calcium hydroxide TS*.

B. Solutions of bicarbonates yield no precipitate with *magnesium sulfate TS* at room temperature, but on boiling the mixture, a white precipitate is produced.

Bismuth Salts

A. To 500 mg of bismuth salts add 10 ml of 2 M *hydrochloric acid* or use 10 ml of the prescribed solution. Boil for 1 minute, cool, filter if necessary and to 1 ml of the resulting solution add 20 ml of *water*: a white or slightly yellow precipitate is produced which turns brown on addition of 1 or 2 drops of s*odium sulfide TS*.

B. To 40 to 50 mg of bismuth salts add 10 ml of 2 M *nitric acid* or use 10 ml of the prescribed solution. Boil for 1 minute, allow to cool, filter if necessary and to 5 ml of the resulting solution add 2 ml of a 10 per cent w/v solution of *thiourea*: an orange-yellow colour or an orange precipitate is produced. Add 4 ml of a 2.5 per cent w/v solution of *sodium fluoride*: the solution is not decolorized within 30 minutes.

Bisulfites

See Sulfites.

Borate

A. To 1 ml of a borate solution, acidified with *hydrochloric acid* to litmus, add 3 or 4 drops of *iodine TS* and 3 or 4 drops of a 2 per cent v/v solution of *polyvinyl alcohol*: an intense blue colour is produced.

B. When a borate is treated with *sulfuric acid*, *methanol* is added, and the mixture is ignited, it burns with a green-bordered flame.

Bromides

A. Solutions of bromides yield, with *silver nitrate TS*, a yellowish white precipitate, somewhat soluble in *strong ammonia solution* but slightly soluble in *ammonia TS* and in *nitric acid*.

B. Solutions of bromides, upon the addition of *chlorine TS*, dropwise, liberate bromine, which is dissolved by shaking with *chloroform*, colouring the chloroform red to reddish brown.

Calcium Salts

A. To 0.2 ml of a neutral solution containing the equivalent of about 0.04 mg of calcium or to 0.2 ml of the prescribed solution, add 0.5 ml of a 0.2 per cent w/v solution of *glyoxal bis(2-hydroxyanil)* in *ethanol*, 4 drops of 2 M *sodium hydroxide*, and 4 drops of 1 M *sodium carbonate*. Extract with 1 to 2 ml of *chloroform* and add 1 to 2 ml of *water*: the chloroform layer is red.

B. Dissolve 20 mg of calcium salts or the prescribed quantity in 5 ml of 5 M *acetic acid*. Add 0.5 ml of *potassium hexacyanoferrate(II) TS*: the solution remains clear. Add 50 mg of *ammonium chloride*: a white crystalline precipitate forms.

C. To a solution of calcium salts add a few drops of a solution of *ammonium oxalate*: a white precipitate is obtained that is only sparingly soluble in 6 M *acetic acid* but is soluble in *hydrochloric acid*.

D. Moisten calcium salts with *hydrochloric acid* and introduce on a platinum wire into an open flame: a brick-red colour develops.

Carbonates

A. Carbonates effervesce with dilute acids, liberating carbon dioxide: the gas is colourless and produces a white precipitate in *calcium hydroxide TS* (common with bicarbonates).

B. Solutions of carbonates produce, at room temperature, a white precipitate with *magnesium sulfate TS* (distinction from bicarbonates).

Chlorides

A. Solutions of chlorides yield with *silver nitrate TS* a white, curdy precipitate, which is insoluble in *nitric acid*, but is soluble in *ammonia TS*.

B. Dry chlorides, when mixed with an equal weight of *manganese(IV) oxide*, moistened with *sulfuric acid*, and gently heated, evolve chlorine which is recognizable by its odour and by the production of a blue colour with moistened *starch-iodide paper*. Carry out the reaction preferably under a hood.

Citrates

A. Upon the addition of a few mg of a citrate to a mixture of 15 ml of *pyridine* and 5 ml of *acetic anhydride*: a carmine-red colour is produced.

B. Neutral solutions of citrates, when treated with *calcium chloride TS*, do not yield a precipitate; on boiling they yield a white precipitate which is soluble in *acetic acid*.

C. Solutions of citrates, when boiled with an excess of *mercury(II) sulfate TS* and filtered if necessary, yield a solution which, after being boiled and treated with a few drops of *potassium permanganate TS*, decolorizes the reagent and yields a white precipitate.

Copper Salts

A. Solutions of copper(II) compounds, acidified with *hydrochloric acid*, deposit a red film of metallic copper upon a bright, untarnished surface of *metallic iron*.

B. An excess of *ammonia TS*, added to a solution of a copper(II) salt, produces first a bluish precipitate and then a deep blue-coloured solution.

C. With *potassium hexacyanoferrate(II) TS*, solutions of copper(II) salts yield a reddish brown precipitate, insoluble in diluted acids.

Esters

To 30 mg of the test substance or to the prescribed quantity add 0.5 ml of a 7 per cent w/v solution of *hydroxylamine hydrochloride* in *methanol* and 0.5 ml of a 10 per cent w/v solution of *potassium hydroxide* in

ethanol. Heat to boiling, cool, acidify with 2 M *hydrochloric acid*, and add 4 drops of a 1 per cent w/v solution of *iron(III) chloride*: a bluish red or red colour is produced.

Iodides

A. Solutions of iodides yield, with *silver nitrate TS*, a yellow, curdy precipitate, practically insoluble in *ammonia TS* and in *nitric acid*.

B. Solutions of iodides, upon the addition of *chlorine TS*, dropwise, liberate iodine, which colours the solution yellow to red. When the solution is shaken with *chloroform*, the latter is coloured violet.

Iron Salts

Iron(II) and iron(III) compounds in solution yield a black precipitate with *ammonium sulfide TS*. This precipitate is dissolved by cold *dilute hydrochloric acid* with the evolution of hydrogen sulfide.

IRON(II) SALTS

A. Solutions of iron(II) salts yield a dark blue precipitate with *potassium hexacyanoferrate(III) TS*. This precipitate is insoluble in *dilute hydrochloric acid*, but is decomposed by *sodium hydroxide TS*.

B. Solutions of iron(II) salts yield with *sodium hydroxide TS* a greenish white precipitate, the colour rapidly changing to green and then to brown when shaken.

IRON(III) SALTS

A. Acid solutions of iron(III) salts yield a dark blue precipitate with *potassium hexacyanoferrate(II) TS*.

B. With an excess of *sodium hydroxide TS*, solutions of iron(III) salts yield a reddish brown precipitate.

C. Solutions of iron(III) salts produce with *ammonium thiocyanate TS*: a deep red colour which is not destroyed by dilute mineral acids.

Lactates

A. Lactates in solution, heated with *dilute sulfuric acid* and *potassium permanganate TS*, evolve acetaldehyde recognizable by its odour.

B. Lactates, treated with *iodine and potassium iodide TS* in the presence of a slight excess of *sodium hydroxide TS*, give a yellowish precipitate of iodoform which, after extraction with *ether* and spontaneous evaporation, is characterized by its crystalline form and its odour: it melts at 118° to 124° (Appendix 4.3).

C. Lactates, shaken with a few drops of *water* and 2 ml of *sulfuric acid* and treated with 2 drops of *guaiacol TS*, slowly give a red colour.

Lead and Lead Compounds

A. To 100 mg of the substance being examined dissolved in 1 ml of 5 M *acetic acid* or to 1 ml of the prescribed solution add 2 ml of *potassium chromate TS*: a yellow precipitate is produced which is soluble in 2 ml of 10 M *sodium hydroxide*.

B. To 50 mg of the substance being examined dissolved in 1 ml of 5 M *acetic acid* or to 1 ml of the prescribed solution add 10 ml of *water* and 4 drops of *potassium iodide TS*: a yellow precipitate is produced. Heat to boiling for 1 to 2 minutes and allow to cool: the precipitate reappears as glistening, yellow plates.

Lignin

A. Lignified cell walls are coloured bright red by soaking them in *phloroglucinol TS* and adding 2 to 4 drops of *hydrochloric acid*.

B. Lignified tissues are coloured yellow by *aniline hydrochloride TS*.

Magnesium

A. Dissolve 10 to 15 mg of magnesium salts in 2 ml of *water* or use 2 ml of the prescribed solution. Add 1 ml of *ammonia TS*: a white precipitate forms that is redissolved by adding 1 ml of 2 M *ammonium chloride*. Add 1 ml of 0.25 M *disodium hydrogenphosphate*: a white crystalline precipitate forms.

B. To 0.5 ml of a neutral or slightly acidic solution of magnesium salts, add 4 drops of a 0.1 per cent w/v solution of *titan yellow* and 0.5 ml of 0.1 M *sodium hydroxide*: a bright red turbidity forms, which gradually settles to give a bright red precipitate.

Manganese Salts

A. Solutions of manganese(II) salts yield, with *ammonium sulfide TS*, an orange-pink precipitate, which dissolves in *acetic acid*.

B. To 100 mg of manganese(II) salts, add 2 g of *lead(IV) oxide* and 5 ml of *nitric acid*, boil gently for a few minutes, add 100 ml of *water* and filter: a purple solution is produced.

Mercury and Mercury Compounds

MERCURY Solutions of mercury salts, free from an excess of nitric acid, when applied to bright copper foil, yield a deposit that, upon rubbing, becomes bright and silvery in appearance. With *hydrogen sulfide*, solutions of mercury compounds yield a black precipitate that is insoluble in *ammonium sulfide TS* and in boiling 2 M *nitric acid*.

MERCURY(I) SALTS Mercury(I) compounds are decomposed by 1 M *sodium hydroxide*, producing a black colour. Solutions of mercury(I) salts yield with *hydrochloric acid* a white precipitate that is blackened by *ammonium TS*. With *potassium iodide TS*, a yellow precipitate, that may become green upon standing, is formed.

MERCURY(II) SALTS Solutions of mercury(II) salts yield a yellow precipitate with 1 M *sodium hydroxide*. They yield also, in neutral solutions with *potassium iodide TS*, a scarlet precipitate that is very soluble in an excess of the reagent.

Nitrates

A. Mix a solution of nitrates with an equal volume of *sulfuric acid*, cool the mixture, and superimpose a solution of *iron(II) sulfate*: a brown colour is produced at the junction of the two liquids.

B. Nitrates heated with *sulfuric acid* and *copper* liberate brownish red fumes.

C. Nitrates do not decolourize acidified *potassium permanganate TS* (distinction from nitrites).

Penicillins

To 2 mg of a penicillin, add 2 mg of *chromotropic acid sodium salt* and 2 ml of *sulfuric acid*. Immerse in an oil-bath at 150°: the solution, when shaken and examined every 30 seconds, exhibits the colours stated in the table below.

Time (Min)	Ampicillin Ampicillin Sodium Ampicillin Trihydrate	Carbenicillin Disodium	Cloxacillin Sodium Dicloxacillin Sodium	Methicillin Sodium	Oxacillin Sodium	Penicillin G Benzathine Penicillin G Potassium Penicillin G Sodium	Penicillin V Potassium
0	Colourless	Colourless	Colourless	Yellow	Colourless	Yellow	Colourless
½	Colourless	Light brown	Pale yellow	Greenish yellow	Greenish yellow	Yellow	Colourless
1	Colourless	Yellowish brown	Greenish yellow	Greenish yellow	Olive-green	Yellow	Colourless
1½	Colourless	Greenish brown	Green	Greenish yellow	Green	Orange-yellow	Pale pink
2	Purple	Greenish brown	Greenish purple	Yellowish green	Greenish purple	Orange-yellow	Purple
2½	Deep purple	Brown	Purple	Yellowish green	Purple	Orange-yellow	Purple
3	Violet	Dark brown	Purple	Yellowish brown	Purple	Pale orange	Bluish violet
3½	Violet	Dark brown	Purple	Yellowish brown	Purple	Orange or brown	Dark blue
4	Charred	Dark brown	Purple	Charred	Purple	Orange or brown	Dark blue
4½	Charred	Dark brown	Purple	Charred	Purple	Orange or brown	Dark blue
5	Charred	Dark brown	Purple	Charred	Charred	Orange or brown	Dark blue

Permanganates

Solutions of permanganates acidified with *sulfuric acid* are decolorized by *hydrogen peroxide TS* and by *sodium metabisulfite TS*, in the peroxide cold, and by *oxalic acid TS*, in hot solution.

Peroxides

Solutions of peroxides slightly acidified with *sulfuric acid* yield a deep blue colour upon the addition of *potassium dichromate TS*. On shaking the mixture with an equal volume of *ether* and allowing the liquids to separate, the blue colour is found in the ether layer.

Phosphates (Orthophosphates)

A. Solutions of phosphates, when neutralized to about pH 7 treated with *silver nitate TS*, yield a light yellow precipitate which is readily soluble in *ammonia TS* and in *dilute nitric acid:*

B. Solutions of phosphates in *dilute nitric acid*, when treated with and equal volume of *ammonium molybdate TS* and warmed, yield a bright canary-yellow precipitate.

Potassium Salts

A. Ignite a small quantity of potassium salts, dissolve the residue in *water*, filter and make alkaline with *sodium hydroxide TS*. Treat the solution so obtained with *sodium tetraphenylborate TS*: a white precipitate is produced.

B. To 1 ml of a 5 per cent w/v solution of potassium salts, add 1 ml of 2 M *acetic acid* and 1 ml of a freshly prepared 10 per cent w/v solution of *sodium hexanitrocobaltate(III)*: a yellow or orange-yellow precipitate forms immediately.

C. Potassium compounds impart a violet colour to a nonluminous flame, but the presence of small quantities of sodium masks the colour unless being viewed through a cobalt-blue glass.

Salicylates

A. To 1 ml of a 10 per cent w/v neutral solution of salicylates, add 0.5 ml of *iron(III) chloride TS*: a violet colour is produced which persists after the addition of 2 drops of 5 M *acetic acid*.

B. Dissolve 500 mg of salicylates in 10 ml of *water* or use 10 ml of the prescribed solution. Add 0.5 ml of *hydrochloric acid*: the precipitate obtained, after recrystallization from hot *water* and drying in vacuum, melts between 156° and 161° (Appendix 4.3).

Silicates

In a lead or platinum crucible mix to a thin slurry using a copper wire the prescribed quantity of the test substance with 10 mg of *sodium fluoride* and 4 drops of *sulfuric acid*. Cover the crucible with a thin, transparent plate of plastic from which a drop of *water* is suspended and warm gently: a white ring is produced around the drop of water within a short time.

Silver Salts

A. Dissolve 10 mg of silver salts in 10 ml of *water* or use 10 ml of the prescribed solution. Add 6 drops of 7 M *hydrochloric acid*: a curdy, white precipitate is produced that is soluble in 3 ml of *ammonia TS*. Add *potassium iodide TS*: a yellow precipitate is produced.

B. A solution of a silver salt to which *ammonia TS* and a small quantity of *formaldehyde solution* are added deposits, upon warming, a mirror of metallic silver on the inner walls of the tube.

Sodium Salts

A. Ignite a small quantity of sodium compounds and dissolve the residue in *dilute acetic acid*, filter, if necessary, and treat with *zinc uranyl(IV) acetate TS*: a yellow crystalline precipitate is formed.

B. Sodium compounds impart an intense yellow colour to a nonluminous flame.

Sulfates

A. Solutions of sulfates yield with *barium chloride TS* a white precipitate, which is insoluble in *hydrochloric acid* and in *nitric acid*.

B. Solutions of sulfates, when treated with *lead(II) acetate TS*, yield a white precipitate, which is soluble in an 8 per cent w/v solution of *ammonium acetate* and in *sodium hydroxide TS*.

Sulfites

When treated with 3 M *hydrochloric acid*, sulfites and bisulfites yield sulfur dioxide, which blackens filter paper moistened with *mercury(II) nitrate TS*.

Tartrates

A. Add a few mg of a tartrate to a mixture of 15 ml of *pyridine* and 5 ml of *acetic anhydride*: an emerald-green colour is produced.

B. To 5 ml of a 0.4 per cent w/v solution of the substance being examined, add 1 drop of a 1 per cent w/v solution of *iron(II) sulfate* and 1 drop of *hydrogen peroxide TS (10 volumes)*: a transient yellow colour is produced. When 2 M *sodium hydroxide* is added dropwise, an intense blue colour is produced.

C. Mix a few ml of *sulfuric acid* with a few drops of a 2 per cent w/v solution of *resorcinol* and a few drops of a 10 per cent w/v solution of *potassium bromide*, and add 2 or 3 drops of the solution being examined. Warm the mixture on a water-bath for 5 to 10 minutes: an intense blue colour is produced that changes to red when the solution is cooled and poured into *water*.

Thiocyanates

With *iron(III) chloride TS* solutions of thiocyanates yield a red colour that is not destroyed by moderately concentrated mineral acids.

Thiosulfates

With *hydrochloric acid* solutions of thiosulfates yield a white precipitate that soon turns yellow, and sulfur dioxide, which blackens filter paper moistened with *mercury(II) nitrate TS*. The addition of *iron(III) chloride TS* to solutions of thiosulfates produces a dark violet colour that quickly disappears.

Xanthines

Mix a few mg of xanthines or the prescribed quantity with 0.1 ml of *hydrogen peroxide TS (100 volumes)* and 6 drops of 2 M *hydrochloric acid*. Heat to dryness on a water bath until a yellowish red residue is produced and add 2 drops of 2 M *ammonia*: the colour of the residue changes to violet-red.

Zinc Salts

A. Solutions of zinc salts, prepared if necessary by the addition of *hydrochloric acid*, when treated with. solutions of alkali hydroxides, yield a white precipitate which is soluble in an excess of the solution of alkali hydroxide; the solution remains clear on the addition of *ammonium chloride TS* but yields a white, flocculent precipitate on the addition of *sodium sulfide TS*.

B. Solutions of zinc salts, when treated with *potassium hexacyanoferrate(II) TS*, yield a white precipitate which is insoluble in *dilute hydrochloric acid*.

C. Solutions of zinc salts, when acidified with *dilute sulfuric acid* and treated with 1 drop of a 0.1 per cent w/v solution of *copper(II) sulfate* and 2 ml of *ammonium mercuri-thiocyanate TS*, yield a violet precipitate.

5.2 LIMIT TESTS

ALUMINIUM

Extract the prescribed solution with successive portions of 20, 20 and 10 ml of a 0.5 per cent w/v solution of *8-quinolinol* in *chloroform* and dilute the combined extracts to 50.0 ml with *chloroform*. Unless otherwise stated in the monograph, use as the blank solution a mixture of 10 ml of *acetate buffer pH 6.0* and 100 ml of *water* treated in the same manner and as the standard solution a mixture of 2.0 ml of *aluminium standard solution (2 ppm Al)*, 10 ml of *acetate buffer pH 6.0* and 98 ml of *water* treated in the same manner. Measure the fluorescence of the test solution and of the standard solution (Appendix 2.4), using an excitation wavelength of 392 nm and a secondary filter with a transmission band centred at 518 nm, or a monochromator set to transmit at this wavelength, and setting the instrument to zero with the blank solution in each case. The fluorescence of the test solution is not greater than that of the standard solution.

HEAVY METALS

This test is provided to demonstrate that the content of metallic impurities that are coloured by sulfide ion, under the specified test conditions, does not exceed the heavy metals limit specified in the individual monograph in terms of parts per million (ppm), or, when the limit exceeds 100 ppm, in terms of a percentage (by weight), of lead in the test substance, as determined by concomitant visual comparison with a control prepared from a lead standard solution.

Method I is used for substances that yield clear, colourless preparations under the specified test conditions. Method II is used for substances that do not yield clear, colourless preparations under the test conditions specified for Method I, or for substances that, by virtue of their complex nature, interfere with the precipitation of metals by sulfide ion, or for fixed and volatile oils.

Reagent

Thioacetamide reagent To 1.0 ml of a 4 per cent w/v solution of *thioacetamide*, add 5 ml of a mixture of 15 ml of 1 M *sodium hydroxide*, 5 ml of *water* and 20 ml of *glycerol*. Heat on a water-bath for 20 seconds, cool, and use immediately.

Method I

Standard Preparation Transfer 10.0 ml of *lead standard solution* (*1 ppm Pb* or *2 ppm Pb*), as prescribed, to a 50-ml comparison tube, add 2.0 ml of the solution prepared from the test as described in the individual monograph, add 2 ml of *acetate buffer pH 3.5*, and mix.

Test Preparation Into a 50-ml comparison tube place 12.0 ml of the solution prepared for the test as directed in the individual monograph, add 2 ml of *acetate buffer pH 3.5*, and mix.

Procedure To each of the tubes containing the standard preparation and the test preparation, add 1.2 ml of thioacetamide reagent, mix, allow to stand for 2 minutes, and view downward over a white surface: the colour of the solution from the test preparation is not darker than that of the solution from the standard preparation.

Method II

Standard Preparation Transfer 10.0 ml of *lead standard solution* (*1 ppm Pb* or *2 ppm Pb*), as prescribed, to a 50-ml comparison tube, add 2.0 ml of solution A (see under test preparation below). Add 2 ml of *acetate buffer pH 3.5* and mix.

Test Preparation Accurately weigh the prescribed quantity of the substance being examined, in a suitable crucible, preferably made of silica, and carefully ignite at a low temperature until the contents are thoroughly charred. The crucible may be loosely covered with a lid during the charring. Add to the contents of the crucible 2 ml of *nitric acid* and 5 drops of *sulfuric acid*, and heat cautiously until white fumes are no longer evolved. Ignite, preferably in a muffle furnace, at 500° to 600°, until the carbon is completely burned off. Cool, add 4 ml of 6 M *hydrochloric acid*, cover, digest on a water-bath for 15 minutes, uncover, and slowly evaporate on a water-bath to dryness. Moisten the residue with 1 drop of *hydrochloric acid*, add 10 ml of hot *water*, and digest for 2 minutes. Add 0.1 ml of diluted *phenolphthalein TS* (1 in 10) and 6 M *ammonia* dropwise, until a pink colour is obtained. Cool, add *glacial acetic acid* until the solution is decolorized and add a further 0.5 ml. Filter if necessary, and dilute the solution to 20.0 ml with *water* (solution A). To 12.0 ml of solution A add 2 ml of *acetate buffer pH 3.5* and mix.

Procedure To each of the tubes containing the standard preparation and the test preparation, add 1.2 ml of thioacetamide reagent, mix, allow to stand for 2 minutes, and view downward over a white surface: the colour of the solution from the test preparation is not darker than that of the solution from the standard preparation.

Method III

Standard Preparation Prepare a standard solution similar to solution A as described under test preparation, but using the prescribed volume of *lead standard solution (10 ppm Pb)* in place of the test substance. Transfer 10.0 ml of the resulting solution to a 50-ml comparison tube and add 2.0 ml of solution A. Add 2 ml of *acetate buffer pH 3.5* and mix.

Test Preparation Place the prescribed quantity of the test substance in a silica crucible with 4 ml of a 25 per cent w/v solution of *magnesium sulfate* in 1 M *sulfuric acid*. Mix using a fine glass rod, and heat cautiously. If the mixture is liquid, evaporate gently to dryness on a water-bath. Progressively heat to ignition, not allowing the temperature to exceed 800°, and continue heating until a white or greyish residue is obtained. Allow to cool, moisten the residue with a few drops of 1 M *sulfuric acid*, evaporate, ignite again, and allow to cool. The total period of ignition should not exceed 2 hours. Dissolve the residue using two 5-ml portions of 2 M *hydrochloric acid*. Add 0.1 ml of *dilute phenolphthalein TS* and 13.5 M *ammonia* until a pink colour is obtained. Cool, add *glacial acetic acid* until the solution is decolorized and add a further 0.5 ml. Filter if necessary, and dilute the solution with *water* to 20.0 ml (solution A). To 12.0 ml of solution A, add 2 ml of *acetate buffer pH 3.5* and mix.

Procedure To each of the tubes containing the standard preparation and the test preparation, add 1.2 ml of thioacetamide reagent, mix, allow to stand for 2 minutes, and view downward over a white surface: the colour of the solution from the test preparation is not darker than that of the solution from the standard preparation.

AMMONIUM

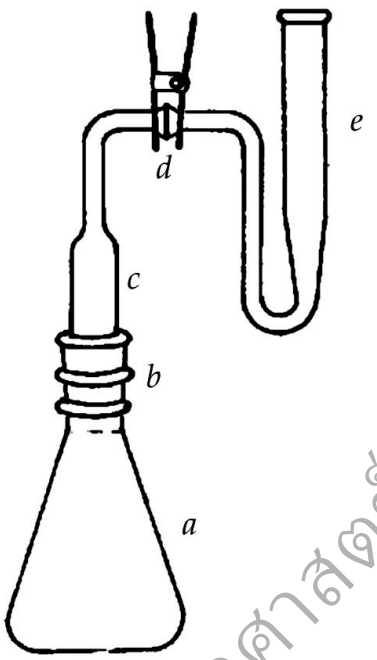
Dissolve the prescribed quantity of the test substance in 14 ml of *water* in a test-tube, if necessary make alkaline with 2 M *sodium hydroxide* and dilute to 15.0 ml with *water*. Add 0.3 ml of *alkaline mercuric-potassium iodide TS*, stopper the tube, mix, and allow to stand for 5 minutes. Any yellow colour produced is not more intense than that obtained by treating a mixture of 10.0 ml of *ammonium standard solution (1 ppm NH_4)* and 5.0 ml of *water* in the same manner, unless otherwise specified.

ARSENIC

This photometric procedure, based upon the reaction between silver diethyldithiocarbamate and arsine, is provided to demonstrate that the content of arsenic does not exceed the limit given in the individual monograph.

Apparatus

The apparatus (see illustration) consists of an arsine generator (*a*) fitted with a scrubber unit (*c*) and an absorber tube (*e*) with standard-taper or ground glass ball-and-socket joints (*b* and *d*) between the units. However, any other suitable apparatus, embodying the principle of the assembly described and illustrated, may be used.



Arsenic Test Apparatus

Standard Preparation

Transfer 3.0 ml of *arsenic standard solution* (*1 ppm As*) to the generator flask (*a*) and add *water* to make 35 ml.

If the test preparation was prepared as directed in the general procedure for organic compounds, mix the portion of *arsenic standard solution* (*1 ppm As*) with 2 ml of *sulfuric acid* and the total amount of *hydrogen peroxide TS* (*100 volumes*) used in the oxidation of the test specimen. Then heat the mixture to strong fuming. Cool, add cautiously 10 ml of *water*, again evaporate to strong fuming, and cool. Dilute with *water* to 35 ml.

Test Preparation

Caution Some substances may react with explosive violence when digested with hydrogen peroxide. Exercise safety precautions at all times.

(Note If halogen-containing compounds are present, use a lower temperature while heating the substance to be examined with *sulfuric acid*. Avoid boiling the mixture and add the hydrogen peroxide with caution, before charring begins, to prevent loss of trivalent arsenic.)

Test preparations of inorganic compounds are prepared as directed in the individual monograph.

Test preparations of organic compounds are prepared according to the following general procedure, unless otherwise directed in the individual monograph.

Transfer the prescribed quantity of the test substance to a 125-ml Kjeldahl flask. Add 5 ml of *sulfuric acid* and a few glass beads, and digest in a fume hood, preferably on a hot plate, at a temperature not exceeding 120° until charring begins. (Additional *sulfuric acid* may be necessary to wet some specimens completely, but the total volume added should not exceed 10 ml.) After the test substance has been initially decomposed by the acid, cautiously add, dropwise, *hydrogen peroxide TS* (*100 volumes*), allowing the reaction to subside and again heating between drops. Add the first few drops very slowly with sufficient mixing, in order to prevent a rapid reaction. Discontinue heating if foaming becomes excessive. When the reaction has abated, heat cautiously, rotating the flask occasionally to prevent the specimen from caking on glass exposed to the heating unit. **Maintain oxidizing conditions at all time during the digestion by adding small quantities of the hydrogen peroxide solution whenever the mixture turns brown or darkens.** Continue the digestion until the organic matter is destroyed. Gradually raise the temperature of the hot plate to between 250° and 300°, until fumes of sulfur trioxide are copiously evolved and the solution becomes colourless or retains only a light straw colour. Cool, add cautiously 10 ml of *water*, mix, and again evaporate to strong fuming. Repeat this procedure, if necessary, to remove any trace of hydrogen peroxide. Cool, add cautiously 10 ml of *water*, and transfer quantitatively to the generator flask. Dilute with *water* to 35 ml.

Procedure

To each of the generator flasks containing the standard preparation and the test preparation, add 20 ml of diluted *sulfuric acid* (1 in 5), 2 ml of *potassium iodide TS* (16.5 per cent w/v), and 0.5 ml of *stronger acid tin(II) chloride TS* and mix. Allow to stand at room temperature for 30 minutes. Pack each scrubber unit (*c*) with two pledgets of cotton that have been soaked in saturated *lead acetate* solution, freed from excess solution by expression, and dried in vacuum at room temperature, leaving a small space between the two pledgets. Lubricate the joints (*b* and *d*) with a suitable stopcock grease (designed for use with organic solvents), and connect each scrubber unit to its absorber tube (*e*). Transfer 3.0 ml of *silver diethyldithiocarbamate TS* to each absorber tube. Alternatively, a larger accurately measured volume of the *silver diethyldithiocarbamate TS* may be employed, provided that the same volume is used for the control test and that the apparatus will accommodate the larger volume. Add 3.0 g of *granulated zinc* to

the mixture in each flask and immediately connect the assembled scrubber unit. Place the generator flasks in a water-bath, maintained at a temperature of 25°±3°, and allow the evolution of hydrogen and the colour development to proceed for 45 minutes, swirling the flasks gently at 10-minute intervals. (If necessary, to obtain a more uniform rate of gas evolution, 1 ml of *2-propanol* may be added to each generator.) Disconnect each absorber tube from the generator and scrubber units, and transfer the absorbing solutions to the 1-cm absorption cells. Determine the absorbances of the solutions at the wavelength of maximum absorbance between 535 and 540 nm, with a suitable spectrophotometer, using *silver diethyldithiocarbamate TS* as the blank. The absorbance due to any red colour from the Test Preparation does not exceed that produced by the Standard preparation.

Interfering Chemicals

Metals or salts of metals such as chromium, cobalt, copper, mercury, molybdenum, nickel, palladium, and silver may interfere with the evolution of arsine. Antimony, which forms stibine, produces a positive interference in the colour development with *silver diethyldithiocarbamate TS*; when the presence of antimony is suspected, the red colours produced in the two silver diethyldithiocarbamate solutions may be compared at the wavelength of maximum absorbance between 535 and 540 nm, since at this wavelength the interference due to stibine is negligible.

CALCIUM

The solutions used for this test should be prepared with *distilled water*. To 0.20 ml of *ethanolic calcium standard solution* (*100 ppm Ca*) add 1.0 ml of a 4.0 per cent w/v solution of *ammonium oxalate*. After 1 minute add 1.0 ml of 2 M *acetic acid* and 15.0 ml of the test solution prepared as directed in the individual monograph, and shake. After 15 minutes any opalescence produced is not more intense than that of a standard prepared in the same manner using a mixture of 10.0 ml of *calcium standard solution* (*10 ppm Ca*) and 5.0 ml of *water* in place of the test solution.

CHLORIDE

Dissolve the specified quantity of the substance in 30 to 40 ml of *water*, or, when the substance is already in solution, add *water* to make a total volume of 30 to 40 ml; or prepare a solution as directed in the monograph, and transfer to a comparison tube. Add 1 ml of *nitric acid*, except where nitric acid is used in the preparation of the solution. If, after acidification, the solution is not perfectly clear, filter it through a filter paper that gives negative test for chloride. Add 1 ml of *silver nitrate TS*, and sufficient *water* to make 50 ml. Mix, allow to stand for 5 minutes protected from direct sunlight. The opalescence produced is not greater than the standard opalescence, when viewed transversely.

Standard Opalescence

Measure the volume of 0.020 M *hydrochloric acid*, as directed in the individual monograph, into another comparison tube, dilute to a volume of 30 to 40 ml with *water*, add 1 ml of *nitric acid*, 1 ml of *silver nitrate TS*, and sufficient *water* to make 50 ml. Mix well, and allow to stand for 5 minutes.

FLUORIDE

(**Note** Use plasticware throughout this test.)

Reagent

pH 5.25 buffer Dissolve 110 g of *sodium chloride* and 1 g of *sodium citrate dihydrate* in 700 ml of *water* in a 2000-ml volumetric flask. Cautiously add 150 g of *sodium hydroxide*, and dissolve with shaking. Cool to room temperature, and while stirring, cautiously add 450 ml of *glacial acetic acid* to the cooled solution. Cool, add 600 ml of *2-propanol*, dilute with *water* to volume, and mix: the pH of this solution is between 5.0 and 5.5.

Standard Stock Solution

Transfer 221 mg of *sodium fluoride*, previously dried at 150° for 4 hours, to a 100-ml volumetric flask, add 20 ml of *water* and mix to dissolve. Add 1.0 ml of 0.01 M *sodium hydroxide*, dilute with *water* to volume, and mix. Each ml of this solution contains 1 mg of fluoride ions. Store in a tightly closed, plastic container.

Standard Preparation

Dilute portions of the standard stock solution quantitatively and stepwise with pH 5.25 buffer to obtain 100-ml solutions having concentrations of 1, 3, 5, and 10 µg per ml.

Test Preparation

Unless otherwise specified in the monograph, prepare a test preparation as follows.

Transfer 1.0 g of the test substance, accurately weighed, to a 100-ml volumetric flask, dissolve in pH 5.25 buffer, dilute with pH 5.25 buffer to volume, and mix.

Procedure

Concomitantly measure the potential in millivolts, of the standard preparations and of the test preparation, with a pH meter capable of a minimum reproducibility of ±0.2 mV, equipped with a glass-sleeved calomel-fluoride specific-ion electrode system. (**Note** When taking measurements, immerse the electrodes in the solution, which has been transferred to a 150-ml beaker containing a polytetrafluoroethylene-coated stirring bar. Allow to stir on a magnetic stirrer having an insulated top (or a thin asbestos sheet to prevent heat transfer) until equilibrium is attained (1 to 2 minutes), and record the potential. Rinse and dry the electrodes between measurements, being careful to avoid damaging the crystals of the specific-ion electrode.) Plot the logarithm

of the fluoride-ion concentrations, in µg per ml, of the standard preparations versus the potential in millivolts. From the measured potential of the test preparation and the standard curve, determine the concentration, in µg per ml, of fluoride in the test preparation.

IRON

Reagent

Ammonium thiocyanate solution Dissolve 30 g of *ammonium thiocyanate* in *water* to make 100 ml.

Standard Preparation

Measure 1.0 ml of *iron standard solution* (*10 ppm Fe*) (Appendix 1.3) into a 50-ml comparison tube, add 40 ml of *water* and 2 ml of *hydrochloric acid*. Dilute with *water* to 50 ml, mix.

Test Preparation

Use the solution prepared as directed in the test for Iron in the individual monograph, and transfer it to a 50-ml comparison tube. Dilute with *water* to 50 ml, mix.

Procedure

To each of the tubes containing the standard preparation and the test preparation add 50 mg of *ammonium peroxydisulfate* crystals and 3 ml of ammonium thiocyanate solution, and mix, the colour of the solution from the test preparation is not darker than that of the solution from the standard preparation.

LEAD

The imposition of stringent limits on the amounts of lead that may be present in pharmaceutical products has resulted in the use of two methods, of which the one set forth following depends upon extraction of lead by solutions of dithizone. For determination of the content of heavy metals generally, expressed as a lead equivalent, see "Limit Test for Heavy Metals" (Appendix 5.2).

Select all reagents for this test to have as low a content of lead as practicable, and store all reagent solutions in containers of borosilicate glass. Rinse thoroughly all glassware with a warm 50 per cent v/v solution of *nitric acid*, followed by *water*.

Reagents

Ammonia-cyanide solution Dissolve 2 g of *potassium cyanide* in 15 ml of *strong ammonia solution*, and dilute with *water* to 10 ml.

Ammonium citrate solution Dissolve 40 g of *citric acid* in 90 ml of *water*. Add 2 or 3 drops of *phenol red TS*, then cautiously add *strong ammonia solution* until the solution acquires a reddish colour. Remove any lead that may be present by extracting the solution with 20-ml portions of dithizone extraction solution, until the dithizone solution retains its green colour.

Diluted lead standard solution Dilute an accurately measured volume of *lead standard solution* (*10 ppm Pb*) with 9 volumes of a 1 per cent v/v solution of *nitric acid* to obtain a solution that contains 1 µg of lead per ml.

Dithizone extraction solution Dissolve 30 mg of *dithizone* in 1000 ml of *chloroform*, and add 5 ml of *ethanol*. Store the solution in a refrigerator.

Before use, shake a suitable volume of the dithizone extraction solution with about half its volume of a 1 per cent v/v solution of *nitric acid*, discarding the nitric acid.

Hydroxylamine hydrochloride solution Dissolve 20 g of *hydroxylamine hydrochloride* in sufficient *water* to make approximately 65 ml. Transfer to a separator, add 5 drops of *thymol blue TS*, then add *strong ammonia solution* until the solution assumes a yellow colour. Add 10 ml of a 4 per cent w/v solution of *sodium diethyldithiocarbamate*, mix, and allow to stand for 5 minutes. Extract this solution with successive 10-to 15-ml portions of *chloroform* until a 5-ml portion of the chloroform extract does not assume a yellow colour when shaken with *copper(II) sulfate TS*. Add 3 M *hydrochloric acid* until the solution is pink (if necessary, add 1 or 2 drops more of *thymol blue TS*), and then dilute with *water* to 100 ml.

Potassium cyanide solution Dissolve 50 g of *potassium cyanide* in sufficient *water* to make 100 ml. Remove the lead from this solution by extraction with successive portions of dithizone extraction solution, as described under ammonium citrate solution above, then extract any dithizone remaining in the cyanide solution by shaking with *chloroform*, finally dilute the cyanide solution with sufficient *water* so that each 100 ml contains 10 g of potassium cyanide.

Standard dithizone solution Dissolve 10 mg of *dithizone* in 1000 ml of *chloroform*. Keep the solution in a glass-stoppered, lead-free bottle, suitably wrapped to protect it from light and store in a refrigerator.

Test Preparation

Unless otherwise specified in the monograph, prepare a test preparation as follows:

> ***Caution*** Exercise safety precautions in this procedure, as some substances may react with explosive violence when digested with hydrogen peroxide.

Transfer 1.0 g of the substance under test to a suitable flask, add 5 ml of *sulfuric acid* and a few glass beads, and digest on a hot plate in a hood until charring begins. Other suitable means of heating may be substituted. (Add additional sulfuric acid if necessary, to wet the substance completely, but do not add more than a total of 10 ml.) After the substance has been initially decomposed by the acid, add dropwise and with caution, *strong hydrogen peroxide solution*, allowing the reaction to subside and again heating between drops. Add the first few drops very slowly, mix carefully to prevent a rapid reaction, and discontinue heating if foaming becomes excessive. Swirl the solution in the

flask to prevent unreacted substance from caking on the walls of the flask. (**Note** Add peroxide whenever the mixture turns brown or darkens.) Continue the digestion until the substance is completely destroyed, copious fumes of sulfur trioxide are evolved, and the solution is colourless. Cool, cautiously add 10 ml of *water*, evaporate until sulfur trioxide again is evolved, and cool.

Procedure

Transfer the test preparation or the volume of the prepared sample specified in the monograph to a separator and, unless otherwise directed in the monograph, add 6 ml of ammonium citrate solution and 2 ml of hydroxylamine hydrochloride solution. (For the determination of lead in iron salts use 10 ml of ammonium citrate solution.) Add 2 drops of *phenol red TS*, and make the solution just alkaline (red in colour) by the addition of *strong ammonia solution*. Cool the solution if necessary, and add 2 ml of potassium cyanide solution. Immediately extract the solution with 5-ml portions of dithizone extraction solution, draining off each extract into another separator, until the dithizone solution retains its green colour. Shake the combined dithizone solutions for 30 seconds with 20 ml of a 1 per cent w/v solution of *nitric acid*, and discard the chloroform layer. Add to the acid solution 5.0 ml of standard dithizone solution and 4 ml of ammonia-cyanide solution, and shake for 30 seconds: the colour of the chloroform layer is of no deeper shade of violet than that of a control made with a volume of diluted lead standard solution equivalent to the amount of lead permitted in the sample under examination, and with the same quantities of the same reagents and in the same manner as in the test with the sample.

SELENIUM

Reagent

Diaminonaphthalene solution Dissolve 100 mg of *2,3-diaminonaphthalene* and 500 mg of *hydroxylamine hydrochloride* in 0.1 M *hydrochloric acid* to make 100 ml. Prepare this solution freshly on the day of use.

Standard Preparation

Pipette 6 ml of *selenium standard solution* (*1 ppm Se*) into a 150-ml beaker, and add 50 ml of a 1.6 per cent v/v solution of *nitric acid*.

Test Preparation

Using a 1000-ml combustion flask and using 25 ml of a 3.3 per cent v/v solution of *nitric acid* as the absorbing liquid, proceed as directed under "Oxygen Flask Combustion" (Appendix 6.2). Upon completion of the combustion, place a few ml of water in the cup, loosen the stopper, and rinse the stopper, the sample holder, and the sides of the flask with about 10 ml of *water*. Transfer the solution with the aid of about 20 ml of *water* to a 150-ml beaker, and heat gently to the boiling temperature. Boil for 10 minutes, and allow the solution to cool to room temperature.

Procedure

Treat the standard preparation, the test preparation, and the reagent blank consisting of 50 ml of a 1.6 per cent v/v solution of *nitric acid*, concomitantly and in parallel, as follows. Add a 50 per cent v/v solution of *strong ammonia solution* to adjust to a pH of 2.0±0.2. Dilute with *water* to 60.0 ml, and transfer to a low-actinic separator with the aid of 10.0 ml of *water*, adding the 10.0 ml of rinsings to the separator. Add 200 mg of *hydroxylamine hydrochloride*, swirl to dissolve, insert the stopper and swirl to mix. Allow the solution to stand at room temperature for 100 minutes. Add 5.0 ml of s*cyclohexane*, shake vigorously for 2 minutes, and allow the layers to separate. Discard the aqueous layer, and centrifuge the cyclohexane extract to remove any traces of water. Determine the absorbance of each solution in a 1-cm cell at the wavelength of maximum absorbance at about 380 nm, with a suitable spectrophotometer, using *cyclohexane* as the blank, and compare the absorbances: the absorbance of the test preparation is not greater than of the standard preparation where a 200-mg test sample has been taken, or is not more than one-half that of the standard preparation where a 100-mg test sample has been taken.

SULFATE

Dissolve the specified quantity of the substance in 30 to 40 ml of *water*, or where the substance is already in solution, add *water* to make a total volume of 30 to 40 ml; or prepare a solution as directed in the monograph, and transfer to a comparison tube. Add 1 ml of *dilute hydrochloric acid*, except where hydrochloric acid is used in the preparation of the solution. If, after acidification, the solution is not perfectly clear, filter it through a filter paper that gives negative test for sulfate. Add 3 ml of *barium chloride TS*, and sufficient *water* to make 50 ml. Mix, allow to stand for 10 minutes. The turbidity produced is not more than the standard turbidity, when viewed transversely.

Standard Turbidity

Measure the volume of 0.010 M *sulfuric acid*, as directed in the individual monograph, into another comparison tube, dilute to a volume of 30 to 40 ml with *water*, add 1 ml of *dilute hydrochloric acid*, 3 ml of *barium chloride TS*, and sufficient *water* to make 50 ml. Mix well, and allow to stand for 10 minutes.

5.3 DETERMINATION OF SULFATED ASH

Use Method I unless otherwise directed.

Method I

Heat a platinum dish to redness for 10 minutes, allow to cool in a desiccator and weigh. Place about 1 g of the substance, or the quantity specified in the monograph, accurately weighed, in the dish, moisten with *sulfuric acid*, heat gently until white fumes are no longer evolved, again moisten with *sulfuric acid* and ignite at 800°±25° until the carbon is consumed. Allow the crucible to cool in a desiccator over *self-indicating silica gel*, weigh it again and calculate the weight of the residue. If the weight of residue so obtained exceeds the prescribed limit, repeat the moistening with *sulfuric acid* and ignition, as previously, to constant weight, unless otherwise prescribed.

Method II

Ignite a suitable crucible (silica, platinum, porcelain or quartz) at 600°±50° for 30 minutes, allow to cool in a desiccator over *self-indicating silica gel* and weigh. Place the prescribed amount of the substance in the crucible and weigh. Moisten the substance with a small amount of *sulfuric acid* (usually 1 ml) and heat gently at temperature as low as practicable until the sample is thoroughly charred. After cooling, moisten the residue with a small amount of *sulfuric acid*, heat gently until white fumes are no longer evolved and ignite at 600°±50° until the residue is completely incinerated. Ensure that flames are not produced at any time during the procedure. Allow the crucible to cool in a desiccator over *self-indicating silica gel*, weigh it again and calculate the weight of the residue. If the weight of residue so obtained exceeds the prescribed limit, repeat the moistening with *sulfuric acid* and ignition, as previously, to constant weight, unless otherwise prescribed.

5.4 ACID VALUE

The acid value is the number of mg of potassium hydroxide required to neutralize the free acid in 1 g of the substance, when determined by the following method, unless otherwise stated in the monograph.

Procedure

Place about 10 g of the substance, accurately weighed, or the quantity specified in the monograph, in a 250-ml flask, and add 50 ml of a mixture of equal volumes of *ethanol* and *ether* which has been neutralized after the addition of 1 ml of *dilute phenolphthalein TS*. If necessary, heat with caution until the substance is completely dissolved; titrate with 0.1 M *potassium hydroxide VS*, shaking constantly until a pink colour which persists for 15 seconds is obtained. Calculate the acid value from the equation:

$$\text{Acid Value} = \frac{56.11MV}{W}$$

where 56.11 = the molecular weight of potassium hydroxide,
V = the volume, in ml, of 0.1 M *potassium hydroxide VS*,
M = the exact molarity of the potassium hydroxide, and
W = the weight, in g, of the test substance.

5.5 ESTER VALUE

The ester value is the number of mg of potassium hydroxide required to saponify the esters present in 1 g of the substance. If the Saponification Value and the Acid Value have been determined, the difference between these two represents the Ester Value.

Determine the "Acid Value" (Appendix 5.4) of the substance and the "Saponification Value" (Appendix 5.7). Calculate the ester value from the equation:

$$\text{Ester Value} = \text{Saponification Value} - \text{Acid Value}.$$

5.6 IODINE VALUE

The iodine value represents the number of g of iodine absorbed, under the prescribed conditions, by 100 g of the substance. Unless otherwise specified in the individual monograph, determine the Iodine Value by Method I and use the following quantities of the substance for both methods:

Presumed Iodine Value	Quantity of Substance (g)±0.001
<5	3.000
5 to 20	1.000
21 to 50	0.400
51 to 100	0.200
101 to 150	0.130
151 to 200	0.100

Method I

Transfer an accurately weighed quantity of the substance, as determined from the accompanying table, into a 250-ml iodine flask, dissolve it in 10 ml of *chloroform*, add 25.0 ml of *iodine bromide TS*, insert the stopper in the vessel securely, and allow it to stand for 30 minutes protected from light, with occasional shaking. Then add, in the order named, 30 ml of *potassium iodide TS* and 100 ml of *water*, and titrate the liberated iodine with 0.1 M *sodium thiosulfate VS*, shaking thoroughly after each addition of thiosulfate. When the iodine colour becomes quite pale, add 3 ml of *starch TS*, and continue the titration with 0.1 M *sodium thiosulfate VS* until the blue colour is discharged. At the same time, perform a blank test in exactly the same manner (Residual Titrations, Appendix 6.17).

Calculate the iodine value from the equation:

$$\text{Iodine Value} = \frac{126.9(b-a)M}{10W}$$

where 126.9 = the atomic weight of iodine,
b = the volumes, in ml, of 0.1 M *sodium thiosulfate VS* consumed by the blank,
a = the volumes, in ml, of 0.1 M *sodium thiosulfate VS* consumed by the actual test,
M = the exact molarity of the sodium thiosulfate VS, and
W = the weight, in g, of the substance.

(**Note** If more than half of the iodine bromide TS is absorbed by the portion of the substance taken, repeat the determination, using a smaller portion of the substance under examination.)

Method II

Preparation of the sample Melt the substance, if it is not already liquid. (**Note** The temperature during melting should not exceed the melting point of the substance by more than 10°.) Pass through two pieces of filter paper to remove any solid impurities and the last traces of moisture. The filtration may be performed in a hot air oven at 100° but should be completed within 5 minutes ±30 seconds. The substance must be absolutely dry. All glassware must be absolutely clean and completely dry. After filtration, allow the filtrate to achieve a temperature of 68° to 72° befores weighing the substance.

Procedure Immediately weigh the sample into a 500-ml iodine flask, using the weights and weighing accuracy noted in the accompanying table. **(Note** The weight of the substance must be such that there will be an excess of iodochloride TS of 50 to 60 per cent of the amount added, that is, 100 to 150 per cent of the amount absorbed.) Add 15 ml of a fresh mixture of *cyclohexane* and *glacial acetic acid* (1:1), and swirl to dissolve the sample. Add 25.0 ml of *iodinechloride TS*, insert the stopper securely in the flask, and swirl to mix. Allow it to stand at 25°±5°, protected from light, with occasional shaking, for 1.0 or 2.0 hours, depending on the Iodine Value (IV) of the sample: IV less than 150, 1.0 hour; IV equal to or more than 150, 2.0 hours. Then, within 3 minutes after the indicated reaction time, add, in the order named, 20 ml of *potassium iodide TS* and 150 ml of recently boiled and cooled *water*, and mix. Within 30 minutes, titrate the liberated iodine with 0.1 M *sodium thiosulfate VS*, while stirring by mechanical means after each addition of thiosulfate. When the yellow iodine colour has almost disappeared, add 1 to 2 ml of *starch TS*, and continue the titration with 0.1 M *sodium thiosulfate VS* until the blue colour is discharged. At the same time, perform a blank test in exactly the same manner (Residual Titrations, Appendix 6.17). Calculate the iodine value from the equation as described under Method I.

5.7 SAPONIFICATION VALUE

The saponification value is the number of mg of potassium hydroxide required to neutralize the free acids and saponify the esters contained in 1.0 g of the substance.

Procedure

Place about 2 g of the substance, accurately weighed, or the quantity specified in the monograph, in a 250-ml, conical, borosilicate glass flask. Add 25.0 ml of 0.5 M *ethanolic potassium hydroxide* and a few glass beads, attach a reflux condenser and heat in a boiling water-bath for 30 minutes to 1 hour until the substance is completely saponified. This is shown by the formation of a clear, homogeneous solution which does not change on addition of *water*. Add 1 ml of *phenolphthalein TS* and immediately titrate (while the solution is still hot) the excess potassium hydroxide with 0.5 M *hydrochloric acid VS*. At the same time, perform a blank test in exactly the same manner (Residual Titrations, Appendix 6.17).

Calculate the saponification value from the expression:

$$\text{Saponification Value} = \frac{56.11(b-a)M}{W}$$

where 56.11 = the molecular weight of potassium hydroxide,
b = the volumes, in ml, of 0.5 M *hydrochloric acid VS* consumed by the blank,
a = the volumes, in ml, of 0.5 M *hydrochloric acid VS* consumed by the actual test,
M = the exact molarity of hydrochloric acid VS, and
W = the weight, in g, of the substance.

5.8 UNSAPONIFIABLE MATTER

The term "unsaponifiable matter" refers to those substances present in oils or fats that are not saponified by alkali hydroxides and are extractable into ether. The unsaponifiable matter is the percentage content, w/w, of the extracted material not volatile at 100° to 105°.

Use Method I unless otherwise indicated in the monograph. Use ungreased ground-glass glassware for each method.

Method I

To 2.0 to 2.5 g of the substance being examined in a 250-ml flask add 25 ml of 0.5 M *ethanolic potassium hydroxide* and boil under a reflux condenser on a water-bath for 1 hour, swirling frequently. Cool to below 25°, transfer the contents of the flask into a separator with the aid of 50 ml of *water* and extract carefully by shaking

vigorously with three 50-ml portions of *peroxide-free ether*, rinsing the flask with the first portion of ether. Combine the ether extracts in another separator containing 20 ml of *water*. (If the ether solutions contain solid suspended matter, filter them into the separator through a fat-free filter paper and wash the filter paper with *peroxide-free ether*.) Gently rotate the separator for a few minutes without violent shaking, allow the liquids to separate and discard the aqueous layer. Wash the ether solution by shaking vigorously with two 20-ml portions of *water* and then treat with three 20-ml portions of 0.5 M *potassium hydroxide*, shaking vigorously on each occasion, each treatment being followed by washing with 20 ml of *water*. Finally wash with successive 20-ml portions of *water* until the aqueous layer is no longer alkaline to *phenolphthalein TS*. Transfer the ether extract to a weighed flask, rinsing the separator with *peroxide-free ether*, distil off the ether and add 3 ml of *acetone* to the flask. With the aid of a gentle current of air, remove the solvent completely from the flask, which is almost entirely immersed in boiling water and preferably held obliquely and rotated. Dry to constant weight at a temperature not exceeding 80°. Allow to cool in a desiccator and weigh.

Dissolve the residue in 10 ml of freshly boiled *ethanol*, previously neutralized to *phenolphthalein TS*, and titrate with 0.1 M *ethanolic sodium hydroxide VS*.

If the volume of 0.1 M *ethanolic sodium hydroxide* required does not exceed 0.1 ml, the amount of residue weighed is to be taken as the unsaponifiable matter. Calculate the unsaponifiable matter as a percentage of the substance being examined.

If the volume of 0.1 M *ethanolic sodium hydroxide* required exceeds 0.1 ml, the amount of residue weighed cannot be taken as the unsaponifiable matter and the test must be repeated.

Method II

Add 50 ml of 2 M *ethanolic potassium hydroxide* to the prescribed portion of the substance being examined in a 250-ml flask and boil under a reflux condenser on a water-bath for 1 hour, swirling frequently. Cool to below 25°, transfer the contents of the flask into a separator with the aid of 100 ml of *water* and extract carefully with three 100-ml portions of *peroxide-free ether*. Combine the ether extracts in another separator containing 40 ml of *water*, shake gently for a few minutes, allow to separate and discard the aqueous layer. Wash the ether extract with two 40-ml portions of *water* and then with three 40-ml portions of a 3 per cent w/v solution of *potassium hydroxide*, each treatment being followed by washing with 40 ml of *water*. Finally wash the ether extract with successive 40-ml portions of *water* until the aqueous layer is no longer alkaline to *phenolphthalein TS*. Transfer the ether extract to a weighed flask, washing out the separator with *peroxide-free ether*. Distil off the ether with suitable precautions and add 6 ml of *acetone* to the residue. Remove the solvent in a current of air and dry to constant weight at 100° to 105°. Allow to cool in a desiccator and weigh.

Dissolve the residue in 20 ml of *ethanol*, previously neutralized to *phenolphthalein TS*, and titrate with 0.1 M *ethanolic sodium hydroxide VS*.

If the volume of 0.1 M *ethanolic sodium hydroxide* required exceeds 0.2 ml, the separation of the layers has been incomplete; the amount of residue weighed cannot be taken as the unsaponifiable matter and the test must be repeated.

5.9 HYDROXYL VALUE

The hydroxyl value of a substance is the number of mg of potassium hydroxide required to neutralize the acid combined by acylation in 1 g of the substance.

Unless otherwise directed in the individual monograph, use the following quantities of the substance:

Presumed Hydroxyl Value	Quantity of Substance (g)
0 to 20	10
20 to 50	5
50 to 100	3
100 to 150	2
150 to 200	1.5
200 to 250	1.25
250 to 300	1.0
300 to 350	0.75

Procedure

Transfer a quantity of the substance, determined by reference to the accompanying table and accurately weighed, to a glass-stoppered, 250-ml conical flask, and add 5.0 ml of *acetic anhydride-pyridine TS*. Transfer 5.0 ml of *acetic anhydride-pyridine TS* to a second glass-stoppered, 250-ml conical flask to provide the reagent blank. Fit both flasks with suitable glass-jointed reflux condensers, heat on a water-bath for 1 hour, add 10 ml of *water* through each condenser, and heat on the water-bath for 10 minutes more. Cool, and to each add 25 ml of *butanol*, previously neutralized to *phenolphthalein TS* with 0.5 M *ethanolic potassium hydroxide*, by pouring 15 ml through each condenser and, after removing the condensers, washing the sides of both flasks with the remaining 10-ml portions. To each flask add 1 ml of *phenolphthalein TS*, and titrate with 0.5 M *ethanolic potassium hydroxide VS*, recording the volume, in ml, consumed by the residual acid in the test solution as *T* and that consumed by the blank as *B*. In a 125-ml conical flask, mix about 10 g of the substance, accurately weighed, with 10 ml of freshly distilled pyridine, previously neutralized to *phenolphthalein TS*, add 1 ml of *phenolphthalein TS*, and titrate with 0.5 M *ethanolic potassium hydroxide VS*, recording the volume, in ml, consumed by the free acid in the test substance as *A*, or use the acid value to obtain *A*.

Calculate the hydroxyl value from the expression:

$$\text{Hydroxyl Value} = \frac{56.11M}{W}\left(B + \frac{WA}{C} - T\right),$$

in which *W* and *C* are the weights, in g, of the substances taken for the acetylation and for the free acid determination, respectively; *M* is the exact molarity of the ethanolic potassium hydroxide; and 56.11 is the molecular weight of potassium hydroxide.

5.10 SOLIDIFICATION TEMPERATURE OF FATTY ACIDS

Preparation of the Fatty Acids

Heat 75 ml of glycerol-potassium hydroxide solution (made by dissolving 25 g of *potassium hydroxide* in 100 ml of *glycerol*) in an 800-ml beaker to 150°, and add 50 ml of the clarified fat, melted if necessary. Heat the mixture for 15 minutes with frequent stirring, but do not allow the temperature to rise above 150°. Saponification is complete when the mixture is homogeneous, with no particles clinging to the beaker at the meniscus. Pour the contents of the beaker into 500 ml of nearly boiling *water* in an 800-ml beaker or casserole, add slowly 50 ml of diluted *sulfuric acid* (1 in 4), and heat the solution, with frequent stirring, until the fatty acids separate cleanly as a transparent layer. Wash the acids with boiling *water* until free from sulfuric acid, collect them in a small beaker, place on a water-bath until the water has settled and the fatty acids are clear, filter into a dry beaker while hot, and dry at 105° for 20 minutes. Place the warm fatty acids in a suitable container, and cool in an ice bath until they congeal.

Test for Complete Saponification

Place 3 ml of the dry acids in a test-tube, and add 15 ml of *ethanol*. Heat the solution to boiling, and add an equal volume of *ammonia TS*. A clear solution results.

Procedure

Using an apparatus similar to the "Freezing Temperature Apparatus" specified therein, proceed as directed for Procedure under "Determination of Freezing Temperature" (Appendix 4.4), reading "solidification temperature" for "freezing or congealing temperature" (the terms are synonymous). The average of not less than four consecutive readings of the highest point to which the temperature rises is the solidification temperature of the fatty acids.

5.11 DETERMINATION OF ESTERS

Boil a convenient amount of *ethanol* thoroughly to expel carbon dioxide and neutralize it to *phenolphthalein TS*. Unless otherwise directed in the monograph, weigh accurately about 2 g of the test substance, or other suitable quantity, so that the volume of 0.5 M *ethanolic potassium hydroxide VS* to be added is at least twice that theoretically required, dissolve it in 5 ml of the neutralized ethanol contained in a hard-glass flask, and neutralize the free acid in the solution with 0.1 M *ethanolic potassium hydroxide VS*, using 0.2 ml of *phenolphthalein TS* as indicator. Add 25.0 ml of 0.5 M *ethanolic potassium hydroxide VS*, attach the flask to a reflux condenser, and boil on a water-bath for 1 hour. Add 20 ml of *water*, and titrate the excess of alkali with 0.5 M *hydrochloric acid VS*, using a further 0.2 ml of *phenolphthalein TS* as indicator. Repeat the operation without the test substance. The difference between the titrations is equivalent to the alkali required to saponify the esters.

5.12 PEROXIDE VALUE

The peroxide value is the number of milliequivalents of active oxygen that expresses the amount of peroxide contained in 1000 g of the substance, when determined by the following method.

Procedure

Place about 5 g of the test substance, accurately weighed, in a 250-ml glass-stoppered conical flask. Add 30 ml of a mixture of 30 volumes of *glacial acetic acid* and 20 volumes of *chloroform*, shake until dissolved and add 0.5 ml of a saturated solution of *potassium iodide*. Shake for exactly 1 minute, add 30 ml of *water* and titrate slowly, shaking continuously, with 0.01 M *sodium thiosulfate VS* until the yellow colour almost disappears. Add 5 ml of *starch TS* and continue the titration, shaking vigorously, until the blue colour just disappears. Repeat the operation without the test substance.

Calculate the peroxide value from the expression:

$$\text{Peroxide Value} = \frac{100VM}{W},$$

where V = difference, in ml, between the titrations,
M = molarity of 0.01 M *sodium thiosulfate VS*, and
W = weight, in g, of the test substance.

The volume of 0.01 M *sodium thiosulfate VS* used in the blank determination must not exceed 0.1 ml.

5.13 ACID-NEUTRALIZING CAPACITY

(**Note** All tests shall be conducted at a temperature of 37°±3°.)

Standardization of pH Meter

Standardize a pH meter using Potassium Hydrogenphthalate Standard Buffer Solution and Potassium Tetraoxalate Standard Buffer Solution as described under the "Determination of pH" (Appendix 4.11).

Magnetic Stirrer

Transfer 100 ml of *water* to a 250-ml beaker containing a 40- × 10-mm (or other suitable size) magnetic stirring bar that is coated with solid perfluorocarbon and has a spin ring at its centre. Adjust the power setting of the magnetic stirrer to produce a stirring rate of 300±30 rpm when the stirring bar is centred in the beaker, as determined by a suitable optical tachometer.

Test Preparation

Powders Transfer the accurately weighed portion of the substance specified in the individual monograph to a 250-ml beaker, add 70 ml of *water* and mix on the magnetic stirrer for 1 minute.

Effervescent solids Transfer an accurately weighed quantity, equivalent to the minimum labelled dosage, to a 250-ml beaker, add 10 ml of *water*, and swirl the beaker gently while allowing the reaction to subside. Add another 10 ml of *water*, and swirl gently. Wash the walls of the beaker with 50 ml of *water*, and mix on the magnetic stirrer for 1 minute.

Suspensions and other liquids Shake the container until the contents are uniform, and determine the weight per millilitre. Transfer an accurately weighed quantity of the uniform mixture, equivalent to the minimum labelled dosage, to a 250-ml beaker, add *water* to make a total volume of about 70 ml, and mix on the magnetic stirrer for 1 minute.

Non-chewable tablets Weigh not less than 2 tablets, and determine the average tablet weight. Grind the tablets to a fine powder, mix to obtain a uniform mixture, and transfer an accurately weighed quantity of it, equivalent to the minimum labelled dosage, to a 250-ml beaker. If wetting is desired, add not more than 5 ml of *ethanol* (neutralized to an apparent pH of 3.5), and mix to wet the sample thoroughly. Add 70 ml of *water*, and mix on the magnetic stirrer for 1 minute.

Chewable tablets Prepare as directed for Non-chewable tablets.

Tablets that are required to be chewed Transfer 1 tablet to a 250-ml beaker, add 50 ml of *water*, and mix on the magnetic stirrer for 1 minute.

Capsules Weigh accurately not less than 20 capsules. Remove the capsule contents completely, with the aid of a cotton swab if necessary. Accurately weigh the empty capsules, and determine the average weight of the contents per capsule. Mix the combined capsule contents to obtain a uniform mixture, and proceed as directed for Non-chewable tablets, beginning with "transfer an accurately weighed quantity of it, ...".

Procedure

Powders, effervescent solids, suspensions and other liquids, non-chewable tablets, chewable tablets, and capsules Pipette 30 ml of 1 M *hydrochloric acid VS* into the test preparation while continuing to stir with the magnetic stirrer. (**Note** Where the acid-neutralizing capacity of the sample is greater than 25 mEq, use 60.0 ml of 1 M *hydrochloric acid VS*, and make the appropriate modifications in the calculation.) Stir for 15 minutes, accurately timed, after the addition of the acid, begin to titrate immediately, and in a period not to exceed an additional 5 minutes, titrate the excess hydrochloric acid with 0.5 M *sodium hydroxide VS* to attain a stable (for 10 to 15 seconds) pH of 3.5. Calculate the number of mEq of acid consumed by the sample taken by the formula:

$$(V_{HCl} \times M_{HCl}) - (V_{NaOH} \times M_{NaOH}),$$

in which M_{HCl} and M_{NaOH} are the molarities of the hydrochloric acid VS and the sodium hydroxide VS, respectively; and V_{HCl} is the volume of the hydrochloric acid VS added into the test preparation and V_{NaOH} is the volume of the sodium hydroxide VS used for titration. Express the result in terms of mEq of acid consumed.

Tablets that are required to be chewed Pipette 30 ml of 1 M *hydrochloric acid VS* into the test preparation while continuing to stir with the magnetic stirrer for 10 minutes, accurately timed, after the addition of the acid. Discontinue stirring briefly, and without delay remove any gum base from the beaker using a long needle. Promptly rinse the needle with 20 ml of *water*, collecting the washing in the beaker, and resume stirring for 5 minutes, accurately timed, then begin to titrate immediately, and in a period not to exceed an additional 5 minutes, titrate the excess hydrochloric acid with 0.5 M *sodium hydroxide VS* to attain a stable (for 10 to 15 seconds) pH of 3.5. Calculate the number of mEq of acid consumed by the Tablet taken by the formula:

$$(30 \times M_{HCl}) - (V_{NaOH} \times M_{NaOH}),$$

in which the terms are as defined above.

5.14 RELATIONSHIP BETWEEN REACTION OF SOLUTION, APPROXIMATE pH AND COLOUR OF CERTAIN INDICATORS

To 10 ml of the solution to be examined, add 0.1 ml of indicator, unless otherwise prescribed in the following table.

Reaction	pH	Indicator	Colour
Alkaline	>8.0	*Red Litmus paper* *Thymol blue TS**	Blue Grey or violet-blue
Slightly alkaline	8.0-10.0	*Phenolphthalein TS*** *Thymol blue TS**	Colourless to pink Grey
Strongly alkaline	>10.0	*Phenolphthalein paper* *Thymol blue TS**	Red Violet-blue
Neutral	6.0-8.0	*Methyl red TS* *Phenol red TS*	Yellow Yellow or pink
Neutral to dimethyl yellow	>4.0	*Dimethyl yellow TS***	Yellow; red after adding 0.10 ml of 0.10 M *hydrochloric acid*
Neutral to methyl red	4.5-6.0	*Methyl red TS*	Orange-red
Neutral to phenolphthalein	<8.0	*Phenolphthalein TS***	Colourless; pink or red after adding 0.50 ml of 0.10 M *sodium hydroxide*
Acid	<6.0	*Methyl red TS* *Bromothymol blue TS****	Orange or red Yellow
Faintly acid	4.0-6.0	*Methyl red TS* *Bromocresol green TS*	Orange Green or blue
Strongly acid	<4.0	*Congo red paper* *Dimethyl yellow TS***	Green or blue Orange or red

*Use 0.2 ml.

**Use 0.05 ml.

***Use *dilute bromothymol blue TS*

5.15 DETERMINATION OF METHANOL AND 2-PROPANOL

Carry out the determination as described in the "Gas Chromatography" (Appendix 3.4), using the following solutions.

Internal Standard Solution Prepare a solution containing 2.5 per cent v/v of *1-propanol* in *ethanol*.

Test Solution (a) To a certain amount of the distillate add 2.0 ml of *Internal standard solution*; adjust the ethanol content (Appendix 6.5) to 10.0 per cent v/v by dilution to 50 ml with *water* or addition of *ethanol*.

Test Solution (b) Adjust the ethanol content (Appendix 6.5) of a certain amount of the distillate to 10.0 per cent v/v by dilution to 50 ml with *water* or addition of *ethanol*.

Reference Solution (a) Prepare 50 ml of a solution containing 2.0 ml of *Internal standard solution*, 3.0 ml of ethanol, 0.05 per cent v/v of 2-propanol and sufficient *anhydrous methanol* to give a total of 0.05 per cent v/v of methanol taking into account the methanol content of *ethanol*.

Reference Solution (b) Prepare a 10.0 per cent v/v solution of ethanol containing 0.0025 per cent v/v of each *methanol* and *2-propanol*.

Chromatographic system The chromatographic procedure may be carried out using (a) a fused silica column (30 m × 0.53 mm) packed with 7 per cent cyanopropyl-7 per cent phenyl-86 per cent methylpolysiloxane (film thickness 3 µm) and maintained at 35° for 0 to 5 minutes and at 35° to 85° for 5 to 15 minutes, with the inlet port at 250°, the split ratio at 1:10, (b) *helium* as the carrier gas at a flow rate of 2 ml per minute, the split ratio at 1:10 and (c) a flame ionization detector maintained at 250°.

Separately inject 1.0 µl of each solution and calculate the content of methanol and 2-propanol with reference to the original substance.

System suitability

1-PROPANOL There is no peak corresponding to 1-propanol in the chromatogram obtained from *Test solution* (*b*),

PEAK-TO-VALLEY RATIO Minimum 15, where H_p = height above the baseline of the peak due to 2-propanol and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to ethanol in the chromatogram obtained from *Reference solution* (*a*),

SIGNAL-TO-NOISE RATIO Minimum 10 for the peaks due to methanol and 2-propanol in the chromatogram obtained from *Reference solution* (*b*).

5.16 *N,N*-DIMETHYLANILINE (DIMETHYLANILINE)

The following limit test is provided as a general procedure, when specified in the individual monographs for the gas chromatographic determination in compendial articles of traces of *N,N*-dimethylaniline, a hydrochloric acid scavenger that may have been carried over during processing.

Carry out the determination by gas chromatography, Appendix 3.4, using *naphthalene* as the internal standard.

Internal standard solution Unless otherwise specified in the individual monograph, dissolve 50 mg of *naphthalene* in *cyclohexane* and dilute to 50 ml with the same solvent. Dilute 5 ml of this solution to 100 ml with *cyclohexane*.

Standard solution Unless otherwise specified in the individual monograph, transfer 50.0 mg of N,N-*dimethylaniline* to a 50-ml volumetric flask, add 2 ml of *hydrochloric acid* and 20 ml of *water*, shake to dissolve and dilute to volume with *water*. Dilute 5.0 ml of this solution to 250.0 ml with *water*. To 1.0 ml of the latter solution in a ground-glass-stoppered tube add 5 ml of 1 M *sodium hydroxide* and 1.0 ml of the *Internal standard solution*. Stopper the tube and shake vigorously for 1 minute. Centrifuge if necessary and use the upper layer.

Test solution Unless otherwise specified in the individual monograph, transfer 1.00 g of the test substance to a ground-glass-stoppered tube, add 5 ml of 1 M *sodium hydroxide* and 1.0 ml of the *Internal standard solution*. Stopper the tube and shake vigorously for 1 minute. Centrifuge if necessary and use the upper layer.

Chromatographic system The chromatographic procedure may be carried out using (a) a glass column 2 m long and 2 mm in internal diameter packed with *silanized diatomaceous earth for gas chromatography* impregnated with 3 per cent w/w of *polymethylphenyl siloxane*, (b) *nitrogen for chromatography* as the carrier gas at a flow rate of 30 ml per minute, and (c) a flame-ionization detector, maintaining the temperature of the column at 120° and that of the injection port and of the detector at 150°.

Procedure Separately inject 1 µl of *Standard solution* and *Test solution* into the chromatograph, record the chromatograms, and measure the areas of the major peaks. The ratio of the response of any dimethylaniline peak to the response of the naphthalene peak obtained from *Test solution* is not more than that obtained from *Standard solution*.

5.17 ORDINARY IMPURITIES

Limit

This test, where called for in the individual monograph, is provided to evaluate the presence of ordinary impurities in official articles. Ordinary impurities are defined as those species in drug substances and/or drug products that have no significant, undesirable biological activity in the amounts present. These impurities may arise out of the synthesis, preparation, or degradation of compendial articles. In certain instances, impurities that pose a potential health risk may be detected. Because these impurities would not be individually identified by the strict use of this Appendix, a separate evaluation may be necessary to ensure that the detected impurities fit the requirements set forth in the definition of Ordinary Impurities. Selections of tests and assays allow for anticipated amounts of impurities that are unobjectionable for the customary use of the article.

Reporting and specifications The value of 2.0 per cent, unless otherwise specified in the individual monograph, was selected as the general limit for the total amount of ordinary impurities in monographs where documentation did not support adoption of other values.

Where a monograph sets limits on concomitant components and/or specified impurities/degradation products, these species are not to be included in the estimation of ordinary impurities unless so stated in the individual monograph. Concomitant components are defined as species characteristic of many drug substances that are not considered to be impurities in the Pharmacopoeial sense. Examples of concomitant components are geometric and optical isomers (or racemates) and antibiotics that are mixtures. Any component that can be considered a toxic impurity because of significant undesirable biological effect is not considered to be a concomitant component.

Method

Unless otherwise specified in an individual monograph, estimation of the amount and number of ordinary impurities is made by relative methods rather than by strict comparison to individual Reference Substances. Nonspecific detection of ordinary impurities is also consistent with this classification.

Typical evaluation methods used for ordinary impurities are thin-layer chromatographic (TLC) techniques (Appendix 3.1). Tests for related substances or chromatographic purity may also be used to evaluate the presence of ordinary impurities. Other methods (e.g., HPLC, HPTLC, etc.) may also be used with adequate justification as an alternate method. Unless otherwise specified in the individual monograph, use the following method.

Test solution Prepare, in the solvent specified in the monograph, a solution of the substance under test having a known concentration of 10.0 mg per ml.

(**Note** Heat or sonication may be used to dissolve the drug substance where use of such does not adversely affect the compound.)

Standard solutions Prepare, in the solvent specified in the monograph, solutions of the Reference Substance or designated substance having known concentrations of 10, 50, 100, and 200 µg per ml. (**Note** Heat or sonication may be used to dissolve the drug substance where use of such does not adversely affect the compound.)

Procedure

Use a thin-layer chromatographic plate coated with a 0.25-mm layer of *silica gel GF254*, and *mobile phase* specified in the monograph. Apply equal volumes (20 µl) of *Test solution* and *Standard solutions* to the plate, using a stream of *nitrogen* to dry the spots.

Allow the chromatogram to develop in a pre-equilibrated chamber until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the chamber, and air-dry. View the plate using the visualization technique(s) specified. Locate any spots other than the principal spot in the chromatogram of *Test solution*, and determine their relative intensities by comparison with the chromatograms of the appropriate *Standard solutions*. See discussion above with regard to reporting and specifying total ordinary impurities.

Key for Visualization Techniques

(1) Use UV light at 254 nm and at about 366 nm.

(2) Use *iodoplatinate TS*.

(3) Mix 10 ml of *acetic potassium iodobismuthate TS* with 20 ml of *glacial acetic acid*, and dilute with *water* to make 100 ml.

(4) **Ninhydrin spray** Dissolve 200 mg of *ninhydrin* in 100 ml of *ethanol*. Heat the plate after spraying.

(5) **Acid spray** In an ice-bath, add slowly and cautiously, with stirring, 10 ml of *sulfuric acid* to 90 ml of *ethanol*. Spray the plate, and heat until charred.

(6) **Acid-dichromate spray** Add sufficient *potassium dichromate* to 100 ml of *sulfuric acid* to make a saturated solution. Spray the plate, and heat until charred.

(7) **Vanillin** Dissolve 1 g of *vanillin* in 100 ml of *sulfuric acid*.

(8) **Chloramine T-trichloroacetic acid** Mix 10 ml of a 3 per cent w/v solution of *chloramine T* with 40 ml of a 25 per cent w/v solution of *trichloro acetic acid* in *ethanol*. Prepare immediately before use.

(9) **Folin-C** Add 10 g of *sodium tungstate* and 2.5 g of *sodium molybdate* to 70 ml of *water*, add 5 ml of *phosphoric acid* and 10 ml of *hydrochloric acid*, and reflux this solution for 10 hours.

(10) **$KMnO_4$** Dissolve 100 mg of *potassium permanganate* in 100 ml of *water*.

(11) **DAB** Mix 1 g of *4-dimethylaminobenzaldehyde* in 100 ml of 0.6 M *hydrochloric acid*.

(12) **DAC** Mix 100 mg of *4-dimethylaminocinnamaldehyde* in 100 ml of 1 M *hydrochloric acid*.

(13) **Ferricyanide** Mix equal volumes of a 1 per cent w/v solution of *iron(III) chloride* and a 1 per cent w/v solution of *potassium hexacyanoferate (III)*. Use immediately.

(14) **Fast blue B:**

Reagent A Dissolve 500 mg of *Fast Blue B Salt* in 100 ml of *water*.

Reagent B 0.1 M *sodium hydroxide*.

Spray first with A, then with B.

(15) **Alkaline iron(III) cyanide** Dilute 1.5 ml of a 1 per cent w/v solution of *potassium hexacyanoferrate(III)* with *water* to 20 ml, and add 10 ml of 15 per cent w/v solution of *sodium hydroxide*.

(16) **Iodine spray** Prepare a 0.5 per cent w/v solution of *iodine* in *chloroform*.

(17) Expose the plate for 10 minutes to iodine vapours in a pre-equilibrated closed chamber, on the bottom of which there are *iodine* crystals.

(18) **Solution A** Dissolve 0.5 g of *potassium iodide* in 50 ml of *water*.

Solution B Prepare a solution of 0.5 g of *soluble starch* in 50 ml of hot *water*.

Just prior to use, mix equal volumes of Solution A and Solution B.

(19) **PTSS** Dissolve 20 g of *4-toluenesulfonic acid* in 100 ml of *ethanol*, spray the plate, dry for 15 minutes at 110°, and view under UV light at 366 nm.

(20) ***o*-Tolidine Spray** Dissolve 160 mg of *o-tolidine* in 30 ml of *glacial acetic acid*, dilute with *water* to make 500 ml, add 1 g of *potassium iodide*, and mix until the potassium iodide has dissolved.

(21) Mix 3 ml of a 10 per cent w/v solution of *chloroplatinic(IV) acid* with 97 ml of *water*, followed by the addition of 100 ml of a 6 per cent w/v solution of *potassium iodide* to prepare the spray reagent.

(22) **Iodine-Methanol Spray** Prepare a mixture of equal volume of *iodine TS* and *methanol*.

APPENDIX 6 ASSAYS

6.1 NON-AQUEOUS TITRATION

Acids and bases have long been defined by Arrhenius as substances which furnish, when dissolved in water, hydrogen and hydroxyl ions, respectively. A more generalized definition is that of Brönsted, who defined an acid as a proton donor, and a base as a proton acceptor. Even broader is the definition of Lewis, who defined an acid as any material that will accept an electron pair, a base as any material that will donate an electron pair, and neutralization as the formation of a coordination bond between an acid and a base.

The apparent strength of an acid or base is determined by the extent of its reaction with a solvent. In water solution, all strong acids appear equally strong because they react with the solvent to undergo almost complete conversion to hydronium ion (H_3O^+) and the acid anion. In a weakly protophilic solvent such as acetic acid the strength of acids decreases in the series perchloric, hydrobromic, sulfuric, hydrochloric, and nitric. Acetic acid also enhances the basic properties of weak bases and it is possible to titrate such solution of weak bases with perchloric acid in acetic acid. On the other hand, weak acids tend to enhance their acid properties when dissolved in basic solvents and it is possible to titrate weak acids with strong bases. The order of decreasing strength for bases is potassium methoxide, sodium methoxide, lithium methoxide, and tetrabutylammonium hydroxide.

Many water-insoluble compounds behave as weak acids or weak bases in water but acquire enhanced acidic or basic properties when dissolved in appropriate organic solvents. Thus, it is necessary to perform non-aqueous titrations by proper selection of solvent and titrant to increase the sensitivity of the end-points. Most of the pharmaceutical preparations can be titrated directly, but it is sometimes necessary to isolate the active ingredients from interfering excipients and carriers.

The types of compounds that may be titrated as acids include acid halides, acid anhydrides, carboxylic acids, amino acids, enols such as barbiturates and xanthines, imides, phenols, pyrroles, and sulfonamides. The types of compounds that may be titrated as bases include amines, nitrogen-containing heterocyclic compounds, oxazolines, quaternary ammonium compounds, alkali salts of organic acids, alkali salts of weak inorganic acids, and some salts of amines. Many salts of halogen acids may be titrated in acetic acid or acetic anhydride after the addition of mercury(II) acetate, which removes halide ion as the un-ionized mercury(II) halide complex. In the case of hydrochlorides of weak bases not containing acetylatable groupings it is also possible to titrate in acetic anhydride without the addition of mercury(II) acetate and using an indicator such as malachite green or crystal violet.

In the titration of a basic compound, a volumetric solution of perchloric acid in glacial acetic acid is preferred, although perchloric acid in dioxane is used in special cases. In the titration of an acidic compound, a volumetric solution of sodium methoxide in a mixture of methanol and toluene is preferred, although lithium methoxide in the methanol-toluene solvent may be used for those compounds yielding a gelatinous precipitate on titration with sodium methoxide.

Care should be taken to avoid subsequent exposure to the atmospheric carbon dioxide for titration of acid substances. Absorption of carbon dioxide may be determined by performing a blank titration. The blank generally should not exceed 0.01 ml of a 0.1 M titrant per ml of solvent.

The end-point may be determined visually by colour change, or potentiometrically (Appendix 6.4). If the calomel reference electrode is used, it is advantageous to replace the aqueous potassium chloride salt bridge with 0.1 M lithium perchlorate in glacial acetic acid for titrations in acidic solvents, or potassium chloride in methanol for titrations in basic solvents.

Where these or other mixtures are specified in individual monographs, the calomel reference electrode is modified by first removing the aqueous potassium chloride solution and residual potassium chloride, if any, by rinsing with *water*, then eliminating residual water by rinsing with the required non-aqueous solvent, and finally filling the electrode with the designated non-aqueous mixture.

When using titrants prepared with solvents of high coefficient of expansion, correction should be made to compensate for differences in temperature between the titration and standardization time.

Systems for Non-aqueous Titrations

Type of Solvent	Solvent*	Indicator	Electrodes
Acidic (for titration of bases and their salts)	Acetic Anhydride Glacial Acetic Acid Formic Acid Propionic Acid Sulfuryl Chloride	Alphazurine 2-G Crystal Violet Malachite Green *p*-Naphtholbenzein Thymol Quinaldine Red Blue	Glass-Calomel Glass-Silver-silver chloride Mercury-Mercuric acetate
Relatively Neutral (for differential titration of bases)	Acetonitrile Alcohols Benzene Chlorobenzene Chloroform Dioxane Ethyl Acetate	Methyl Orange Methyl Red *p*-Naphtholbenzein	Glass-Calomel Calomel-Silver-silver chloride
Basic (for titration of acids)	*n*-Butylamine Dimethylformamide Ethylenediamine Morpholine Pyridine	Azo Violet *p*-Hydroxyazobenzene *o*-Nitroaniline Quinaldine Red Thymol Blue Thymolphthalein	Antimony-Antimony** Antimony-Calomel Antimony-Glass Glass-Calomel Platinum-Calomel
Relatively Neutral (for differential titration of acids)	Acetone Acetonitrile 2-Methyl-2-propanol 2-Butanone 4-Methyl-2-pentanone	Azo Violet Bromothymol Blue *p*-Hydroxyazobenzene Thymol Blue	Antimony-Calomel Glass-Calomel Glass-Platinum**

*Relatively neutral solvents of low dielectric constant such as benzene, chloroform, or dioxane may be used in conjunction with any acidic or basic solvent in order to increase the sensitivity of the titration end-points.
**In titrant.

6.2 OXYGEN FLASK COMBUSTION

The oxygen flask combustion procedure is provided as the preparatory step in the determination of bromine, chlorine, fluorine, iodine, selenium and sulfur in some Pharmacopoeial articles. Combustion of the material under test (usually organic) yields water-soluble inorganic products, which are determined for specific elements directed for the individual monograph or general appendix.

The caution statement given under Procedure of Combustion covers minimum safety precautions only, and serves to emphasize the need for exceptional care throughout.

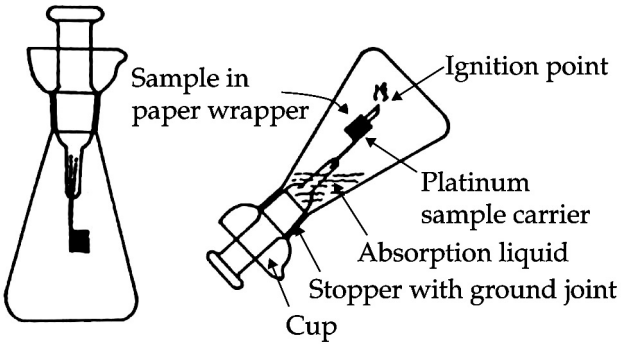
Apparatus

The apparatus consists of a heavy-welled conical, deeply lipped or cupped 500-ml flask (unless a larger flask is specified), fitted with a ground-glass stopper to which is fused a test sample carrier consisting of heavy-gauge platinum wire and a piece of welded platinum gauze measuring about 1.5 cm × 2 cm, or of a platinum basket or cylinder made of platinum woven gauze.

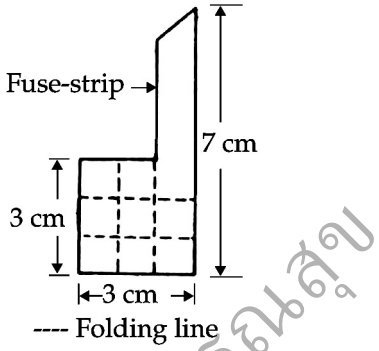
Preparation of Sample

For solid samples Accurately weigh the quantity of the sample specified in the individual monograph on the centre of halide-free filter paper measuring about 3 cm square, illustrated in the figure. Wrap the sample carefully along the dotted line without scattering, and place the parcel in a sample carrier, leaving its fuse-strip outside. (**Note** Pulverizable substances should be finely ground and thoroughly mixed before the specified quantity is weighed.) Ointment should be enclosed in grease-proof paper before wrapping in filter paper.

For liquid samples Liquid substances are weighed in tared capsules, polycarbonate capsules being used for liquids in volumes not exceeding 200 µl, and gelatin capsules being satisfactory for use for larger volumes. (**Note** Gelatin capsules may contain significant amounts of combined halide or sulfur. If such capsules are used, perform a blank determination, and make any necessary correction.) Place the sample, together with a filter paper fuse-strip, in the platinum gauze sample carrier.



Apparatus for Oxygen Flask Combustion



Filter Paper

Procedure of Combustion

Caution The analyst should wear safety glasses and use a suitable safety shield between himself and the apparatus. The flask must be scrupulously clean, free from all traces of organic solvents.

Place the specified absorbing liquid in the flask, moisten the joint of the stopper with *water*, and flush the air from the flask with a stream of rapidly flowing *oxygen*, swirling the liquid to favour its taking up oxygen. (**Note** Saturation of the liquid with *oxygen* is essential for the successful performance of the combustion procedure.) Ignite the fuse-strip by suitable means. If the strip is ignited outside the flask, immediately plunge the sample holder into the flask, carefully tilt the flask so that the absorbing liquid makes a seal around the stopper as illustrated in the figure, and hold the stopper firmly in place. If the ignition is carried out in a closed system, the inversion of the flask may be omitted. After combustion is complete, shake the flask vigorously, and allow to stand for not less than 10 minutes with intermittent shaking. Then proceed as directed in the individual monograph or general appendix.

6.3 COMPLEXOMETRIC TITRATIONS

Complexing agents of value as titrants are aminopolycarboxylic acids that possess the characteristic group, $N(CH_2COOH)_2$. Such compounds are capable of forming chelate complexes with many cations in which the cation is bound in a ring structure. The ring results from the formation of a salt-like bond between the cation and the carboxyl groups together with a coordinate bond through the lone pair of electrons of the nitrogen atom. If the ring is five-membered, the chelate thus formed is likely to have high stability, so the most useful chelating titrants are those that favour the formation of such rings. This is the case with edetic acid (ethylenediaminetetraacetic acid, EDTA); the commonly used disodium salt is known as disodium edetate. With most metals carrying more than unit positive charge, edetic acid forms highly water-soluble 1:1 complexes of such a structure that at least three five-membered chelate rings are formed, thus conferring high stability on the complex.

The stability of such complexes is markedly dependent on pH. Most divalent metals form complexes that are stable in alkaline solution but alkaline earth chelates decompose below about pH 8, whereas many divalent metal complexes (zinc and lead, for example) are also stable in quite acid solution. Trivalent metal complexes, by virtue of the additional stability conferred by an increased number of chelate rings, are often stable even in strongly acid solution. In alkaline solution, however, some of these metals may be precipitated as hydroxides in the presence of edetic acid, not because of instability of the complex but because of the more powerful effect of the very low solubility product of the metal hydroxide.

In order to determine the equivalence point in titration of metal ions with edetic acid, it is necessary to use a suitable indicator that will react to the presence of free metal ions in solution. The indicator originally used by Schwarzenbach for titration of calcium ions was murexide (ammonium purpurate) but this is now rarely used. Perhaps the most widely used of indicators has been mordant black 11 (also known under several trade names). This has a blue colour in ammoniacal solution but yields red complexes with many metal ions in such solutions; the metal complexes so formed are generally weaker than the corresponding edetic acid complexes, so that titration with edetates will readily remove the metal from the indicator complex and a colour change to full blue signifies total chelation of the metal present in solution. Mordant black 11 is frequently used as a mixture with methyl orange, which serves to provide a screened (more readily detectable) end-point.

Many other substances have been proposed and used as indicators for complexometric titrations, but the present discussion must be limited to a consideration of those that are of potential value in pharmaceutical applications. Hydroxynaphthol blue gives a very sharp colour change from reddish pink to full blue when a calcium solution is titrated with sodium edetate in the pH range of 12 to 13. If magnesium is present it is precipitated as hydroxide at this pH and, providing the alkali is added before the indicator, does not interfere.

Another widely used indicator is xylenol orange; this is a conventional acid-base indicator into which iminodiacetic acid groups have been introduced, thus permitting the substance to act as a metal-complexing indicator. The indicator gives a clear colour change from pink-violet to yellow at the end-point in titrations of aluminium, bismuth, lead, mercury, and zinc, and may be used from pH 2 to 6 according to the metal being titrated.

ALUMINIUM

Use Method I unless otherwise directed.

Method I To 20.0 ml of the prescribed solution add 25.0 ml of 0.05 M *disodium edetate VS* and 10 ml of a mixture of equal volumes of 2 M *ammonium acetate* and 2 M *acetic acid*. Heat to boiling for 2 minutes, cool, and add 50 ml of *ethanol* and 3 ml of freshly prepared *dithizone TS*. Titrate the excess of disodium edetate with 0.05 M *zinc sulfate VS* until the colour changes from greenish blue to reddish violet. Each ml of 0.05 M *disodium edetate* is equivalent to 1.349 mg of Al.

Method II Dissolve the prescribed quantity of the substance being examined in a mixture of 2 ml of 1 M *hydrochloric acid* and 50 ml of *water*, add 50.0 ml of 0.05 M *disodium edetate VS* and neutralize with 1 M *sodium hydroxide* using *methyl red TS* as indicator. Heat the solution to boiling, allow to stand for 10 minutes on a water-bath, cool rapidly, add about 50 mg of *xylenol orange mixture* and 5 g of *methenamine*, and titrate the excess of disodium edetate with 0.05 M *lead(II) nitrate VS* until the solution becomes red. Each ml of 0.05 M *disodium edetate* is equivalent to 1.349 mg of Al.

BISMUTH

Method I should be used unless otherwise directed.

Method I Dilute the prescribed solution to 250 ml with *water* and unless otherwise directed add, with shaking, *strong ammonia solution* dropwise until cloudiness is first observed. Add 0.5 ml of *nitric acid* and heat to 70°, maintaining the solution at this temperature until the solution becomes completely clear. Add about 50 mg of *xylenol orange mixture* and titrate with 0.05 M *disodium edetate VS* until the colour changes from pinkish violet to lemon-yellow. Each ml of 0.05 M *disodium edetate* is equivalent to 10.45 mg of Bi.

Method II Dissolve the prescribed quantity of the substance being examined in the minimum volume of 2 M *nitric acid*, add 50 ml of *water* and adjust the pH to 1 to 2 by adding dropwise, with shaking, 2 M *nitric acid* or 5 M *ammonia*. Add about 30 mg of *xylenol orange mixture* and titrate with 0.05 M *disodium edetate VS* until the colour changes from pinkish violet to full yellow. Each ml of 0.05 M *disodium edetate* is equivalent to 10.45 mg of Bi.

CALCIUM

Dilute the prescribed solution with *water* to 150 ml, add 15 ml of 1 M *sodium hydroxide* and 300 mg of *hydroxynaphthol blue* or 100 mg of *calcon mixture* or *calconcarboxylic acid mixture* may be used in place of *hydroxynaphthol blue*. Titrate with 0.05 M *disodium edetate VS* until the colour changes to full blue. Each ml of 0.05 M *disodium edetate* is equivalent to 2.004 mg of Ca.

LEAD

Dilute the prescribed solution with *water* to 200 ml, or dissolve the prescribed quantity of the substance being examined in 5 to 10 ml of *water*, or in the minimum volume of 5 M *acetic acid*, and dilute to 50 ml with *water*. To the resulting solution add about 50 mg of *xylenol orange mixture* and sufficient *methenamine* to produce a violet-pink coloration. Titrate with 0.05 M *disodium edetate VS* until the colour changes to lemon-yellow. Each ml of 0.05 M *disodium edetate* is equivalent to 10.36 mg of Pb.

MAGNESIUM

Dilute the prescribed solution to 300 ml with *water*, or dissolve the prescribed quantity of the substance being examined in 5 to 10 ml of *water*, or in the minimum volume of 2 M *hydrochloric acid*, and dilute to 50 ml with *water*. To the resulting solution add 10 ml of *ammonia buffer pH 10.0* and about 50 mg of *mordant black 11 mixture*. Titrate with 0.05 M *disodium edetate VS*, until the colour changes from violet to full blue. Each ml of 0.05 M *disodium edetate* is equivalent to 1.215 mg of Mg.

ZINC

Dilute the prescribed solution to 200 ml with *water*, or dissolve the prescribed quantity of the substance being examined in the prescribed quantity of the substance being examined in the prescribed amount of 2 M *acetic acid* and dilute to 50 ml with *water*. To the solution thus obtained add about 50 mg of *xylenol orange mixture* and sufficient *methenamine* to produce a violet-pink coloration. Add a further 2 g of *methenamine*, and titrate with 0.05 M *disodium edetate VS* until the colour changes to yellow. Each ml of 0.05 M *disodium edetate* is equivalent to 3.269 mg of Zn.

6.4 POTENTIOMETRY

Potentiometry is an electrochemical method based on the measurement of the electrical potential between a pair of suitable electrodes immersed in the solution to be analyzed. The apparatus required consists of an indicator electrode, a reference electrode and a device for measuring potential.

6.4.1 POTENTIOMETRIC TITRATION

In a potentiometric titration, the end-point of the titration is determined by following the variation of the potential difference between two electrodes (either one indicator electrode and one reference electrode or two indicator electrodes) immersed in the solution being examined as a function of the quantity of titrant added.

The potential is usually measured at zero or practically zero current.

Apparatus

The apparatus used (a simple potentiometer or electronic device) comprises a voltmeter allowing readings to the nearest millivolt. The choice of indicator electrode depends on the substance being examined and may be a glass or metal electrode (for example, platinum, gold, silver or mercury). The reference electrode is generally a calomel or a silver-silver chloride electrode. For acid-base titrations and unless otherwise prescribed, a glass-calomel or glass-silver-silver chloride electrode combination is used.

Several acceptable electrode systems for potentiometric titrations are summarized in Table 1.

Method

Plot a graph of the variation of potential difference as a function of the quantity of the titrant added, i.e. for an acid-base titration, pH versus ml of titrant added, for a precipitimetric, complexometric, or oxidation-reduction titration, mv versus ml of titrant added, continuing the addition of the titrant beyond the presumed equivalence point. The end-point corresponds to a sharp variation of potential difference. The equivalence point may also be determined mathematically without plotting a graph. Two types of automatic electrometric titrators are available. The first is one that carries out titrant addition automatically and records the electrode potential differences during the course of titration as a sigmoid curve. In the second type, titrant addition is performed automatically until a preset potential or pH, representing the end-point, is reached, at which point the titrant addition ceases.

Table 1 Potentiometric Titration Electrode System

Titration	Indicating Electrode	Equation*	Reference Electrode	Applicability**
Acid-base	Glass	$E = k + 0.0591$ pH	Calomel or silver-silver chloride	Titration of acids and bases
Precipitimetric (silver)	Silver	$E = E_0 + 0.0591 \log [Ag^+]$	Calomel (with potassium nitrate salt bridge)	Titration with or of silver involving halides or thiocyanate
Complexometric	Mercury-mercury(II)	$E = E_0 + 0.0296(\log k' - pM)$	Calomel	Titration of various metals (M), e.g. Mg^{+2}, Ca^{+2}, Al^{+3}, Bi^{+3}, with EDTA
Oxidation-reduction	Platinum	$E = E_0 + \frac{0.0591}{n} \log \frac{[ox]}{[red]}$	Calomel or silver-silver chloride	Titrations with arsenite, bromine, cerate, dichromate, hexacyanoferrate(III), iodate, nitrite, permanganate, thiosulfate

*Appropriate form of Nernst equation describing the indicating electrode system: k = glass electrode contant; k' = constant derived from Hg–Hg(II)–EDTA equilibrium; M = any metal undergoing EDTA titration; pM = –log[M]; [ox] and [red] from the equation, ox + $ne \leftrightharpoons$ red.
**Listing is representative but not exhaustive.

6.4.2 POTENTIOMETRIC DETERMINATION OF IONIC CONCENTRATION USING ION-SELECTIVE ELECTRODES

The potentiometric determination of the ion concentration is carried out by measuring the potential difference (E) between two suitable electrodes immersed in the solution to be examined; the indicator electrode is selective for the ion to be determined and the other is a reference electrode.

The potential E of an ion-selective electrode varies linearly with the logarithm of the activity a_i of a given ion, as expressed by the Nernst equation:

$$E = E_0 + 2.303 \frac{RT}{z_i F} \log a_i,$$

where E_0 = a constant called the standard electrode potential, which is characteristic for each half-reaction,
R = gas constant,
T = absolute temperature,
F = Faraday's number, and
z_i = charge number of the ion including its sign.

At a constant ionic strength, the following holds:

$$E = E_0 + \frac{k}{z_i} \log fC_i,$$

where C_i = molar concentration of the ion,
f = the activity coefficient ($a_i = fC_i$), and
$k = \frac{RT}{F}$

If: $E_0 + \frac{k}{z_i} log\, f = E_0'$ and $S = \frac{k}{z_i}$

where S = slope of the calibration curve of the electrode,

the following holds: $E = E_0' + S\log C_i$
and for $-\log C_i = pC_i$: $E = E_0' - SpC_i$

Apparatus

Use a voltmeter allowing measurements to the nearest 0.1 millivolt and whose input impedance is at least 100 times greater than that of the electrodes used. Ion-selective electrodes may be primary electrodes with a crystal or non-crystal membrane or with a rigid matrix (for example, glass electrodes), or electrodes with charged (positive or negative) or uncharged mobile carriers, or sensitized electrodes (enzymatic substrate electrodes, gas-indicator electrodes). The reference electrode is generally a silver-silver chloride electrode or a caomel electrode, with suitable junction liquids, producing no interference.

Procedure

Carry out each measurement at a temperature constant to ±0.5°, taking into account the variation of the slope of the electrode with temperature (Table 2). Adjust the ionic strength and possibly the pH of the solution to be analyzed using the buffer reagent described in the monograph and equilibrate the electrode by immersing it in the solution to be analyzed, under slow and uniform stirring, until a constant reading is obtained.

If the electrode system is used frequently, check regularly the repeatability and the stability of responses, and the linearity of the calibration curve or the calculation algorithm in the range of concentrations of the test solution; if not, carry out the test before each set of measurements. The response of the electrode may be regarded as linear if the slope S of the calibration curve is approximately equal to k/z_i per unit of pC_i.

Table 2 Values of *k* at Different Temperatures

Temperature (°)	*k*
20	0.0582
25	0.0592
30	0.0602

Method I (Direct Calibration)

Measure at least three times in succession the potential of at least three reference solutions spanning the expected concentration of the test solution. Calculate the calibration curve or plot on a chart the mean potential E obtained against the concentration of the ion to be determined expressed as $-\log C_i$ or pC_i.

Prepare the test solution as prescribed in the monograph; measure the potential three times and, from the mean potential, calculate the concentration of the ion to be determined using the calibration curve.

Method II (Multiple Standard Additions)

Prepare the test solution as prescribed in the monograph. Measure the potential at equilibrium E_T of a volume V_T of this solution of unknown concentration C_T of the ion to be determined. Make at least three consecutive additions of a volume V_S negligible compared to V_T ($V_S \le 0.01V_T$) of a reference solution of a concentration C_S known to be within the linear part of the calibration curve. After each addition, measure the potential and calculate the difference of potential ΔE between the measured potential and E_T. ΔE is related to the concentration of the ion to be determined by the equation:

$$\Delta E = S \log \left(1 + \frac{C_S V_S}{C_T V_T} \right),$$

or

$$10^{\Delta E/S} = 1 + \frac{C_S V_S}{C_T V_T},$$

where V_T = volume of the test solution,
C_T = concentration of the ion to be determined in the test solution,
V_S = added volume of the reference solution,
C_S = concentration of the ion to be determined in the reference solution, and
S = slope of the electrode determined experimentally, at constant temperature, by measuring the difference between the potentials obtained from two references whose concentrations differ by a factor of 10 and are situated within the range where the calibration curve is linear.

Plot on a graph $10^{\Delta E/S}$ (y-axis) against V_S (x-axis) and extrapolate the line obtained until it intersects the x-axis. At the intersection, the concentration C_T of the test solution in the ion to be determined is given by the equation:

$$C_T = \frac{C_S V_S}{V_T}$$

Method III (Single Standard Addition)

To a volume V_T of the test solution prepared as prescribed in the monograph, add a volume V_S of a reference solution containing an amount of the ion to be determined known to give a response situated in the linear part of the calibration curve. Prepare a blank solution in the same conditions. Measure at least three times the potentials of the test solution and the blank solution, before and after adding the reference solution. Calculate the concentration C_T of the ion to be analyzed

using the following equation and making the necessary corrections for the blank:

$$C_T = \frac{C_S V_S}{10^{\Delta E/S}(V_T + V_S) - V_T},$$

where V_T = volume of the test solution or the blank,
C_T = concentration of the ion to be determined in the test solution,
V_S = added volume of the reference solution,
C_S = concentration of the ion to be determined in the reference solution,
ΔE = difference between the average potentials measured before and after adding V_S, and
S = slope of the electrode determined experimentally, at constant temperature, by measuring the difference between the potentials obtained from two reference solutions whose concentrations differ by a factor of 10 and are situated within the range where the calibration curve is linear.

6.5 DETERMINATION OF ETHANOL

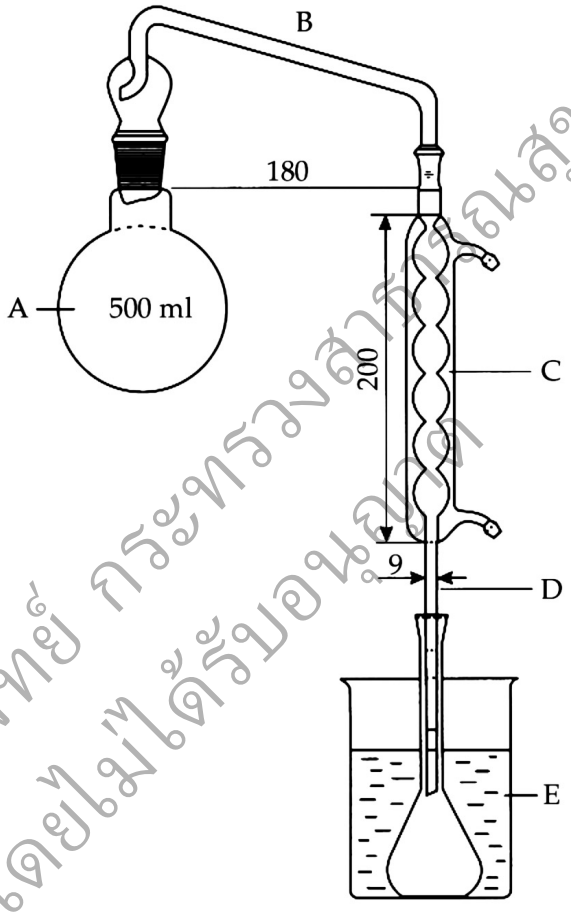
Method I Distillation Method

Method I is to be used for the determination of ethanol, unless otherwise specified in the individual monograph. It is suitable for examining liquid pharmaceutical preparation such as fluid extracts and tinctures, provided the capacity of the distilling flask is sufficient (commonly two to four times the volume of the liquid to be heated) and the rate of distillation is such that clear distillates are produced. Cloudy distillates may be clarified by agitation with *talc*, or with *calcium carbonate*, and filtered, after which the temperature of the filtrate is adjusted and the ethanol content determined from the specific gravity. During all manipulations, take precautions to minimize the loss of ethanol by evaporation.

Apparatus The apparatus (see Figure) consists of a round-bottomed flask (*A*) fitted with a distillation head (*B*) with a steam trap and attached to a vertical condenser (*C*). The latter is fitted at its lower part with a tube (*D*) which carries the distillate into the lower part of a 100-ml or 250-ml volumetric flask. The volumetric flask is immersed in a mixture of ice and water (*E*) during the distillation. A disc having a circular aperture 6 cm in diameter is placed under flask (*A*) to reduce the risk of charring of any dissolved substances.

FROTHING Treat liquids that froth to a troublesome extent during distillation by rendering them strongly acid with phosphoric, sulfuric, or tannic acid, or treat with a slight excess of calcium chloride solution, or with a small amount of paraffin or silicone oil before starting the distillation.

BUMPING Prevent bumping during distillation by adding porous chips of insoluble material such as silicon carbide, or beads.



Apparatus for the Determination of Ethanol Content
Dimensions in mm

For liquids presumed to contain 30 per cent of ethanol or less By means of a pipette, transfer to a suitable distilling apparatus not less than 25 ml of the liquid in which the ethanol is to be determined, and note the temperature at which the volume was measured. Add an equal volume of *water*, distil, and collect a volume of distillate about 2 ml less than the volume taken of the original test liquid. Adjust to the temperature at which the original test liquid was measured, add sufficient *water* to measure exactly the original volume of the test liquid, and mix. The distillate is clear or not more than slightly cloudy, and does not contain more than traces of volatile substances other than ethanol and water. Determine the specific gravity of the liquid at 25° (Appendix 4.9), using this result to ascertain the percentage, by volume, of C_2H_5OH contained in the liquid examined by reference to the *Alcoholometric Table*, p. 496.

For liquids presumed to contain more than 30 per cent of ethanol Proceed as directed in the foregoing paragraph, except: dilute the specimen with about

twice its volume of *water*, and collect a volume of distillate about 2 ml less than twice the volume of the original test liquid. Bring to the temperature at which the original liquid was measured, add sufficient *water* to measure exactly twice the original volume of the test liquid, mix, and determine its specific gravity. The proportion of C_2H_5OH by volume, in this distillate, as ascertained from its specific gravity, equals one-half that in the liquid examined.

Special Treatment

VOLATILE ACIDS AND BASES Render preparations containing volatile bases slightly acidic with *dilute sulfuric acid* before distilling. If volatile acids are present, render the preparation slightly alkaline with *sodium hydroxide TS*.

GLYCEROL To liquids that contain glycerol add sufficient *water* so that the residue, after distillation, contains not less than 50 per cent of water.

IODINE Treat all solutions containing free iodine with *zinc powder* before the distillation, or decolorize with just sufficient 10 per cent w/v solution of *sodium thiosulfate*, followed by a few drops of *sodium hydroxide TS*.

OTHER VOLATILE SUBSTANCES Spirits, elixirs, tinctures, and similar preparations that contain appreciable proportions of volatile materials other than ethanol and water, such as volatile oils, chloroform, ether, camphor, etc., require special treatment, as follows:

For liquids presumed to contain 50 per cent of ethanol or less Mix 25 ml of the sample under examination, accurately measured, with about an equal volume of *water* in a separator. Saturate this mixture with *sodium chloride*, then add 25 ml of *petroleum ether* (*boiling range, 40° to 60°*), and shake the mixture to extract the interfering volatile ingredients. Draw off the separated, lower layer into a second separator, and repeat the extraction twice with two further 25-ml portions of *petroleum ether* (*boiling range, 40° to 60°*). Extract the combined petroleum ether solutions with three 10-ml portions of a saturated solution of *sodium chloride*. Combine the saline solutions, and distil in the usual manner, collecting a volume of distillate having a simple ratio to the volume of the original sample.

For liquids presumed to contain more than 50 per cent of ethanol Adjust the sample under examination to a concentration of approximately 25 per cent of ethanol by diluting it with *water*. Then proceed as directed in the preceding paragraph, beginning with "Saturate this mixture with *sodium chloride*...".

In preparing *Collodion* or *Flexible Collodion* for distillation, use *water* in place of the saturated solution of *sodium chloride* directed above.

If volatile oils are present in small proportions only and a cloudy distillate is obtained, the petroleum ether treatment not having been employed, the distillate may be clarified and rendered suitable for the specific gravity determination by shaking it with about one-fifth its volume of *petroleum ether* (*boiling range, 40° to 60°*), or by filtering it through a thin layer of *talc*.

Method II Gas Chromatographic Method

Method II is to be used where specified in the individual monograph.

Carry out the determination as described in the "Gas Chromatography" (Appendix 3.4).

Standard preparation Dilute 5.0 ml of *absolute ethanol* and 5.0 ml of *1-propanol* with *water* to produce 100.0 ml.

Assay preparation Prepare the following two solutions.

SOLUTION (A) Dilute a volume of the preparation being examined with *water* to obtain a solution containing between 4.0 and 6.0 per cent v/v of ethanol.

SOLUTION (B) Dilute a volume of the preparation being examined and a volume of *1-propanol* with *water* to obtain a solution containing between 4.0 and 6.0 per cent v/v of ethanol and 5.0 per cent v/v of 1-propanol.

Chromatographic system The chromatographic procedure may be carried out using (a) a glass column (1.5 m × 4 mm) packed with porous polymer beads (100 to 120 mesh), maintained at 150° (either Porapak Q or Chromosorb 101 is suitable), (b) *nitrogen* as the carrier gas, and (c) a flame ionization detector; maintain the injection port and the detector at 170°.

System suitability In a suitable chromatogram, the resolution factor is not less than 2, the symmetry factor of the ethanol peak is not more than 2.0, and six replicate injections of the Standard preparation show a relative standard deviation of not more than 2.0 per cent in the ratio of the peak of ethanol to the peak of 1-propanol.

Calculation Calculate the content of C_2H_5OH from the peak areas or peak heights obtained.

ALCOHOLOMETRIC TABLE

Based upon data appearing in *Bull. Natl. Bur. Std.*, 1913, *9*(3), 424-425.

Percentage of C_2H_5OH		Specific Gravity in Air at $\frac{25^o}{25^o}$	Percentage of C_2H_5OH		Specific Gravity in Air at $\frac{25^o}{25^o}$
By Volume at 15.56°	By weight		By weight	By Volume at 15.56°	
0	0.00	1.0000	0	0.00	1.0000
1	0.80	0.9985	1	1.26	0.9981
2	1.59	0.9970	2	2.51	0.9963
3	2.39	0.9956	3	3.76	0.9945
4	3.19	0.9941	4	5.00	0.9927
5	4.00	0.9927	5	6.24	0.9911
6	4.80	0.9914	6	7.48	0.9894
7	5.61	0.9901	7	8.71	0.9879
8	6.42	0.9888	8	9.94	0.9863
9	7.23	0.9875	9	11.17	0.9848
10	8.05	0.9862	10	12.39	0.9833
11	8.86	0.9850	11	13.61	0.9818
12	9.68	0.9838	12	14.83	0.9804
13	10.50	0.9826	13	16.05	0.9789
14	11.32	0.9814	14	17.26	0.9776
15	12.14	0.9802	15	18.47	0.9762
16	12.96	0.9790	16	19.68	0.9748
17	13.79	0.9778	17	20.88	0.9734
18	14.61	0.9767	18	22.08	0.9720
19	15.44	0.9756	19	23.28	0.9706
20	16.27	0.9744	20	24.47	0.9692
21	17.10	0.9733	21	25.66	0.9677
22	17.93	0.9721	22	26.85	0.9663
23	18.77	0.9710	23	28.03	0.9648
24	19.60	0.9698	24	29.21	0.9633
25	20.44	0.9685	25	30.39	0.9617
26	21.29	0.9673	26	31.56	0.9601
27	22.13	0.9661	27	32.72	0.9585
28	22.97	0.9648	28	33.88	0.9568
29	23.82	0.9635	29	35.03	0.9551
30	24.67	0.9622	30	36.18	0.9534
31	25.52	0.9609	31	37.32	0.9516
32	26.38	0.9595	32	38.46	0.9498
33	27.24	0.9581	33	39.59	0.9480
34	28.10	0.9567	34	40.72	0.9461
35	28.97	0.9552	35	41.83	0.9442
36	29.84	0.9537	36	42.94	0.9422
37	30.72	0.9521	37	44.05	0.9402
38	31.60	0.9506	38	45.15	0.9382
39	32.48	0.9489	39	46.24	0.9362
40	33.36	0.9473	40	47.33	0.9341

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ALCOHOLOMETRIC TABLE (continued)

Based upon data appearing in *Bull. Natl. Bur. Std.*, 1913, *9*(3), 424-425.

Percentage of C_2H_5OH		Specific Gravity in Air at $\frac{25^o}{25^o}$	Percentage of C_2H_5OH		Specific Gravity in Air at $\frac{25^o}{25^o}$
By Volume at 15.56°	By weight		By weight	By Volume at 15.56°	
41	34.25	0.9456	41	48.41	0.9320
42	35.15	0.9439	42	49.48	0.9299
43	36.05	0.9421	43	50.55	0.9278
44	36.96	0.9403	44	51.61	0.9256
45	37.87	0.9385	45	52.66	0.9235
46	38.78	0.9366	46	53.71	0.9213
47	39.70	0.9348	47	54.75	0.9191
48	40.62	0.9328	48	55.78	0.9169
49	41.55	0.9309	49	56.81	0.9147
50	42.49	0.9289	50	57.83	0.9124
51	43.43	0.9269	51	58.84	0.9102
52	44.37	0.9248	52	59.85	0.9079
53	45.33	0.9228	53	60.85	0.9056
54	46.28	0.9207	54	61.85	0.9033
55	47.25	0.9185	55	62.84	0.9010
56	48.21	0.9164	56	63.82	0.8987
57	49.19	0.9142	57	64.80	0.8964
58	50.17	0.9120	58	65.77	0.8941
59	51.15	0.9098	59	66.73	0.8918
60	52.15	0.9076	60	67.79	0.8895
61	53.15	0.9053	61	68.64	0.8871
62	54.15	0.9030	62	69.59	0.8848
63	55.17	0.9006	63	70.52	0.8824
64	56.18	0.8983	64	71.46	0.8801
65	57.21	0.8959	65	72.38	0.8777
66	58.24	0.8936	66	73.30	0.8753
67	59.28	0.8911	67	74.21	0.8729
68	60.33	0.8887	68	75.12	0.8706
69	61.38	0.8862	69	76.02	0.8682
70	62.44	0.8837	70	76.91	0.8658
71	63.51	0.8812	71	77.79	0.8634
72	64.59	0.8787	72	78.67	0.8609
73	65.67	0.8761	73	79.54	0.8585
74	66.77	0.8735	74	80.41	0.8561
75	67.87	0.8709	75	81.27	0.8537
76	68.98	0.8682	76	82.12	0.8512
77	70.10	0.8655	77	82.97	0.8488
78	71.23	0.8628	78	83.81	0.8463
79	72.38	0.8600	79	84.64	0.8439
80	73.53	0.8572	80	85.46	0.8414
81	74.69	0.8544	81	86.28	0.8389
82	75.86	0.8516	82	87.08	0.8364
83	77.04	0.8487	83	87.89	0.8339
84	78.23	0.8458	84	88.68	0.8314
85	79.44	0.8428	85	89.46	0.8288

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ALCOHOLOMETRIC TABLE (continued)

Based upon data appearing in *Bull. Natl. Bur. Std.*, 1913, *9*(3), 424-425.

Percentage of C_2H_5OH		Specific Gravity in Air at $\frac{25^o}{25^o}$	Percentage of C_2H_5OH		Specific Gravity in Air at $\frac{25^o}{25^o}$
By Volume at 15.56°	By weight		By weight	By Volume at 15.56°	
86	80.66	0.8397	86	90.24	0.8263
87	81.90	0.8367	87	91.01	0.8237
88	83.14	0.8335	88	91.77	0.8211
89	84.41	0.8303	89	92.52	0.8184
90	85.69	0.8271	90	93.25	0.8158
91	86.99	0.8237	91	93.98	0.8131
92	88.31	0.8202	92	94.70	0.8104
93	89.65	0.8167	93	95.41	0.8076
94	91.03	0.8130	94	96.10	0.8048
95	92.42	0.8092	95	96.79	0.8020
96	93.85	0.8053	96	97.46	0.7992
97	95.32	0.8011	97	98.12	0.7962
98	96.82	0.7968	98	98.76	0.7932
99	98.38	0.7921	99	99.39	0.7902
100	100.00	0.7871	100	100.00	0.7871

6.6 DETERMINATION OF METHOXYL

Apparatus

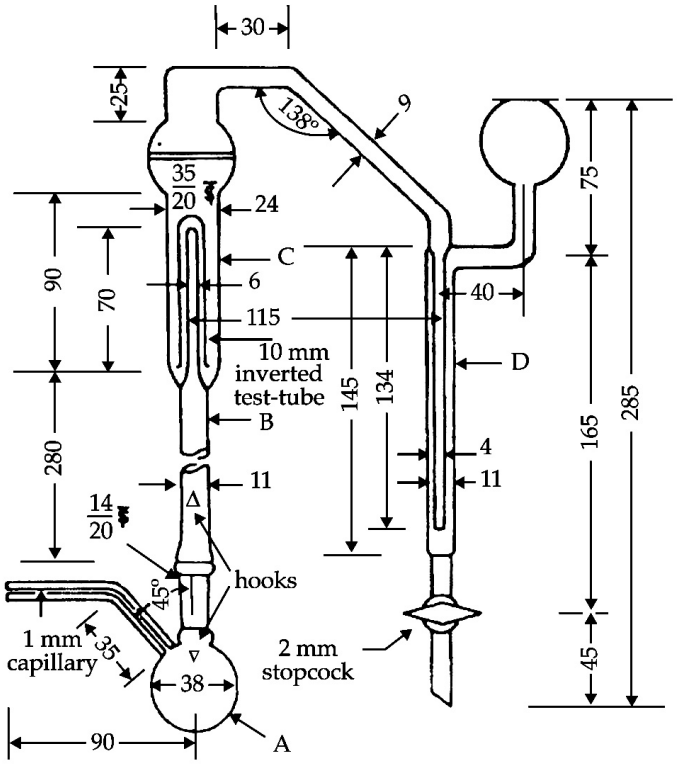
The apparatus for methoxyl determination is diagrammatically in the accompanying figure. The boiling flask, *A*, is fitted with a capillary side-arm for the introduction of carbon dioxide or nitrogen and is connected to a column, *B*, which serves to separate aqueous hydriodic acid from the more volatile methyl iodide. The methyl iodide passes through water in a scrubber trap, *C*, and is finally absorbed in the bromine-acetic acid solution in an absorption tube, *D*. The carbon dioxide or nitrogen is introduced through a pressure-regulating device and connected to the apparatus by a small capillary containing small cotton pledget. (**Note** Avoid the use of organic solvents in cleaning this apparatus, since traces remaining may interfere with the determination.) This test is used also for ethoxyl determination with an 80-minute reaction time and a titrant equivalent of 0.751 mg of ethoxyl moiety (OC_2H_5).

For greater convenience in use and cleaning, a ground-glass ball joint connects the two upright columns of the apparatus. The top of the scrubber *C* consists of a 35/20 ball joint, the upper half of which is connected to the side-arm leading into tube *D*. This permits taking the apparatus apart and facilitates adding the water to the trap. Also, it allows access to the loose inverted (10-mm) test-tube that serves as the trap over the inner tube of the scrubber.

Procedure

Prepare the apparatus by disconnecting the ball joint and pouring *water* into trap *C* until it is half-full. Connect the two parts, using a minimal amount of a suitable silicone grease to seal the ball joint. Add 7 ml of *acetic bromine TS* to absorption tube *D*. Weigh the sample in a tared gelatin capsule, and add it to the boiling flask along with a few boiling chips or pieces of porous plate. Finally add 6 ml of *hydriodic acid* and attach the flask to the column, using a minimal amount of suitable silicone grease to seal the junction. Bubble *carbon dioxide* through the apparatus at the rate of two bubbles per second, place the boiling flask in an oil-bath or heating mantle heated to 150°, and continue the reaction for 40 minutes. Drain the contents of the absorption tube into a 500-ml conical flask containing 10 ml of a 25 per cent w/v solution of *sodium acetate*. Rinse the tube with *water*, adding the rinsings to the flask, and finally dilute with *water* to about 125 ml. Add *formic acid*, dropwise, with swirling until the reddish brown colour of the bromine is discharged, and then add 3 additional drops. A total of 12 to 15 drops usually is required. Allow to stand for 3 minutes, and add 15 ml of *dilute sulfuric acid* and 3 g of *potassium iodide*. Titrate immediately with 0.1 M *sodium thiosulfate VS*, using *starch TS* as indicator near the end of the titration. Perform a blank determination, including also a gelatin capsule, and make any necessary correction. Each ml of

0.1 M *sodium thiosulfate* is equivalent to 0.517 mg of methoxyl moiety (OCH_3).



Apparatus for Determination of Methoxyl
Dimensions in mm

6.7 DETERMINATION OF NITROGEN

Some alkaloids and other nitrogen-containing organic compounds fail to yield all of their nitrogen upon digestion with sulfuric acid therefore these methods cannot be used for the determination of nitrogen in all organic compounds.

Method I

NITRATES AND NITRITES ABSENT Place about 1 g of the substance, accurately weighed, in a 500-ml Kjeldahl flask of hard borosilicate glass. The material to be tested, if solid or semisolid, may be wrapped in a sheet of nitrogen-free filter paper for convenience in transferring it to the flask. Add 10 g of powdered *potassium sulfate* or *anhydrous sodium sulfate*, 500 mg of powdered *copper(II) sulfate*, and 20 ml of *sulfuric acid*. Incline the flask at an angle of about 45°, and gently heat the mixture, keeping the temperature below the boiling point until frothing has ceased. Increase the heat until the acid boils briskly, and continue the heating until the solution has been clear green in colour or almost colourless for 30 minutes. Allow to cool, add 150 ml of *water*, mix the contents of the flask, and again cool. Add cautiously 100 ml of a 40 per cent w/v solution of *sodium hydroxide*, in such a manner as to cause the solution to flow down the inner side of the flask to form a layer under the acid solution. Immediately add a few pieces of granulated *zinc*, and without delay connect the flask to a Kjeldahl connecting bulb (trap), previously attached to a condenser, the delivery tube from which dips beneath the surface of 100 ml of a 4 per cent w/v solution of *boric acid* contained in a conical flask or a wide-mouth bottle of about 500-ml capacity. Mix the contents of the Kjeldahl flask by gentle rotation, and distill until about four-fifths of the contents of the flask has distilled over. Titrate with 0.25 M *sulfuric acid VS*, determining the end-point potentiometrically. Perform a blank determination, and make any necessary correction. Each ml of 0.25 M *sulfuric acid* is equivalent to 7.003 mg of N.

When the nitrogen content of the substance is known to be low, the 0.25 M *sulfuric acid VS* may be replaced by 0.05 M *sulfuric acid VS*. Each ml of 0.05 M *sulfuric acid* is equivalent to 1.401 mg of N.

NITRATES AND NITRITES PRESENT Place a quantity of the substance, accurately weighed, corresponding to about 150 mg of N, in a 500-ml Kjeldahl flask of hard borosilicate glass, and add 25 ml of *sulfuric acid* in which 1 g of *salicylic acid* previously has been dissolved. Mix the contents of the flask, and allow the mixture to stand for 30 minutes with frequent shaking. To the mixture add 5 g of powdered *sodium thiosulfate*, again mix, and add 500 mg of powdered *copper(II) sulfate*. Proceed as directed under Nitrates and Nitrites Absent, beginning with "Incline the flask at an angle of about 45°."

When nitrogen content of the substance is known to exceed 10 per cent, 500 mg to 1 g of *benzoic acid* may be added, prior to digestion, to facilitate the decomposition of the substance.

Method II
(Determination of Protein in Blood Products)

For dried blood products prepare a solution of the preparation as directed in the monograph.

To an accurately measured volume of the preparation being examined expected to contain about 100 mg of protein add sufficient *saline TS* to produce 20.0 ml. To 2.0 ml of the resulting solution, in a 75-ml boiling tube, add 2 ml of a solution containing 75 per cent v/v of *nitrogen-free sulfuric acid*, 4.5 per cent w/v of *potassium sulfate* and 0.5 per cent w/v of *copper(II) sulfate*, mix and loosely stopper the tube. Heat gradually to boiling, boil vigorously for one and a half hours and cool. If the solution is not clear, add 0.25 ml of *hydrogen peroxide TS (20 volumes)*, continue heating until a clear solution is produced and cool. During heating, caution should be taken to ensure that the upper part of the tube is not overheated.

Transfer the solution to a distillation apparatus, with the aid of three 3-ml portions of *water*. Add 10 ml of 10 M *sodium hydroxide* and distil rapidly for 4 minutes, collecting the distillate in a mixture of 5 ml of a saturated solution of *boric acid* and 5 ml of *water* and keeping the tip of the condenser below the level of the acid. Lower the collection flask so that the condenser can drain freely and continue the distillation for a further 1 minute. Titrate with 0.02 M *hydrochloric acid VS* using *methyl red-methylene blue TS* as indicator (V_1 ml).

To a further volume of the preparation being examined, or of the solution prepared from it, expected to contain about 100 mg of protein, add 12 ml of *saline TS*, 2 ml of a 7.5 per cent w/v solution of *sodium molybdate* and 2 ml of mixture of 1 volume of *nitrogen-free sulfuric acid* and 30 volumes of *water*. Shake, allow to stand for 15 minutes, add sufficient *water* to produce 20.0 ml, shake again, and centrifuge.

Using 2 ml of the clear supernatant liquid thus obtained repeat the procedure described above beginning with "in a 75-ml boiling tube..." (V_2 ml). Calculate the protein content in mg per ml of the preparation being examined, using the expression $6.25 \times 0.280 (V_1 - V_2)$ and taking into account the initial dilution.

Method III
(Lowry Method for Determination of Protein)

Dilute the sample, if necessary, to obtain a solution containing 20 to 120 µg/ml of protein. Pipette 1 ml of the resulting solution into a centrifuge tube, add 1 ml of a 10 per cent w/v solution of *trichloroacetic acid* and heat for 15 minutes in a water-bath. Cool and centrifuge for 20 minutes at over $1400 \times g$. Add 2 ml of a 5 per cent w/v solution of *trichloroacetic acid* to the resulting precipitate, shake well and centrifuge. Decant the supernatant liquid and dissolve the precipitate in 2.5 ml of alkaline-copper TS, shake well and allow to stand for at least 10 minutes. To the solution, add 2.5 ml of *water* and 0.5 ml of *sodium molybdotungstophosphate TS* and allow to stand at 37° for 30 minutes. Measure the absorbance of the resulting solution at the maximum at about 750 nm (Appendix 2.2) against the reagent blank. Calculate the protein content from the calibration curve of standard solutions, containing 50 µg per ml, 100 µg per ml and 150 µg per ml of albumin simultaneously treated in the same manner as the sample.

Reagent

ALBUMIN Use a suitable grade of bovine serum albumin for protein standard.

ALKALINE-COPPER TS Mix 0.5 ml of a 2 per cent w/v solution of *copper(II) sulfate* with 0.5 ml of a 4 per cent w/v solution of *sodium tartrate*, add 50 ml of a 4 per cent w/v solution of *sodium hydrogencarbonate* in 0.2 M *sodium hydroxide*, and shake throughly.

CITRATE BUFFER pH 5.0, 0.75 M Dissolve 223.6 g of *trisodium citrate* in sufficient *water* to produce 1000 ml and adjust the pH with *hydrochloric acid*.

CITRATE BUFFER pH 5.0, 0.375 M Mix equal volumes of 0.75 M citrate buffer pH 5.0 and *water* and adjust the pH, if necessary, with *hydrochloric acid*.

6.10 BIOLOGICAL ASSAY OF ANTIBIOTICS

The activity (potency) of antibiotics may be demonstrated under suitable conditions by their inhibitory effect on micro-organisms. A reduction in antimicrobial activity also will reveal subtle changes not demonstrable by chemical methods. Accordingly, microbial or biological assays remain generally the standard for resolving doubt with respect to possible loss of activity.

Two general methods are employed, the diffusion, cylinder-plate or "plate" assay and the turbidimetric or "tube" assay. The first depends upon diffusion of the antibiotic from a vertical cylinder through a solidified agar layer in a Petri dish or plate to an extent such that growth of the added micro-organism is prevented entirely in a circular area or "zone" around the cylinder containing a solution of the antibiotic. The turbidimetric method depends upon the inhibition of growth of a microbial culture in a uniform solution of the antibiotic in a fluid medium that is favorable to its rapid growth in the absence of the antibiotic.

Apparatus

All equipment is to be thoroughly cleansed before and after each use. Glasswares and other receptacles used in the test must be free from micro-organisms and interfering substances. Carefully clean all apparatus used for holding and transferring test organisms and sterilize by dry heat, or by steam as appropriate.

Temperature control Thermostatic control is required in several stages of a microbial assay, when culturing a micro-organism and preparing its inoculum, and during incubation in plate and tube assays. Maintain the temperature of assay plates at ±0.5° of the temperature selected. Closer control of the temperature (±0.1° of the selected temperature) is imperative during incubation in a tube assay, and may be achieved in either circulated air or water, the greater heat capacity of water lending it some advantage over circulating air.

Spectrophotometer Measuring transmittance within a fairly narrow frequency band requires a suitable spectrophotometer in which the wavelength of the light source can be varied or restricted by the use of a 580-nm filter for preparing inocula of the required density, or of a 530-nm filter for reading the absorbance in a tube method (turbidimetric method). For the latter purpose, the instrument may be arranged to accept the tube in which the incubation takes place, to accept a modified cell fitted with a drain that facilitates rapid change of content, or preferably, fixed with a flow-through cell for a continuous flow-through analysis. Set the instrument at zero absorbance with clear, uninoculated broth prepared as specified for the particular antibiotic, including the same amount of test solution and formaldehyde as found in each sample. (**Note** Either absorbance or transmittance measurement may be used for preparing inocula.)

Receptacles for diffusion method For assay plates, use glass or plastic Petri dishes (approximately 20 mm × 100 mm) or square plates (18 mm × 243 mm × 243 mm) having covers of suitable material on the outside. For assay cylinders, use stainless steel, glass, or porcelain cylinders of uniform size with the following dimensions, each dimension having a tolerance of ±0.1 mm: outside diameter 8 mm; inside diameter 6 mm; and length 10 mm. The cylinders used for the plate method have to be of inert material. For particular case of strictly light sensitive antibiotic solution, the use of brown glass or plastic is recommended. Carefully clean the cylinders to remove all residues and sterilize before subsequent use. An acid bath, e.g. 2 M *nitric acid* or *chromic acid cleansing mixture*, is occasionally needed. Alternatively, the cavities prepared in the agar of uniform size may be used as the assay receptacles.

Receptacles for turbidimetric method For assay tubes, use glass or plastic test-tubes, e.g. 16 mm × 125 mm or 18 mm × 150 mm that are relatively uniform in length, diameter and thickness and substantially free from surface blemishes and scratches. Tubes that are to be placed in the spectrophotometer are matched and are without scratches or blemishes. Cleanse thoroughly to remove all antibiotic residues and traces of cleaning solution, and sterilize tubes that have been used previously, before subsequent use.

Media and Diluents

Media The media required for the preparation of test organism inocula are made from the ingredients listed herein. Minor modifications of the individual ingredients, or reconstituted dehydrated media, may be substituted, provided the resulting media possess equal or better growth-promoting properties and give a similar standard curve response.

Dissolve the ingredients in *water* to make 1 litre, and adjust the solutions with either 1 M *sodium hydroxide* or 1 M *hydrochloric acid* as required, so that after steam sterilization the pH is as specified.

MEDIUM 1

Peptone	6.0	g
Pancreatic digest of casein	4.0	g
Beef extract	1.5	g
Yeast extract	3.0	g
Dextrose monohydrate	1.0	g
Agar	15.0	g
Water	1000	ml

pH after sterilization: 6.6±0.1

MEDIUM 2

Peptone	5.0	g
Yeast extract	1.5	g
Beef extract	1.5	g
Sodium chloride	3.5	g
Dextrose monohydrate	1.0	g
Dipotassium hydrogenphosphate	3.68	g
Potassium dihydrogenphosphate	1.32	g
Water	1000	ml

pH after sterilization: 7.0±0.05

MEDIUM 3

Peptone	6.0	g
Yeast extract	3.0	g
Beef extract	1.5	g
Agar	15.0	g
Water	1000	ml

pH after sterilization: 5.9±0.1

MEDIUM 4

Same as Medium 1, except that the final pH after sterilizaion is 8.3±0.1

MEDIUM 5

Peptone	9.4	g
Yeast extract	4.7	g
Beef extract	2.4	g
Sodium chloride	10.0	g
Dextrose monohydrate	10.0	g
Agar	23.5	g
Water	1000	ml

pH after sterilization: 6.1±0.1

MEDIUM 6

Same as Medium 1, except for the additional ingredient 0.3 g of *Manganese Sulfate*

MEDIUM 7

Same as Medium 2, except that the final pH after sterilization is 7.9±0.1

MEDIUM 8

Peptone	6	g
Beef extract	1.5	g
Yeast extract	3	g
Sodium chloride	3.5	g
Dextrose monohydrate	1	g
Dipotassium hydrogenphosphate	3.68	g
Potassium dihydrogenphosphate	1.32	g
Water	1000	ml

pH after sterilization: 7.0±0.1

MEDIUM 9

Heart extract	1.5	g
Yeast extract	1.5	g
Peptone-casein	5	g
Dextrose monohydrate	1	g
Sodium chloride	3.5	g
Dipotassium hydrogenphosphate	3.68	g
Potassium dihydrogenphosphate	1.32	g
Potassium nitrate	2	g
Water	1000	ml

ลิขสิทธิ์ของสำนักงานคณะกรรมการอาหารและยา ห้ามทำซ้ำ ดัดแปลง นำไปใช้ในเชิงพาณิชย์ โดยไม่ได้รับอนุญาต

pH after sterilization: 7.0±0.1

MEDIUM 10

Peptone	5	g
Meat extract	3	g
Disodium hydrogenphosphate	26.9	g
Agar	10	g
Water	1000	ml

pH after sterilization: 7.9±0.1

The disodium hydrogenphosphate is added as a sterile solution after sterilization of the medium.

Phosphate buffers and other solutions Prepare as follows, or by other suitable means, the potassium phosphate buffers required for the antibiotic under asssay. Dissolve the ingredients in sufficient *water* to make 1 litre, and adjust with *phosphoric acid* or 10 M *potassium hydroxide* to yield the required pH. The buffers are sterilized after preparation, and the pH specified in each case is the pH after sterilization.

BUFFER 1, 1 PER CENT, pH 6.0±0.05

Dipotassium hydrogenphosphate	2.0	g
Potassium dihydrogenphosphate	8.0	g
Water	1000	ml

BUFFER 2, 0.1 M, pH 8.0±0.1

Dipotassium hydrogenphosphate	16.73	g
Potassium dihydrogenphosphate	0.523	g
Water	1000	ml

BUFFER 3, 0.1 M, pH 4.5±0.05

Dipotassium hydrogenphosphate	13.61	g
Water	1000	ml

BUFFER 4, 10 PER CENT, pH 6.0±0.05

Dipotassium hydrogenphosphate	20.0	g
Potassium dihydrogenphosphate	80.0	g
Water	1000	ml

BUFFER 5, 0.1 M, pH 7.0±0.2

Dipotassium hydrogenphosphate	13.6	g
Potassium dihydrogenphosphate	4.0	g
Water	1000	ml

Reference Substances and Units

The reference substances used in the assay are substances whose activity has been precisely determined with reference to the corresponding International Biological Standard or International Biological Reference Preparation. The Reference Substances are available from the Bureau of Drug and Narcotic, Department of Medical Sciences, Ministry of Public Health or other recognized institutions.

The potency of an antibiotic is expressed in International Units. An International Unit (IU) is the specific activity contained in such an amount (weight) of the relevant International Biological Standard or International Biological Reference Preparation that the WHO Expert Committee on Biological Standardization may, from time to time, indicate as the quantity exactly equivalent to the unit accepted for international use. In some cases, a defined number of international units may be assigned to the total contents of some material, since difficulties are experienced in weighing with adequate accuracy small amounts of the relevant International Biological Standard or International Biological Reference Preparation.

The potency of some antibiotics may be expressed in microgram (µg) of activities. The µg of activity does not necessarily correspond to the µg (weight) of the antibiotic substance, since the latter may not consist entirely of a pure single chemical entity of that individual antibiotic.

Preparation of the Standard

To prepare a stock solution, dissolve a quantity of the Reference Substance of a given antibiotic, accurately weighed, or the entire contents of a vial of Reference Substance, where appropriate, in the solvent specified in Table 1, and then dilute to the required concentration as indicated. Store in a refrigerator, and use within the period indicated. On the day of the assay, prepare from the stock solution five or more test dilutions, the successive solutions increasing stepwise in concentration, usually in the ratio of 1:1.25 for a cylinder-plate assay or smaller for a turbidimetric assay. Use the final diluent specified and a sequence such that the middle or median has the concentration designated.

Preparation of the Sample

From the information available for the preparation to be assayed (the "Unknown"), assign to it an assumed potency per unit weight or volume, and on this assumption prepare on the day of the assay a stock solution and test dilution as specified for each antibiotic but with the same final diluent as used for the Reference Substance. The assay with five levels of the Standard requires only one level of the Unknown at a concentration assumed equal to the median level of the Standard.

Organisms and Inoculum

Test organisms The test organism for each antibiotic is listed in Table 2. The method of assay is given for each in Table 1. Maintain a culture on slants of the medium and under the incubation conditions specified in Tables 3 and 4 and transfer weekly to fresh slants. For *Klebsiella pneumoniae* use a noncapsulated culture.

Table 1 Preparation of Stock Solutions and Test Dilutions of Reference Substances

Antibiotic and Type of Assay (Cylinder-plate (CP) or Turbidimetric (T))	Stock Solution			Test Dilution	
	Initial Solvent (and initial concentration where specified); Further Diluent, If different	Final Stock Concentration per ml	Use Within	Final Diluent	Median Dose (μg of activity or Units per ml)
Erythromycin (CP)	*Methanol* (10 mg/ml); Buffer 2	1 mg	14 days	Buffer 2	1.0 μg
Gentamicin (CP)	Buffer 2	1 mg	30 days	Buffer 2	0.1 μg
Neomycin (CP)	Buffer 2	1 mg	14 days	Buffer 2	1.0 μg
Neomycin (T)	Buffer 2	100 μg	14 days	Buffer 2	1.0 μg
Nystatin (CP)	*Dimethylformamide*	1000 IU	Same day	Buffer 4	20 IU
Streptomycin (T)	*Water*	1 mg	30 days	*Water*	30 μg
Vancomycin (CP)	*Water*	1 mg	7 days	Buffer 3	10 μg

Notes For neomycin turbidimetric assay, dilute the 100-μg-per-ml stock solution quantitatively with Buffer 2 to obtain a solution having a concentration equivalent to 25.0 μg of neomycin per ml. To separate 50-ml volumetric flasks add 1.39, 1.67, 2.00, 2.40, and 2.88 ml of this solution, add 5.0 ml of 0.01 M *hydrochloric acid* to each flask, dilute with Buffer 3 to volume, and mix to obtain solutions having concentrations of 0.69, 0.83, 1.0, 1.2, and 1.44 μg of neomycin per ml. Use these solutions to prepare the standard response line.

For nystatin, further dilute the stock solution with *dimethylformamide* to give concentrations of 256, 320, 400, 500, and 624 Units per ml prior to making the test dilutions. Prepare the standard response line solutions simultaneously with dilutions of the sample to be tested. The *Test Dilution* of the sample should contain the same amount of dimethylformamide as the test dilutions of the Standard. Use red low-actinic glassware.

Preparation of inoculum

Bacillus subtilis; Bacillus pumilus Spore suspensions of the organisms to be used as inocula are prepared as follows. Grow the organism at 35° to 37° for 7 days[1] on the surface of Medium 6. Using sterile *water*, wash off the growth, which consists mainly of spores. Heat the suspension at 70° for 30 minutes and dilute to give an appropriate concentration of spores, usually 10×10^6 to 100×10^6 per ml. The spore suspensions may be stored for long periods at a temperature not exceeding 4°. Alternatively, spore suspensions may be prepared by cultivating the organisms in Medium 8 at 26° for 4 to 6 days, then adding, aseptically, sufficient *manganese sulfate* to give a concentration of 0.001 g per litre and incubating for a further 48 hours. Examine the suspension microscopically to ensure that adequate spore formation has taken place (about 80 per cent) and centrifuge. Resuspend the sediment in sterile *water* to give a concentration of 10×10^6 to 100×10^6 spores per ml, and then heat to 70° for 30 minutes. Store the suspension at a temperature not exceeding 4°.

Klebsiella pneumoniae; *Micrococcus luteus*; *Staphylococcus aureus*; *Staphylococcus epidermidis* Grow the test organism on Medium 1 at 32° to 35° for 24 hours[2] and adjust the opacity to one which has been shown to produce a satisfactory dose-response relationship in the turbidimetric assay, or to produce clearly defined zones of inhibition of convenient diameter in the diffusion assay, as appropriate.

Saccharomyces cerevisiae; *Candida tropicalis* Grow the test organism on Medium 5 at 29° to 31° for 48 hours[3]. Wash off the growth with sterile *saline TS*. Dilute to a suitable opacity with the same solution. Determine by trial the quantity of stock suspension to be used as the inoculum, starting with the volume suggested in Table 2. The trial tests should be incubated for the times indicated in the section Turbidimetric method. Adjust the quantity of inoculum on a daily basis, if necessary, to obtain the optimum dose-response relationship from the amount of growth of the test organism in the assay tubes and the length of the time of incubation. At the completion of the incubation periods described in the section Turbidimetric method, tubes containing the median dose of the Standard should have absorbances of at least 0.3 absorbance unit.

For the cylinder-plate assay, determine by trial the proportions of stock suspension to be incorporated in the inoculum, starting with the volumes indicated in Table 2, that result in satisfactory demarcation of the zones of inhibition of about 14 to 16 mm in diameter and giving a reproducible dose relationship. Prepare the inoculum by adding a portion of stock suspension to a sufficient amount of agar medium that has been melted and cooled to 45° to 50°, and swirling to attain a homogeneous suspension.

Procedure

Assay designs The potency of an antibiotic is estimated by comparing the inhibition of growth of sensitive micro-organisms produced by known concentrations of the antibiotic to be examined and a reference substance.

[1]Except for *Bacillus subtilis* (incubate at 32° to 35° for 5 days).

[2]Except for *Klebsiella pneumoniae* (incubate at 36° to 37.5° for 16 to 24 hours).

[3]Except for *Candida tropicalis* (incubate at 30° to 37° for 24 hours).

Table 2 Preparation of Inoculum

Test Organism	Incubation Conditions			Suggested Inoculum Composition		Antibiotic and Type of Assay (Cylinder-plate (CP) or Turbidimetric (T))
	Medium	Temperature (°)	Time	Medium	Amount (ml per 100 ml medium)	
Bacillus pumilus NCTC 8241	6	35 - 37	7 days	1 1 10	As required	Erythromycin (CP) Gentamicin (CP) Neomycin (CP)
Bacillus subtilis ATCC 6633 DMST 15896 NCIB 8054 NCTC 8236, 10400	6	32 - 35	5 days	1 10 1 3	As required	Erythromycin (CP) Neomycin (CP) Streptomycin (CP) Vancomycin (CP)
Candida tropicalis NCYC 1393	5	30 - 37	24 hours	5	As required	Nystatin (CP)
Klebsiella pneumoniae ATCC 10031 NCIB 9111 NCTC 7427	1	36 - 37.5	16-24 hours	9 7 2	As required 2 0.1	Erythromycin (T) Neomycin (T) Streptomycin (T)
Micrococcus luteus (*Kocuria rhizophila*) ATCC 9341 DMST 15503	1	32 - 35	24 hours	4	1.5	Erythromycin (CP)
Saccharomyces cerevisiae ATCC 2601, 9763 NCYC 87	5	29 - 31	48 hours	5	1.0	Nystatin (CP)
Staphylococcus aureus ATCC 6538 P DMST 6512 NCTC 7447	1	32 - 35	24 hours	8	As required	Erythromycin (T) Gentamicin (T) Neomycin (T) Vancomycin (T)
Staphylococcus epidermidis ATCC 12228 DMST 15505 NCIB 8853	1	32 - 35	24 hours	1 4	0.03 0.4	Gentamicin (CP) Neomycin (CP)

ATCC — American Type Culture Collection
10801 University Boulevard, Manassas VA 20110-2209, USA (http://www.atcc.org)

DMST — Department of Medical Sciences, Ministry of Public Health, Nonthaburi 11000, Thailand

NCIB — National Collection of Industrial Bacteria
Torry Research Station, PO Box 31, 135 Abbey Road, Aberdeen AB9 8DG, Scotland
(**Note** In 1983, the management of the collection of industrial bacteria under NCIB was transferred to the National Collection of Industrial and Marine Bacteria Ltd (NCIMB), 23 St Machar Drive, Aberdeen AB2 1RY, Great Britain.)

NCTC — National Collection of Type Cultures
Central Public Health Laboratory, Colindale Avenue, London NW9 5HT, Great Britain

NCYC — National Collection of Yeast Cultures
AFRC Food Research Institute, Colney Lane, Norwich NR4 7UA, Great Britain

ลิขสิทธิ์ กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข
ห้ามมิให้ทำซ้ำ ดัดแปลง แก้ไข โดยไม่ได้รับอนุญาต

The assay must be designed in a way that will permit examination of the validity of the mathematical model on which the potency equation is based. If a parallel-line model is chosen, the two log dose-response (or transformed response) lines of the preparation being examined and the reference preparation must be parallel; they must be linear over the range of doses used in the calculation. These conditions must be verified by validity tests for a given probability, usually $P = 0.05$. Other mathematical models, such as the slope ratio model, may be used provided that proof of validity is demonstrated (Appendix 9).

Unless otherwise stated in the monograph, the confidence limits ($P = 0.95$) of the assay for potency are not less than 95 per cent and not more than 105 per cent of the estimated potency.

Carry out the assay by the diffusion or turbidimetric method as listed in Tables 3 and 4.

Diffusion method (Cylinder-plate method) Liquefy a medium suitable for the conditions of the assay and inoculate it at a suitable temperature, for example, 48° to 50° for vegetative forms, with a known quantity of a suspension of micro-organisms sensitive to the antibiotic being examined, such that clearly defined zones of inhibition of suitable diameter are produced with the concentrations of the antibiotic used for the assay. Immediately pour into Petri dishes or large rectangular dishes a quantity of the inoculated medium to form a uniform layer 2 to 5 mm thick. Alternatively, the medium may consist of two layers, only the upper layer being inoculated.

Store the dishes so that no appreciable growth or death of the micro-organisms occurs before the dishes are used and so that the surface of the medium is dry at the time of use.

Using the solvent and the buffer solution indicated in Table 3, prepare solutions of the reference substance and of the antibiotic being examined having known concentrations and presumed to be of equal activity. Apply the solutions to the surface of the medium, for example, in sterile cylinders of porcelain, stainless steel or other suitable material, or in cavities prepared in the agar. The same volume of solution must be added to each cylinder or cavity. Alternatively, use sterile absorbent paper discs of suitable quality; impregnate the discs with the solutions of the reference substance or the solutions of the antibiotic to be examined and place on the surface of the agar.

In order to assess the validity of the assay, use not less than three doses of the reference substance and three doses of the antibiotic being examined having the same presumed activity as the doses of the reference substance. It is preferable to use a series of doses in geometric progression. In routine assays when the linearity of the system has been demonstrated over an adequate number of experiments using a three-point assay, a two-point assay may be sufficient, subject to agreement by the competent authority. However, in all cases of dispute, a three-point assay as described above must be applied.

Arrange the solutions on each Petri dish or on each rectangular dish according to a statistically suitable design, except for small Petri dishes that cannot accommodate more than six solutions, arrange the solutions of the antibiotic to be examined and the solutions of the reference substance in an alternate manner to avoid interaction of the more concentrated solutions.

Incubate at a suitable temperature for about 18 hours. A period of diffusion prior to incubation, usually 1 to 4 hours, at room temperature or at about 4°, as appropriate, may be used to minimize the effects of the variation in time between the application of the solutions and to improve the regression slope.

Measure the diameters with a precision of at least 0.1 mm or the areas of the circular inhibition zones with a corresponding precision and calculate the potency using appropriate statistical methods.

Use in each assay the number of replications per dose sufficient to ensure the required precision. The assay may be repeated and the results combined statistically to obtain the required precision and to ascertain whether the potency of the antibiotic to be examined is not less than the minimum required.

For the 1-level assay with a standard curve, prepare dilution representing five test levels of the Standard (S_1 to S_5) and a single test level of the Unknown U_3 corresponding to S_3 of the standard curve defined under Preparation of the Standard and Preparation of the Sample. For deriving the standard curve, fill alternate cylinders as each of three plates with the median test dilution (S_3) of the Standard and each of the remaining nine cylinders with one of the other dilutions of the Standard. Repeat the process for the three dilutions of the Standard. For each Unknown, fill alternate cylinders on each three plates with the median test dilution of the Standard (S_3), and the remaining nine cylinders with the corresponding test dilution of the Unknown (U_3).

ESTIMATION OF POTENCY To establish a standard response line (standard curve), average the 36 inhibition zone diameters of the standard median concentration in all the four groups of plates. Use this value as the "corrected, median point" of the standard curve. Average also the nine inhibition zone diameters of the standard median concentration in each of the four groups of three plates. Correct the average diameter obtained for each concentration of the standard test solutions to the figure it would be if the average median concentration diameter for that group of three plates were the same as the corrected median point. Thus, if in correcting the figure for the highest concentration of the standard curve, the average of the 36 diameters of the median concentration, the corrected median point, is 16.5 mm, and if the average of the median concentration of the group of three plates containing the highest concentration of the standard test solution is 16.3 mm, the correction is +0.2 mm. If the average reading of the inhibition zone diameter of the highest concentration of

Table 3 Diffusion Method

Antibiotic	Reference Substance	Solvent to Be Used in Preparing the Stock Solution	Buffer Solution	Micro-organism	Medium	Incubation Temperature (°)
Erythromycin Estolate	Erythromycin RS	*Methanol* (see the monographs)	Buffer 2	*Bacillus pumilus* NCTC 8241	1	32-35
				Bacillus subtilis ATCC 6633 DMST 15896 NCTC 10400	1	32-35
				Micrococcus luteus ATCC 9341 DMST 15503	1	32-35
Erythromycin Stearate	Erythromycin RS	*Methanol*	Buffer 2	*Bacillus pumilus* NCTC 8241	1	32-35
				Bacillus subtilis ATCC 6633 DMST 15896 NCTC 10400	1	32-35
				Micrococcus luteus ATCC 9341 DMST 15503	4	32-35
Gentamicin Sulfate	Gentamicin Sulfate RS	*Water*	Buffer 2	*Bacillus pumilus* NCTC 8241	1	32-35
				Staphylococcus epidermidis ATCC 12228 DMST 15505 NCIB 8853	1	32-35
Neomycin Sulfate	Neomycin Sulfate for Microbiological Assay RS	*Water*	Buffer 2	*Bacillus pumilus* NCTC 8241	10	32-35
				Bacillus subtilis ATCC 6633 DMST 15896 NCTC 10400	10	32-35
				Staphylococcus epidermidis ATCC 2228 DMST 15505	4	32-35
Nystatin	Nystatin RS	*Dimethylformamide*	Buffer 1 containing 5 per cent v/v of *dimethylformamide*	*Candida tropicalis* NCYC 1393	5	30-37
				Saccharomyces cerevisiae ATCC 9763, 2601 NCYC 87	5	29-31
Streptomycin Sulfate	Streptomycin Sulfate RS	*Water*	Buffer 2	*Bacillus subtilis* ATCC 6633 DMST 15896 NCTC 10400	1	32-35
Vancomycin Hydrochloride	Vancomycin Hydrochloride RS	*Water*	Buffer 2	*Bacillus subtilis* ATCC 6633 DMST 15896 NCIB 8054 NCTC 8236	3	37-39

Note For abbreviations, see under Table 2.

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the standard test solution of these same three plates is 16.9 mm, the corrected diameter is then 17.1 mm. Plot these corrected diameters, including the corrected median point, on a two-cycle semilog paper, using the concentrations of the antibiotic in micrograms or units per ml as the ordinate (logarithmic scale) and the diameters of the inhibition zones as the abscissa (arithmatic scale). Thus, the response line is drawn through points plotted for the highest and lowest zone diameters obtained by means of the following equations:

$$L = \frac{3a + 2b + c - e}{5},$$

$$H = \frac{3e + 2d + c - a}{5},$$

where L = calculated zone diameter for the lowest concentration of the standard response line,

H = calculated zone diameter for the highest concentration of the standard response line,

c = average zone diameter of 36 readings of the median point standard solution,

a, b, d, and e = corrected average values for the other standard solutions, lowest to highest concentration, respectively.

To estimate the potency of the Unknown, average the zone diameters of the Standard and the zone diameters of the Unknown on the three plates used. If the average zone diameter of the Unknown is larger than that of the Standard, add the difference between them to the median concentration diameter of the standard response line. If the average zone diameter of the Unknown is lower than that of the Standard, subtract the difference between them from the median concentration diameter of the standard response line. From the response line, read the concentration corresponding to these corrected values of zone diameters. Multiply the concentration by the appropriate dilution factor to obtain the antibiotic content of the Unknown.

Turbidimetric method (Tube method) Inoculate a suitable medium with a suspension of the chosen microorganism having a sensitivity to the antibiotic being examined such that a sufficiently large inhibition of microbial growth occurs in the conditions of the test. Use a known quantity of the suspension chosen so as to obtain a readily measurable opacity after an incubation period of about 4 hours.

Use the inoculated medium immediately after its preparation.

Using the solvent and the buffer solution indicated in Table 4 prepare solutions of the reference substance and of the antibiotic being examined having known concentrations presumed to be of equal activity.

In order that the validity of the assay may be assessed, use not less than three doses of the reference substance and three doses of the antibiotic to be examined having the same presumed activity as the doses of the reference substance. It is preferable to use a series of doses in geometric progression. In order to obtain the required linearity, it may be necessary to select from a large number three consecutive doses, using corresponding doses for the reference substance and the antibiotic being examined.

Distribute an equal volume of each of the solutions into identical test-tubes and add to each tube an equal volume of inoculated medium (for example, 1 ml of the solution and 9 ml of the medium). Prepare at the same time two control tubes without antibiotic both containing the inoculated medium and to one of which is added immediately 0.5 ml of *formaldehyde solution*. These tubes are used to set the optical apparatus used to measure the growth.

Place all the tubes, randomly distributed or in a Latin square or randomized block arrangement, in a water-bath or other suitable apparatus fitted with a means of bringing all the tubes rapidly to the appropriate incubation temperature and maintain them at that temperature for 3 to 4 hours, taking precautions to ensure uniformity of temperature and identical incubation time.

After incubation, stop the growth of the microorganisms by adding 0.5 ml of *formaldehyde solution* to each tube or by heat treatment and measure the opacity to three significant figures using suitable optical apparatus. Alternatively, use a method which allows the opacity of each tube to be measured after exactly the same period of incubation.

Calculate the potency using appropriate statistical methods (Appendix 9).

Linearity of the dose-response relationship, transformed or untransformed, is often obtained only over a very limited range. It is this range which must be used in calculating the activity and it must include at least three consecutive doses in order to permit linearity to be verified. In routine assays when the linearity of the system has been demonstrated over an adequate number of experiments using a three-point assay, a two-point assay may be sufficient, subject to agreement by the competent authority. However, in all cases of dispute, a three-point assay must be applied.

Use in each assay the number of replications per dose sufficient to ensure the required precision. The assay may be repeated and the results combined statistically to obtain the required precision and to ascertain whether the potency of the antibiotic being examined is not less than the minimum required.

For the 1-level assay with a standard curve, prepare dilutions representing five test levels of the Standard (S_1 to S_5) and a single use level (U_3) of each of up to 20 Unknowns corresponding to S_3 of the Standard. Prepare also an extra S_3 as a test of growth. Add 1 ml of each test dilution to three tubes and 1 ml of antibiotic-free diluent to six tubes as controls. Distribute one complete set, including two tubes of controls to a tube rack, intermingling them at random. Add 9.0 ml of inoculum, incubate, add 0.5 ml of diluted *formaldehyde*

Table 4 Turbidimetric Method

Antibiotic	Reference Substance	Solvent to Be Used in Preparing the Stock Solution	Buffer Solution	Micro-organism	Medium	Incubation Temperature (°)
Erythromycin Estolate	Erythromycin RS	*Methanol* (see the monographs)	Buffer 2	*Klebsiella pneumoniae* ATCC 10031 NCIB 9111 NCTC 7427	9	36-37.5
Erythromycin Ethylsuccinate				*Staphylococcus aureus* ATCC 6538 P DMST 6512 NCTC 7447	8	35-37
Erythromycin Stearate	Erythromycin RS	*Methanol*	Buffer 2	*Klebsiella pneumoniae* ATCC 10031 NCIB 9111 NCTC 7427	9	36-37.5
				Staphylococcus aureus ATCC 6538 P DMST 6512 NCTC 7447	8	35-37
Gentamicin Sulfate	Gentamicin Sulfate RS	*Water*	Buffer 5	*Staphylococcus aureus* ATCC 6538 P DMST 6512 NCTC 7447	8	35-37
Neomycin Sulfate	Neomycin Sulfate for Microbiological Assay RS	*Water*	Buffer 2	*Staphylococcus aureus* ATCC 6538 P DMST 6512 NCTC 7447	8	35-37
				Klebsiella pneumoniae ATCC 10031 NCIB 9111 NCTC 7427	7	36-37.5
Streptomycin Sulfate	Streptomycin Sulfate RS	*Water*	Buffer 2	*Klebsiella pneumoniae* ATCC 10031 NCIB 9111 NCTC 7427	2	36-37.5
Vancomycin Hydrochloride	Vancomycin Hydrochloride RS	*Water*	Buffer 2	*Staphylococcus aureus* ATCC 6538 P DMST 6512 NCTC 7447	8	37-39

Note For abbreviation, see under Table 2.

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solution (1 in 3), and complete the assay as directed above. Determine the exact duration of incubation by observation of growth in the reference concentration (median dose) of the dilutions of the Standard (S_3).

ESTIMATION OF POTENCY To prepare the standard response line, plot the average transmittance or absorbance values for each concentration of the standard response line on a one-cycle semilog paper, with the transmittance or absorbance values on the arithmatic scale and the concentrations on the logarithmic scale. The response line is drawn through points plotted for the highest and lowest transmittance or absorbance values by means of the equations stated in Plate method.

To estimate the potency of the Unknown, average the transmittance or absorbance values of the Unknown and determine the antibiotic concentration from the standard response line. Multiply the concentration by the appropriate dilution factor to obtain the antibiotic content of the Unknown.

6.17 RESIDUAL TITRATIONS

Some assays require the addition of a measured volume of a volumetric solution, in excess of the amount actually needed to react with the substance being assayed, the excess of this solution then being titrated with a second volumetric solution. This constitutes a residual titration and is known also as a "back titration". The quantity of the substance being titrated may be calculated from the difference between the volume of the volumetric solution originally added and that consumed by the titrant in the back titration, due allowance being made for the respective molarity factors of the two solutions, and the equivalence factor for the substance given in the individual monograph.

6.22 CONTENT OF ANTIMICROBIAL AGENTS

An essential component of injections preserved in multiple-dose containers is the agent or agents present to reduce the hazard of having introduced, in the course of removing some of the contents, accidental microbial contamination of the contents remaining. It is a Pharmacopoeial requirement that the presence and amount added of such agent(s) be declared on the label of the container. The methods provided herein for the most commonly used agents are to be used to demonstrate that the declared agent is present but does not exceed the labelled amount by more than 20 per cent of the labelled amount.

The concentration of an antimicrobial preservative added to a multiple-dose or single-dose parenteral, ear, nose, and eye preparation may diminish during the shelf-life of the product. Because it is recognized that the antimicrobial preservative concentration in a given preparation may decrease during the product's shelf-life, the manufacturer shall determine the lowest level at which the preservative is effective, and the product should be so formulated as to assure that this level is exceeded throughout the product's shelf-life. At the time of its manufacture, the product should contain the declared amount of antimicrobial preservative (within ±20 per cent to allow for manufacturing and analytical variations). The quantitative label statement of the preservative content is not intended to mean that the labelled quantity is retained during the shelf-life of the product; rather, it is a statement of the amount added, within process limits, and which is not exceeded by more than 20 per cent. An example of such a label statement is "___(unit) added as preservative." (**Note** "___(unit)" would be a number followed by the unit of measurement, e.g., 0.015 mg per ml or 0.1 per cent.)

The most commonly used agents include the two mercurials, phenylmercuric nitrate and thiomersal and the four homologous esters of *p*-hydroxybenzoic acid, phenol, benzyl alcohol, and chlorobutanol. The methods for the first two names are polarographic, while quantitative gas chromatography is employed in the determination of the other agents.

GENERAL GAS CHROMATOGRAPHIC METHOD

The general procedures set forth in the following paragraphs are applicable to the quantitative determination of benzyl alcohol, chlorobutanol, phenol, and the methyl, ethyl, propyl, and butyl esters of *p*-hydroxybenzoic acid, the latter being treated as a group, the individual members of which, if present, are capable of separate determination. Prepare the internal standard solution and the standard preparation for each agent as directed individually below. Unless otherwise directed below, prepare the test preparation from accurately measured portions of the internal standard solution and the sample under test, of such size that the concentration of the agent and the composition of the solvent correspond closely to the concentration and composition of the standard preparation.

Benzyl Alcohol

Carry out determination as described in the "Gas Chromatography" (Appendix 3.4).

Internal standard solution Dissolve about 380 mg of *phenol* in 10 ml of *methanol* contained in a 200-ml volumetric flask. Add *water* to volume, and mix.

Standard preparation Dissolve about 180 mg of *benzyl alcohol*, accurately weighed, in 20.0 ml of *methanol* contained in a 100-ml volumetric flask. Add internal standard solution to volume, and mix.

Chromatographic system The chromatographic procedure may be carried out using (a) a glass column (1.8 m × 3 mm), packed with 5 per cent liquid phase of polyethyleneglycol compound (av. mol. wt. about 15,000) (Carbowax 20M or equivalent is suitable) on *diatomaceous support* (80- to 100- or 100- to 120-mesh), maintained at 140° (**Note** The *diatomaceous support* is *acid-washed* and then water-washed until neutral, but it is not *alkali-washed*. It may be *silanized*.), and (b) *helium* or *nitrogen* as the carrier gas at a flow rate of 50 ml per minute.

Procedure Separately inject equal volumes (about 5 µl) of the standard preparation and the test preparation into the chromatograph, and record the chromatograms. Measure the areas under the peaks for benzyl alcohol and phenol of the chromatogram for the standard preparation, designating them P_1 and P_2, respectively. Similarly, determine the corresponding values p_1 and p_2 for the test preparation.

Calculation Calculate the content, in mg per ml, of benzyl alcohol (C_7H_8O) in the sample taken by the expression:

$$100(C/V)\,(p_1/p_2)\,(P_2/P_1),$$

in which C is the concentration, in mg per ml, of benzyl alcohol in the standard preparation, and V is the volume, in ml, of the sample under test used in preparing each 100 ml of the test preparation.

Chlorobutanol

Carry out the determination as described in the "Gas Chromatography" (Appendix 3.4).

Internal standard solution Transfer about 140 mg of *benzaldehyde* to a 100-ml volumetric flask, add 10 ml of *methanol*, and swirl to dissolve. Dilute with *water* to volume, and mix.

Standard preparation Transfer about 125 mg of *chlorobutanol*, accurately weighed, to a 25-ml volumetric flask. Add 2 ml of *methanol*, swirl to dissolve, dilute with *water* to volume, and mix. Transfer 5.0 ml of this solution and 5.0 ml of internal standard solution to a 25-ml flask, and mix to obtain a solution having a known concentration of about 2.5 mg of chlorobutanol per ml.

Test preparation Dilute, if necessary, an accurately measured volume of the sample under test quantitatively with *methanol* to obtain a solution containing not more than about 5.0 mg of chlorobutanol per ml. Combine 3.0 ml of this solution with 3.0 ml of internal standard solution, and mix.

Chromatographic system The chromatographic procedure may be carried out using (a) a glass column (1.8 m × 2 mm), packed with 5 per cent liquid phase of polyethylene glycol compound (av. mol. wt. about 15,000) (Carbowax 20M or equivalent is suitable) on *diatomaceous support* (80- to 100- or 100- to 120-mesh), maintained at 110° (**Note** The *diatomaceous support* is *acid-washed* and then water-washed until neutral, but it is not *alkali-washed*. It may be *silanized*.), (b) *helium* or *nitrogen* as the carrier gas at a flow rate of 20 ml per minute, (c) the injection port is maintained at 180°, and (d) the detector is maintained at 220°. Chromatograph the standard preparation, and record the peak responses as directed under Procedure: the resolution factor between the benzaldehyde and the chlorobutanol peak is not less than 2.0, and the relative standard deviation for replicate injections is not more than 2.0 per cent.

Procedure (**Note** Use peak areas where peak responses are indicated.) Separately inject equal volumes (about 1 µl) of the standard preparation and the test preparation into the chromatograph, record the chromatograms, and measure the responses of the major peaks. The relative retention times are about 0.8 for benzaldehyde and 1.0 for chlorobutanol.

Calculation Calculate the quantity, in mg, of chlorobutanol ($C_4H_7Cl_3O$) in each ml of the sample under test by the expression:

$$C(L/D)\,(R_u/R_s),$$

in which C is the concentration, in mg per ml, of chlorobutanol calculated on the anhydrous basis, in the standard preparation, L is the labelled quantity, in mg, of chlorobutanol in each ml of the sample under test, D is the concentration, in mg per ml, of chlorobutanol in the test preparation, based on the volume of specimen under test taken and the extent of dilution, and R_u and R_s are the ratios of the chlorobutanol peak to the benzaldehyde peak obtained from the test preparation and the standard preparation, respectively.

Phenol

Carry out the determination as described in the "Gas Chromatography" (Appendix 3.4).

Internal standard solution Pipette 1 ml of *benzyl alcohol* into a 500-ml volumetric flask, add *methanol* to volume, and mix.

Standard preparation Dissolve about 75 mg of *phenol*, accurately weighed, in 7.5 ml of *methanol* contained in a 100-ml volumetric flask. Add 20.0 ml of internal standard solution, then add *water* to volume, and mix.

Chromatographic system The chromatographic procedure may be carried out using (a) a glass column (1.2 m × 3 mm), packed with 5 per cent liquid phase of polyethylene glycol compound (av. mol. wt. about 15,000) (Carbowax 20M or equivalent is suitable) on *diatomaceous support* (80- to 100- or 100- to 120-mesh), maintained at 145° (**Note** The *diatomaceous support* is *acid-washed* and then water-washed until neutral, but it is not *alkali-washed*. It may be *silanized*.), and (b) *helium* or *nitrogen* as the carrier gas at a flow rate of 50 ml per minute.

Procedure Separately inject equal volumes (about 3 µl) of the standard preparation and the test preparation into the chromatograph, and record the chromatograms. Measure the areas under the peaks for phenol and benzyl alcohol of the chromatogram for the standard preparation, designating them P_1 and P_2, respectively. Similarly, measure the corresponding values p_1 and p_2 for the test preparation.

Calculation Calculate the content, in mg per ml, of phenol (C_6H_6O) in each ml of the sample taken by the expression:

$$100(C/V)\,(p_1/p_2)\,(P_2/P_1),$$

in which C is the concentration, in mg per ml, of phenol in the standard preparation, and V is the volume, in ml, of the sample under test used in preparing each 100 ml of the test preparation.

Methylparaben and Propylparaben

Carry out the determination as described in the "Gas Chromatography" (Appendix 3.4).

Internal standard solution Place about 200 mg of benzophenone in a 250-ml volumetric flask, add *ether* to volume, and mix.

Standard preparation Place about 100 mg of *methylparaben* and 10 mg of *propylparaben*, each accurately weighed, in a 200-ml volumetric flask, add internal standard solution to volume, and mix. Place 10.0 ml of this solution in a 25-ml conical flask, and proceed as directed under Test preparation, beginning with "Add 3 ml of *pyridine*".

Test preparation Pipette 10 ml of the sample under test and 10 ml of the internal standard solution into a small separator, and transfer the ether layer into a small flask through a funnel containing *anhydrous sodium sulfate*. Extract the aqueous layer with two 10-ml portions of ether, also filtering the extracts through *anhydrous sodium sulfate*. Evaporate the combined extracts under a current of dry air until the volume is reduced to about 10 ml, and then transfer the residue to a 25-ml conical flask. Add 3 ml of *pyridine*, complete the evaporation of the ether, and boil on a hot plate until the volume is reduced to about 1 ml. Cool, and add 1.0 ml of a suitable agent, such as bis(trimethylsilyl) trifluoroacetamide, bis(trimethylsilyl) acetamide, or a mixture of hexamethlyldisilazane and trimethylchlorosilane [2:1 or 3:1 (v/v)]. Mix and allow to stand for not less than 15 minutes.

Chromatographic system The chromatographic procedure may be carried out using (a) a glass column (1.8 m × 2 mm), packed with 5 per cent liquid phase of dimethylpolysiloxane gum on *diatomaceous support* (80- to 100- or 100- to 120-mesh), maintained at 150° (**Note** The *diatomaceous support* is *acid-washed* and then water-washed until neutral, but it is not *alkali-washed*. It may be *silanized*.), and (b) *helium* or *nitrogen* as the carrier gas at a flow rate of 20 ml per minute.

Procedure Separately inject equal volumes (about 2 µl) of the silanized solution from the standard preparation and the test preparation into the chromatograph, and record the chromatograms. Measure the areas under the peaks of methylparaben, propylparaben, and benzophenone, designating them P_1, P_2, and P_3 respectively. Similarly, measure the corresponding areas for the silanized solution from the test preparation, designating them p_1, p_2, and p_3, respectively.

Calculation Calculate the content, in µg per ml, of methylparaben ($C_8H_8O_3$) in the sample under test by the expression:

$$100(C_M/V)\,(p_1/p_3)\,(P_3/P_1),$$

in which C_M is the concentration, in µg per ml, of methylparaben in the standard preparation, and V is the volume, in ml, of the sample taken. Similarly, calculate the content, in µg per ml, of propylparaben ($C_{10}H_{12}O_3$) in the sample under test by the expression:

$$100(C_P/V)\,(p_2/p_3)\,(P_3/P_2),$$

in which C_P is the concentration, in µg per ml, of propylparaben in the standard preparation. Ethylparaben and Butylparaben may be determined in a similar manner.

POLAROGRAPHIC METHOD

Phenylmercuric Nitrate

Standard preparation Dissolve about 100 mg of *phenylmercuric nitrate*, accurately weighed, in *sodium hydroxide* solution (1 in 250) contained in a 1000-ml volumetric flask, warming if necessary to effect solution, add the sodium hydroxide solution to volume, and mix. Pipette 10 ml of this solution into a 25-ml volumetric flask, and proceed as directed under Test preparation, beginning with "add 2 ml of a 1 per cent w/v solution of *potassium nitrate*".

Test preparation Pipette 10 ml of the sample under test into a 25-ml volumetric flask, add 2 ml of a 1 per cent w/v solution of *potassium nitrate* and 10 ml of *borate buffer pH 9.2* and adjust to a pH of 9.2 if necessary, by the addition of 2 M *nitric acid*. Add 1.5 ml of a freshly prepared 0.1 per cent w/v solution of *gelatin*, then add *borate buffer pH 9.2* to volume, and mix.

Procedure Pipette a portion of the test preparation into the polarographic cell, and deaerate by bubbling *nitrogen* through the solution for 15 minutes. Insert the dropping mercury electrode of a suitable polarograph (Appendix 6.25), and record the polarogram from –0.6 to –1.5 volts versus the saturated calomel electrode. Determine the diffusion current of the test preparation, $(i_d)_U$, as the difference between the residual current and the limiting current. Similarly and concomitantly determine the diffusion current, $(i_d)_S$, of the standard preparation. Calculate the quantity, in µg, of phenyl mercuric nitrate ($C_6H_5HgNO_3.C_6H_5HgOH$) in each ml of the sample taken by the expression:

$$2.5C[(i_d)_U/(i_d)_S],$$

in which C is the concentration, in µg per ml, of phenylmercuric nitrate in the standard preparation.

Thiomersal

Standard preparation On the day of use, place about 25 mg of *thiomersal*, accurately weighed, in a 250-ml volumetric flask, add *water* to volume, and mix. Protect from light. Pipette 15 ml of this solution into a 25-ml volumetric flask, add 1.5 ml of a freshly prepared 0.1 per cent w/v solution of *gelatin*, then add a 1 per cent w/v solution of *potassium nitrate* to volume, and mix.

Test preparation Pipette 15 ml of the sample under test into a 25-ml volumetric flask, add 1.5 ml of a freshly prepared 0.1 per cent w/v solution of *gelatin*, add a 1 per cent w/v solution of *potassium nitrate* to volume, and mix.

Procedure Transfer a portion of the test preparation to a polarographic cell, and deaerate by bubbling *nitrogen* through the solution for 15 minutes. Insert the dropping mercury electrode of a suitable polarograph

(Appendix 6.25), and record the polarogram from –0.2 to –1.4 volts versus the saturated calomel electrode. Determine the diffusion current, $(i_d)_U$, as the difference between the residual current and the limiting current. Similarly and concomitantly determine the diffusion current, $(i_d)_S$, of the standard preparation. Calculate the quantity, in μg of thiomersal ($C_9H_9HgO_2S.Na$) in each ml of the test specimen taken by the expression:

$$1.667C[(i_d)_U/(i_d)_S],$$

in which C is the concentration, in μg per ml, of thiomersal in the standard preparation and the other terms are as defined therein.

6.25 POLAROGRAPHY

Polarography is an electrochemical method of analysis based on the measurement of the current flow resulting from the electrolysis of a solution at a polarizable microelectrode, as a function of an applied voltage. The polarogram obtained by this measurement provides qualitative and quantitative information on electro-reducible and electro-oxidizable substances. The normal concentration range for substances being analyzed is from 10^{-2} to 10^{-5} M.

In direct current (dc) polarography, the microelectrode is a dropping mercury electrode (DME) consisting of small reproducible drops of mercury flowing from the orifice of a capillary tube connected to a mercury reservoir. A saturated calomel electrode (SCE) with a large surface area is the most commonly employed reference electrode. As the voltage applied to the cell increases, only a very small residual current flows until the substance under assay undergoes reduction or oxidation. Then the current increases, at first gradually, then almost linearly with voltage, and it gradually reaches a limiting value. On the initial rising portion of the polarographic wave, the increased flow of current results in a decrease in the concentration of the electro-active species at the electrode surface. As the voltage and current increase, the concentration of the reactive species decreases further to a minimal value at the electrode surface. The current is then limited by the rate at which the reacting species can diffuse from the bulk of the solution to the surface of the microelectrode. The final current rise is caused by the reaction of the supporting electrolyte. This large concentration of electrolyte is inert within the potential range used in the analysis, and it prevents the reactive species from reaching the electrode by electrical migration, thus assuring that the limiting current is diffusion-controlled.

Since, in the case of the DME, the electrode surface is being constantly renewed in a cyclic fashion, the current increases from a small value as the drop begins to form to a maximum value as the drop falls. By the use of a suitable recorder to measure the current, the characteristic saw-toothed record is obtained. The limiting current is the sum of the residual and the diffusion currents. The residual current is subtracted from the limiting current to give the wave height.

Ilkovic equation The linear relationship between the diffusion current (i_d) and the concentration of electro-active species is shown by the Ilkovic equation:

$$i_d = 708nD^{1/2}Cm^{2/3}t^{1/6},$$

in which i_d is the maximum current in microamperes, n is the number of electrons required per molecule of electro-active substance, D is its diffusion coefficient, in square cm per second, C is the concentration, in millimoles per liter, m is the rate of mercury flow from the DME, in mg per second, and t is the drop time, in seconds.

Apparatus The apparatus used comprises a potentiometer connected to two electrodes via a microammeter. Many types of electrodes systems are available but the simplest and most convenient is the DME, which consists of a mercury reservoir connected to a short length of very fine capillary tubing (diameter of aperture 20 to 80 μm) in conjunction with a mercury pool anode. R_1 is a shunt for the galvanometer and R_2 is often a calibrated potentiometer, previously standardized with a Weston Standard Cell in the usual way. Alternatively an uncalibrated potentiometer may be used, in which case the applied voltage, V, may be measured by means of a high resistance voltmeter (Fig. 1).

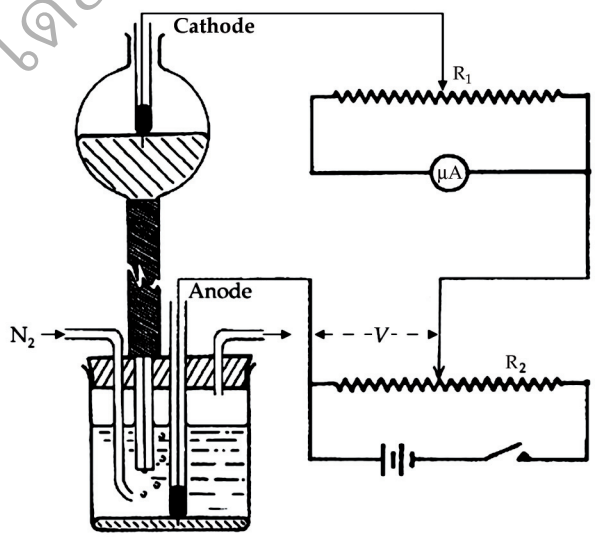


Fig. 1 Apparatus for Obtaining Polarograms

Modern polarographs are equipped with recorders capable of following the current during the latter portion of the drop life, consequently, the maximum of the oscillations is the measure of the current. When the current is measured only at the end of the drop life, the technique is termed sampled dc polarography. In this case, only the maximum currents are recorded and oscillations due to drop growth are not observed.

For instruments equipped with galvanometers to measure the current or recorders operated in a damped mode, the saw-toothed waves correspond to oscillations about the average current. In the latter case, the average of the oscillations is the measure of the current. For polarograms obtained in this manner, the i_d given by the Ilkovic equation is the average current in microamperes observed during the life of the drop, when the coefficient 708 is replaced by 607.

Control of the diffusion current The Ilkovic equation identifies the variables that must be controlled to ensure that the diffusion current is directly proportional to the concentration of electro-active material. At 25° the diffusion coefficients for aqueous solutions of many ions and organic molecules increase 1 per cent to 2 per cent per degree rise in temperature. Thus the temperature of the polarographic cell must be controlled to within ±0.5°. The quantities m and t depend upon the dimensions of the capillary and the height of the mercury column above the electrode. Although results obtained with different capillaries can be compared if the product $m^{2/3}\,t^{1/6}$ is known, it is advisable to use the same capillary with a constant head of mercury during a series of analyses. The diffusion current is proportional to the square root of the height of the mercury column. A mercury reservoir with a diameter greater than 4 cm prevents any significant drop in the mercury level during a series of runs.

The capillary for the DME has a bore of approximately 0.04 mm and a length of 6 to 15 cm. The height of the mercury column, measured from the tip of the capillary to the top of the mercury pool, ranges from 40 to 80 cm. The exact length of the capillary and the height of the mercury column are adjusted to give a drop-time of between 3 and 5 seconds at open circuit with the capillary immersed in the test solution.

Equipment is available that allows controlled drop-times of fractions of a second to several seconds. As detail within a polarogram is related to the number of drops delivered during a given potential change, such short drop-times allow more rapid recording of the polarogram.

The current flowing through the test solution during the recording of a polarogram is in the microampere range. Thus, the current flow produces negligible changes in the test solution and several polarograms can be run on the same test solution without significant differences.

Half-wave potential The half-wave potential ($E_{1/2}$) occurs at the point on the polarogram one-half the distance between the residual current and the limiting current plateau. This potential is characteristic of the electro-active species and is largely independent of its concentration or the capillary used to obtain the wave. It is dependent upon the solution composition and may change with variations in the pH or in the solvent system or with the addition of complexing agents. The half-wave potential thus serves as a criterion for the qualitative identification of a substance.

The potential of the DME is equal to the applied voltage versus the reference electrode after correction for the iR drop (that potential need to pass the current, i, through the solution with a resistance R). It is especially important to make this correction for non-aqueous solutions, which ordinarily possess high resistance, if an accurate potential for the DME is needed. Correction of the half-wave potential is not required for quantitative analysis. Unless otherwise indicated, it is to be understood that potentials represent measurements made against the SCE.

Removal of dissolved oxygen In as much as oxygen is reduced at the DME in two steps, first to hydrogen peroxide and then to water, it interferes where polarograms are to be made at potentials more negative than about 0 volt versus SCE, and must be removed. This may be accomplished by bubbling oxygen-free nitrogen through the solution for 10 to 15 minutes immediately before recording the wave, the nitrogen first having been "conditioned" to minimize changes due to evaporation, by being passed through a separate portion of the solution.

It is necessary that the solution be quiet and vibration-free during the time the wave is recorded, to ensure that the current is diffusion-controlled. Therefore, the nitrogen aeration should be stopped and the gas be directed to flow over the surface of the solution before a polarogram is recorded.

In alkaline media, sodium metabisulfite may be added to remove oxygen, provided the reagent does not react with other components of the system.

Measurement of wave height To use a polarogram quantitatively, it is necessary to measure the height of the wave. Since this is a measure of the magnitude of the diffusion current, it is measured vertically. To compensate for the residual current, the segment of the curve preceding the wave is extrapolated beyond the rise in the wave. For a well-formed wave where this extrapolation parallels the limiting current plateau, the measurement is unambiguous. For less well-defined waves, the following procedure may be used unless otherwise directed in the individual monograph. Both the residual current and the limiting current are extrapolated with straight lines, as shown by the graph (Fig. 2). The wave height is taken as the vertical distance between these lines measured at the half-wave potential.

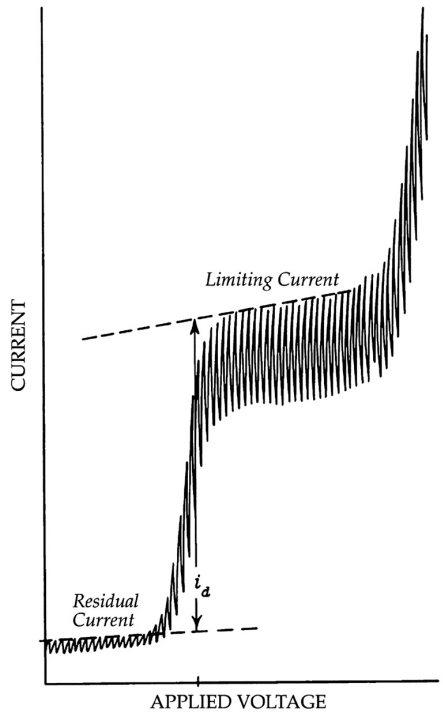


Fig. 2 Typical Polarogram Showing Change in Current Flow with Increasing Potential Applied to the Dropping Mercury Electrode

Procedure

Caution Mercury vapour is poisonous, and metallic mercury has a significant vapour pressure at room temperature. The work area in which mercury is used should be constructed in such a way that any spilled or spattered droplets of mercury can be completely recovered with relative ease. Scrupulously clean up mercury after each use of the instrument. Work in a well-ventilated laboratory, taking care to clean up any spilled mercury.

Transfer a portion of the final dilution of the substance being assayed to suitable polarographic cell immersed in a water-bath regulated to 25°±0.5°. Pass a stream of *nitrogen* through the solution for 10 to 15 minutes to remove dissolved oxygen. Start the mercury dropping from the capillary, insert the capillary into the test solution, and adjust the height of the mercury reservoir. Switch the flow of nitrogen to pass over the surface of the solution, and record the polarogram over the potential range indicated in the individual monograph, using the appropriate recorder or galvanometer sensitivity to give a suitable wave. Measure the height of the wave, and unless otherwise directed in the monograph, compare this with the wave height obtained with the appropriate Reference Substance, measured under the same conditions.

Pulse Polarography In conventional dc polarography, the current is measured continuously as potential is applied as a linear ramp (Fig. 3). This current is composed of two elements. The first, the diffusion (faradaic) current, is produced by the substance undergoing reduction or oxidation at the working electrode, and is directly proportional to the concentration of this substance. The second element is the capacitive current (charging of the electrochemical double layer). The changes in these currents as the mercury drop varies in size produce the oscillations present in typical dc polarograms.

In normal pulse polarography, a potential pulse is applied to the mercury electrode near the end of the drop life, with the drop being held at the initial potential during growth period (Fig. 4). Each succeeding drop has a slightly higher pulse applied to it, with the rate of increase being determined by the selected scan rate. The current is measured at the end of the pulse where the capacitive current is nearly zero, and thus primarily faradaic current is measured (Fig. 5). In addition, since the pulse is applied for only a short duration, the diffusion layer is not depleted as extensively as in dc polarography and larger current levels are obtained for equivalent concentrations. Concentrations as low as 10^{-6} M can be measured, providing approximately a tenfold increase in sensitivity over that with dc polarography. Limiting current values are more easily measured, since the waves are free from oscillations.

Differential pulse polarography is a technique whereby a fixed-height pulse applied at the end of the life of each drop is superimposed on a linear increasing dc ramp (Fig. 6). Current flow is measured just before application of the pulse and again at the end of the pulse. The difference between these two currents is measured and presented to the recorder. Such a differential signal provides a curve approximating the derivative of the polarographic wave, and gives a peak presentation. The peak potential is equivalent to:

$$E_{1/2} - \Delta E/2,$$

where ΔE is the pulse height. The peak height is directly proportional to concentration at constant scan rates and constant pulse heights. This technique is especially sensitive (levels of 10^{-7} M may be determined) and affords improved resolution between closely spaced waves.

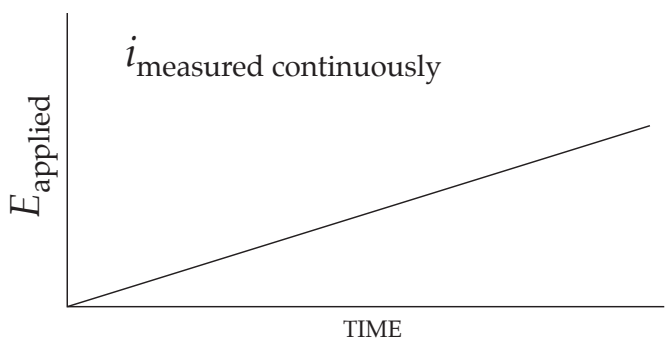


Fig. 3 Direct Current (dc) Polarography

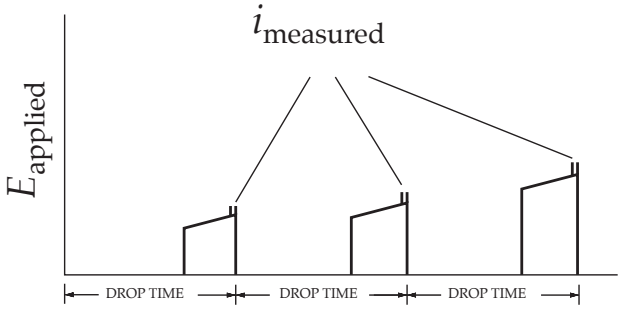


Fig. 4 Pulse Polarography

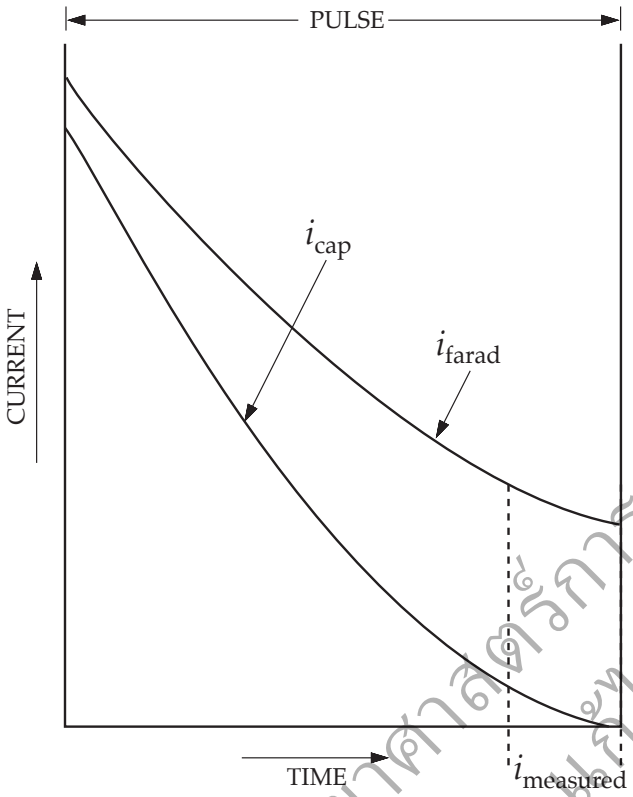


Fig. 5 Plot of Current Versus Time in Pulse Polarography

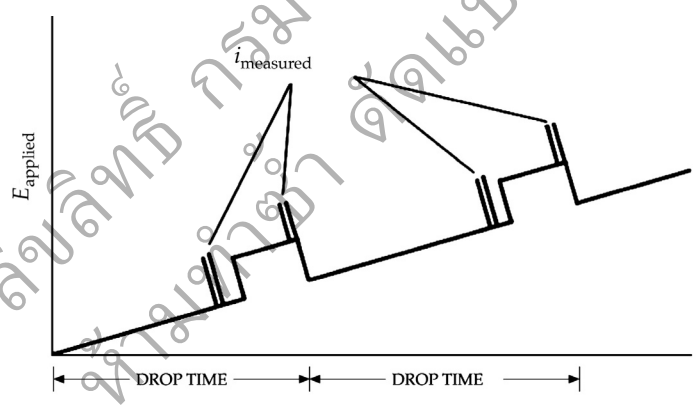


Fig. 6 Differential Pulse Polarography

Anodic stripping voltammetry Anodic stripping voltammetry is an electrochemical technique whereby trace amounts of substances in solution are concentrated (reduced) onto an electrode and then stripped (oxidized) back into solution by scanning the applied voltage anodically. The measurement of the current flow as a function of this voltage and scanning rate provides qualitative and quantitative information on such substances. The concentration step permits analyses at 10^{-7} to 10^{-9} M levels.

Basic instrumentation includes a voltage ramp generator, current-measuring circuitry; a cell with working, reference, and counter electrodes; and a recorder or other read-out device. Instruments having dc or pulse-polarographic capabilities are generally quite adequate for stripping application. The working electrode commonly used is the hanging mercury drop electrode (HMDE), although the mercury thin-film electrode (MTFE) has acquired acceptance. For analysis of metals such as silver, platinum, and gold, whose oxidation potentials are more positive than mercury, and mercury itself, the use of solid electrodes such as platinum, gold, or carbon is required. A saturated calomel electrode or a silver-silver chloride electrode serves as the reference except for the analysis of mercury or silver. A platinum wire is commonly employed as the counter electrode.

Test samples containing suitable electrolyte are pipetted into the cell. Dissolved oxygen is removed by bubbling *nitrogen* through the cell for 5 to 10 minutes.

Generally, an electrolysis potential equivalent to 200 to 300 mV more negative than the half-wave potential of the material to be analyzed is applied (although this potential is to be determined experimentally), with stirring for 1 to 10 minutes. For reproducible results, maintain constant conditions (i.e., deposition time, stirring rate, temperature, specimen volume, and drop size if HMDE is used).

After deposition, the stirring is discontinued and the solution and electrode are allowed to equilibrate for a short period. The potential is then rapidly scanned anodically (10 mV/second or more in dc polarography and 5 mV/second in differential pulse polarography). As in polarography, the limiting current is proportional to concentration of the species (wave height in dc and pulse; peak height in differential pulse), while the half-wave potential (dc, pulse) or peak potential (differential pulse) identifies the species. It is imperative that the choice of supporting electrolyte be made carefully in order to obtain satisfactory behavior. Quantitation is usually achieved by a standard addition or calibration method.

This technique is appropriate for trace-metal analysis, but has limited use in organic determinations, since many of these reactions are irreversible. In analyzing substances such as chloride, cathodic stripping voltammetry may be used. The technique is the same as anodic stripping voltammetry, except that the substance is deposited anodically and then stripped by a cathodic voltage scan.

APPENDIX 7 CRUDE DRUGS

7.1 SAMPLING

Sampling of material in bulk

Unless otherwise specified, the sample should be taken by the following methods, and, if necessary, preserved in tightly closed containers.

1. Examine the uniformity and the integrity of the containers (or of the outer wrapping), the markings and the labelling in the whole batch.

2. If on external examination the batch is considered as homogeneous, take individual samples from the number of packages as indicated in the following table.

Schedule Recommended for Sampling

Number of Packages in the Batch	Number of Packages to Be Sampled (n)
1 to 5	All
6 to 50	5
>50*	10 per cent

*Round calculated "n" to next highest whole number.

3. From each container or package selected, take three original samples, taking care to avoid fragmentation. Samples should be taken from the top, middle and bottom of the container. In the case of sacks and packages, the three samples should be taken by hand, the first from a depth of not less than 10 cm from the top and the second and third from the middle and bottom after cutting into the side of the package. Samples of seeds should be withdrawn with a grain probe. Material in boxes should first be sampled from the upper layer; then approximately half of the contents should be removed and a second sample taken. Finally after further removal of material, another sample should be taken from the bottom. Samples should be as uniform as possible in mass. The three original samples should then be combined into a pooled sample which should be mixed carefully.

The average sample is obtained by quartering. From the pooled sample, adequately mixed, into an even and square-shaped heap, and divide it diagonally into four equal parts. Take two diagonally opposite parts and mix carefully. Repeat the process as necessary until the required quantity, to within ±10 per cent, is obtained. Any remaining material should be returned to the batch.

Using the same quartering procedure, divide the average sample into four final samples, taking care that each portion is representative of the bulk material.

Sampling of material in retail packages

From each wholesale container (boxes, cartons, etc.) selected for sampling, take at random two consumer packages. From small batches (1 to 5 boxes), take ten consumer packages. Prepare the pooled sample by mixing the contents of the selected consumer packages and proceed as described above to obtain the final sample.

7.2 FOREIGN MATTER

Vegetable drugs should be free from moulds, insects and other animal contamination.

Foreign matter is material consisting of any or all of the following:

1. *Foreign organs*: matter coming from the source plant but not defined as the drug.

2. *Foreign elements*: matter not coming from the source plant and of either vegetable or mineral origin.

Method

Weigh 100 to 500 g of the substance being examined or the quantity specified in the monograph and spread it in a thin layer. Separate the foreign matter by hand as completely as possible, weigh it and calculate the percentage present.

7.3 DETERMINATION OF VOLATILE OIL

Volatile oils are characterized by their odour, oil-like appearance and ability to volatilize at room temperature. Chemically, they are usually composed of mixtures of, for example, monoterpenes, sesquiterpenes and their oxygenated derivatives. Aromatic compounds predominate in certain volatile oils. Because they are considered to be the "essence" of the vegetable drugs, and are often biologically active, they are also known as "essential oils". The term "volatile oil" is preferred because it is more specific and describes the physical properties.

The determination of volatile oil in vegetable drugs is carried out by steam distillation in a special apparatus in the conditions described below. The distillate is collected in its graduated tube using xylene to take up the volatile oil; the aqueous phase is automatically recirculated into the distillation flask.

Apparatus

The apparatus (see Fig. 1) is constructed of resistant glass of low coefficient of expansion and has the following dimensions:

(a) a round-bottomed flask (*A*) of suitable capacity with a short, ground-glass neck having an internal diameter of about 29 mm at the wide end;

(b) a condenser assembly that closely fits the flask and consists of the following parts fused into one piece:

— a vertical tube (*BD*), 215 to 265 mm long and 14 to 16 mm in internal diameter,

— a bent tube (*DEF*) in which the distances *DE* and *EF* are each 145 to 155 mm long and 9 to 10 mm in internal diameter,

— a bulb-condenser (*GH*), 145 to 155 mm long and 9 to 10 mm in diameter at the restrictions,

— a vented stopper (*M*) and a tube (*N*) with an orifice of diameter about 1 mm that coincides with the vent. The wide end of the tube (*N*) is of ground-glass, having an internal diameter of 10 mm,

— a tube (*HJ*), 75 to 85 mm long and 9 to 10 mm in internal diameter, making a 30° to 40° angle (*HJN*) with the tube (*JN*),

— a graduated tube (*LP*), graduated over 105 to 115 mm to give 1 ml subdivided in 0.01 ml. Above the graduation are two circular marks (*K* and *L*),

— a pear-shaped swelling (*S*), about 3 ml in capacity,

— a bulb-shaped swelling (*P*), about 2 ml in capacity,

— a three-way tap (*Q*), and

— a connecting tube (*CQ*), 7 to 8 mm in internal diameter, fitted in the middle with a filling funnel (*R*). The junction (*C*) is at a level 20 mm higher than uppermost graduation;

(c) a suitable heating device, allowing a fine control; and

(d) a vertical support with horizontal ring covered with insulating material.

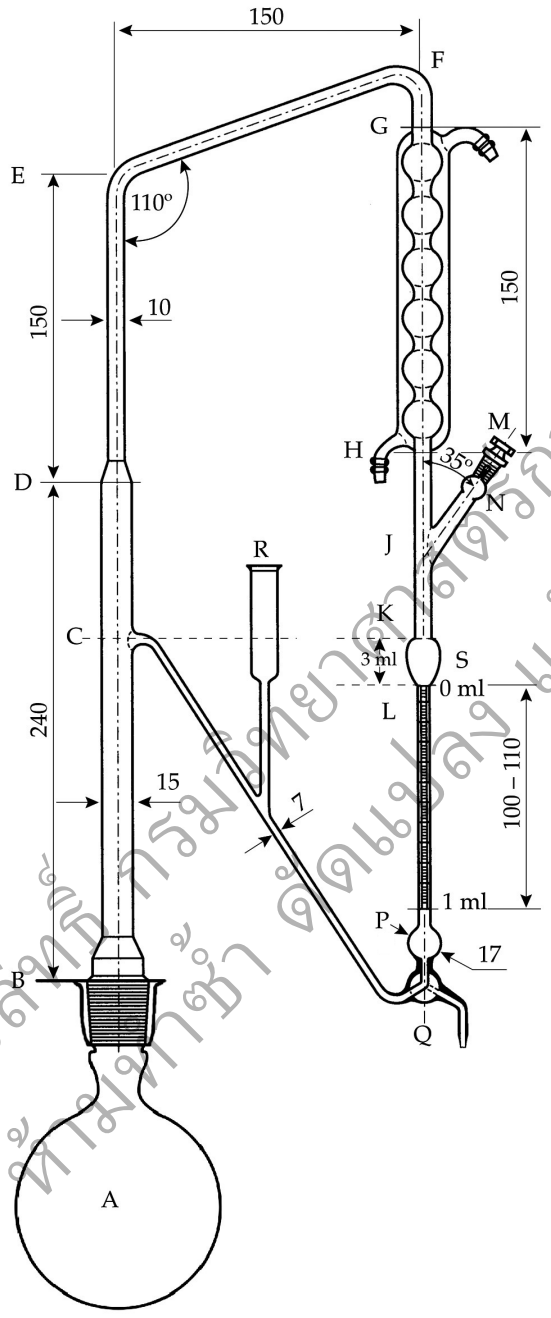


Fig. 1 Apparatus for the Determination of Volatile Oil
Dimensions in mm

Before use, clean the apparatus by successive washings with *acetone*, *water* and *chromic acid cleansing mixture* inverting several times, and rinse with *water*. Drain the apparatus and mount it in a place protected from a draught.

Procedure

Carry out the assay according to the nature of the drug to be examined.

Place the prescribed volume of distillation liquid in the flask, add a few pieces of porous porcelain and attach the condenser assembly. Introduce *water* through the filling funnel (*R*) until it is at the level (*C*). Remove the stopper (*M*) and introduce the prescribed quantity of *xylene*, using a pipette with its tip at the bottom of the tube (*N*). Replace the stopper (*M*) and ensure that the orifice is coincidental with the vent. Heat the liquid in the flask to boiling and adjust the distillation rate to 2 to 3 ml per minute, unless otherwise prescribed.

To determine the rate of distillation, lower during distillation the level of the water by means of the three-way tap (*Q*) until the meniscus is in level with the lower mark (*L*) (see Fig. 2). Close the tap and record the time taken for the meniscus to reach the upper mark (*K*). Open the tap and continue the distillation, modifying the heat to regulate the distillation rate. Distil for the time prescribed in the monograph. Stop the heating, and after at least 10 minutes read off the volume of xylene in the graduated tube.

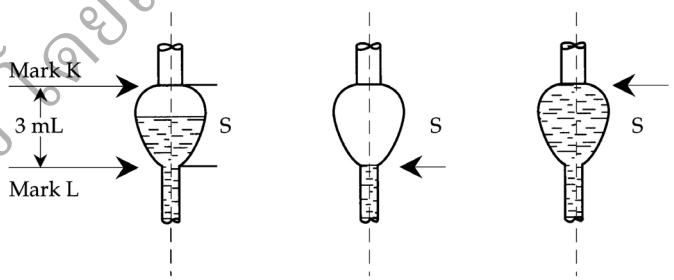


Fig. 2

Introduce into the flask, the prescribed quantity of the drug and continue the distillation as described above for the time and at the rate prescribed. After a further 10 minutes, read the volume of xylene previously noted. The difference represents the quantity of volatile oil in the weight of the drug taken. Calculate the result as millilitres per 100 g of drug.

When the volatile oil is to be used for other analytical purposes, the water-free mixture of xylene and volatile oil may be recovered as follows. Remove the stopper (*M*) and introduce 0.1 ml of a 0.1 per cent w/v solution of *sodium fluoresceinate* and 0.5 ml of *water*. Lower the level of xylene and volatile oil mixture into the bulb-shaped swelling (*P*) by means of the three-way tap, allow to stand for 5 minutes and lower the mixture layer slowly until it just reaches the tap (*Q*). Turn the tap clockwise so that the water flows out of the connecting tube (*CQ*). Wash the tube with *acetone* and with a little *toluene* introduced through the filling funnel (*R*).

Turn the tap clockwise in order to recover the mixture of xylene and volatile oil in an appropriate flask.

7.6 ACID-INSOLUBLE ASH

Use Method I unless otherwise indicated in the monograph.

Method I

Boil the total ash for 5 minutes with 25 ml of *dilute hydrochloric acid*, collect the insoluble matter on an ashless filter paper, wash with hot *water* until the filtrate is neutral, and ignite at about 500°. Calculate the percentage of acid-insoluble ash with reference to the air-dried substance.

Method II

Place the total ash or the sulfated ash, as directed in the monograph, in a crucible, add 15 ml of *water* and 10 ml of *hydrochloric acid*, cover with a watch glass, and boil for 10 minutes; allow to cool. Collect the insoluble matter on an ashless filter paper, wash with hot *water* until the filtrate is neutral, ignite to dull redness (550° to 700°), cool in a desiccator and weigh. Reheat until the difference between two successive weighings is not more than 1 mg. Calculate the percentage of acid-insoluble ash with reference to the air-dried substance.

7.7 TOTAL ASH

Use Method I unless otherwise directed in the monograph.

Method I

For vegetable drugs Incinerate 2 to 3 g of the ground drug in a tared platinum or silica dish at a temperature not exceeding 450° until free from carbon, cool and weigh. If a carbon-free ash cannot be obtained in this way, exhaust the charred mass with hot *water*, collect the residue on an ashless filter paper, incinerate the residue and filter paper, add the filtrate, evaporate to dryness and ignite at a temperature not exceeding 450°. Calculate the percentage of total ash with reference to the air-dried drug.

For other substances Carry out the above method using 1 g, unless otherwise stated. Calculate the percentage of total ash.

Method II

Heat a silica or platinum crucible to red heat for 30 minutes, allow to cool in a desiccator and weigh. Unless otherwise specified in the monograph, evenly distribute 1 g of the substance being examined in the crucible, dry at 100° to 105° for 1 hour and ignite to constant weight in a muffle furnace at 600°±25°. Allow the crucible to cool in a desiccator after each ignition. Flames should not be produced at any time during the procedure. If after prolonged ignition a carbon-free ash cannot be obtained, take up with hot *water*, filter through an ashless filter paper and ignite the residue and the filter paper. Combine the filtrate with the ash, carefully evaporate to dryness and ignite to constant weight.

7.8 DETERMINATION OF CINEOLE

Weigh 3.00 g of the oil, recently dried with *anhydrous sodium sulfate*, into a dry test-tube and add 2.10 g of melted o-*cresol*. Place the tube in the apparatus for the "Determination of Freezing Temperature" (Appendix 4.4), and allow to cool, stirring continuously. When crystallization takes place, there is a small rise in temperature. Note the highest temperature reached (t_1).

Remelt the mixture on a water-bath at a temperature that does not exceed t_1 by more than 5° and place the tube in the apparatus, maintained at a temperature 5° below t_1. When crystallization takes place, or when the temperature of the mixture has fallen 3° below t_1, stir continuously. Note the highest temperature at which the mixture crystallizes (t_2). Repeat the operation until two highest values obtained for t_2 do not differ by more than 0.2°. If supercooling occurs, induce crystallization by adding a small crystal of the complex consisting of 3.00 g of *cineole* and 2.10 g of melted o-*cresol*. If t_2 is below 27.4°, repeat the determination after the addition of 5.10 g of the complex.

The content of cineole, corresponding to the highest temperature observed (t_2) in the Table is obtained, where necessary, by interpolation.

If 5.10 g of the complex has been added, calculate the cineole content in per cent w/w from the expression:

$$2(A - 50)$$

Where A is the value found in the Table

t_2 (°)	Cineole Per Cent w/w	t_2 (°)	Cineole Per Cent w/w
24	45.5	40	67.0
25	47.0	41	68.5
26	48.5	42	70.0
27	49.5	43	72.5
28	50.5	44	74.0
29	52.0	45	76.0
30	53.5	46	78.0
31	54.5	47	80.0
32	56.0	48	82.0
33	57.0	49	84.0
34	58.5	50	86.0
35	60.0	51	88.5
36	61.0	52	91.0
37	62.5	53	93.5
38	63.5	54	96.0
39	65.0	55	99.0

7.9 DETERMINATION OF ALDEHYDES

To 1 g of the oil, accurately weighed, in a glass-stoppered tube (approximately 150 mm × 25 mm) add 5 ml of *toluene* and 15 ml of *hydroxylamine in ethanol (60 per cent) TS*, shake vigorously and titrate immediately with 0.5 M *potassium hydroxide in ethanol (60 per cent) VS* until the red colour changes to yellow. Continue shaking and neutralizing until the full yellow colour of the indicator is permanent in the lower layer after shaking vigorously for 2 minutes and allowing to separate; the reaction is complete in about 15 minutes. This procedure gives an approximate value for the aldehyde content of the oil.

Repeat this procedure, using as the colour standard for the end-point of the titration the titrated liquid of the first determination with the addition of 0.5 ml of 0.5 M *potassium hydroxide in ethanol (60 per cent) VS*. Calculate the content of aldehydes from the second determination, using the equivalent given in the monograph.

7.10 SWELLING INDEX

The swelling index is the volume in ml occupied by 1 g of a drug, including any adhering mucilage, after it has swollen in an aqueous liquid for 4 hours.

Place 1 g, accurately weighed, of the drug, whole or of the degree of comminution prescribed in the monograph, in a 25-ml ground-glass stoppered cylinder graduated over a height of 120 to 130 mm in 0.5-ml divisions. Unless otherwise specified, moisten the drug with 1.0 ml of *ethanol*, add 25 ml of *water* and close the cylinder. Shake vigorously every 10 minutes for 1 hour and then allow to stand for 3 hours. At 90 minutes after the beginning of the test, release any large volumes of liquid retained in the layer of the drug and any particles of the drug floating at the surface of the liquid by rotating the cylinder about a vertical axis. Measure the volume occupied by the drug, including any adhering mucilage. Carry out three tests at the same time. Calculate the swelling index from the mean of the three tests.

7.11 WATER-SOLUBLE ASH

Boil the *total ash* for 5 minutes with 25 ml of *water*, collect the insoluble matter in a sintered-glass crucible or on an ashless filter paper, wash with hot *water* and ignite for 15 minutes at a temperature not exceeding 450°. Subtract the weight of the residue from the weight of the ash. The difference in weight is taken to be the water-soluble ash. Calculate the percentage of water-soluble ash with reference to the air-dried drug.

7.12 EXTRACTIVES

Ethanol-soluble Extractive

Use Method I unless otherwise indicated in the monograph.

Method I Macerate 5 g of the air-dried drug, coarsely powdered and accurately weighed, with 100.0 ml of *ethanol* of the specified strength in a closed flask for 24 hours, shaking frequently during the first 6 hours and then allowing to stand for 18 hours. Filter rapidly, taking precautions against loss of ethanol, evaporate 20.0 ml of the filtrate to dryness in a tared, flat-bottomed, shallow dish and dry at 105° to constant weight. Calculate the percentage of ethanol-soluble extractive with reference to the air-dried drug.

Method II Transfer about 4 g of the air-dried drug, coarsely powdered and accurately weighed, to a glass-stoppered conical flask. Add 100 ml of *ethanol* of the specified strength, and weigh the flask. Shake and allow to stand for 1 hour. Attach a reflux condenser to the flask. Boil gently for 1 hour, cool, and weigh. Readjust to the original weight with ethanol. Shake, and filter rapidly through a dry filter. Transfer 25.0 ml of the filtrate to a tared flat-bottomed dish, and evaporate on a water-bath to dryness. Dry at 105° for 6 hours, cool in a desiccator for 30 minutes, and weigh without delay. Calculate the percentage of ethanol-soluble extractive with reference to the air-dried drug.

Water-soluble Extractive

Use Method I unless otherwise indicated in the monograph.

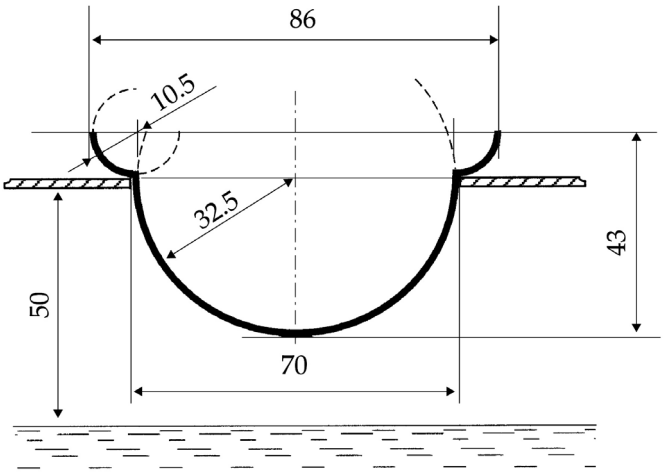
Method I Proceed as directed in Method I under Ethanol-soluble Extractive but using *chloroform water* in place of ethanol.

Method II Proceed as directed in Method II under Ethanol-soluble Extractive but using *chloroform water* in place of ethanol.

7.13 RESIDUE ON EVAPORATION OF VOLATILE OILS

The residue on evaporation is the percentage by weight of the oil that remains after evaporation when determined by the following method. Unless otherwise specified in the monograph, place 5 g of the oil, accurately weighed, in a heat-resistant glass evaporating dish, which has been weighed after heating on a water-bath for 1 hour and cooling in a desiccator; place the evaporating dish over a water-bath on a cover with holes 70 mm in diameter and maintain the water level

in the water-bath so that it is about 50 mm below the cover throughout the test. Vigorously boil the water in the water-bath in a draught-free atmosphere for the time specified in the monograph. Allow the evaporating dish to cool in a desiccator and weigh.



Apparatus for Determination of Residue on Evaporation of Volatile Oils
Dimensions in mm

7.21 TOTAL SOLIDS

The term "total solids" is applied to the residue obtained when the prescribed amount of the preparation is dried to constant weight under the conditions specified below.

Apparatus

A shallow, flat-bottomed, flanged dish, about 75 mm in diameter and about 25 mm deep, made of suitable metal of high heat conductivity and low specific heat and which is not affected by the preparation such as nickel or stainless steel, with a well-fitting cover.

Method

Place the quantity stated in the monograph in a tared dish, evaporate at as low a temperature as possible until the ethanol is removed and heat on a water-bath until the residue is apparently dry. Transfer to an oven operating without a fan and dry to constant weight at 105° unless otherwise stated in the monograph. It may be necessary, for residues of a hygroscopic nature, to use a dish provided with a well-fitting cover and to cool in a desiccator.

APPENDIX 8 BIOLOGICAL TESTS AND METHODS

Test Animals

Healthy animals of different species and unquestioned strains are required for biological tests. They should not have previously been used for any test purpose, unless otherwise specified.

ANIMAL ROOM Unless otherwise specified, animal room should be maintained at the temperature of 25° to 30° and the relative humidity within a range of 30 to 70 per cent. Air-conditioning is highly recommended since it promotes environmental stability. If air-conditioning is not available, alternative methods of providing for heat dissipation must be sought.

FEED Bulk supplies for food and bedding should not be stored in animal rooms. Commercially prepared laboratory animal feeds are preferred, if available. The special daily requirement for vitamin C for guinea-pigs may be met by the use of 50 mg per litre of ascorbic acid daily in the drinking water. Great care must be taken to ensure against contamination in foods with antibiotics or other harmful substances which may have been used in the preparation of livestock feeds, since such contaminations may confuse the results. Food and drinking water should be given *ad libitum*.

CAGE AND BEDDING Cages are made up of appropriate material which can be cleaned easily and permit free ventilation. Cleaning and disinfecting facilities should be adequately provided. Animal colonies should not be crowded.

Care should be ensured that animal colonies are not contaminated by field rodents or other vermins. If available, heat sterilization of bedding before and/or after the use is preferred. Bedding should be changed at least once a week.

Diluents

DILUENT A: PYROGEN-FREE WATER Prepare pyrogen-free water by collecting freshly distilled water and sterilizing it in an autoclave at 121° for not less than 20 minutes. Pyrogen-free water meets the requirements for the absence of pyrogens as described in the "Pyrogen Test" (Appendix 8.2).

DILUENT B: PYROGEN-FREE SALINE SOLUTION Prepare an isotonic solution of sodium chloride by dissolving 9.0 g of pyrogen-free sodium chloride (prepared by heating *sodium chloride* at 200° for 2 hours) in pyrogen-free distilled water to make 1000 ml. Sterilize in an autoclave at 121° for not less than 20 minutes. Pyrogen-free saline solution meets the requirements for the absence of pyrogens as described in the "Pyrogen Test" (Appendix 8.2).

DILUENT C: STERILE DISTILLED WATER Prepare freshly distilled water. Sterilize in an autoclave at 121° for 20 minutes.

DILUENT D: STERILE SALINE SOLUTION Dissolve 9.0 g of *sodium chloride* in distilled water to make 1000 ml. Sterilize in an autoclave at 121° for 20 minutes.

DILUENT E: PYROGEN-FREE SODIUM CARBONATE SOLUTION Dissolve 25.6 g of pyrogen-free anhydrous sodium carbonate (prepared by heating *anhydrous sodium carbonate* at 170° for 4 hours) in pyrogen-free distilled water to make 1000 ml. Sterilize in an autoclave at 121° for not less than 20 minutes. Pyrogen-free sodium carbonate solution meets the requirements for the absence of pyrogens as described in the "Pyrogen Test" (Appendix 8.2).

8.1 ABNORMAL TOXICITY TEST

The test is designed to reveal the presence of toxicity not inherited, unexpected or unacceptable biological reactivity.

Test for General Products

Select five healthy mice not previously used for testing, weighing between 17 and 24 g. During the testing period, the animals should be housed in a suitable container and given adequate food and water. A constant temperature environment is desirable at all times.

Prepare the solution of the test substance as specified in the monograph. Select the route of administration and proceed as directed. If the product is packaged for dispensing in a combination package with a diluent, dilute the product as directed in the labelling.

Use sterile glassware, syringes and needles. To each of five mice, administer the appropriate test dose by one of the following routes of administration as specified in the monograph:

(a) **Intravenous** Use a 26-gauge needle, three-quarter inch in length. Inject the test dose into a tail vein of each mouse. The injection should be made at the rate of 0.1 ml per second.

(b) **Intraperitoneal** Use a 26-gauge needle, one-quarter inch or one-half inch in length. Inject the test dose through the abdominal wall into the peritoneal cavity of each animal.

(c) **Subcutaneous** Use a 26-gauge needle, one-half inch in length. Inject the test dose subcutaneously at a site on the abdominal or dorsal surface.

(d) **Oral** By means of a cannula or other suitable device, administer a test dose orally.

Observe the animals over the 48 hours following the injection. If, at the end of 48 hours, all of the animals survive and not more than one of the animals shows outward symptoms of a reaction not normally expected of the level of toxicity related to the tested material, the requirements of this test are met. If one or more animals die or if more than one of the animals shows signs of abnormal or untoward toxicity of the tested material under test, repeat the test using at least another 10 mice similar to those used in the initial test, but weighing 19 to 21 g. In either case, if all of the animals survive for 48 hours and show no symptoms of a reaction indicative of an abnormal or undue level of toxicity of the tested material, the requirements of the test are met.

Tests for Biological Products (Immunosera and Vaccines)

Unless otherwise prescribed, inject intraperitoneally one human dose but not more than 1.0 ml of liquid product or of the reconstituted product, into each of five healthy mice, weighing 17 to 24 g and one human dose but not more than 5.0 ml of the liquid product or of the reconstituted product, into each of two guinea-pigs, weighing 250 to 400 g. (The human dose is that stated on the label of the product to be examined or on the accompanying leaflet.)

The requirements of this test are met if none of the animals shows signs of ill health within 7 days following the inoculation. If more than one of the animals die during the time specified, the product fails the test. If one of the animals dies or shows signs of ill health during the time specified, repeat the test with a new group of unused one or both species in which the requirements were not met. The product passes the test if none of the animals in the second group dies or shows signs of ill health within the time interval specified.

8.2 PYROGEN TEST

Materials intended for parenteral use should be free from undue amounts of pyrogenic substances. The pyrogen test is designed to limit to an acceptable level of the risks of febrile reaction in the patient to the administration, by injection, of the product concerned. The test involves measuring the rise in temperature of rabbits following the intravenous injection of a test solution. The dose specific for the test is usually related to that generally given to the patient, but for practical reasons, it does not exceed 10 ml per kg of body weight of the test animal, injected intravenously within a period of not more than 10 minutes. For products that require preliminary preparation or are subject to special conditions of administration, additional directions given in the individual monograph are to be followed.

Test Animals

Use healthy, adult rabbits weighing not less than 1.5 kg. House the rabbits individually in an area of uniform temperature between 20° and 24° and free from disturbances likely to excite them. The temperature varies not more than ±3° from the selected temperature. The animals are given *ad libitum* water and food, commonly used for laboratory animals. Not more than seven days before using a rabbit that has not previously been used for a pyrogen test, perform a sham test to condition the animal by conducting a training exercise as described under Procedure, omitting the injection.

Do not use animals for pyrogen tests more frequently than once every 72 hours. After a pyrogen test in the course of which a rabbit's temperature has risen by 0.5° or more, or after a rabbit has been given a test substance that was adjudged pyrogenic, at least 2 weeks

must be allowed to elapse before the animal is used again.

Temperature Recording

Use an accurate temperature-sensing device such as a clinical thermometer, or thermistor probes or similar probes that have been calibrated to assure an accuracy of ±0.1° and have been tested to determine that a maximum reading is reached in less than 5 minutes. Insert the temperature-sensing probe into the rectum of the test rabbit to a depth of 7.5 to 9 cm, and, after a period of time not less than that previously determined as sufficient, record the rabbit's body temperature.

Apparatus and Diluents

Render the syringes, needles and glassware free from pyrogens by heating at 250° for not less than 30 minutes or at 200° for not less than 1 hour or by any other suitable method. Treat all diluents and solutions for washing and rinsing of devices or parenteral injection assemblies in a manner that will assure that they are sterile and pyrogen-free. Periodically perform control pyrogen tests on representative portions of the diluents and solutions for washing or rinsing of the apparatus.

Procedure

Perform the test using three rabbits in a separate area designated solely for pyrogen testing and under environmental conditions similar to those under which the animals are housed and free from disturbances likely to excite them. Food is withheld from the rabbits overnight until the end of the test, and water withheld during the test. The animals should be placed under the conditions of the test at least 1 hour before the injection. If the temperature-sensing device is to remain inserted throughout the sensing period, restrain the rabbit in such a way that allows it to assume a natural resting position.

Not more than 30 minutes prior to the injection of the test dose, determine the "control temperature" of each rabbit: this is the base for the determination of any temperature increase resulting from the injection of a test solution. In any one group of test rabbits, use only those rabbits whose control temperatures do not vary by more than 1° from each other, and do not use any rabbit having temperature exceeding 39.8°.

Unless otherwise specified in the individual monograph, inject into an ear vein of each of three rabbits 10 ml of the test solution per kg of body weight, completing each injection within 10 minutes after start of administration. The test solution is either the product, constituted if necessary as directed in the labelling, or the material under test treated as directed in the individual monograph and injected in the dose specified the rein. For pyrogen testing of devices or injection assemblies, use washings or rinsings of the surfaces that come in contact with the parenterally administered material or with the injection site or internal tissues of the patient. Assure that all test solutions are protected from contamination. Perform the injection after warming the test solution to a temperature of 37°±2°. Record the temperature at 30-minute intervals between 1 and 3 hours subsequent to the injection.

Test Interpretation and Retest

Consider any temperature decreases as zero rise. If no rabbit shows an individual rise in temperature of 0.5° or more above its respective control temperature, the tested material meets the requirements for the absence of pyrogens. If any rabbit shows an individual temperature rise of 0.5° or more, continue the test using five other rabbits. If not more than three of the eight rabbits show individual rises in temperature of 0.5° or more and if the sum of the eight individual maximum temperature rises does not exceed 3.3°, the tested material under examination meets the requirements for the absence of pyrogens.

8.5 TEST FOR BACTERIAL ENDOTOXINS

The test for bacterial endotoxins is designed to detect or quantify bacterial endotoxins of gram-negative bacterial origin that may be present in or on the sample to which the test is applied. It uses Limulus Amoebocyte Lysate (LAL) obtained from the aqueous extracts of circulating amoebocytes of horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*) which has been prepared and characterized for use as an LAL Reagent or a lysate reagent.

There are two types of techniques for this test: the gel-clot techniques, which are based on gel formation, and the photometric techniques. The latter include a turbidimetric method, which is based on the development of turbidity after cleavage of an endogenous substrate, and a chromogenic method, which is based on the development of colour after cleavage of a synthetic peptide-chromogen complex. Any one of these techniques for the test may be followed. In the event of doubt or dispute, the final decision is made based on the gel-clot techniques, unless otherwise indicated.

The test is carried out in a manner that avoids microbial contamination.

Apparatus and Glassware

Depyrogenate all glassware and other heat-stable materials in a hot-air oven using a validated process. Commonly used time and temperature setting are not less than 30 minutes at 250° or not less than 1 hour at 200°. If employing plastic apparatus, such as multi-well plates and tips for micropipettes, use only that which has been shown to be free of detectable endotoxin and not to interfere with the test.

Preparation of the Solutions

Preparation of the standard endotoxin stock solution Prepare standard endotoxin stock solution from an endotoxin reference standard that has been calibrated against the International Standard or equivalence. Endotoxin is expressed in Endotoxin Unit (EU). One

Endotoxin Unit (EU) is equal to one International Unit (IU) of endotoxin as well.

Follow the specifications in the package leaflet and on the label for preparation and storage of the standard endotoxin stock solution.

Preparation of the standard endotoxin solution After vigorously mixing the standard endotoxin stock solution as recommended by the manufacturer, prepare appropriate serial dilutions of this solution using water for bacterial endotoxins test (LAL Reagent Water). Use the solutions as soon as possible to avoid loss of activity.

Preparation of sample solutions Unless otherwise specified, prepare sample solutions by dissolving or diluting drugs or extracting medical devices using LAL Reagent Water. Some substances or preparations may be more appropriately dissolved, diluted, or extracted in other aqueous solutions. If necessary, adjust the pH of the solution to be examined so that the pH of the mixture of the LAL Reagent and sample solution falls within the specified pH range for the LAL Reagent to be used. This usually applies to a sample solution with a pH in the range of 6.0 to 8.0. The pH may be adjusted using an acid, base, or suitable buffer as recommended by the LAL Reagent manufacturer. Buffers must be validated to be free of detectable endotoxin and interfering factors.

Determination of Maximum Valid Dilution (MVD)

The Maximum Valid Dilution is the maximum allowable dilution of a sample solution at which the endotoxin limit can be determined. The general equation to determine MVD is:

$$\text{MVD} = \frac{\text{Endotoxin limit} \times \text{Concentration of sample solution}}{\lambda},$$

Endotoxin limit The endotoxin limit for active substances administered parenterally, defined on the basis of dose, is equal to: K/M, where K is a threshold pyrogenic dose of endotoxin per kilogram of body weight (EU/kg) in a single hour period, and M is equal to maximum recommended dose of product per kilogram of body weight in a single hour period. (**Note** K is 5 EU/kg for any route of administration other than intrathecal (for which K is 0.2 EU/kg body weight). For radiopharmaceutical products not administered intrathecally the endotoxin limit is calculated as $175/V$, where V is the maximum recommended dose in ml. For intrathecally administered radiopharmaceuticals, the endotoxin limit is obtained by the formula $14/V$. For formulations (usually anticancer products) administered on a per square metre of body surface area, the formula is K/M, where $K = 5$ EU/kg and M is the (maximum dose/m^2/hour × 1.80 m^2)/70 kg).

Concentration of sample solution

- in mg/ml if the endotoxin limit is specified by mass (EU/mg),
- in mEq/ml if the endotoxin limit is specified by equivalent (EU/mEq),
- in Units/ml if the endotoxin limit is specified by biological unit (EU/Unit),
- in ml/ml if the endotoxin limit is specified by volume (EU/ml)

λ The labelled lysate sensitivity in the gel-clot techniques (EU/ml) or the lowest point used (EU/ml) in the standard curve of the turbidimetric or chromogenic techniques.

Gel-Clot Techniques

The gel-clot techniques detect or quantify endotoxins based on clotting of the LAL Reagent in the presence of endotoxin. The concentration of endotoxin required to cause the lysate to clot under standard conditions is the labelled sensitivity of LAL Reagent. To ensure both the precision and validity of the test, perform the tests for confirming the labelled LAL Reagent sensitivity and for interfering factors as described under Preparatory testing for the gel-clot techniques.

Preparatory testing for the gel-clot techniques

TEST FOR CONFIRMATION OF LABELLED LAL REAGENT SENSITIVITY Confirm in four replicates the *labelled lysate sensitivity* λ, expressed in EU/ml, of the lysate solution prior to use in the test. Confirmation of the lysate sensitivity is carried out when a new batch of lysate is used or when there is any change in the experimental conditions which may affect the outcome of the test.

Prepare standard solutions having four concentrations equivalent to 2λ, 1λ, 0.5λ and 0.25λ by diluting the Standard Endotoxin Stock Solution with LAL Reagent Water. Prepare the lysate solution by dissolving the LAL Reagent with LAL Reagent Water or a suitable buffer. Mix a volume of the lysate solution with an equal volume of one of the standard solutions (usually, 0.1 ml aliquots) in each tube. When single test vials or ampoules containing lyophilized lysate are used, add solutions directly to the vial or ampoule. Incubate the reaction mixture for a constant period according to the recommendations of the lysate manufacturer (usually at 37°±1° for 60±2 minutes), avoiding vibration. To test the integrity of the gel after incubation, invert each tube or container through about 180° in one smooth motion. If a firm gel has formed that remains in place upon inversion, record the result as positive. A result is negative if either a firm gel is not formed, or if a fragile gel has formed but flows out upon inversion.

The test is not valid unless the lowest concentration of the standard solutions (0.25λ) shows a negative result in all replicate tests.

The end-point is the last positive test in the series of decreasing concentrations of endotoxin. Calculate the mean value of the logarithms of the end-point concentration and then the antilogarithm of the mean value using the following equation:

$$\text{Geometric Mean End-point Concentration} = \text{antilog}\,(\Sigma e/f),$$

where Σe is the sum of the log end-point concentrations of the dilution series used, and f is the number of replicate test-tubes.

The geometric mean end-point concentration is the measured sensitivity of the lysate solution (EU/ml). If the geometric mean end-point concentration is not less than 0.5λ and not more than 2λ, the labelled lysate sensitivity is confirmed.

TEST OF INTERFERING FACTORS FOR THE GEL-CLOT TECHNIQUES The test is performed to check for the presence of enhancing or inhibiting factors for the reaction in sample solutions. Prepare solutions A, B, C, and D as shown in Table 1, and use the sample solutions at a dilution less than the MVD, not containing any detectable endotoxins, following the procedure in the Test for confirmation of labelled LAL Reagent sensitivity above. The geometric mean end-point concentrations of solutions B and C are determined using the equation in that test.

The test must be repeated when any changes are made to the experimental conditions that are likely to influence the result of the test. The test is not valid unless all replicates of Solutions A and D show no reaction and the result of Solution C confirms the labelled lysate sensitivity.

If the sensitivity of the lysate determined with Solution B is not less than 0.5λ and not greater than 2λ, the sample solution does not contain interfering factors under the experimental conditions used. Otherwise, the sample solution to be examined interferes with the test.

If the sample under test does not comply with the test at a dilution less than the MVD, repeat the test using a greater dilution, not exceeding the MVD. The use of a more sensitive lysate permits a greater dilution of the sample to be examined and this may contribute to the elimination of interference.

Interference may be overcome by suitable treatment, such as filtration, neutralization, dialysis, or heating. To establish that the chosen treatment effectively eliminates interference without loss of endotoxins, repeat the test for interfering factors using the preparation being examined to which the standard endotoxin has been added and which has been subjected to the selected treatment.

Table 1 Preparation of Solutions for the Inhibition/Enhancement Test for Gel-Clot Techniques

Solution*	Endotoxin Concentration/ Solution to Which Endotoxin Is Added	Diluent	Dilution Factor	Initial Endotoxin Concentration	Number of Replicates
A	none/sample solution	–	–	–	4
B	2λ/sample solution	Sample Solution	1	2 λ	4
			2	1 λ	4
			4	0.5 λ	4
			8	0.25 λ	4
C	2λ/LAL Reagent Water	LAL Reagent Water	1	2 λ	2
			2	1 λ	2
			4	0.5 λ	2
			8	0.25 λ	2
D	none/LAL Reagent Water	–	–	–	2

*Solution A: a sample solution of the preparation under test that is free of detectable endotoxins; Solution B: test for interference; Solution C: control for labelled LAL Reagent sensitivity; Solution D: LAL Reagent Water (negative control).

Gel-clot limit test Based on the formation of a firm gel in the presence of endotoxin at above labelled LAL Reagent sensitivity, this method tests whether a sample solution contains endotoxin not greater than the endotoxin limit.

PROCEDURE Prepare Solutions A, B, C, and D as shown in Table 2, and perform the test on these solutions following the procedure in the Test for confirmation of labelled LAL Reagent sensitivity under Preparatory testing for the gel-clot techniques.

INTERPRETATION The test is not valid unless both replicates of positive control Solutions B and C are positive and those of negative control Solution D are negative. The sample complies with the test when a negative result is found for both tubes containing Solution A. The sample does not comply with the test when a positive result is found for both tubes containing Solution A.

Repeat the test when a positive result is found for one tube containing Solution A and a negative result for the other one. The sample complies with the test when a negative result is found for both tubes containing Solution A in the repeat result. If the test is positive for the sample at a dilution less than the MVD, the test may be repeated at a dilution not greater than the MVD.

Gel-clot assay The test measures endotoxin concentrations of sample solution by titration to an end-point of gel formation.

PROCEDURE Prepare Solutions A, B, C, and D as shown in Table 3, and test these solutions by following the procedure in the Test for confirmation of labelled LAL reagent sensitivity under Preparatory testing for the gel-clot techniques.

CALCULATION AND INTERPRETATION The test is not valid unless the following conditions are met: (1) both replicates of negative control Solution D are negative; (2) both replicates of positive product control Solution B are positive; and (3) the geometric mean end-point concentration of Solution C is in the range of 0.5λ to 2λ.

Table 2 Preparation of Solutions for the Gel-Clot Limit Test

Solution*	Endotoxin Concentration/Solution to Which Endotoxin Is Added	Number of Replicates
A	none/diluted sample solution	2
B	2λ/diluted sample solution	2
C	2λ/LAL Reagent Water	2
D	none/LAL Reagent Water	2

*Prepare Solution A and positive product control Solution B using a dilution not greater than the MVD and treatments as directed in the Test of interfering factors for the gel-clot techniques under Preparatory testing for the gel-clot techniques. Positive control Solutions B and C contain the standard endotoxin preparation at a concentration corresponding to twice the labelled LAL Reagent sensitivity. The negative control Solution D is LAL Reagent Water.

Table 3 Preparation of Solutions for the Gel-Clot Assay

Solution*	Endotoxin Concentration/ Solution to Which Endotoxin Is Added	Diluent	Dilution Factor	Initial Endotoxin Concentration	Number of Replicates
A	none/sample solution	LAL Reagent Water	1	–	2
			2	–	2
			4	–	2
			8	–	2
B	2λ/sample solution	–	1	2 λ	2
C	2λ/LAL Reagent Water	LAL Reagent Water	1	2 λ	2
			2	1 λ	2
			4	0.5 λ	2
			8	0.25 λ	2
D	none/LAL Reagent Water	–	–	–	2

*Solution A: a sample solution under test at the dilution, not to exceed the MVD, with which the Test of interfering factors for the gel-clot techniques was completed. Subsequent dilution of the sample solution must not exceed the MVD. Use LAL Reagent Water to make dilution series of four tubes containing the sample solution under test at concentrations of 1, 1/2, 1/4, and 1/8 relative to the dilution with which the Test of interfering factors for the gel-clot techniques was completed. Other dilutions may be used as appropriate; Solution B: Solution A containing standard endotoxin at a concentration of 2λ (positive product control); Solution C: two series of four tubes of LAL Reagent Water containing the standard endotoxin at a concentration of 2λ , 1λ , 0.5λ, and 0.25λ, respectively; Solution D: LAL Reagent Water (negative control).

To determine the endotoxin concentration of Solution A, calculate the end-point concentration for each replicate series of dilutions by multiplying each end-point dilution factor by λ.

The endotoxin concentration in the sample is the geometric mean end-point concentration of the replicates (see the equation given in the Test for confirmation of labelled LAL reagent sensitivity under Preparatory testing for the gel-clot techniques). If the test is conducted with a diluted sample solution, calculate the concentration of endotoxin in the original sample solution by multiplying by the dilution factor.

If none of the dilutions of the sample solution is positive in a valid assay, report the endotoxin concentration as less than λ (or, if the diluted sample was tested, less than λ × the lowest dilution factor of the sample). If all dilutions are positive, the endotoxin concentration is reported as equal to or greater than the greatest dilution factor multiplied by λ.

The sample meets the requirements of the test if the endotoxin concentration is less than that specified in the individual monograph.

Photometric Techniques

Photometric techniques which include turbidimetric and chromogenic require the establishment of a standard regression curve and the endotoxin content of the sample is determined by interpolation from the curve. The procedures include incubation for a preselected time of reacting endotoxin and control solutions with the LAL Reagent and reading of the spectrophotometric light absorbance at suitable wavelengths.

The turbidimetric technique measures the endotoxin concentrations of sample solution based on the measurement of turbidity change accompanying gel formation of the LAL Reagent. The technique is classified as either endpoint-turbidimetric or kinetic-turbidimetric. The endpoint-turbidimetric technique is based on the quantitative relationship between the concentration of endotoxins and the turbidity (absorbance or transmission) of the reaction mixture at the end of an incubation period. The kinetic-turbidimetric technique is based on the quantitative relationship between the concentration of endotoxins and either the time needed to reach a

predetermined turbidity of the reaction mixture or the rate of turbidity development.

The chromogenic method measures the endotoxin concentrations of sample solutions based on the measurement of chromophore released from a suitable chromogenic peptide by the reaction of endotoxins with the LAL Reagent. This technique is classified as either end-point-chromogenic or kinetic-chromogenic. The end-point-chromogenic technique is based on the quantitative relationship between the concentration of endotoxins and the release of chromophore at the end of an incubation period. The kinetic-chromogenic technique is based on the quantitative relationship between the concentration of endotoxins and either the time needed to reach a predetermined absorbance (or transmittance) of the reaction mixture or the rate of colour development.

All photometric tests are carried out at the incubation temperature recommended by the LAL Reagent manufacturer, which is usually 37°±1°.

Preparatory testing for the photometric techniques To assure the precision or validity of the turbidimetric and chromogenic techniques, preparatory tests are conducted to verify that the criteria for the standard curve are valid and that the sample solution does not inhibit or enhance the reaction. Revalidation for the test method is required when conditions that are likely to influence the test result change.

VERIFICAITON OF CRITERIA FOR THE STANDARD CURVE Using the Standard Endotoxin Solution, prepare at least three endotoxin concentrations to generate the standard curve. Perform the test using at least three replicates of each standard endotoxin concentration according to the manufacturer's instructions for the LAL Reagent (with regard to volume ratios, incubation time, temperature, pH, etc.). If the desired range in the kinetic methods is greater than two logs, additional standards should be included to bracket each log increase within the range of the standard curve.

The absolute value of the correlation coefficient, $|r|$, must be greater than or equal to 0.980 for the range of endotoxin concentrations set up.

INTERFERING FACTORS TEST FOR THE PHOTOMETRIC TECHNIQUES Select an endotoxin concentration at or near the middle of the endotoxin standard curve. Prepare Solutions A, B, C, and D as shown in Table 4. Perform the test on Solutions A, B, C, and D at least in duplicate following the instructions for the LAL Reagent used (with regard to volume of sample and LAL Reagent, volume ratio of sample to LAL Reagent, incubation time, etc.).

Calculate the mean recovery of the added endotoxin by subtracting the mean endotoxin concentration in the solution (if any) from that containing the added endotoxin. In order to be considered free of interfering factors under the conditions of the test, the measured concentration of the endotoxin added to the sample solution must be within 50 per cent to 200 per cent of the known added endotoxin concentration after subtraction of any endotoxin detected in the solution without added endotoxin.

When the endotoxin recovery is out of the specified ranges, the interfering factors must be removed as described in the Test of interfering factors for the gel-clot techniques under Preparatory testing for the gel clot techniques. The efficiency of the treatment is verified by repeating the Test of interfering factors for the gel-clot techniques.

Table 4 Preparation of Solutions for the Inhibition/Enhancement Test for Photometric Techniques

Solution*	Endotoxin Concentration	Solution to Which Endotoxin Is Added	Number of Replicates
A	none	sample solution	not less than 2
B	middle concentration of the standard curve	sample solution	not less than 2
C	at least 3 concentrations (lowest concentration is designated λ)	LAL Reagent Water	each not less than 2
D	none	LAL Reagent Water	not less than 2

*Solution A: the sample solution may be diluted not to exceed MVD; Solution B: the preparation under test at the same dilution as Solution A, containing added endotoxin at a concentration equal to or near the middle of the standard curve; Solution C: the standard endotoxin at the concentrations used in the validation of the method described in Verification of criteria for the standard curve under Preparatory testing for the photometric techniques (positive control series); Solution D: LAL Reagent Water (negative control).

Procedure for the photometric techniques Follow the procedure described in the Test of interfering factors for the photometric techniques under Preparatory testing for the photometric techniques.

Calculation for the photometric techniques Calculate the endotoxin concentration of each of the replicates of test Solution A using the standard curve generated by positive control series C. The test is not valid unless the following conditions are met: (1) the results of control series C comply with the requirements for validation defined under Verification of criteria for the standard curve under Preparatory testing for the photometric techniques; (2) the endotoxin recovery, calculated from the concentration found in Solution B after subtracting the endotoxin concentration found in Solution A is within 50 to 200 per cent; and (3) the result of negative control series D does not exceed the limit of the blank value required in the description of the LAL Reagent used.

Interpretation of results from the photometric techniques In photometric assays, the preparation under test complies with the test if the mean endotoxin concentration of the replicates of Solution A, after correction for dilution and concentration, is less than the endotoxin limit for the product.

Reagents

AMOEBOCYTE LYSATE (LAL) is a lyophilized product obtained for amoebocyte lysate from horseshoe crab. The reagent refers only to a product manufactured in accordance with the regulations of the competent authority. Amoebocyte lysate reacts with some β-glucans in addition to endotoxins. Some preparations which do not react with glucans are available; they are prepared by removing from amoebocyte lysate the G factor, which reacts with glucans, or by inhibiting the G factor reacting system of amoebocyte lysate. These preparations may be used for endotoxin testing in the presence of glucans.

LAL REAGENT WATER (water for bacterial endotoxin test) is Sterile Water for Injection or other types of water that show no reaction with the LAL Reagent used, at the detection limit of the reagent.

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APPENDIX 9 STATISTICAL ANALYSIS OF RESULTS OF BIOLOGICAL ASSAYS AND TESTS

1. Introduction

This appendix provides guidance for the design of biological assays prescribed in the Thai pharmacopoeia and for analysis of their results. It is intended for use by those whose primary training and responsibilities are not in statistics, but who have responsibility for analysis or interpretation of the results of these assays, often without the help and advice of a statistician. The methods of calculation described in this appendix are not mandatory for the biological assays which themselves constitute a mandatory part of the Thai Pharmacopoeia. Alternative methods may be used, provided that they are not less reliable than those described here. A wide range of computer software is available and may be useful depending on the facilities available to, and the expertise of, the analyst.

Professional advice should be obtained in situations where: a comprehensive treatment of design and analysis suitable for research or development of new products is required; the restrictions imposed on the assay design by this appendix are not satisfied (for example, particular laboratory constraints may require customized assay designs, or equal numbers of equally spaced doses may not be suitable); analysis is required for extended non-linear dose-response curves (for example, as may be encountered in immunoassays). An outline of extended dose-response curve analysis for one widely used model is nevertheless included and a simple example is given (See Section 3.1.4).

GENERAL DESIGN AND PRECISION

Biological methods are described for the assay of certain substances and preparations whose potency cannot be adequately assured by chemical or physical analysis. The principle applied wherever possible throughout these assays is that of comparison with a standard preparation so as to determine how much of the substance to be examined produces the same biological effect as a given quantity, the *Unit*, of the standard preparation. It is an essential condition of such methods of biological assay that the tests on the standard preparation and on the substance to be examined be carried out at the same time and under identical conditions.

For certain assays (determination of virus titre for example) the potency of the test sample is not expressed relative to a standard. This type of assay is dealt with in Section 3.2.4.

Any estimate of potency derived from a biological assay is subject to random error due to the inherent variability of biological responses and calculations of error should be made, if possible, from the results of each assay, even when the official method of assay is used. Methods for the design of assays and the calculation of their errors are, therefore, described below. In every case, before a statistical method is adopted, a preliminary test is to be carried out with an appropriate number of assays, in order to ascertain the applicability of this method.

Estimates of error are themselves subject to appreciable error if they are not based on a very large number of observations. The calculations may thus lead to false conclusions unless some allowance is made for this fact, as may be done by the calculation of fiducial limits. Since most of the responses to the biological assays are normally distributed, the fiducial limits are therefore replaced by the confidence limits.

The confidence interval for the potency gives an indication of the precision with which the potency has been estimated in the assay. It is calculated with due regard to the experimental design and the sample size. The 95 per cent confidence interval is usually chosen in biological assays. Mathematical statistical methods are used to calculate these limits so as to warrant the statement that there is a 95 per cent probability that these limits include the true potency. Whether this precision is acceptable to the Thai Pharmacopoeia depends on the requirements set in the monograph for the preparation concerned.

The following terms are used in this appendix to indicate the corresponding concepts:

Term	Definition
Stated potency	A nominal value assigned from knowledge of the potency of the bulk material, in the case of a formulated product; the potency estimated by the manufacturer, in the case of bulk material.
Labelled potency	The same as stated potency.
Assigned potency	The potency of the standard preparation.
Assumed potency	The provisionally assigned potency of a preparation to be examined which forms the basis of calculating the doses that would be equipotent with the doses to be used of the standard preparation.
Potency ratio of an unknown preparation	The ratio of equipotent doses of the standard preparation and the unknown preparation under the conditions of the assay.
Estimated potency	The potency calculated from assay data.
Experimental unit	A subject or a set of subjects, received a treatment at a time.

Glossary of symbols is a tabulation of the more important uses of symbols throughout this appendix (See Section 6). Where the text refers to a symbol or uses a symbol to denote a different concept, this is defined in that part of the text.

2. Randomization and Independence of Individual Treatments

The allocation of the different treatments to different experimental units (animals, tubes, etc.) should be made by some strictly random process. Any other choice of experimental conditions that is not deliberately allowed for in the experimental design should also be made randomly. Examples are the choice of positions for cages in a laboratory and the order in which treatments are administered. In particular, a group of animals receiving the same dose of any preparation should not be treated together (at the same time or in the same position) unless there is strong evidence that the relevant source of variation (for example, between times, or between positions) is negligible. Random allocations may be obtained from computers by using the built-in randomization function. The analyst must check whether a different series of numbers is produced every time the function is started.

The preparations allocated to each experimental unit should be as independent as possible. Within each experimental group, the dilutions allocated to each treatment are not normally divisions of the same dose, but should be prepared individually. Without this precaution, the variability inherent in the preparation will not be fully represented in the experimental error variance. The result will be an underestimation of the residual error leading to:

1) an unjustified increase in the stringency of the test for the analysis of variance.
2) an underestimation of the true confidence limits for the test which are calculated from the estimate of s^2, the residual error mean square.

3. Assay Design and Analysis

Numerous assay designs and analyses described herein (Fig. 1) can be chosen under certain conditions. There are three types of assays: the parallel-line model, the slope-ratio model, and the dose-response curve. The biological responses may be quantitative or quantal. Quantitative responses are the measurement of the effect on each experimental unit on a quantitative scale and quantal responses are on a qualitative scale. Under the parallel-line model with quantitative responses, there are four assay designs prescribed: the completely randomized design, the randomized block design, the Latin square design and the cross-over design. For the quantal response of parallel-line model, the probit and logit transformations are performed. The design used in the slope-ratio model is the completely randomized design, and the median effective dose is shown for the dose-response curve.

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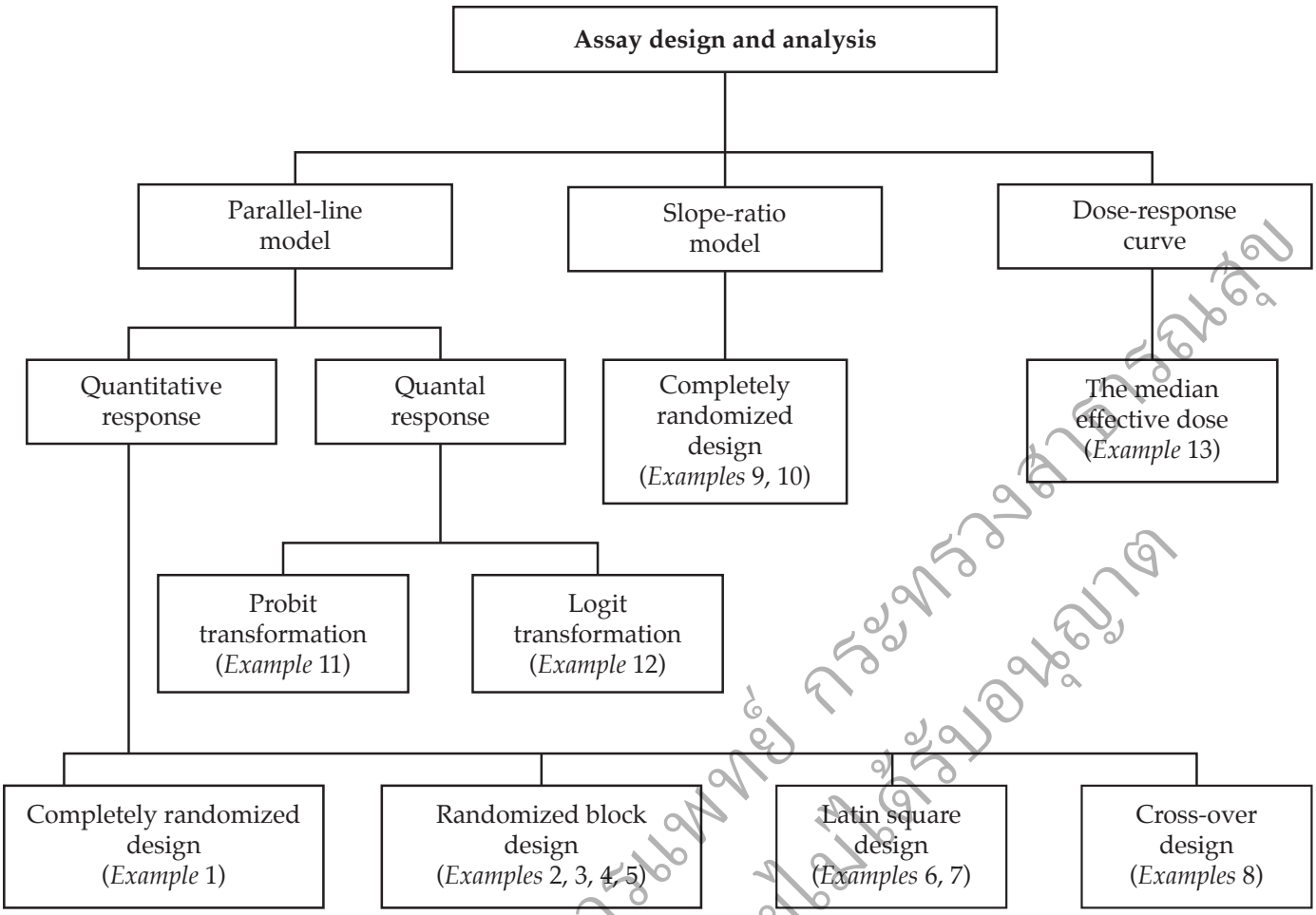

Fig. 1 Assay Design and Analysis for Biological Assays

3.1 ASSAYS DEPENDING UPON QUANTITATIVE RESPONSES

3.1.1 Statistical models

3.1.1.1 General principles

The biological assays included in the Thai Pharmacopoeia have been conceived as "dilution assays", which means that the unknown preparation to be assayed is supposed to contain the same active principle as the standard preparation, but in a different ratio of active and inert components. In such a case the unknown preparation may in theory be derived from the standard preparation by dilution with inert components. To check whether any particular assay may be regarded as a dilution assay, it is necessary to compare the dose-response relationships of the standard and unknown preparations. If these dose-response relationships differ significantly, then the theoretical dilution assay model is not valid. Significant differences in the dose-response relationships for the standard and unknown preparations may suggest that one of the preparations contains, in addition to the active principle, other components which are not inert but which influence the measured responses.

To make the effect of dilution in the theoretical model apparent, it is useful to transform the dose-response relationship to a linear function on the widest possible range of doses. Two statistical models are of interest as models for the biological assays prescribed: the parallel-line model and the slope-ratio model.

The application of either is dependent on the fulfillment of the following conditions:

Condition 1: the different treatments have been randomly assigned to the experimental units,

Condition 2: the responses to each treatment are normally distributed,

Condition 3: the standard deviations of the responses within each treatment group of both standard and unknown preparations do not differ significantly from one another.

When an assay is being developed for use, the analyst has to determine that the data collected from many assays meet these theoretical conditions.

— Condition 1 can be fulfilled by an efficient use of Section 2.

— Condition 2 is an assumption which in practice is almost always fulfilled. Minor deviations from this assumption will in general not introduce serious flaws in the analysis as long as several replicates per treatment are

included. In case of doubt, a test for deviations from normality (e.g., the Shapiro-Wilk test[1]) may be performed.

— Condition 3 can be checked with a test for homogeneity of variances (e.g., Bartlett's test[2], Cochran's test[3]). Inspection of graphical representations of the data can also be very instructive for this purpose.

When conditions 2 and/or 3 are not met, a transformation of the responses may bring a better fulfillment of these conditions. Examples are ln y, $\sqrt{y}$, y^2

— Logarithmic transformation of the responses y to ln y can be useful when the homogeneity of variances is not satisfactory. It can also improve the normality if the distribution is skewed to the right.

— The transformation of y to $\sqrt{y}$ is useful when the observations follow a Poisson distribution i.e. when they are obtained by counting.

— The square transformation of y to y^2 can be useful if, for example, the dose is more likely to be proportional to the area of an inhibition zone rather than the measured diameter of that zone.

For some assays depending on quantitative responses, such as immunoassays or cell-based *in vitro* assays, a large number of doses is used. These doses give responses that completely span the possible response range and produce an extended non-linear dose-response curve. Such curves are typical for all biological assays, but for many assays the use of a large number of doses is not ethical (for example, *in vivo* assays) or practical, and the aims of the assay may be achieved with a limited number of doses. It is therefore customary to restrict doses to that part of the dose-response range which is linear under suitable transformation, so that the methods of Section 3.1.2 or 3.1.3 apply. However, in some cases analysis of extended dose-response curves may be desirable. An outline of one model which may be used for such analysis is given (See Section 3.1.4).

There is another category of assays in which the response cannot be measured in each experimental unit, but in which only the fraction of units responding to each treatment can be counted. This category is dealt with in Section 3.2.

3.1.1.2 Routine assays

When an assay is in routine use, it is seldom possible to check systematically for conditions 1 to 3, because the limited number of observations per assay is likely to influence the sensitivity of the statistical tests. Fortunately, statisticians have shown that, in symmetrical balanced assays, small deviations from homogeneity of variances and normality do not seriously affect the assay results. The applicability of the statistical model needs to be questioned only if a series of assays shows doubtful validity. It may then be necessary to perform a new series of preliminary investigations as discussed (See Section 3.1.1.1).

Two other necessary conditions depend on the statistical model to be used:

FOR THE PARALLEL-LINE MODEL (*Condition A*):

Condition 4A: the relationship between the logarithm of the dose and the response can be represented by a straight line over the range of doses used,

Condition 5A: for any unknown preparation in the assay the straight line is parallel to that for the standard.

FOR THE SLOPE-RATIO MODEL (*Condition B*):

Condition 4B: the relationship between the dose and the response can be represented by a straight line for each preparation in the assay over the range of doses used,

Condition 5B: for any unknown preparation in the assay the straight line intersects the y-axis (at zero dose) at the same point as the straight line of the standard preparation (i.e., the response functions of all preparations in the assay must have the same intercept as the response function of the standard).

Conditions 4A and 4B can be verified only in assays in which at least three dilutions of each preparation have been tested. The use of an assay with only one or two dilutions may be justified when experience has shown that linearity and parallelism or equal intercept are regularly fulfilled.

After having collected the results of an assay, and before calculating the relative potency of each test sample, an analysis of variance is performed, in order to check whether conditions 4A and 5A (or 4B and 5B) are fulfilled. For this, the total sum of squares is subdivided into a certain number of sum of squares corresponding to each condition which has to be fulfilled. The remaining sum of squares represents the residual experimental error to which the absence or existence of the relevant sources of variation can be compared by a series of F-ratios.

When validity is established, the potency of each unknown relative to the standard may be calculated and expressed as a potency ratio or converted to some unit relevant to the preparation under test, e.g. an International Unit. Confidence limits may also be estimated from each set of assay data.

Assays based on the parallel-line model are discussed (See Section 3.1.2) and those based on the slope-ratio model (See Section 3.1.3).

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[1]M. B. Wilk and S. S. Shapiro, "The Joint Assessment of Normality of Several Independent Samples," *Technometrics*, *10*, 1968, pp. 825-839.

[2]M. S. Bartlett, *Properties of sufficiency and statistical tests*, Series A, Proc. Roy. Soc., London, 1937, pp. 160, 280-281.

[3]W. G. Cochran, "Testing a Linear Relation Among Variances," *Biometrics*, 7, 1951, pp. 17-32.

If any of the 5 conditions (1, 2, 3, 4A, 5A or 1, 2, 3, 4B, 5B) are not fulfilled, the methods of calculation described here are invalid and an investigation of the assay technique should be made.

The analyst should not adopt another transformation unless it is shown that non-fulfillment of the requirements is not incidental but is due to a systematic change in the experimental conditions. In this case, testing as described (See Section 3.1.1.1) should be repeated before a new transformation is adopted for the routine assays.

Excess numbers of invalid assays due to non-parallelism or non-linearity, in a routine assay carried out to compare similar materials, are likely to reflect assay designs with inadequate replication. This inadequacy commonly results from incomplete recognition of all sources of variability affecting the assay, which can result in underestimation of the residual error leading to large *F*-ratios.

It is not always feasible to take account of all possible sources of variation within one single assay (e.g., day-today variation). In such a case, the confidence intervals from repeated assays on the same sample may not satisfactorily overlap, and care should be exercised in the interpretation of the individual confidence intervals. In order to obtain a more reliable estimate of the confidence interval it may be necessary to perform several independent assays and to combine these into one single potency estimate and confidence interval (See Section 4).

For the purpose of quality control of routine assays it is recommended to keep record of the estimates of the slope of regression and of the estimate of the residual error in control charts.

— An exceptionally high residual error may indicate some technical problem. This should be investigated and, if it can be made evident that something went wrong during the assay procedure, the assay should be repeated. An unusually high residual error may also indicate the presence of an occasional outlying or aberrant observation. A response that is questionable because of failure to comply with the procedure during the course of an assay is rejected. If an aberrant value is discovered after the responses have been recorded, but can then be traced to assay irregularities, omission may be justified. The arbitrary rejection or retention of an apparently aberrant response can be a serious source of bias. In general, the rejection of observations solely because a test for outliers is significant is discouraged.

— An exceptionally low residual error may once in a while occur and cause the *F*-ratios to exceed the critical values. In such a case it may be justified to replace the residual error estimated from the individual assay, by an average residual error based on historical data recorded in the control charts.

3.1.1.3 Calculations and restrictions

According to general principles of good design the following three restrictions are normally imposed on the assay design. They have advantages both for ease of computation and for precision.

a. Each preparation in the assay must be tested with the same number of dilutions.

b. In the parallel-line model, the ratio of adjacent doses must be constant for all treatments in the assay; in the slope-ratio model, the interval between adjacent doses must be constant for all treatments in the assay.

c. There must be an equal number of experimental units to each treatment.

If a design is used which meets these restrictions, the calculations are simple. The formulae are given (See Sections 3.1.2 and 3.1.3). It is recommended to use software which has been developed for this special purpose. There are several programs in existence which can easily deal with all assay-designs described in the monographs. Not all programs may use the same formulae and algorithms, but they should all lead to the same results.

Assay designs not meeting the above mentioned restrictions may be both possible and correct, but the necessary formulae are too complicated to describe in this text.

The formulae for the restricted designs given in this text may be used, for example, to create *ad hoc* programs in a spreadsheet. The examples in Sections 3 and 4 can be used to clarify the statistics and to check whether such a program gives correct results.

An assay design is such that one or more sources of variation are eliminated from comparisons of means of several treatments. A statistical examination of the results must be such that a proper allowance is made for the elimination of the source of variation. With some experimental designs this can be done by the method called the "Analysis of Variance or ANOVA". It can be said that an analysis of variance is an aid to examine the validity of the assay. Analysis of variance is based on a partition of a total sum of squares of deviations of all the values of a variate from their mean into several sums of squares that correspond to different sources of variation and a residual sum of squares that corresponds to the variation that has not been eliminated. The method can, therefore, be used for estimation of the experimental error.

The precision of experimental results and the ease of computation depend on the assay design and the experimental design.

3.1.2 The parallel-line model

3.1.2.1 Introduction

For a drug that is assayed biologically, the response should plot as a straight line against the log-dose over an adequate range of doses. The straight line representing its relationship with the response of the assay must parallel to that for the standard.

The parallel-line model is illustrated in Fig. 2. The logarithm of the doses are represented on the horizontal axis with the lowest concentration on the left and the highest concentration on the right. The responses are indicated on the vertical axis. The individual responses to each treatment are indicated with black dots. The two lines are the calculated ln (dose)-response relationship for the standard and the unknown.
(**Note** The natural logarithm, ln or $\log_e$, is used throughout this text. Wherever the term "antilogarithm", antiln or antilog_e, is used, the quantity e^x is meant. However, the Briggs or "common" logarithm, log or $\log_{10}$, can equally well be used. In this case the corresponding antilogarithm is 10^x.)

For a satisfactory assay the assumed potency of the unknown must be close to the true potency. On the basis of this assumed potency and the assigned potency of the standard, equipotent dilutions (if feasible) are prepared, i.e. corresponding doses of standard and unknown are expected to give the same response. If no information on the assumed potency is available, preliminary assays are carried out over a wide range of doses to determine the range where the curve is linear.

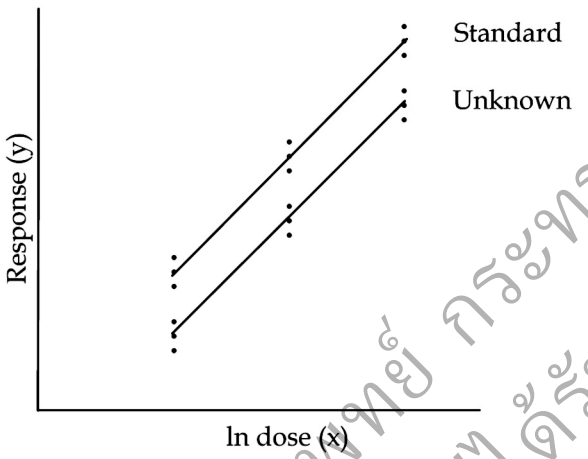

Fig. 2 The Parallel-line Model for a 3 + 3 Assay

The more nearly correct the assumed potency of the unknown, the closer the two lines will be together, for they should give equal responses at equal doses. The horizontal distance between the lines represents the "true" potency of the unknown, relative to its assumed potency. The greater the distance between the two lines, the poorer the assumed potency of the unknown. If the line of the unknown is situated to the right of the standard, the assumed potency was overestimated, and the calculations will indicate an estimated potency lower than the assumed potency. Similarly, if the line of the unknown is situated to the left of the standard, the assumed potency was underestimated, and the calculations will indicate an estimated potency higher than the assumed potency.

3.1.2.2 Assay design

The following considerations will be useful in optimizing the precision of the assay design:

— the ratio between the slope and the residual error should be as large as possible,
— the range of doses should be as large as possible,
— the lines should be as close together as possible, i.e. the assumed potency should be a good estimate of the true potency.

The allocation of experimental units (animals, tubes, etc.) to different treatments may be made as follows:

— completely randomized design (See Section 3.1.2.4),
— randomized block design (See Section 3.1.2.5),
— Latin square design (See Section 3.2.1.6), and
— cross-over design (See Section 3.1.2.7).

3.1.2.3 Tests of validity

Assay results are said to be "statistically valid" if the outcome of the analysis of variance is as follows.

a. The linear regression term is significant, i.e. the calculated probability is less than 0.05. If this criterion is not met, it is not possible to calculate 95 per cent confidence limits.

b. The term for non-linearity is not significant, i.e. the calculated probability is not less than 0.05. This indicates that Condition 4A, Section 3.1.1.2, is satisfied.

c. The term for non-parallelism is not significant, i.e. the calculated probability is not less than 0.05. This indicates that Condition 5A, Section 3.1.1.2, is satisfied.

A significant deviation from parallelism in a multiple assay may be due to the inclusion in the assay design of a preparation to be examined that gives an ln (dose)-response line with a slope different from those for the other

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preparations. Instead of declaring the whole assay invalid, it may then be decided to eliminate all data relating to that preparation and to restart the analysis from the beginning.

When statistical validity is established, potencies and confidence limits may be estimated by the methods described in the example under each design.

3.1.2.4 Completely randomized design

If the totality of experimental units appears to be reasonably homogeneous with no indication that variability in response will be smaller within certain recognizable sub-groups, the allocation of the units to the different treatments should be made randomly.

If units in sub-groups such as physical positions or experimental days are likely to be more homogeneous than the totality of the units, the precision of the assay may be increased by introducing one or more restrictions into the design. A careful distribution of the units over these restrictions permits irrelevant sources of variation to be eliminated. The statistical method of calculation is illustrated in Example 1.

Example 1 Three-Dose Single Assay, Completely Randomized Design

The assay is composed of the standard of three concentrations, designated s_1, s_2 and s_3 and one unknown of similar three concentrations, designated u_1, u_2 and u_3, respectively.

All six concentrations of standard and unknown are allocated randomly using a standard table of random numbers.

Table 1 Response y (Absorbance*)

	Standard *S*			Unknown *U*		
	s_1	s_2	s_3	u_1	u_2	u_3
Individual Response	0.840 0.790 0.796	1.237 1.210 1.228	1.737 1.674 1.696	0.794 0.765 0.760	1.228 1.190 1.183	1.637 1.607 1.623
Total Response	2.426 (S_1)	3.675 (S_2)	5.107 (S_3)	2.319 (U_1)	3.601 (U_2)	4.867 (U_3)

*In case of assay of Calcium Pantothenate.

Table 2 Response Totals and Contrasts

	Standard *S*	Unknown *U*
Low Dose	$S_1 = 2.426$	$U_1 = 2.319$
Medium Dose	$S_2 = 3.675$	$U_2 = 3.601$
High Dose	$S_3 = 5.107$	$U_3 = 4.867$
Preparation Total	$S = S_1 + S_2 + S_3$ $= 11.208$	$U = U_1 + U_2 + U_3$ $= 10.787$
Linear Contrast	$L_S = S_3 - S_1$ $= 2.681$	$L_U = U_3 - U_1$ $= 2.548$
Quadratic Contrast	$Q_S = S_1 - 2S_2 + S_3$ $= 0.183$	$Q_U = U_1 - 2U_2 + U_3$ $= -0.016$

Table 3 Analysis of Variance

Source of Variation	df	Sum of Squares (SS)	Mean Square	$F_{(cal)}$	$F_{(tab)}$ at $\alpha = 0.05$	p-value
Preparations	1	0.0098	0.0098			
Regression	1	2.2785	2.2785	4,402.4969	4.75	<0.01
Parallelism	1	0.0014	0.0014	2.8482	4.75	>0.05
Quadratic Curvature	1	0.0007	0.0007	1.4968	4.75	>0.05
Difference of Quadratics	1	0.0011	0.0011	2.1254	4.75	>0.05
Treatments	5	2.2917	0.4583	885.5986	3.11	<0.01
Residual Error	12	0.0062	0.0005			
Total	17	2.2979				

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Mean Square = Sum of Squares / df

$F_{(cal)}$ = Mean Square / Mean Square of Residual Error

Given statistic values for the next steps:

$N = 18$

$n = 3$

$h = 2$

$d = 3$

dose ratio = 2

Correction term, K $= \dfrac{(\Sigma y)^2}{N}$

$= \dfrac{(0.840 + 0.790 + \cdots + 1.623)^2}{18}$

$= 26.8766$

Calculation of sums of squares (SS) of the variations:

SS preparations $= \dfrac{S^2 + U^2}{3n} - K$

$= \dfrac{(11.208)^2 + (10.787)^2}{(3)(3)} - 26.8766$

$= 0.0098$

SS regression, E $= \dfrac{(L_S + L_U)^2}{2nh}$

$= \dfrac{(2.681 + 2.548)^2}{(2)(3)(2)}$

$= 2.2785$

SS parallelism $= \dfrac{L_S^2 + L_U^2}{2n} - E$

$= \dfrac{(2.681)^2 + (2.548)^2}{(2)(3)} - 2.2785$

$= 0.0014$

SS quadratic curvature, Q $= \dfrac{(Q_S + Q_U)^2}{6nh}$

$= \dfrac{[(0.183) + (-0.016)]^2}{(6)(3)(2)}$

$= 7.0 \times 10^{-4}$

SS difference of quadratics $= \dfrac{Q_S^2 + Q_U^2}{6n} - Q$

$= \dfrac{(0.183)^2 + (-0.016)^2}{(6)(3)} - 7.0 \times 10^{-4}$

$= 1.1 \times 10^{-4}$

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$$\text{SS treatments} = \frac{\Sigma(\text{Total response})^2}{n} - K$$

$$= \frac{(2.426)^2 + (3.675)^2 + \cdots + (4.867)^2}{(3)} - 26.8766$$

$$= 2.2917$$

$$\text{SS total} = \Sigma y^2 - K$$

$$= \left((0.840)^2 + (0.790)^2 + \cdots + (1.623)^2\right) - 26.8766$$

$$= 2.2979$$

$$\text{SS residual error} = \text{SS total} - \text{SS treatments}$$

$$= 2.2979 - 2.2917$$

$$= 0.0062$$

Calculation of potency ratio and confidence limits:

Calculate slope, b:

$$b = \frac{L_S + L_U}{2nh(\text{ln dose ratio})}$$

$$= \frac{2.681 + 2.548}{(2)(3)(0.6931)}$$

$$= 0.6286$$

Calculate ln potency ratio, M:

$$M = \frac{U - S}{ndb}$$

$$= \frac{10.787 - 11.208}{(3)(3)(0.6286)}$$

$$= -0.0744$$

$$\text{Potency ratio} = \text{antiln } M$$

$$= 0.9283$$

Given statistic values for the next steps:

From Table 73 with 12 df of error, $\left(\frac{\alpha}{2} = 0.025\right)$, t = 2.1788

Mean square of residual error, $s^2 = 5.1756 \times 10^{-4}$

$N_S = 9$

$N_U = 9$

Calculate and apply ln confidence limits to the ln potency ratio: (M_L, M_U)

Confidence limits are given by antiln M_L and antiln M_L: antiln $M_L = 0.8943$ and anitn $M_U = 0.9635$.

$$M_L, M_U = M \pm \frac{ts}{b}\sqrt{\left(\frac{1}{N_S} + \frac{1}{N_U}\right) + \frac{M^2}{2nh(\text{ln dose ratio})^2}}$$

$$= (-0.0744) \pm \frac{(2.1788)(\sqrt{5.1756 \times 10^{-4}})}{0.6286}\sqrt{\left(\frac{1}{9} + \frac{1}{9}\right) + \frac{(-0.0744)^2}{(2)(3)(2)(0.6931)^2}}$$

$$= -0.1117, -0.0371$$

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Thus, the potency is estimated to be 92.83 per cent of the stated potency, with confidence limits at 89.43 per cent and 96.35 per cent of the stated potency.

If the precision of the assay is limited to be not less than 95.0 per cent and not more than 105.0 per cent of the estimated potency, the confidence limits of error will be 88.19 and 97.47 per cent of the estimated potency.

Therefore, they imply such assay precision.

3.1.2.5 Randomized block design

In this design it is possible to segregate an identifiable source of variation, such as the sensitivity variation between litters of experimental animals or the variation between Petri dishes in a diffusion microbiological assay. The design requires that every treatment be applied an equal number of times in every block (litter or Petri dish) and is suitable only when the block is large enough to accommodate all treatments once. This is illustrated in Examples 2 to 5. It is also possible to use a randomized design with repetitions. The treatments should be allocated randomly within each block.

Example 2 Two-Dose Single Assay, Randomized Block Design

The assay is composed of one standard and two concentrations, designated s_1 and s_2 and one unknown of similar two concentrations, designated u_1 and u_2 respectively.

Table 4 Response y (Degree of Calcification*)

Block	Standard *S*		Unknown *U*		Block Total
	s_1	s_2	u_1	u_2	
1	1.80	3.75	2.25	4.50	12.30
2	2.20	3.37	2.00	4.50	12.07
3	2.12	3.75	2.75	4.00	12.62
4	2.25	4.50	1.85	3.75	12.35
5	2.50	4.50	1.90	4.75	13.65
6	1.75	4.00	2.87	3.50	12.12
7	2.00	4.50	2.50	4.75	13.75
8	1.37	3.37	2.47	4.75	11.96
Total Response	15.99 (S_1)	31.74 (S_2)	18.59 (U_1)	34.50 (U_2)	100.82

*In case of assay of Vitamin D.

Table 5 Response Totals and Contrasts

	Standard *S*	Unknown *U*
Low Dose	$S_1 = 15.99$	$U_1 = 18.59$
High Dose	$S_2 = 31.74$	$U_2 = 34.50$
Preparation Total	$S = S_1 + S_2$	$U = U_1 + U_2$
	$= 47.73$	$= 53.09$
Linear Contrast	$L_S = S_2 - S_1$	$L_U = U_2 - U_1$
	$= 15.75$	$= 15.91$

Table 6 Analysis of Variance

Source of Variation	df	Sum of Squares (SS)	Mean Square	$F_{(cal)}$	$F_{(tab)}$ at $\alpha = 0.05$	p-value
Preparations	1	0.8978	0.8978			
Regression	1	31.3236	31.3236	149.9072	4.32	<0.01
Parallelism	1	0.0008	0.0008	0.0038	4.32	>0.05
Treatments	3	32.2222	10.7407	51.0597	3.07	<0.01
Blocks	7	0.8746	0.1249	0.5940	2.49	>0.05
Residual Error	21	4.4174	0.2103			
Total	31	37.5143				

Mean Square = Sum of Squares / df

$F_{(cal)}$ = Mean Square / Mean Square of Residual Error

Given statistic values for the next steps:

$$N = 32$$
$$n = 8$$
$$h = 2$$
$$d = 2$$
$$\text{dose ratio} = 32$$

$$\text{Correction term, K} = \frac{(\Sigma y)^2}{N} = \frac{(1.80 + 2.20 + \cdots + 4.75)^2}{32} = 317.6460$$

Calculation of sums of squares (SS) of the variations:

$$\text{SS preparations} = \frac{S^2 + U^2}{2n} - K = \frac{(47.73)^2 + (53.09)^2}{(2)(8)} - 317.6460 = 0.8978$$

$$\text{SS regression, E} = \frac{(L_S + L_U)^2}{2nh} = \frac{(15.75 + 15.91)^2}{(2)(8)(2)} = 31.3236$$

$$\text{SS parallelism} = \frac{L_S^2 + L_U^2}{2n} - E = \frac{(15.75)^2 + (15.91)^2}{(2)(8)} - 31.3236 = 8.0 \times 10^{-4}$$

$$\text{SS treatments} = \frac{\Sigma(\text{Total response})^2}{n} - K = \frac{(15.99)^2 + (31.74)^2 + \cdots + (34.50)^2}{8} - 317.6460 = 32.2222$$

$$\text{SS blocks} = \frac{\Sigma(\text{Block total})^2}{hd} - K = \frac{(12.30)^2 + (12.07)^2 + \cdots + (11.96)^2}{(2)(2)} - 317.6460 = 0.8746$$

$$\text{SS total} = \Sigma y^2 - K = \left((1.80)^2 + (2.20)^2 + \cdots + (4.75)^2\right) - 317.6460 = 37.5143$$

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SS residual error = SS total – SS treatments – SS blocks

= 37.5143 – 32.2222 – 0.8746

= 4.4174

Calculation of potency ratio and confidence limits:

Calculate slope, b:

$$b = \frac{L_S + L_U}{nh(\text{ln dose ratio})}$$

$$= \frac{15.75 + 15.91}{(8)(2)(0.6931)}$$

$$= 2.8547$$

Calculate ln potency ratio, M:

$$M = \frac{U - S}{ndb}$$

$$= \frac{53.09 - 47.73}{(8)(2)(2.8547)}$$

$$= 0.1173$$

Potency ratio = antiln M

= 1.1245

Given statistic values for the next steps:

From Table 73 with 21 df of error, $\left(\frac{\alpha}{2} = 0.025\right)$, t = 2.0796

Mean square of residual error, s^2 = 0.2103

N_S = 16

N_U = 16

Calculate and apply ln confidence limits to the ln potency ratio: (M_L, M_U)

$$M_L, M_U = M \pm \frac{ts}{b}\sqrt{\left(\frac{1}{N_S} + \frac{1}{N_U}\right) + \frac{M^2}{2nh(\text{ln dose ratio})^2}}$$

$$= 0.1173 \pm \frac{(2.0796)(\sqrt{0.2103})}{2.8547}\sqrt{\left(\frac{1}{16} + \frac{1}{16}\right) + \frac{(0.1173)^2}{(2)(8)(2)(0.6931)^2}}$$

$$= -0.0011, 0.2359$$

Confidence limits are given by antiln M_L and antiln M_U: antiln M_L = 0.9989 and antiln M_U = 1.2660

Thus, the potency is estimated to be 112.45 per cent of the stated potency, with confidence limits at 99.89 per cent and 126.60 per cent of the stated potency.

If the precision of the assay is limited to be not less than 95.0 per cent and not more than 105.0 per cent of the estimated potency, the confidence limits of error will be 106.83 and 118.07 per cent of the estimated potency.

Therefore, they do not imply such assay precision. The assay must be repeated and the combination of potency estimates is required.

Example 3. Three-Dose Single Assay, Randomized Block Design

The assay is composed of the standard of three concentrations, designated s_1, s_2 and s_3 for 5.0, 10.0 and 20.0 μg per ml, respectively; and one unknown of similar three concentrations, designated u_1, u_2 and u_3.

The assay requires six plates (20 mm × 100 mm), each containing six cylinders arranged in a radius pattern. In each plate, all six concentrations of standard and unknown are assigned as in Table 7.

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Table 7 A Pattern of Arrangement of Treatments

Block (Plate Number)	Position Number					
	1	2	3	4	5	6
1	s_3	u_2	s_1	u_3	s_2	u_1
2	s_2	u_1	s_3	u_2	s_1	u_3
3	s_1	u_3	s_2	u_1	s_3	u_2
4	u_3	s_2	u_1	s_3	u_2	s_1
5	u_2	s_1	u_3	s_2	u_1	s_3
6	u_1	s_3	u_2	s_1	u_3	s_2

Table 8 Response y (Diameters of Inhibition Zones*, in mm × 10)

Block (Plate Number)	Standard *S*			Unknown *U*			Block Total
	s_1	s_2	s_3	u_1	u_2	u_3	
1	156	180	202	158	176	204	1,076
2	157	182	202	152	178	206	1,077
3	152	184	204	158	181	205	1,084
4	154	178	203	152	180	206	1,073
5	156	178	204	156	182	204	1,080
6	158	180	202	158	179	203	1,080
Total Response	933 (S_1)	1,082 (S_2)	1,217 (S_3)	934 (U_1)	1,076 (U_2)	1,228 (U_3)	6,470

*In case of assay of antibiotics.

Table 9 Response Totals and Contrasts

	Standard *S*	Unknown *U*
Low Dose	$S_1 = 933$	$U_1 = 934$
Medium Dose	$S_2 = 1{,}082$	$U_2 = 1{,}076$
High Dose	$S_3 = 1{,}217$	$U_3 = 1{,}228$
Preparation Total	$S = S_1 + S_2 + S_3$	$U = U_1 + U_2 + U_3$
	$= 3{,}232$	$= 3{,}238$
Linear Contrast	$L_S = S_3 - S_1$	$L_U = U_3 - U_1$
	$= 284$	$= 294$
Quadratic Contrast	$Q_S = S_1 - 2S_2 + S_3$	$Q_U = U_1 - 2U_2 + U_3$
	$= -14$	$= 10$

Table 10 Analysis of Variance

Source of Variation	df	Sum of Squares (SS)	Mean Square	$F_{(cal)}$	$F_{(tab)}$ at $\alpha = 0.05$	p-value
Preparations	1	1.0000	1.0000			
Regression	1	13,920.1667	13,920.1667	2,963.1386	4.24	<0.01
Parallelism	1	4.1667	4.1667	0.8869	4.24	>0.05
Quadratic Curvature	1	0.2222	0.2222	0.0473	4.24	>0.05
Difference of Quadratics	1	8.0000	8.0000	1.7029	4.24	>0.05
Treatments	5	13,933.5556	2,786.7111	593.1977	2.60	<0.01
Blocks	5	12.2222	2.4444	0.5203	2.60	>0.05
Residual Error	25	117.4444	4.6978			
Total	35	14,063.2222				

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Mean Square = Sum of Squares / df

$F_{(cal)}$ = Mean Square / Mean Square of Residual Error

Given statistic values for the next steps:

$N = 36$

$n = 6$

$h = 2$

$d = 3$

dose ratio = 2

Correction term, K $= \dfrac{(\Sigma y)^2}{N}$

$= \dfrac{(156 + 157 + \cdots + 203)^2}{36}$

$= 1{,}162{,}802.7778$

Calculation of sums of squares (SS) of the variations:

SS preparations $= \dfrac{S^2 + U^2}{3n} - K$

$= \dfrac{(3{,}232)^2 + (3{,}238)^2}{(3)(6)} - 1{,}162{,}802.7778$

$= 1.0000$

SS regression, E $= \dfrac{(L_S + L_U)^2}{2nh}$

$= \dfrac{(284 + 294)^2}{(2)(6)(2)}$

$= 13{,}920.1667$

SS parallelism $= \dfrac{L_S^2 + L_U^2}{2n} - E$

$= \dfrac{(284)^2 + (294)^2}{(2)(6)} - 13{,}920.1167$

$= 4.1667$

SS quadratic curvature, Q $= \dfrac{(Q_S + Q_U)^2}{6nh}$

$= \dfrac{[(-14) + (10)]^2}{(6)(6)(2)}$

$= 0.2222$

SS difference of quadratics $= \dfrac{Q_S^2 + Q_U^2}{6n} - Q$

$= \dfrac{(-14)^2 + (10)^2}{(6)(6)} - 0.2222$

$= 8.0000$

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$$\text{SS treatments} = \frac{\Sigma(\text{Total response})^2}{n} - K$$

$$= \frac{(933)^2 + (1{,}082)^2 + \cdots + (1{,}228)^2}{6} - 1{,}162{,}802.7778$$

$$= 13{,}933.5556$$

$$\text{SS blocks} = \frac{\Sigma(\text{Block total})^2}{hd} - K$$

$$= \frac{(1{,}076)^2 + (1{,}077)^2 + \cdots + (1{,}080)^2}{(2)(3)} - 1{,}162{,}802.7778$$

$$= 12.2222$$

$$\text{SS total} = \Sigma y^2 - K$$

$$= \left[(156)^2 + (157)^2 + \cdots + (203)^2\right] - 1{,}162{,}802.7778$$

$$= 14{,}063.2222$$

$$\text{SS residual error} = \text{SS total} - \text{SS treatments} - \text{SS blocks}$$

$$= 14{,}063.2222 - 13{,}933.5556 - 12.2222$$

$$= 117.4444$$

Calculation of potency ratio and confidence limits:

Calculate slope, b:

$$b = \frac{L_S + L_U}{2nh(\ln \text{dose ratio})}$$

$$= \frac{284 + 294}{(2)(6)(2)(0.6931)}$$

$$= 34.7449$$

Calculate ln potency ratio, M:

$$M = \frac{U - S}{ndb}$$

$$= \frac{3{,}238 - 3{,}232}{(6)(3)(34.7449)}$$

$$= 0.0096$$

$$\text{Potency ratio} = \text{antiln } M$$

$$= 1.0096$$

Given statistic values for the next steps:

From Table 73 with 25 df of error, $\left(\frac{\alpha}{2} = 0.025\right)$, $t = 2.0595$

Mean square of residual error, $s^2 = 4.6978$

$N_S = 18$

$N_U = 18$

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Calculate and apply ln confidence limits to the ln potency ratio: (M_L, M_U):

$$M_L, M_U = M \pm \frac{ts}{b}\sqrt{\left(\frac{1}{N_S}+\frac{1}{N_U}\right)+\frac{M^2}{2nh(\ln \text{ dose ratio})^2}}$$

$$= 0.0096 \pm \frac{(2.0596)(\sqrt{4.6978})}{34.7449}\sqrt{\left(\frac{1}{18}+\frac{1}{18}\right)+\frac{(0.0096)^2}{(2)(6)(2)(0.6931)^2}}$$

$$= -0.0332, 0.0524$$

Confidence limits are given by antiln M_L and antiln M_U: antiln $M_L = 0.9673$ and antiln $M_U = 1.0538$

Thus, the potency is estimated to be 100.96 per cent of the stated potency, with confidence limits at 96.73 and 105.38 per cent of the stated potency.

If the precision of the assay is limited to be not less than 95.0 per cent and not more than 105.0 per cent of the estimated potency, the confidence limits of error will be 95.92 and 106.01 per cent of the estimated potency.

Therefore, they imply such assay precision.

Example 4 Three-Dose Multiple Assay, Randomized Block Design

The assay is composed of one standard of three concentrations, designated s_1, s_2, and s_3, and two unknowns of similar three concentrations, designated u_1, u_2 and u_3 to z_1, z_2 and z_3, respectively. Standard doses to be administered were 2, 4 and 8 units, and equivalent test doses are prepared assuming the potencies of the test preparations are identical to that of the standard.

The assay required six plates (20 mm × 150 mm) each containing nine cylinders which are randomly arranged in a radius pattern as in Table 11.

Table 11 A Pattern of Arrangement of Treatments

Block (Plate Number)	Position Number								
	1	2	3	4	5	6	7	8	9
1	s_3	u_2	z_1	u_3	z_2	s_1	z_3	s_2	u_1
2	s_2	u_1	z_3	u_2	z_1	s_3	z_2	s_1	u_3
3	s_1	u_3	z_2	u_1	z_3	s_2	z_1	s_3	u_2
4	u_3	z_2	s_1	z_3	s_2	u_1	s_3	u_2	z_1
5	u_2	z_1	s_3	z_2	s_1	u_3	s_2	u_1	z_3
6	u_1	z_3	s_2	z_1	s_3	u_2	s_1	u_3	z_2

Table 12 Response y (Diameters of Inhibition Zones*, in mm × 10)

Block (Plate Number)	Standard *S*			Unknown *U*			Unknown *Z*			Block Total
	s_1	s_2	s_3	u_1	u_2	u_3	z_1	z_2	z_3	
1	176	205	235	174	202	232	176	205	234	1,839
2	178	208	238	175	206	234	176	207	237	1,859
3	178	207	237	177	203	236	175	204	236	1,853
4	175	205	235	173	201	232	175	208	236	1,840
5	176	206	235	174	204	231	176	206	237	1,845
6	174	204	236	170	202	229	178	205	234	1,832
Total Response	1,057 (S_1)	1,235 (S_2)	1,416 (S_3)	1,043 (U_1)	1,218 (U_2)	1,394 (U_3)	1,056 (Z_1)	1,235 (Z_2)	1,414 (Z_3)	11,068

*In case of assay of antibiotics.

Table 13 Response Totals and Contrasts

	Standard *S*	Unknown *U*	Unknown *Z*
Low Dose	$S_1 = 1{,}057$	$U_1 = 1{,}043$	$Z_1 = 1{,}056$
Medium Dose	$S_2 = 1{,}235$	$U_2 = 1{,}218$	$Z_2 = 1{,}235$
High Dose	$S_3 = 1{,}416$	$U_3 = 1{,}394$	$Z_3 = 1{,}414$
Preparation Total	$S = S_1 + S_2 + S_3$ $= 3{,}708$	$U = U_1 + U_2 + U_3$ $= 3{,}655$	$Z = Z_1 + Z_2 + Z_3$ $= 3{,}705$
Linear Contrast	$L_S = S_3 - S_1$ $= 359$	$L_U = U_3 - U_1$ $= 351$	$L_Z = Z_3 - Z_1$ $= 358$
Quadratic Contrast	$Q_S = S_1 - 2S_2 + S_3$ $= 3$	$Q_U = U_1 - 2U_2 + U_3$ $= 1$	$Q_Z = Z_1 - 2Z_2 + Z_3$ $= 0$

Table 14 Analysis of Variance

Source of Variation	df	Sum of Squares (SS)	Mean Square	$F_{(cal)}$	$F_{(tab)}$ $\alpha = 0.05$	p-value
Preparations	2	98.4815	49.2407			
Regression	1	31,684.0000	31,684.0000	16,757.4535	4.08	<0.01
Parallelism	2	3.1667	1.5833	0.8374	3.23	>0.05
Quadratic Curvature	1	0.1481	0.1481	0.0784	4.08	>0.05
Difference of Quadratics	2	0.1296	0.0648	0.0343	3.23	>0.05
Treatments	8	31,785.9259	3,973.2407	2,101.4202	2.18	<0.01
Blocks	5	54.3704	10.8741	5.7512	2.45	<0.01
Residual Error	40	75.6296	1.8907			
Total	53	31,915.9259				

Mean Square = Sum of Squares / df

$F_{(cal)}$ = Mean Square / Mean Square of Residual Error

Given statistic values for the next steps:

$N = 54$

$n = 6$

$h = 3$

$d = 3$

dose ratio $= 2$

Correction term, K $= \dfrac{(\Sigma y)^2}{N}$

$= \dfrac{(176 + 178 + \cdots + 234)^2}{54}$

$= 2{,}268{,}530.0741$

Calculation of sums of squares (SS) of the variations:

SS preparations $= \dfrac{S^2 + U^2 + Z^2}{3n} - K$

$= \dfrac{(3{,}708)^2 + (3{,}655)^2 + (3{,}705)^2}{(3)(6)} - 2{,}268{,}530.0741$

$= 98.4815$

SS regression, E $= \dfrac{(L_S + L_U + L_Z)^2}{2nh}$

$= \dfrac{(359 + 351 + 358)^2}{(2)(6)(3)}$

$= 31{,}684.0000$

SS parallelism $= \dfrac{L_S^2 + L_U^2 + L_Z^2}{2n} - E$

$= \dfrac{(359)^2 + (351)^2 + (358)^2}{(2)(6)} - 31{,}684.0000$

$= 3.1667$

SS quadratic curvature, Q $= \dfrac{(Q_S + Q_U + Q_Z)^2}{6nh}$

$= \dfrac{(3 + 1 + 0)^2}{(6)(6)(3)}$

$= 0.1481$

SS difference of quadratics $= \dfrac{Q_S^2 + Q_U^2 + Q_Z^2}{6n} - Q$

$= \dfrac{3^2 + 1^2 + 0^2}{(6)(6)} - 0.1481$

$= 0.1296$

SS treatments $= \dfrac{\Sigma(\text{Total response})^2}{n} - K$

$= \dfrac{(1{,}057)^2 + (1{,}235)^2 + \cdots + (1{,}414)^2}{6} - 2{,}268{,}530.0741$

$= 31{,}785.9259$

SS blocks $= \dfrac{\Sigma(\text{Block total})^2}{hd} - K$

$= \dfrac{(1{,}839)^2 + (1{,}859)^2 + \cdots + (1{,}832)^2}{(3)(3)} - 2{,}268{,}530.0741$

$= 54.3704$

SS total $= \Sigma y^2 - K$

$= \left((176)^2 + (178)^2 + \cdots + (234)^2\right) - 2{,}268{,}530.0741$

$= 31{,}915.9259$

SS residual error $=$ SS total – SS treatments – SS blocks

$= 31{,}915.9259 - 31{,}785.9259 - 54.3704$

$= 75.6296$

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Calculation of potency ratio and confidence limits:

Calculate slope, b:

$$b = \frac{L_S + L_U + L_Z}{2nh(\text{ln dose ratio})}$$

$$= \frac{359 + 351 + 358}{(2)(6)(3)(0.6931)}$$

$$= 42.7999$$

Calculate ln potency ratio, M:

$$M = \frac{U - S}{ndb}$$

$$= \frac{3{,}655 - 3{,}708}{(6)(3)(42.7999)}$$

$$= -0.0688$$

$$\text{Potency ratio} = \text{antiln } M$$

$$= 0.9335$$

Given statistic values for the next steps:

From Table 73 with 40 df of error, $\left(\frac{\alpha}{2} = 0.025\right)$, $t = 2.0211$

Mean square of residual error, $s^2 = 1.8907$

$N_S = 18$

$N_U = 18$

Calculate and apply ln confidence limits to the ln potency ratio (M_L, M_U):

$$M_L, M_U = M \pm \frac{ts}{b}\sqrt{\left(\frac{1}{N_S} + \frac{1}{N_U}\right) + \frac{M^2}{2nh(\text{ln dose ratio})^2}}$$

$$= -0.0688 \pm \frac{(2.0211)(\sqrt{1.8907})}{42.7999}\sqrt{\left(\frac{1}{18} + \frac{1}{18}\right) + \frac{(-0.0688)^2}{(2)(6)(3)(0.6931)^2}}$$

$$= -0.0904, -0.0471$$

Confidence limits are given by antiln M_L and antiln M_U: antiln $M_L = 0.9135$ and antiln $M_U = 0.9540$

Thus, the potency is estimated to be 93.35 per cent of the stated potency, with confidence limits at 91.35 and 95.40 per cent of the stated potency.

Using the same procedure, the potency for unknown *Z* is 99.61 per cent of the stated potency with confidence limits at 97.48 and 101.79 per cent of the stated potency.

If the precision of the assay is limited to be not less than 95.0 per cent and not more than 105.0 per cent of the estimated potency, the confidence limits of error of unknown *U* will be 88.68 and 98.02 per cent of the estimated potency, and of unknown *Z* will be 94.63 and 104.59 per cent of the estimated potency.

Therefore, they imply such assay precision.

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Example 5 Four-Dose Multiple Assay, Randomized Block Design

The assay is composed of one standard of four concentrations, designated s_1, s_2, s_3 and s_4, and two or more unknowns of similar four concentrations, designated u_1, u_2, u_3 and u_4 to z_1, z_2, z_3 and z_4, respectively.

Table 15 Response y (Fluorescence Intensity*)

Block (Row Number)	Standard *S*				Unknown *U*				Unknown *Z*				Block Total
	s_1	s_2	s_3	s_4	u_1	u_2	u_3	u_4	z_1	z_2	z_3	z_4	
1	123	241	326	452	110	227	361	455	161	202	334	468	3,460
2	121	248	350	454	138	214	341	445	150	223	358	456	3,498
3	136	259	345	443	146	244	341	424	134	246	385	476	3,579
4	147	243	324	464	155	250	325	463	152	239	331	427	3,520
Total Response	527 (S_1)	991 (S_2)	1,345 (S_3)	1,813 (S_4)	549 (U_1)	935 (U_2)	1,368 (U_3)	1,787 (U_4)	597 (Z_1)	910 (Z_2)	1,408 (Z_3)	1,827 (Z_4)	14,057

*In case of assay of Tetracosactrin.

Table 16 Response Totals and Contrasts

	Standard *S*	Unknown *U*	Unknown *Z*
Low Dose	$S_1 = 527$	$U_1 = 549$	$Z_1 = 597$
Medium Low Dose	$S_2 = 991$	$U_2 = 935$	$Z_2 = 910$
Medium High Dose	$S_3 = 1{,}345$	$U_3 = 1{,}368$	$Z_3 = 1{,}408$
High Dose	$S_4 = 1{,}813$	$U_4 = 1{,}787$	$Z_4 = 1{,}827$
Preparation Total	$S = S_1 + S_2 + S_3 + S_4$ $= 4{,}676$	$U = U_1 + U_2 + U_3 + U_4$ $= 4{,}639$	$Z = Z_1 + Z_2 + Z_3 + Z_4$ $= 4{,}742$
Linear Contrast	$L_S = 3S_4 + S_3 - S_2 - 3S_1$ $= 4{,}212$	$L_U = 3U_4 + U_3 - U_2 - 3U_1$ $= 4{,}147$	$L_Z = 3Z_4 + Z_3 - Z_2 - 3Z_1$ $= 4{,}188$
Quadratic Contrast	$Q_S = S_1 - S_2 - S_3 + S_4$ $= 4$	$Q_U = U_1 - U_2 - U_3 + U_4$ $= 33$	$Q_Z = Z_1 - Z_2 - Z_3 + Z_4$ $= 106$
Cubic Contrast	$C_S = S_4 - 3S_3 + 3S_2 - S_1$ $= 224$	$C_U = U_4 - 3U_3 + 3U_2 - U_1$ $= -61$	$C_Z = Z_4 - 3Z_3 + 3Z_2 - Z_1$ $= -264$

Table 17 Analysis of Variance

Source of Variation	df	Sum of Squares (SS)	Mean Square	$F_{(cal)}$	$F_{(tab)}$ at $\alpha = 0.05$	p-value
Preparations	2	340.2917	170.1562			
Regression	1	655,946.7042	655,946.7042	2,404.5090	4.14	<0.01
Parallelism	2	27.0083	13.5042	0.0495	3.28	>0.05
Quadratic Curvature	1	426.0208	426.0208	1.5617	4.14	>0.05
Difference of Quadratics	2	345.2917	172.6458	0.6329	3.28	>0.05
Cubic Curvature	1	42.5042	42.5042	0.1558	4.14	>0.05
Difference of Cubics	2	1,502.4083	751.2042	2.7537	3.28	>0.05
Treatments	11	658,630.2292	59,875.4754	219.4860	2.09	<0.01
Blocks	3	619.3958	206.4653	0.7568	2.89	>0.05
Residual Error	33	9,002.3542	272.7986			
Total	47	668,251.9792				

Mean Square = Sum of Squares / df

$F_{(cal)}$ = Mean Square / Mean Square of Residual Error

Given statistic values for the next steps:

N = 48

n = 4

h = 3

d = 4

dose ratio = 2

Correction term, K $= \dfrac{(\Sigma y)^2}{N}$

$= \dfrac{(123 + 121 + \cdots + 427)^2}{48}$

$= 4{,}116{,}651.0208$

Calculation of sums of squares (SS) of the variations:

SS preparations $= \dfrac{S^2 + U^2 + Z^2}{4n} - K$

$= \dfrac{(4{,}676)^2 + (4{,}639)^2 + (4{,}742)^2}{(4)(4)} - 4{,}116{,}651.0208$

$= 340.2917$

SS regression, E $= \dfrac{(L_S + L_U + L_Z)^2}{20nh}$

$= \dfrac{(4{,}212 + 4{,}147 + 4{,}188)^2}{(20)(4)(3)}$

$= 655{,}946.7042$

SS parallelism $= \dfrac{L_S^2 + L_U^2 + L_Z^2}{20n} - E$

$= \dfrac{(4{,}212)^2 + (4{,}147)^2 + (4{,}188)^2}{(20)(4)} - 655{,}946.7042$

$= 27.0083$

SS quadratic curvature, Q $= \dfrac{(Q_S + Q_U + Q_Z)^2}{4nh}$

$= \dfrac{(4 + 33 + 106)^2}{(4)(4)(3)}$

$= 426.0208$

SS cubic curvature, C $= \dfrac{(C_S + C_U + C_Z)^2}{20nh}$

$= \dfrac{[(224) + (-61) + (-264)]^2}{(20)(4)(3)}$

$= 42.5042$

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SS difference of cubics $= \dfrac{C_S^2 + C_U^2 + C_Z^2}{20n} - C$

$= \dfrac{(224)^2 + (-61)^2 + (-264)^2}{(20)(4)} - 42.5042$

$= 1{,}502.4083$

SS treatments $= \dfrac{\Sigma(\text{Total response})^2}{n} - K$

$= \dfrac{(527)^2 + (991)^2 + \cdots + (1{,}827)^2}{(4)} - 4{,}116{,}651.0208$

$= 658{,}630.2292$

SS blocks $= \dfrac{\Sigma(\text{Block total})^2}{hd} - K$

$= \dfrac{(3{,}460)^2 + (3{,}498)^2 + \cdots + (3{,}520)^2}{(3)(4)} - 4{,}116{,}651.0208$

$= 619.3958$

SS total $= \Sigma y^2 - K$

$= \left((123)^2 + (121)^2 + \cdots + (427)^2\right) - 4{,}116{,}651.0208$

$= 668{,}251.9792$

SS residual error $=$ SS total – SS treatments – SS blocks

$= 668{,}251.9792 - 658{,}630.2292 - 619.3958$

$= 9{,}002.3542$

Calculation of potency ratio and confidence limits:

Calculate slope, b:

$$b = \frac{L_S + L_U + L_Z}{10nh(\text{ln dose ratio})}$$

$$= \frac{4{,}212 + 4{,}147 + 4{,}188}{(10)(4)(3)(0.6931)}$$

$$= 150.8458$$

Calculate ln potency ratio, M:

$$M = \frac{U - S}{ndb}$$

$$= \frac{4{,}639 - 4{,}676}{(4)(4)(150.8458)}$$

$$= -0.0153$$

Potency ratio $=$ antiln M

$= 0.9847$

Given statistic values for the next steps:

From Table 73 with 33 df of error, $\left(\dfrac{\alpha}{2} = 0.025\right)$, t $= 2.0345$

Mean square of residual error, $s^2 = 272.7986$

$N_S = 16$

$N_U = 16$

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Calculate and apply ln confidence limits to the ln potency ratio (M_L, M_U):

$$M_L, M_U = M \pm \frac{ts}{b}\sqrt{\left(\frac{1}{N_S}+\frac{1}{N_U}\right)+\frac{M^2}{2nh(\ln \text{dose ratio})^2}}$$

$$= -0.0153 \pm \frac{(2.0345)(\sqrt{272.7986})}{150.8458}\sqrt{\left(\frac{1}{16}+\frac{1}{16}\right)\frac{(-0.0153)^2}{(2)(4)(3)(0.6931)^2}}$$

$$= -0.0904, 0.0634$$

Confidence limits are given by antiln M_L and antiln M_U: antiln $M_L = 0.9102$ and antiln $M_U = 1.0655$.

Thus, the potency is estimated to be 98.48 per cent of the stated potency, with confidence limits at 91.02 and 106.55 per cent of the stated potency.

Using the same procedure, the potency for unknown Z is 102.77 per cent of the stated potency with confidence limits at 94.99 and 111.20 per cent of the stated potency.

If the precision of the assay is limited to be not less than 95.0 per cent and not more than 105.0 per cent of the estimated potency, the confidence limits of error of unknown *U* will be 93.55 and 103.40 per cent of the estimated potency, and of unknown Z will be 97.63 and 107.91 per cent of the estimated potency.

Therefore, they do not imply such assay precision. The assay must be repeated and the combination of potency estimates is required.

3.1.2.6 Latin square design

This design is appropriate when the response may be affected by two different sources of variation each of which can assume *k* different levels or positions. For example, in a plate assay of an antibiotic the treatments may be arranged in a $k \times k$ array on a large plate, each treatment occurring once in each row and each column. The design is suitable when the number of rows, the number of columns and the number of treatments are equal. Responses are recorded in a square format known as a Latin square. Variations due to differences in response among the *k* rows and among the *k* columns may be segregated, thus reducing the error. This design is illustrated in Examples 6 to 7.

Example 6 Three-Dose Single Assay, Latin Square Design

The assay is composed of the standard of three concentrations, designated s_1, s_2 and s_3 for 5.0, 10.0 and 20.0 μg per ml, respectively; and one unknown of similar three concentrations, designated u_1, u_2 and u_3.

All six concentrations of the standard and the unknown are assigned to a standard square plate with a 6 × 6 pattern in such a way that each column and each row consists of all six treatments.

Table 18 A Pattern of Arrangement of Treatments

Row	Column					
	1	2	3	4	5	6
1	s_3	u_2	u_3	u_1	s_1	s_2
2	u_2	s_2	s_3	s_1	u_1	u_3
3	s_2	u_3	u_1	u_2	s_3	s_1
4	s_1	u_1	u_2	u_3	s_2	s_3
5	u_1	s_1	s_2	s_3	u_3	u_2
6	u_3	s_3	s_1	s_2	u_2	u_1

Table 19 Measured Inhibition Zones in mm × 10

Row	Column						Row Total
	1	2	3	4	5	6	
1	124	110	126	93	94	111	658
2	110	110	127	94	94	129	664
3	111	126	95	111	129	98	670
4	93	92	110	127	110	128	660
5	93	93	112	128	127	112	665
6	124	127	93	112	112	95	663
Column Total	655	658	663	665	666	673	3,980

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Table 20 Response y (Diameters of Inhibition Zones*, in mm × 10)

Row	Standard *S*			Unknown *U*		
	s_1	s_2	s_3	u_1	u_2	u_3
1	94	111	124	93	110	126
2	94	110	127	94	110	129
3	98	111	129	95	111	126
4	93	110	128	92	110	127
5	93	112	128	93	112	127
6	93	112	127	95	112	124
Total Response	565 (S_1)	666 (S_2)	763 (S_3)	562 (U_1)	665 (U_2)	759 (U_3)

*In case of assay of antibiotics.

Table 21 Response Totals and Contrasts

	Standard *S*	Unknown *U*
Low Dose	$S_1 = 565$	$U_1 = 562$
Medium Dose	$S_2 = 666$	$U_2 = 665$
High Dose	$S_3 = 763$	$U_3 = 759$
Preparation Total	$S = S_1 + S_2 + S_3$	$U = U_1 + U_2 + U_3$
	$= 1{,}994$	$= 1{,}986$
Linear Contrast	$L_S = S_3 - S_1$	$L_U = U_3 - U_1$
	$= 198$	$= 197$
Quadratic Contrast	$Q_S = S_1 - 2S_2 + S_3$	$Q_U = U_1 - 2U_2 + U_3$
	$= -4$	$= -9$

Table 22 Analysis of Variance

Source of Variation	df	Sum of Squares (SS)	Mean Square	$F_{(cal)}$	$F_{(tab)}$ at $\alpha = 0.05$	p-value
Preparations	1	1.7778	1.7778			
Regression	1	6,501.0417	6,501.0417	8,541.5146	4.35	<0.01
Parallelism	1	0.0417	0.0417	0.0547	4.35	>0.05
Quadratic Curvature	1	2.3472	2.3472	3.0839	4.35	>0.05
Difference of Quadratics	1	0.3472	0.3472	0.4562	4.35	>0.05
Treatments	5	6,505.5556	1,301.1111	1,709.4891	2.71	<0.01
Rows	5	14.5556	2.9111	3.8248	2.71	<0.05
Columns	5	33.5556	6.7111	8.8176	2.71	<0.01
Residual Error	20	15.2222	0.7611			
Total	35	6,568.8889				

Mean Square = Sum of Squares / df

$F_{(cal)}$ = Mean Square / Mean Square of Residual Error

Given statistic values for the next steps:

N = 36

n = 6

h = 2

d = 3

dose ratio = 2

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$$\text{Correction term, K} = \frac{(\Sigma y)^2}{N}$$
$$= \frac{(124 + 110 + \cdots + 95)^2}{36}$$
$$= 440{,}011.1111$$

Calculation of sums of squares (SS) of the variations:

$$\text{SS preparations} = \frac{S^2 + U^2}{3n} - K$$
$$= \frac{(1{,}994)^2 + (1{,}986)^2}{(3)(6)} - 440{,}011.1111$$
$$= 1.7778$$

$$\text{SS regression, E} = \frac{(L_S + L_U)^2}{2nh}$$
$$= \frac{(198 + 197)^2}{(2)(6)(2)}$$
$$= 6{,}501.0417$$

$$\text{SS parallelism} = \frac{L_S^2 + L_U^2}{2n} - E$$
$$= \frac{(198)^2 + (197)^2}{(2)(6)} - 6{,}501.0417$$
$$= 0.0417$$

$$\text{SS quadratic curvature, Q} = \frac{(Q_S + Q_U)^2}{6nh}$$
$$= \frac{[(-4) + (-9)]^2}{(6)(6)(2)}$$
$$= 2.3472$$

$$\text{SS difference of quadratics} = \frac{Q_S^2 + Q_U^2}{6n} - Q$$
$$= \frac{(-4)^2 + (-9)^2}{(6)(6)} - 2.3472$$
$$= 0.3472$$

$$\text{SS treatments} = \frac{\Sigma(\text{Total response})^2}{n} - K$$
$$= \frac{(565)^2 + (666)^2 + \cdots + (759)^2}{6} - 440{,}011.1111$$
$$= 6{,}505.5556$$

$$\text{SS rows} = \frac{\Sigma(\text{Row total})^2}{hd} - K$$
$$= \frac{(658)^2 + (664)^2 + \cdots + (663)^2}{(2)(3)} - 440{,}011.1111$$
$$= 14.5556$$

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$$\text{SS columns} = \frac{\Sigma(\text{Column total})^2}{hd} - K$$
$$= \frac{(655)^2 + (658)^2 + \cdots + (673)^2}{(2)(3)} - 440{,}011.1111$$
$$= 33.5556$$

$$\text{SS total} = \Sigma y^2 - K$$
$$= \left((94)^2 + (94)^2 + \cdots + (124)^2\right) - 440{,}011.1111$$
$$= 6{,}568.8889$$

$$\text{SS residual error} = \text{SS total} - \text{SS treatments} - \text{SS rows} - \text{SS columns}$$
$$= 6{,}568.8889 - 6{,}505.5556 - 14.5556 - 33.5556$$
$$= 15.2222$$

Calculation of potency ratio and confidence limits:

Calculate slope, b:

$$b = \frac{L_S + L_U}{2nh(\text{ln dose ratio})}$$
$$= \frac{198 + 197}{(2)(6)(2)(0.6931)}$$
$$= 23.7443$$

Calculate ln potency ratio, M:

$$M = \frac{U - S}{ndb}$$
$$= \frac{1{,}986 - 1{,}994}{(6)(3)(23.7443)}$$
$$= -0.0187$$

$$\text{Potency ratio} = \text{antiln } M$$
$$= 0.9815$$

Given statistic values for the next steps:

From Table 73 with 20 df of error, $\left(\frac{\alpha}{2} = 0.025\right)$, $t = 2.0860$

Mean square of residual error, $s^2 = 0.7611$

$N_S = 18$

$N_U = 18$

Calculate and apply ln confidence limits to the ln potency ratio (M_L, M_U):

$$M_L, M_U = M \pm \frac{ts}{b}\sqrt{\left(\frac{1}{N_S} + \frac{1}{N_U}\right) + \frac{M^2}{2nh(\text{ln dose ratio})^2}}$$
$$= -0.0187 \pm \frac{(2.0860)(\sqrt{0.7611})}{23.7443}\sqrt{\left(\frac{1}{18} + \frac{1}{18}\right) + \frac{(-0.0187)^2}{(2)(6)(2)(0.6931)^2}}$$
$$= -0.0442, 0.0068$$

Confidence limits are given by antiln M_L and antiln M_U: antiln $M_L = 0.9567$ and antiln $M_U = 1.0069$.

Thus, the potency is estimated to be 98.15 per cent of the stated potency, with confidence limits at 95.67 and 100.69 per cent of the stated potency.

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If the precision of the assay is limited to be not less than 95.0 per cent and not more than 105.0 per cent of the estimated potency, the confidence limits of error will be 93.24 and 103.05 per cent of the estimated potency.

Therefore, they imply such assay precision.

Example 7 Three-Dose Multiple Assay, Latin Square Design

Standard doses to be administered were 3, 6 and 12 units, and equivalent unknown doses are prepared assuming the potencies of the unknown and the standard preparations are equal.

Table 23 A Pattern of Arrangement of Treatments

Block (Plate Number)	Column								
	1	2	3	4	5	6	7	8	9
1	u_3	s_3	z_1	u_2	z_3	z_2	s_1	s_2	u_1
2	u_1	s_1	u_2	z_3	z_2	s_3	s_2	u_3	z_1
3	z_1	z_3	z_2	s_3	s_2	s_1	u_3	u_1	u_2
4	s_3	u_2	u_3	z_2	s_1	z_1	u_1	z_3	s_2
5	z_3	u_1	s_3	s_2	u_2	u_3	z_1	s_1	z_2
6	s_2	z_1	s_1	u_3	s_3	u_1	z_2	u_2	z_3
7	z_2	s_2	u_1	s_1	z_1	u_2	z_3	s_3	u_3
8	u_2	z_2	z_3	u_1	u_3	s_2	s_3	z_1	s_1
9	s_1	u_3	s_2	z_1	u_1	z_3	u_2	z_2	s_3

Table 24 Measured Inhibition Zones in mm × 10

Row	Column									Row Total
	1	2	3	4	5	6	7	8	9	
1	218	218	163	183	220	182	168	188	164	1,704
2	163	163	1,952	17	186	214	203	224	171	1,736
3	162	228	203	223	192	162	230	178	194	1,772
4	216	200	229	200	169	175	175	236	206	1,806
5	233	167	230	195	205	227	175	180	211	1,823
6	194	175	175	228	230	171	209	210	237	1,829
7	204	207	179	165	169	194	236	237	237	1,828
8	199	199	233	171	237	201	238	180	172	1,830
9	166	233	196	165	167	225	207	210	224	1,793
Column Total	1,755	1,790	1,803	1,747	1,775	1,751	1,841	1,843	1,816	16,121

Table 25 Response y (Diameters of Inhibition Zones*, in mm × 10)

Row	Standard *S*			Unknown *U*			Unknown *Z*		
	s_1	s_2	s_3	u_1	u_2	u_3	z_1	z_2	z_3
1	168	188	218	164	183	218	163	182	220
2	163	203	214	163	195	224	171	186	217
3	162	192	223	178	194	230	162	203	228
4	169	206	216	175	200	229	175	200	236
5	180	195	230	167	205	227	175	211	233
6	175	194	230	171	210	228	175	209	237
7	165	207	237	179	194	237	169	204	236
8	172	201	238	171	199	237	180	199	233
9	166	196	224	167	207	233	165	210	225
Total Response	1,520 (S_1)	1,782 (S_2)	2,030 (S_3)	1,535 (U_1)	1,787 (U_2)	2,063 (U_3)	1,535 (Z_1)	1,804 (Z_2)	2,065 (Z_3)

*In case of assay of antibiotics.

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Table 26 Response Totals and Contrasts

	Standard *S*	Unknown *U*	Unknown *Z*
Low Dose	$S_1 = 1{,}520$	$U_1 = 1{,}535$	$Z_1 = 1{,}535$
Medium Dose	$S_2 = 1{,}782$	$U_2 = 1{,}787$	$Z_2 = 1{,}804$
High Dose	$S_3 = 2{,}030$	$U_3 = 2{,}063$	$Z_3 = 2{,}065$
Preparation Total	$S = S_1 + S_2 + S_3$ $= 5{,}332$	$U = U_1 + U_2 + U_3$ $= 5{,}385$	$Z = Z_1 + Z_2 + Z_3$ $= 5{,}404$
Linear Contrast	$L_S = S_3 - S_1$ $= 510$	$L_U = U_3 - U_1$ $= 528$	$L_Z = Z_3 - Z_1$ $= 530$
Quadratic Contrast	$Q_S = S_1 - 2S_2 + S_3$ $= -14$	$Q_U = U_1 - 2U_2 + U_3$ $= 24$	$Q_Z = Z_1 - 2Z_2 + Z_3$ $= -8$

Table 27 Analysis of Variance

Source of Variation	df	Sum of Squares (SS)	Mean Square	$F_{(cal)}$	$F_{(tab)}$ at $\alpha = 0.05$	p-value
Preparations	2	103.1358	51.5679			
Regression	1	45,530.0741	45,530.0741	2,921.3854	4.01	<0.01
Parallelism	2	13.4815	6.7408	0.4325	3.16	>0.05
Quadratic Curvature	1	0.0247	0.0247	0.0016	4.01	>0.05
Difference of Quadratics	2	15.4568	7.7284	0.4959	3.16	>0.05
Treatments	8	45,662.1728	5,707.7716	366.2327	2.11	<0.01
Rows	8	1,837.9506	229.7438	14.7413	2.11	<0.01
Columns	8	1,229.0617	153.6327	9.8577	2.11	<0.01
Residual Error	56	872.7655	15.5851			
Total	80	49,601.9506				

Mean Square = Sum of Squares / df

$F_{(cal)}$ = Mean Square / Mean Square of Residual Error

Given statistic values for the next steps:

$N = 81$

$n = 9$

$h = 3$

$d = 3$

dose ratio = 2

Correction term, $K = \dfrac{(\Sigma y)^2}{N}$

$= \dfrac{(168 + 163 + \cdots + 225)^2}{81}$

$= 3{,}208{,}477.0494$

Calculation of sums of squares (SS) of the variations:

SS preparations $= \dfrac{S^2 + U^2 + Z^2}{3n} - K$

$= \dfrac{(5{,}332)^2 + (5{,}385)^2 + (5{,}404)^2}{(3)(9)} - 3{,}208{,}477.0494$

$= 103.1358$

SS regression, E $= \dfrac{(L_S + L_U + L_Z)^2}{2nh}$

$= \dfrac{(510 + 528 + 530)^2}{(2)(9)(3)}$

$= 45{,}530.0741$

SS parallelism $= \dfrac{L_S^2 + L_U^2 + L_Z^2}{2n} - E$

$= \dfrac{(510)^2 + (528)^2 + (530)^2}{(2)(9)} - 45{,}530.0741$

$= 13.4815$

SS quadratic curvature, Q $= \dfrac{(Q_S + Q_U + Q_Z)^2}{6nh}$

$= \dfrac{[(-14) + (24) + (-8)]^2}{(6)(9)(3)}$

$= 0.0247$

SS difference of quadratics $= \dfrac{Q_S^2 + Q_U^2 + Q_Z^2}{6n} - Q$

$= \dfrac{(-14)^2 + (24)^2 + (-8)^2}{(6)(9)} - 0.0247$

$= 15.4568$

SS treatments $= \dfrac{\Sigma(\text{Total response})^2}{n} - K$

$= \dfrac{(1{,}520)^2 + (1{,}782)^2 + \cdots + (2{,}065)^2}{9} - 3{,}208{,}477.0494$

$= 45{,}662.1728$

SS rows $= \dfrac{\Sigma(\text{Row total})^2}{hd} - K$

$= \dfrac{(1{,}704)^2 + (1{,}736)^2 + \cdots + (1{,}793)^2}{(3)(3)} - 3{,}208{,}477.0494$

$= 1{,}837.9506$

SS columns $= \dfrac{\Sigma(\text{Column total})^2}{hd} - K$

$= \dfrac{(1{,}755)^2 + (1{,}790)^2 + \cdots + (1{,}816)^2}{(3)(3)} - 3{,}208{,}447.0494$

$= 1{,}229.0617$

SS total $= \Sigma y^2 - K$

$= \left((168)^2 + (163)^2 + \cdots + (225)^2\right) - 3{,}208{,}477.0494$

$= 49{,}601.9506$

SS residual error $=$ SS total – SS treatments – SS rows – SS columns

$= 49{,}601.9506 - 45{,}662.1728 - 1{,}837.9506 - 1{,}229.0617$

$= 872.7655$

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Calculation of potency ratio and confidence limits:

Calculate slope, b:

$$b = \frac{L_S + L_U + L_Z}{2nh(\text{ln dose ratio})}$$

$$= \frac{510 + 528 + 530}{(2)(9)(3)(0.6931)}$$

$$= 41.8916$$

Calculate ln potency ratio, M:

$$M = \frac{U - S}{ndb}$$

$$= \frac{5{,}385 - 5{,}332}{(9)(3)(41.8916)}$$

$$= 0.0468$$

$$\text{Potency ratio} = \text{antiln M}$$

$$= 1.0480$$

Given statistic values for the next steps:

From Table 73 with 56 df of error, $\left(\frac{\alpha}{2} = 0.025\right)$, $t = 2.0032$

Mean square of residual error, $s^2 = 15.5851$

$N_S = 27$

$N_U = 27$

Calculate and apply ln confidence limits to the ln potency ratio (M_L, M_U):

$$M_L, M_U = M \pm \frac{ts}{b}\sqrt{\left(\frac{1}{N_S} + \frac{1}{N_U}\right) + \frac{M^2}{2nh(\text{ln dose ratio})^2}}$$

$$= 0.0468 \pm \frac{(2.0032)(\sqrt{15.5851})}{41.8916}\sqrt{\left(\frac{1}{27} + \frac{1}{27}\right) + \frac{(0.0468)^2}{(2)(9)(3)(0.6931)^2}}$$

$$= -0.0045, 0.0983$$

Confidence limits are given by antiln M_L and antiln M_U: antiln $M_L = 0.9955$ and antiln $M_U = 1.1033$

Thus, the potency is estimated to be 104.80 per cent of the stated potency, with confidence limits at 99.55 and 110.33 per cent of the stated potency.

Using the same procedure, the potency for unknown *Z* is 106.57 per cent of the stated potency with confidence limits at 101.23 and 112.20 per cent of the stated potency.

If the precision of the assay is limited to be not less than 95.0 per cent and not more than 105.0 per cent of the estimated potency, the confidence limits of error of unknown *U* will be 99.56 and 110.04 per cent of the estimated potency, and of unknown *Z* will be 101.24 and 111.90 per cent of the estimated potency.

Therefore, they do not imply such assay precision. The assay must be repeated and the combination of potency estimates is required.

3.1.2.7 Cross-over design

This design is useful when the experiment can be sub-divided into blocks but it is possible to apply only two treatments to each block. For example, a block may be a single unit that can be tested on two occasions. The design is intended to increase precision by eliminating the effects of differences between units while balancing the effect of any difference between general levels of response at the two occasions. If two doses of a standard (s_1, s_2) and of an unknown (u_1, u_2) preparation are tested, this is known as a twin cross-over test.

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The experiment is divided into two parts separated by a suitable time interval. Units are divided into four groups and each group receives one of the four treatments in the first part of the test. Units that received one preparation in the first part of the test receive the other preparation on the second occasion, and units receiving small doses in one part of the test receive large doses in the other. The arrangement of doses is shown in Table 28. The statistical method of calculation is illustrated in Example 8.

Table 28 Arrangement of Doses in Cross-over Design

Group of Units	Time I	Time II
1	s_1	u_2
2	s_2	u_1
3	u_1	s_1
4	u_2	s_2

Example 8 Two-Dose Single Assay, Twin Cross-Over Design

The assay is composed of the standard of two concentrations, designated s_1 and s_2 and one unknown of similar two concentrations, u_1 and u_2, respectively.

Table 29 A Pattern of Arrangement of Treatments

Group of Rabbits	First Injection (Day I)	Second Injection (Day II)
1	s_1	u_2
2	s_2	u_1
3	u_1	s_2
4	u_2	s_1

Table 30 Response y (Blood Glucose Readings, mg%)* at 1 (y_1) and 2 (y_2) Hours

Rabbit Number	Group 1				Group 2				Group 3				Group 4			
	s_1		u_2		s_2		u_1		u_1		s_2		u_2		s_1	
	y_1	y_2	y_1	y_2	y_1	y_2	y_1	y_2	y_1	y_2	y_1	y_2	y_1	y_2	y_1	y_2
1	77	73	62	73	47	63	73	103	48	51	48	56	41	40	55	68
2	39	61	35	43	40	42	51	54	56	56	43	46	57	54	67	69
3	64	73	64	54	59	65	73	104	51	69	47	50	60	54	70	69
4	55	79	51	52	60	48	60	94	61	88	55	100	71	66	62	113
5	54	60	43	63	61	53	48	79	75	75	46	56	57	63	67	78
6	55	51	32	26	39	52	35	88	68	66	42	46	44	48	59	94

*In case of assay of Insulin.

Table 31 Response y (Sum of Blood Glucose Concentrations, mg%, at 1 and 2 Hours)

Rabbit Number	Group 1			Group 2			Group 3			Group 4		
	s_1	u_2	T	s_2	u_1	T	u_1	s_2	T	u_2	s_1	T
1	150	135	285	110	176	286	99	104	203	81	123	204
2	100	78	178	82	105	187	112	89	201	111	136	247
3	137	118	255	124	177	301	120	97	217	114	139	253
4	134	103	237	108	154	262	149	155	304	137	175	312
5	114	106	220	114	127	241	150	102	252	120	145	265
6	106	58	164	91	123	214	134	88	222	92	153	245
Total Response	741 (S_{1I})	598 (U_{2II})		629 (S_{2I})	862 (U_{1II})		764 (U_{1I})	635 (S_{2II})		655 (U_{2I})	871 (S_{1II})	

Table 32 Response Totals and Contrasts

	Standard S	Unknown U	Total
Day I	$S_I = S_{1I} + S_{2I}$	$U_I = U_{1I} + U_{2I}$	$D_I = S_I + U_I$
	$= 1{,}370$	$= 1{,}419$	$= 2{,}789$
Day II	$S_{II} = S_{1II} + S_{2II}$	$U_{II} = U_{1II} + U_{2II}$	$D_{II} = S_{II} + U_{II}$
	$= 1{,}506$	$= 1{,}460$	$= 2{,}966$
Preparation Total	$S = S_I + S_{II}$	$U = U_I + U_{II}$	$\sum y = S + U$
	$= 2{,}876$	$= 2{,}879$	$= 5{,}755$
Linear Contrast			
Day I	$L_{SI} = S_{2I} - S_{1I}$	$L_{UI} = U_{2I} - U_{1I}$	$L_I = L_{SI} + L_{UI}$
	$= -112$	$= -109$	$= -221$
Day II	$L_{SII} = S_{2II} - S_{1II}$	$L_{UII} = U_{2II} - U_{1II}$	$L_{II} = L_{SII} + L_{UII}$
	$= -236$	$= -264$	$= -500$
Total	$L_S = L_{SI} + L_{SII}$	$L_U = L_{UI} + L_{UII}$	
	$= -348$	$= -373$	

Table 33 Analysis of Variance

Source of Variation	df	Sum of Squares (SS)	Mean Square	$F_{(cal)}$	$F_{(tab)}$ at $\alpha = 0.05$	p-value
Parallelism	1	13.0208	13.0208	0.0154	4.35	>0.05
Days × Preparations	1	188.0209	188.0209	0.2227	4.35	>0.05
Days × Regression	1	1,621.6875	1,621.6875	1.9208	4.35	>0.05
Error (I)	20	16,885.2500	844.2625			
Blocks (rabbits)	23	18,707.9792	813.3904			
Preparations	1	0.1875	0.1875			
Regression	1	10,830.0208	10,830.0208	64.2811	4.35	<0.01
Days	1	652.6875	652.6875	3.8740	4.35	>0.05
Days × Parallelism	1	20.0209	20.0209	0.1188	4.35	>0.05
Error (II)	20	3,369.5833	168.4792			
Total	47	33,580.4792				

Given statistic values for the next steps:

$$N = 48$$
$$n = 12$$
$$h = 2$$
$$d = 2$$
$$\text{dose ratio} = 2$$
$$\text{Correction term, K} = \frac{(\Sigma y)^2}{N}$$
$$= \frac{(150 + 100 + \cdots + 153)^2}{48}$$
$$= 690{,}000.5208$$

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Calculation of sums of squares (SS) of the variations:

SS total $= \Sigma y^2 - K$

$= \left((150)^2 + (100)^2 + \cdots + (153)^2\right) - 690{,}000.5208$

$= 33{,}580.4792$

SS block, B $= \dfrac{\Sigma(T^2)}{h} - K$

$= \dfrac{(285)^2 + (178)^2 + \cdots + (245)^2}{2} - 690{,}000.5208$

$= 18{,}707.9792$

SS preparations, P $= \dfrac{S^2 + U^2}{2n} - K$

$= \dfrac{(2{,}876)^2 + (2{,}879)^2}{(2)(12)} - 690{,}000.5208$

$= 0.1875$

SS regression, E $= \dfrac{(L_S + L_U)^2}{2nh}$

$= \dfrac{\left((-384) + (-373)\right)^2}{(2)(12)(2)}$

$= 10{,}830.0208$

SS days, D $= \dfrac{D_I^2 + D_{II}^2}{2n} - K$

$= \dfrac{(2{,}789)^2 + (2{,}966)^2}{(2)(12)} - 690{,}000.5208$

$= 652.6875$

SS parallelism, A $= \dfrac{L_S^2 + L_U^2}{2n} - E$

$= \dfrac{(-348)^2 + (-373)^2}{(2)(12)} - 10{,}830.0208$

$= 13.0208$

SS (days × preparations) $= \dfrac{S_I^2 + S_{II}^2 + U_I^2 + U_{II}^2}{n} - K - D - P$

$= \dfrac{(1{,}370)^2 + (1{,}506)^2 + (1{,}419)^2 + (1{,}460)^2}{12} - 690{,}000.5208 - 652.6875 - 0.1875$

$= 188.0209$

SS (days × regression) $= \dfrac{L_I^2 + L_{II}^2}{2n} - E$

$= \dfrac{(-221)^2 + (-500)^2}{(2)(12)} - 10{,}830.0208$

$= 1{,}621.6875$

SS (days × parallelism) $= \dfrac{L_{SI}^2 + L_{SII}^2 + L_{UI}^2 + L_{UII}^2}{n} - E - A - SS\ (days \times regression)$

$= \dfrac{(-112)^2 + (-236)^2 + (-109)^2 + (-264)^2}{12} - 10{,}830.0208 - 13.0208 - 1{,}621.6875$

$= 20.0209$

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SS error (I) = B – A – SS (days × preparations) – SS (days × regression)

= 18,707.9792 – 13.0208 – 188.0209 – 1,621.6875

= 16,885.2500

SS error (II) = SS total – B – P – E – D – SS (days × parallelism)

= 33,580.4792 – 18,707.9792 – 0.1875 – 10,830.0208 – 652.6875 – 20.0209

= 3,369.5833

Calculation of potency ratio and confidence limits:

Calculate slope, b:

$$b = \frac{L_S + L_U}{nh(\text{ln dose ratio})}$$

$$= \frac{(-348) + (-373)}{(12)(2)(0.6931)}$$

$$= -43.3409$$

Calculate ln potency ratio, M:

$$M = \frac{U - S}{ndb}$$

$$= \frac{2{,}879 - 2{,}876}{(12)(2)(-43.3409)}$$

$$= -0.0029$$

Potency ratio = antiln M

= 0.9971

Given statistic values for the next steps:

From Table 73 with 20 df of error, $\left(\frac{\alpha}{2} = 0.025\right)$, t = 2.0860

Mean square of residual error, s^2_d = 168.4792

N_S = 24

N_U = 24

Calculate and apply ln confidence limits to the ln potency ratio (M_L, M_U):

$$M_L, M_U = M \pm \frac{ts}{b}\sqrt{\left(\frac{1}{N_S} + \frac{1}{N_U}\right) + \frac{M^2}{2nh(\text{ln dose ratio})^2}}$$

$$= -0.0029 \pm \frac{(2.0860)(\sqrt{168.4792})}{-43.3409}\sqrt{\left(\frac{1}{24} + \frac{1}{24}\right) + \frac{(-0.0029)^2}{(2)(12)(2)(0.6931)^2}}$$

$$= -0.1832, 0.1775$$

Confidence limits are given by antiln M_L and antiln M_U: antiln M_L = 0.8326 and antiln M_U = 1.1942.

Thus, the potency is estimated to be 99.71 per cent of the stated potency, with confidence limits at 83.26 and 119.42 per cent of the stated potency.

If the precision of the assay is limited to be not less than 95.0 per cent and not more than 105.0 per cent of the estimated potency, the confidence limits of error will be 94.73 and 104.70 per cent of the estimated potency.

Therefore, they do not imply such assay precision. The assay must be repeated and the combination of potency estimates is required.

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3.1.2.8 Missing values

In a balanced assay, an accident totally unconnected with the applied treatments may lead to the loss of one or more responses, for example, because an animal dies. If it is considered that the accident is in no way connected with the composition of the preparation administered, the exact calculations can still be performed but the formulae are necessarily more complicated and can only be given within the framework of general linear models. However, there exists an approximate method which keeps the simplicity of the balanced design by replacing the missing response by a calculated value. The loss of information is taken into account by diminishing the degrees of freedom for the total sum of squares and for the residual error by the number of missing values and using one of the formulae below for the missing values. It should be borne in mind that this is only an approximate method, and that the exact method is to be preferred.

If more than one observation is missing, the same formulae can be used. The procedure is to make a rough guess at all the missing values except one, and to use the proper formula for this one, using all the remaining values including the rough guesses. Fill in the calculated value. Continue by similarly calculating a value for the first rough guess. After calculating all the missing values in this way the whole cycle is repeated from the beginning, each calculation using the most recent guessed or calculated value for every response to which the formula is being applied. This continues until two consecutive cycles give the same values; convergence is usually rapid.

Provided that the number of values replaced is small relative to the total number of observations in the full experiment (say less than 5 per cent), the approximation implied in this replacement and reduction of degrees of freedom by the number of missing values so replaced is usually fairly satisfactory. The analysis should be interpreted with great care, however, especially if there is a preponderance of missing values in one treatment or block, and a biometrician should be consulted if any unusual features are encountered. Replacing missing values in a test without replication is a particularly delicate operation.

Completely randomized design

In a completely randomized assay the missing value can be replaced by the arithmetic mean of the other responses to the same treatment.

Randomized block design

The missing value (y′) is obtained by the use of:

$$y' = \frac{nB' + kT' - G'}{(n-1)(k-1)}$$

where B′ and T′ are the sum of the remaining responses in the block and treatment, respectively, containing the missing value, G′ is the sum of all remaining responses recorded in the assay, and n and k are the number of blocks and treatments, respectively.

As an example, suppose that the response to dose s_1 in the first block of the results obtained from Example 3 was missing:

Calculate the missing value

$$B' = 180 + 202 + 158 + 176 + 204$$
$$= 920$$
$$T' = 157 + 152 + 154 + 156 + 158$$
$$= 777$$
$$G' = 6{,}314$$

therefore

$$y' = \frac{6(920) + 6(777) - 6{,}314}{(5)(5)}$$
$$= 154.72$$

The value 154.72 would appear in Table 8 in place of 156 and calculation would proceed as in Example 3, but the degrees of freedom for the residual error and for total sum of squares would be 24 and 34, respectively.

Latin square design

The missing value (y′) is obtained by the use of:

$$y' = \frac{k(B' + C' + T') - 2G'}{(k-1)(k-2)},$$

where B′, C′ and T′ are the sums of the remaining responses in the row, column and treatment, respectively, containing the missing value. In this case, number of rows, columns and treatments are equal.

As an example, suppose that the response in the first row and first column (s_3) of the results obtained from Example 6 was missing:

Calculate the missing value

$$B' = 110 + 126 + 93 + 94 + 111$$
$$= 534$$
$$C' = 110 + 111 + 93 + 93 + 124$$
$$= 531$$
$$T' = 127 + 129 + 128 + 128 + 127$$
$$= 639$$
$$G' = 3{,}856$$

therefore

$$y' = \frac{6(534 + 531 + 639) - 2(3{,}856)}{(5)(4)}$$
$$= 125.60$$

The value 125.6 would appear in Tables 19 and 20 in place of 124 and calculation would proceed as in Example 6, but the degrees of freedom for the residual error and for the total sum of squares would be 19 and 34, respectively.

Cross-over design

If an accident leading to loss of values occurs in a cross-over design, a book on statistics should be consulted, because the appropriate formulae depend upon the particular treatment combinations.

3.1.3 The slope-ratio model

3.1.3.1 Introduction

This model is suitable, for example, for some microbiological assays when the independent variable is the concentration of an essential growth factor below the optimal concentration of the medium. The slope-ratio model is illustrated in Fig. 3.

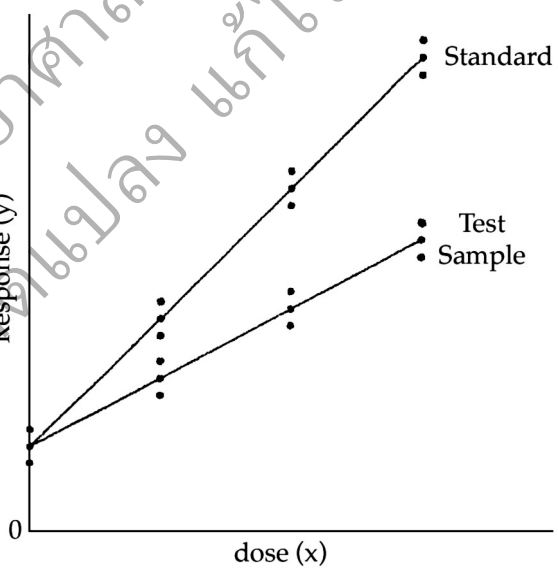

Fig. 3 The Slope-ratio Model for a 2 × 3 + 1 Assay

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The doses are represented on the horizontal axis with zero concentration on the left and the highest concentration on the right. The responses are indicated on the vertical axis. The individual responses to each treatment are indicated with black dots. The two lines are the calculated dose-response relationship for the standard and the unknown under the assumption that they intersect each other at zero-dose. Unlike the parallel-line model, the doses are not transformed to logarithms.

Just as in the case of an assay based on the parallel-line model, it is important that the assumed potency is close to the true potency, and to prepare equipotent dilutions of the test preparations and the standard (if feasible). The more nearly correct the assumed potency, the closer the two lines will be together. The ratio of the slopes represents the "true" potency of the unknown, relative to its assumed potency. If the slope of the unknown preparation is steeper than that of the standard, the potency was underestimated and the calculations will indicate an estimated potency higher than the assumed potency. Similarly, if the slope of the unknown is less steep than that of the standard, the potency was overestimated and the calculations will result in an estimated potency lower than the assumed potency.

In setting up an experiment, all responses should be examined for the fulfillment of conditions 1, 2 and 3 in Section 3.1.1.

3.1.3.2 Assay design

The use of the statistical analysis presented below imposes the following restrictions on the assay:

a. the standard and the test preparations must be tested with the same number of equally spaced dilutions,
b. an extra group of experimental units receiving no treatment may be tested (the blanks),
c. there must be an equal number of experimental units to each treatment.

As already remarked in Section 3.1.1.3, assay designs not meeting these restrictions may be both possible and correct, but the simple statistical analyses presented here are no longer applicable and either expert advice should be sought or suitable software should be used.

A design with two doses per preparation and one blank, the "common zero (2h + 1)-design", is usually preferred, since it gives the highest precision combined with the possibility to check validity within the constraints mentioned above. However, a linear relationship cannot always be assumed to be valid down to zero-dose. With a slight loss of precision a design without blanks may be adopted. In this case three doses per preparation, the "common zero (3h)-design", are preferred to two doses per preparation. The doses are thus given as follows:

1) the standard is given in a high dose, near to but not exceeding the highest dose giving a mean response on the straight portion of the dose-response line,
2) the other doses are uniformly spaced between the highest dose and zero-dose,
3) the test preparations are given in corresponding doses based on the assumed potency of the material.

A completely randomized, a randomized block or a latin square design may be used, such as described in Section 3.1.2.4 to 3.1.2.6. The use of any of these designs necessitates an adjustment to the error sum of squares as described in Examples 1 to 6. The analysis of an assay of one or more test preparations against a standard is described below.

3.1.3.3 Tests of validity

Assay results are said to be "statistically valid" if the outcome of the analysis of variance is as follows:

a) the variation due to blanks in (hd + 1)-designs is not significant, i.e. the calculated probability is not smaller than 0.05. This indicates that the responses of the blanks do not significantly differ from the common intercept and the linear relationship is valid down to zero-dose;

b) the variation due to intersection is not significant, i.e. the calculated probability is not less than 0.05. This indicates that condition 5B, Section 3.1.1 is satisfied;

c) in assays including at least three doses per preparation, the variation due to non-linearity is not significant, i.e. the calculated probability is not less than 0.05. This indicates that condition 4B, Section 3.1.1 is satisfied.

A significant variation due to blanks indicates that the hypothesis of linearity is not valid near zero-dose. If this is likely to be systematic rather than incidental for the type of assay, the (hd)-design is more appropriate. Any response to blanks should then be disregarded.

When statistical validity is established, potencies and confidence limits may be estimated by the methods described in the Examples 9 and 10.

3.1.3.4 Completely randomized design

In this design, it is recommended to test the validity of assay results as described in Section 3.1.3.3. The (hd + 1)-design is applicable, if conditions a), b), and c) are satisfied (See Example 9). If only conditions b) and c) are satisfied (See Example 10), the (hd)-design is more appropriate.

Example 9 Slope-ratio Assay, Completely Randomized (0,3,3)-design

The assay is composed of the standard of three dilutions, designated s_1, s_2 and s_3 and one unknown of similar three dilutions, designated u_1, u_2 and u_3, respectively. In addition a blank is prepared, although a linear dose-response relationship is not expected for low doses.

Eight replications of each dilution are prepared.

Table 34 Response y (Absorbance*)

Concentration (IU/ml)	Blank	Standard *S*			Unknown *U*		
	B	s_1	s_2	s_3	u_1	u_2	u_3
		0.01	0.02	0.03	0.01	0.02	0.03
Individual Response	0.022	0.133	0.215	0.299	0.120	0.188	0.254
	0.024	0.133	0.215	0.299	0.119	0.188	0.253
	0.024	0.131	0.216	0.299	0.118	0.190	0.255
	0.026	0.136	0.218	0.297	0.120	0.190	0.258
	0.023	0.137	0.220	0.297	0.120	0.190	0.257
	0.022	0.136	0.220	0.305	0.121	0.191	0.257
	0.022	0.138	0.219	0.299	0.121	0.191	0.255
	0.023	0.137	0.218	0.302	0.121	0.190	0.254
Total Response	0.186	1.081 (S_1)	1.741 (S_2)	2.397 (S_3)	0.960 (U_1)	1.518 (U_2)	2.043 (U_3)

*In case of a chromogenic assay of factor VIII activity in concentrates.

Table 35 Response Totals and Contrasts

	Standard *S*	Unknown *U*	Total
Low Dose	$S_1 = 1.081$	$U_1 = 0.960$	
Medium Dose	$S_2 = 1.741$	$U_2 = 1.518$	
High Dose	$S_3 = 2.397$	$U_3 = 2.043$	
Preparation Total	$S = S_1 + S_2 + S_3$ $= 5.2190$	$U = U_1 + U_2 + U_3$ $= 4.5210$	$\Sigma y = S + U$ $= 9.7400$
Linear Product	$L_S = S_1 + 2S_2 + 3S_3$ $= 11.7540$	$L_U = U_1 + 2U_2 + 3U_3$ $= 10.1250$	
Intercept Value	$A_S = (4d + 2)S - 6L_S$ $= 2.5420$	$A_U = (4d + 2)S - 6L_U$ $= 2.5440$	
Slope Value	$B_S = 2L_S - (d + 1)S$ $= 2.6320$	$B_U = 2L_U - (d + 1)S$ $= 2.1660$	
Treatment Value	$T_S = S_1^2 + S_2^2 + S_3^2$ $= 9.9452$	$T_U = U_1^2 + U_2^2 + U_3^2$ $= 7.3997$	
Non-linearity	$O_S = T_S - \frac{S^2}{d} - \frac{3B_S^2}{d^3 - d}$ $= 2.6667 \times 10^{-6}$	$O_U = T_U - \frac{U^2}{d} - \frac{3B_U^2}{d^3 - d}$ $= 1.8150 \times 10^{-4}$	

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Table 36 Analysis of Variance

Source of Variation	df	Sum of Squares (SS)	Mean Square	$F_{(cal)}$	$F_{(tab)}$ at $\alpha = 0.05$	p-value
Regression	2	0.1916	0.0958	24,849.5648	3.2199	<0.01
Intersection	1	3.0000×10^{-9}	3.0000×10^{-9}	0.0007	4.7027	>0.05
Non-linearity	2	2.3020×10^{-5}	1.1510×10^{-5}	2.9842	3.2199	>0.05
Treatments	5	0.1917	0.0383	9,941.0197	2.4377	<0.01
Residual Error	42	1.6200×10^{-4}	3.8571×10^{-6}			
Total	47	0.1918				

Mean Square = Sum of Squares / df

$F_{(cal)}$ = Mean Square / Mean Square of Residual Error

Given statistic values for the next steps:

$N = 48$

$n = 8$

$h = 2$

$d = 3$

$$A' = \frac{A_S + A_U}{h(d^2 - d)}$$

$$= \frac{2.5420 + 2.5440}{2(3^2 - 3)}$$

$$= 0.4238$$

$$H' = \frac{n}{4d^3 - 2d^2 - 2d}$$

$$= \frac{8}{4(3)^3 - 2(3)^2 - 2(3)}$$

$$= 0.0952$$

Correction term, K $= \dfrac{(\Sigma y)^2}{N}$

$$= \frac{(9.7400)^2}{48}$$

$$= 1.9764$$

Calculation of sums of squares (SS) of the variations:

SS treatments, T $= \dfrac{\Sigma(\text{Total response})^2}{n} - K$

$$= \frac{(1.081)^2 + (1.741)^2 + \cdots + (2.043)^2}{8} - 1.9764$$

$$= 0.1917$$

SS intersection, I $= \dfrac{H'\left(A_S^2 + A_U^2 - h(d^2 - d)^2 A'^2\right)}{n^2}$

$$= \frac{0.0952\left((2.5420)^2 + (2.5440)^2 - 2(3^2 - 3)^2 (0.4238)^2\right)}{(8)^2}$$

$$= 3.0000 \times 10^{-9}$$

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SS non-linearity, O $= \dfrac{O_S + O_U}{n}$

$= \dfrac{2.6667 \times 10^{-6} + 1.8150 \times 10^{-4}}{8}$

$= 2.3020 \times 10^{-5}$

SS regression $= T - I - O$

$= 0.1917 - \left(3.0000 \times 10^{-9}\right) - \left(2.3020 \times 10^{-5}\right)$

$= 0.1916$

SS total $= \Sigma y^2 - K$

$= \left((0.133)^2 + (0.133)^2 + \cdots + (0.254)^2 \right) - 1.9764$

$= 0.1918$

SS residual error $=$ SS total – SS treatments

$= 0.1918 - 0.1917$

$= 1.6200 \times 10^{-4}$

Calculation of potency ratio and confidence limits:

Calculate slope, b:

$$b_S = \frac{6L_S - 3d(d+1)A'}{n(2d^3 + 3d^2 + d)}$$

$$= \frac{6(11.7540) - 3(3)(3+1)(0.4238)}{8\left(2(3)^3 + 3(3)^2 + 3\right)}$$

$$= 0.0822$$

$$b_U = \frac{6L_U - 3d(d+1)A'}{n(2d^3 + 3d^2 + d)}$$

$$= \frac{6(10.1250) - 3(3)(3+1)(0.4238)}{8\left(2(3)^3 + 3(3)^2 + 3\right)}$$

$$= 0.0676$$

Calculate potency ratio, R:

$$R = \frac{b_U}{b_S}$$

$$= \frac{0.0676}{0.0822}$$

$$= 0.8231$$

Given statistic values for the next steps:

From Table 73 with 42 df of error, $\left(\dfrac{\alpha}{2} = 0.025\right)$, t $= 2.0181$

Mean square of residual error, $s^2 = 3.8571 \times 10^{-6}$

$$V_1' = \frac{6}{nd(2d+1)}\left(\frac{1}{d+1} + \frac{3}{h(d-1)}\right)$$

$$= \frac{6}{8(3)\left[2(3)+1\right]}\left(\frac{1}{3+1} + \frac{3}{2(3-1)}\right)$$

$$= 0.0357$$

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$$V_2' = \frac{3(d+1)}{3(d+1)+h(d-1)} = \frac{3(3+1)}{3(3+1)+2(3-1)} = 0.7500$$

$$X' = \frac{b_S^2}{b_S^2 - s^2 t^2 V_1'} = \frac{(0.0822)^2}{(0.0822)^2 - (3.8571\times10^{-6})(2.0181)^2(0.0357)} = 1.0000$$

$$D' = (X'-1)V_2' = (1.0000-1)(0.7500) = 6.2216\times10^{-5}$$

$$X'R - D' = (1.0000)(0.8231) - 6.2216\times10^{-5} = 0.8231$$

$$(X'-1)(X'R^2+1) = (1.0000-1)\left((1.0000)(0.8231)^2+1\right) = 1.3916\times10^{-4}$$

$$D'(D'-2X'R)s = (6.2216\times10^{-5})\left(6.2216\times10^{-5} - 2(1.0000)(0.8231)\right)\sqrt{3.8571\times10^{-6}} = -1.0243\times10^{-4}$$

Calculate and apply ln confidence limits to the potency ratio (lower limit, upper limit):

$$\text{lower limit, upper limit} = X'R - D' \pm \sqrt{(X'-1)(X'R^2+1) + D'(D'-2X'R)}s$$

$$= 0.8231 \pm \sqrt{1.3916\times10^{-4} - 1.0243\times10^{-4}}$$

$$= 0.8171,\ 0.8292$$

Thus, the potency is estimated to be 82.31 per cent of the stated potency, with confidence limits at 81.71 and 82.92 per cent of the stated potency.

If the precision of the assay is limited to be not less than 95.0 per cent and not more than 105.0 per cent of the estimated potency, the confidence limits of error will be 78.19 and 86.43 per cent of the estimated potency.

Therefore, they imply such assay precision.

Example 10 Slope-ratio Assay, Completely Randomized (0,4,4,4)-design

The assay is composed of the standard of four dilutions, designated s_1, s_2, s_3 and s_4 and two or more unknowns of similar four dilutions, designated u_1, u_2, u_3 and u_4 to z_1, z_2, z_3 and z_4, respectively.

Two replications of each dilution are prepared.

Table 37 Response y (Absorbance*)

Concentration (IU/ml)	Standard *S*				Unknown *U*				Unknown *Z*			
	s_1 7.5	s_2 15.0	s_3 22.5	s_4 30.0	u_1 7.5	u_2 15.0	u_3 22.5	u_4 30.0	z_1 7.5	z_2 15.0	z_3 22.5	z_4 30.0
Individual Response	18.0 18.0	22.8 24.5	30.4 30.4	35.7 36.6	15.1 16.8	23.1 24.2	28.9 27.4	34.4 37.8	15.4 15.7	20.2 18.6	24.2 23.1	27.4 27.0
Total Response	36.0 (S_1)	47.3 (S_2)	60.8 (S_3)	72.3 (S_4)	31.9 (U_1)	47.3 (U_2)	56.3 (U_3)	72.2 (U_4)	31.1 (Z_1)	38.8 (Z_2)	47.3 (Z_3)	54.4 (Z_4)

*In case of assay haemagglutinin antigen content of two influenza vaccines.

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Table 38 Response Total and Contrasts

	Standard *S*	Unknown *U*	Unknown *Z*
Low Dose	$S_1 = 36.0$	$U_1 = 31.9$	$Z_1 = 31.1$
Medium Low Dose	$S_2 = 47.3$	$U_2 = 47.3$	$Z_2 = 38.8$
Medium High Dose	$S_3 = 60.8$	$U_3 = 56.3$	$Z_3 = 47.3$
High Dose	$S_4 = 72.3$	$U_4 = 72.2$	$Z_4 = 54.4$
Preparation Total	$S = S_1 + S_2 + S_3 + S_4$ $= 216.4000$	$U = U_1 + U_2 + U_3 + U_4$ $= 207.7000$	$Z = Z_1 + Z_2 + Z_3 + Z_4$ $= 171.6000$
Linear Product	$L_S = S_1 + 2S_2 + 3S_3 + 4S_4$ $= 602.2000$	$L_U = U_1 + 2U_2 + 3U_3 + 4U_4$ $= 584.2000$	$L_Z = Z_1 + 2Z_2 + 3Z_3 + 4Z_4$ $= 468.2000$
Intercept Value	$A_S = (4d + 2)S - 6L_S$ $= 282.0000$	$A_U = (4d + 2)U - 6L_U$ $= 233.4000$	$A_Z = (4d + 2)Z - 6L_Z$ $= 279.6000$
Slope Value	$B_S = 2L_S - (d + 1)S$ $= 122.4000$	$B_U = 2L_U - (d + 1)U$ $= 129.9000$	$B_Z = 2L_Z - (d + 1)Z$ $= 78.4000$
Treatment Value	$T_S = S_1^2 + S_2^2 + S_3^2 + S_4^2$ $= 12{,}457.2200$	$T_U = U_1^2 + U_2^2 + U_3^2 + U_4^2$ $= 11{,}637.4300$	$T_Z = Z_1^2 + Z_2^2 + Z_3^2 + Z_4^2$ $= 7{,}669.3000$
Non-linearity	$O_S = T_S - \frac{S^2}{d} - \frac{3B_S^2}{d^3 - d}$ $= 0.8920$	$O_U = T_U - \frac{U^2}{d} - \frac{3B_U^2}{d^3 - d}$ $= 8.9070$	$O_Z = T_Z - \frac{Z^2}{d} - \frac{3B_U^2}{d^3 - d}$ $= 0.3320$

Table 39 Analysis of Variance

Source of Variation	df	Sum of Squares (SS)	Mean Square	$F_{(cal)}$	$F_{(tab)}$ at $\alpha = 0.05$	p-value
Regression	3	1,087.6651	362.5550	339.4975	3.4903	<0.01
Intersection	2	3.4738	1.7369	1.6265	3.8853	>0.05
Non-linearity	6	5.0655	0.8442	0.7906	2.9961	>0.05
Treatments	11	1,096.2045	99.6549	93.3172	2.7173	<0.01
Residual Error	12	12.8150	1.0679			
Total	23	1,109.0158				

Mean Square = Sum of Squares / df

$F_{(cal)}$ = Mean Square / Mean Square of Residual Error

Given statistic value for the next steps:

$N = 24$

$n = 2$

$h = 3$

$d = 4$

$$A' = \frac{A_S + A_U + A_Z}{h(d^2 - d)}$$

$$= \frac{282.0000 + 233.4000 + 279.60000}{3(4^2 - 4)}$$

$$= 22.0833$$

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$$H' = \frac{n}{4d^3 - 2d^2 - 2d}$$

$$= \frac{2}{4(4)^3 - 2(4)^2 - 2(4)}$$

$$= 0.0092$$

Correction term, K

$$= \frac{(\Sigma y)^2}{N}$$

$$= \frac{(18.0 + 18.0 + \cdots + 27.0)^2}{24}$$

$$= \frac{(595.7000)^2}{24}$$

$$= 14{,}785.7704$$

Calculation of sums of squares (SS) of the variations:

SS treatments, T

$$= \frac{\Sigma(\text{Total response})^2}{n}$$

$$= \frac{(36.0000)^2 + (47.3000)^2 + \cdots + (54.4000)^2}{2}$$

$$= 1{,}096.2045$$

SS intersection, I

$$= H' \left(\frac{A_S^2 + A_U^2 + A_Z^2 - h(d^2 - d)^2 A'^2}{n^2} \right)$$

$$= \frac{0.0092 \left((282.000)^2 + (233.400)^2 + (279.600)^2 - 3(4^2 - 4)^2 (22.0833)^2 \right)}{(2)^2}$$

$$= 3.4738$$

SS non-linearity, O

$$= \frac{O_S + O_U + O_Z}{n}$$

$$= \frac{0.8920 + 8.9070 + 0.3320}{2}$$

$$= 5.0655$$

SS regression

$$= T - I - O$$

$$= 1{,}096.2045 - 3.4738 - 5.0655$$

$$= 1{,}087.6651$$

SS total

$$= \Sigma y^2 - K$$

$$= \left((18.0000)^2 + (18.0000)^2 + \cdots + (27.0000)^2 \right) - 14{,}785.7704$$

$$= 1{,}109.0158$$

SS residual error

$$= \text{SS total} - \text{SS treatments}$$

$$= 1{,}109.0158 - 1{,}096.2045$$

$$= 12.8150$$

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Calculation of potency ratio and confidence limits:

Calculate slope, b:

$$b_S = \frac{6L_S - 3d(d+1)A'}{n(2d^3 + 3d^2 + d)}$$

$$= \frac{6(602.2000) - 3(4)(4+1)(22.0833)}{2\left(2(4)^3 + 3(4)^2 + 4\right)}$$

$$= 6.3561$$

$$b_U = \frac{6L_U - 3d(d+1)A'}{n(2d^3 + 3d^2 + d)}$$

$$= \frac{6(584.2000) - 3(4)(4+1)(22.0833)}{2\left(2(4)^3 + 3(4)^2 + 4\right)}$$

$$= 6.0561$$

$$b_Z = \frac{6L_Z - 3d(d+1)A'}{n(2d^3 + 3d^2 + d)}$$

$$= \frac{6(468.2000) - 3(4)(4+1)(22.0833)}{2\left(2(4)^3 + 3(4)^2 + 4\right)}$$

$$= 4.1227$$

Calculate potency ratio, R:

$$R_U = \frac{b_U}{b_S}$$

$$= \frac{6.0561}{6.3561}$$

$$= 0.9528$$

$$R_Z = \frac{b_Z}{b_S}$$

$$= \frac{4.1227}{6.3561}$$

$$= 0.6486$$

Given statistic values for the next steps:

From Table 73 with 12 df of error, $\left(\frac{\alpha}{2} = 0.025\right)$, $t = 2.1788$

Mean square of residual error, $s^2 = 1.0679$

$$V_1' = \frac{6}{nd(2d+1)}\left(\frac{1}{d+1} + \frac{3}{h(d-1)}\right)$$

$$= \frac{6}{2(4)\left[2(4)+1\right]}\left(\frac{1}{4+1} + \frac{3}{3(4-1)}\right)$$

$$= 0.0444$$

$$V_2' = \frac{3(d+1)}{3(d+1) + h(d-1)}$$

$$= \frac{3(4+1)}{3(4+1) + 3(4-1)}$$

$$= 0.6250$$

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$$
\begin{aligned}
X' &= \frac{b_S^2}{b_S^2 - s^2 t^2 V_1'} \\
&= \frac{(6.3561)^2}{(6.3561)^2 - (1.0679)(2.1788)^2(0.0444)} \\
&= 1.0056 \\
D' &= (X' - 1)V_2' \\
&= (1.0056 - 1)(0.6250) \\
&= 0.0035 \\
X'R_U - D' &= (1.0056)(0.9528) - 0.0035 \\
&= 0.9546 \\
(X' - 1)(X'R_U^2 + 1) &= (1.0056 - 1)((1.0056)(0.9528)^2 + 1) \\
&= 0.0107 \\
D'(D' - 2X'R_U) &= (0.0035)(0.0035 - 2(1.0056)(0.9528)) \\
&= -0.0067
\end{aligned}
$$

Calculate and apply confidence limits to the potency ratio (lower limit, upper limit):

$$
\begin{aligned}
\text{lower limit, upper limit} &= X'R_U - D' \pm \sqrt{(X'-1)(X'R_U^2 + 1) + D'(D' - 2X'R_U)} \\
&= 0.9546 \pm \sqrt{(0.0107 + (-0.0067)} \\
&= 0.8912, 1.0181
\end{aligned}
$$

Thus, the potency is estimated to be 95.28 per cent of the stated potency, with confidence limits at 89.12 and 101.81 per cent of the stated potency.

Using the same procedure, the potency for unknown Z is 64.86 per cent with confidence limits at 59.03 and 70.73 per cent of the stated potency.

If the precision of the assay is limited to be not less than 95.0 per cent and not more than 105.0 per cent of the estimated potency, the confidence limits of error of unknown U will be 90.52 and 100.04 per cent of the estimated potency, and of unknown Z will be 61.62 and 68.11 per cent of the estimated potency.

Therefore, they do not imply such assay precision. The assay must be repeated and the combination of potency estimates is required.

3.1.4 Extended sigmoid dose-response curves

The model is suitable, for example, for some immunoassays when analysis is required of extended sigmoid dose-response curves. This model is illustrated in Fig. 4.

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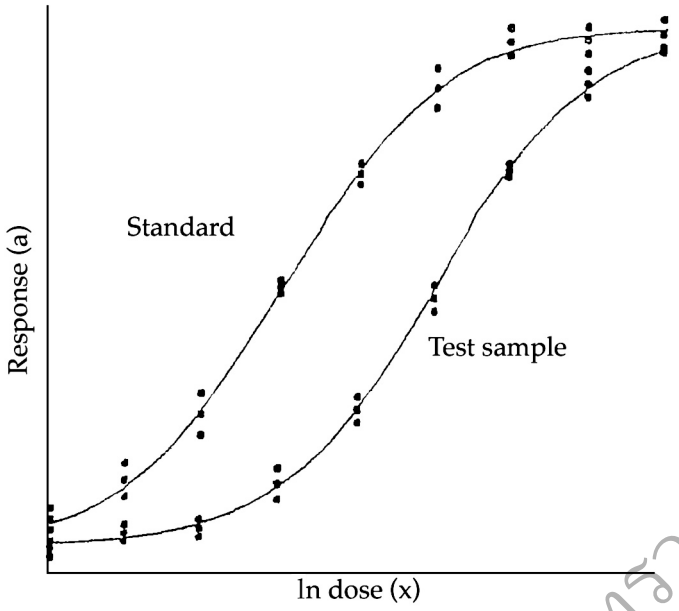

Fig. 4 The Four-parameter Logistic Curve Model

The logarithms of the doses are represented on the horizontal axis with the lowest concentration on the left and the highest concentration on the right. The responses are indicated on the vertical axis. The individual responses to each treatment are indicated with black dots. The two curves are the calculated ln (dose)-response relationship for the standard and the test preparation.

The general shape of the curves, an S-shaped curve can usually be described by a logistic function but other shapes are also possible. Each curve can be characterized by 4 parameters: the upper asymptote (α'), the lower asymptote (δ), the slope-factor (β), and the horizontal location (γ). This model is therefore often referred to as a four-parameter model. A mathematical representation of the ln (dose)-response curve is:

$$u = \delta + \frac{\alpha' - \delta}{1 + e^{-\beta(x-\gamma)}}$$

For a valid assay it is necessary that the curves of the standard and the test preparations have the same slope-factor, and the same maximum and minimum response level at the extreme parts. Only the horizontal location (γ) of the curves may be different. The horizontal distance between the curves is related to the "true" potency of the unknown. If the assay is used routinely, it may be sufficient to test the condition of equal upper and lower response levels when the assay is developed, and then to retest this condition directly only at suitable intervals or when there are changes in materials or assay conditions.

The maximum-likelihood estimates of the parameters and their confidence intervals can be obtained with suitable computer programs. These computer programs may include some statistical tests reflecting validity. For example, if the maximum likelihood estimation shows significant deviations from the fitted model under the assumed conditions of equal upper and lower asymptotes and slopes, then one or all of these conditions may not be satisfied.

The logistic model raises a number of statistical problems which may require different solutions for different types of assays, and no simple summary is possible. A wide variety of possible approaches is described in the relevant literature. Professional advice is therefore recommended for this type of analysis. If professional advice or suitable software is not available, alternative approaches are possible: 1) if "reasonable" estimates of the upper limit (α') and lower limit (δ) are available, select for all preparations the doses with mean of the responses (u) falling between approximately 20 per cent and 80 per cent of the limits, transform responses of the selected doses to and use the parallel line model (Section 3.1.2) for the analysis; 2) select a range of doses for which the responses (u) or suitably transformed responses, the selected doses to $y = \ln\left(\frac{u-\delta}{\alpha'-u}\right)$ and use the parallel line model (Section 3.1.2) for the analysis; 3) select a range of doses for which the responses, for example ln (u), are approximately linear when plotted against ln (dose); the parallel line model (Section 3.1.2) may then be used for analysis.

3.2 ASSAYS DEPENDING UPON QUANTAL RESPONSES

3.2.1 Introduction

In certain assays it is impossible or excessively laborious to measure the effect on each experimental unit on a quantitative scale. Instead, an effect such as death or hypoglycemic symptoms may be observed as either occurring or not occurring in each unit, and the result depends on the number of units in which it occurs. Such assays are called quantal or all-or-none.

The situation is very similar to that described for quantitative assays in Section 3.1.1, but in place of n separate responses to each treatment a single value is recorded, i.e. the fraction of units in each treatment group showing a response. When these fractions are plotted against the logarithms of the doses the resulting curve will tend to be sigmoid (S-shaped) rather than linear. A mathematical function that represents this sigmoid curvature is used to estimate the dose-response curve. The most commonly used function is the cumulative normal distribution function. This function has some theoretical merit, and is perhaps the best choice if the response is a reflection of the tolerance of the units. If the response is more likely to depend upon a process of growth, the logistic distribution model is preferred, although the difference in outcome between the two models is usually very small. The maximum likelihood estimators of the slope and location of the curves can be found only by applying an iterative procedure. There are many procedures which lead to the same outcome, but they differ in efficiency due to the speed of convergence. One of the most rapid methods is direct optimization of the maximum-likelihood function (see Section 5.1), which can easily be performed with computer programs having a built-in procedure for this purpose. The technique described below is not the most rapid, but has been chosen for its simplicity compared to the alternatives. It can be used for assays in which one or more test preparations are compared to a standard. Furthermore, the following conditions must be fulfilled:

1) the relationship between the logarithm of the dose and the response can be represented by a cumulative normal distribution curve,

2) the curves for the standard and the test preparation are parallel, i.e. they are identically shaped and may only differ in their horizontal location,

3) in theory, there is no natural response to extremely low doses and no natural non-response to extremely high doses.

3.2.2 The probit method

The sigmoid curve can be made linear by replacing each response, i.e. the fraction of positive responses per group, by the corresponding value of the cumulative standard normal distribution. This value, often referred to as "normit", ranges theoretically from $-\infty$ to $+\infty$. In the past it was proposed to add 5 to each normit to obtain "probits". This facilitated the hand-performed calculations because negative values were avoided. With the arrival of computers the need to add 5 to the normits has disappeared. The term "normit method" would therefore be better for the method described below. However, since the term "probit analysis" is so widely spread, the term will, for historical reasons, be maintained in this text. Probit analysis requires the iteration process to get the expected probits Y by the fitting of linear regression. All Y are set at zeros for the first iteration. The cycle is repeated until the difference between two cycles has become small (e.g., the maximum difference of Y between two consecutive cycles is smaller than 10^{-8}).

Once the responses have been linearized, it should be possible to apply the parallel-line analysis as described in Section 3.1.2. Unfortunately, the validity condition of homogeneity of variance for each dose is not fulfilled. The variance is minimal at normit = 0 and increases for positive and negative values of the normit. It is therefore necessary to give more weight to responses in the middle part of the curve, and less weight to the more extreme parts of the curve. This method is illustrated in Example 11.

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Example 11 Probit Analysis, An *In-vivo* Assay of a Diphtheria Vaccine

A diphtheria vaccine (assumed potency 140 IU/vial) is assayed against a standard (assigned potency 132 IU/vial). On the basis of this information, equivalent doses are prepared and randomly administered to groups of guinea-pigs. After a given period, the animals are challenged with diphtheria toxin and the number of surviving animals recorded as shown in Table 40.

From Tables 41 to 48, the results are computerized and displayed as four decimal digits, without rounding off, except column (7).

Table 40 Raw Data from a Diphtheria Assay in Guinea-pigs

Standard (S) Assigned Potency 132 IU/vial			Test Preparation (U) Assumed Potency 140 IU/vial		
Dose (IU/ml)	Challenged	Protected	Dose (IU/ml)	Challenged	Protected
1.0	12	0	1.0	11	0
1.6	12	3	1.6	12	4
2.5	12	6	2.5	11	8
4.0	11	10	4.0	11	10

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Table 41 First Working Table in the First Cycle

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)
Vaccine	Dose	n	r	x	p	Y	Φ	Z	y	w	wx	wy	wx^2	wy^2	wxy
S	1.0	12	0	0.0000	0.0000	0.00000000	0.5000	0.3989	–1.2533	7.6394	0.0000	–9.5746	0.0000	12.0000	0.0000
	1.6	12	3	0.4700	0.2500	0.00000000	0.5000	0.3989	–0.6266	7.6394	3.5905	–4.7873	1.6875	3.0000	–2.2500
	2.5	12	6	0.9162	0.5000	0.00000000	0.5000	0.3989	0.0000	7.6394	6.9999	0.0000	6.4139	0.0000	0.0000
	4.0	11	10	1.3862	0.9090	0.00000000	0.5000	0.3989	1.0254	7.0028	9.7079	7.1809	13.4580	7.3636	9.9549
U	1.0	11	0	0.0000	0.0000	0.00000000	0.5000	0.3989	–1.2533	7.0028	0.0000	–8.7767	0.0000	11.0000	0.0000
	1.6	12	4	0.4700	0.3333	0.00000000	0.5000	0.3989	–0.4177	7.6394	3.5905	–3.1915	1.6876	1.3333	–1.5000
	2.5	11	8	0.9162	0.7272	0.00000000	0.5000	0.3989	0.5696	7.0028	6.4166	3.9894	5.8794	2.2727	3.6555
	4.0	11	10	1.3862	0.9090	0.00000000	0.5000	0.3989	1.0254	7.0028	9.7079	7.1809	13.4580	7.3636	9.9549

Table 42 Second Working Table in the First Cycle

(17)	(18)	(19)	(20)	(21)	(22)	(23)	(24)	(25)	(26)	(27)	(28)	(29)	(30)
Vaccine	Σw	Σwx	Σwy	Σwx^2	Σwy^2	Σwxy	S_{xx}	S_{xy}	S_{yy}	$\bar{x}$	$\bar{y}$	Common b	a
S	29.9211	20.2984	–7.1809	21.5596	22.3636	7.7048	7.7891	12.5764	20.6402	0.6783	–0.2399	1.6551	–1.3628
U	28.6478	19.7151	–0.7978	21.0251	21.9697	12.1103	7.4574	12.6594	21.9474	1.6881	–0.0278		–1.1669

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The data entered into the columns in the tables indicated by number:

(1) the vaccine label: S for standard, U for test preparation,
(2) the dose of the standard or the test preparations,
(3) the number n of replicates for each treatment,
(4) the number of positive responding units r per treatment group,
(5) the natural logarithm of the dose, x,
(6) the fraction of positive responses per group, $p = \frac{r}{n}$,

for Vaccine S dose 1.6, $p = \frac{3}{12}$

$= 0.2500$

For the first cycle:

(7) column Y is filled with zeros,
(8) the cumulative standard normal distribution function, $\Phi = \Phi(Y)$,

for Vaccine S dose 1.6, $\Phi = 0.5000$

(9) the compute of Z, $Z = \frac{e^{-\frac{Y^2}{2}}}{\sqrt{2\pi}}$,

for Vaccine S dose 1.6, $Z = \frac{e^0}{\sqrt{2\pi}}$

$= 0.3989$

(10) the compute of y, $y = Y + \frac{p - \Phi}{Z}$,

for Vaccine S dose 1.6, $y = 0 + \frac{0.2500 - 0.5000}{0.3989}$

$= -0.6266$

(11) the compute of w, $w = \frac{nZ^2}{\Phi - \Phi^2}$,

for Vaccine S dose 1.6, $w = \frac{(12)(0.3989)^2}{0.5000 - (0.5000)^2}$

$= 7.6394$

The columns (12) to (16) in the first working table can be calculated from columns (5), (10) and (11) as wx, wy, wx^2, wy^2 and wxy, respectively.

The sums calculated from columns (11) to (16) are transferred to columns (18) to (23) respectively.

(24) the compute of S_{xx}, $S_{xx} = \Sigma wx^2 - \frac{(\Sigma wx)^2}{\Sigma w}$,

for Vaccine S, $S_{xx} = 21.5596 - \frac{(20.2984)^2}{29.9211}$

$= 7.7892$

(25) the compute of S_{xy}, $S_{xy} = \Sigma wxy - \frac{(\Sigma wx)(\Sigma wy)}{\Sigma w}$,

for Vaccine S, $S_{xy} = 7.7048 - \frac{(20.2984)(-7.1809)}{29.9211}$

$= 12.5764$

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(26) the compute of S_{yy}, $S_{yy} = \Sigma wy^2 - \frac{(\Sigma wy)^2}{\Sigma w}$,

for Vaccine S, $S_{yy} = 22.3636 - \frac{(-7.1809)^2}{29.9211}$

$= 20.6402$

(27) the compute of $\bar{x}$, $\bar{x} = \frac{\Sigma wx}{\Sigma w}$,

for Vaccine S, $\bar{x} = \frac{20.2984}{29.9211}$

$= 0.6783$

(28) the compute of $\bar{y}$, $\bar{y} = \frac{\Sigma wy}{\Sigma w}$,

for Vaccine S, $\bar{y} = \frac{(-7.1809)}{29.9211}$

$= -0.2399$

(29) the compute of common b, common $b = \frac{\Sigma S_{xy}}{\Sigma S_{xx}}$,

$b = \frac{(12.5764 + 12.6594)}{(7.7892 + 7.4574)}$

$= 1.6551$

(30) the compute of a, $a = \bar{y} - b\bar{x}$

for Vaccine S, $a = (-0.2399) - (1.6551)(0.6783)$

$= -1.3628$

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Table 43 First Working Table in the Second Cycle

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)
Vaccine	Dose	n	r	x	p	Y	Φ	Z	y	w	wx	wy	wx^2	wy^2	wxy
S	1.0	12	0	0.0000	0.0000	–1.36286919	0.0864	0.1576	–1.9114	3.7738	0.0000	–7.2135	0.0000	13.7884	0.0000
	1.6	12	3	0.4700	0.2500	–0.58492872	0.2792	–0.3362	–0.6720	6.7388	3.1672	–4.5289	1.4886	3.0437	–2.1286
	2.5	12	6	0.9162	0.5000	0.15375663	0.5610	–0.3942	–0.0012	7.5740	6.9400	–0.0092	6.3590	0.0000	–0.0084
	4.0	11	10	1.3862	0.9090	0.93169710	0.8242	0.2584	1.2599	5.0730	7.0327	6.3916	9.7495	8.0530	8.8607
U	1.0	11	0	0.0000	0.0000	–1.16692695	0.1216	0.2019	–1.7691	4.1989	0.0000	–7.4287	0.0000	13.1428	0.0000
	1.6	12	4	0.4700	0.3333	–0.38898649	0.3486	0.3698	–0.4303	7.2291	3.3977	–3.1112	1.5969	1.3390	–1.4623
	2.5	11	8	0.9162	0.7272	0.34969887	0.6367	0.3752	0.5909	6.6974	6.1368	3.9582	5.6231	2.3392	3.6268
	4.0	11	10	1.3862	0.9090	1.12763933	0.8702	0.2112	1.3114	4.3477	6.0272	5.7018	8.3555	7.4775	7.9043

Table 44 Second Working Table in the First Cycle

(17)	(18)	(19)	(20)	(21)	(22)	(23)	(24)	(25)	(26)	(27)	(28)	(29)	(30)
Vaccine	Σw	Σwx	Σwy	Σwx^2	Σwy^2	Σwxy	S_{xx}	S_{xy}	S_{yy}	$\bar{x}$	$\bar{y}$	Common b	a
S	23.1598	17.1401	–5.3600	17.5972	24.8853	6.7236	4.9122	10.6905	23.6447	0.7400	–0.2314	2.2002	–1.8598
U	22.4733	15.5618	–0.8799	15.5756	24.2987	10.0689	4.7997	10.6782	24.2642	0.6924	–0.0391		–1.5627

For the second cycle:

the column (7) of the first working table in the second cycle (Table 43) can be replaced by Y = a + bx (using a and b from the first cycle),
for Vaccine S dose 1.6, Y = –1.3628 + (1.6551) (0.4700) = –0.5849

The next cycles are not shown but they are repeated until the difference in Y between two consecutive cycles has become smaller than 10^{-8}.

Tables 45 and 46 show the cycle before sufficient and Tables 47 and 48 show the sufficient cycle.

The cycles shown in Tables 47 and 48 are sufficient for the estimation.

Table 45 First Working Table in the Second Cycle

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)
Vaccine	Dose	n	r	x	p	Y	Φ	Z	y	w	wx	wy	wx^2	wy^2	wxy
S	1.0	12	0	0.0000	0.0000	–2.05041141	0.0201	0.0487	–2.4639	1.4436	0.0000	–3.5570	0.0000	8.7645	0.0000
	1.6	12	3	0.4700	0.2500	–0.92190250	0.1782	0.2608	–0.6469	5.5724	2.6190	–3.6052	1.2309	2.3324	–1.6945
	2.5	12	6	0.9162	0.5000	0.14966152	0.5594	0.3944	–0.0011	7.5774	6.9431	–0.0085	6.3619	0.0000	–0.0077
	4.0	11	10	1.3862	0.9090	1.27817044	0.8994	0.1762	1.3331	3.7771	5.2362	5.0354	7.2590	6.7128	6.9805
U	1.0	11	0	0.0000	0.0000	–1.72084529	0.0426	0.0907	–2.1906	2.2194	0.0000	–4.8621	0.0000	10.6512	0.0000
	1.6	12	4	0.4700	0.3333	–0.59233637	0.2768	0.3347	–0.4234	6.7171	3.1570	–2.8446	1.4838	1.2046	–1.3370
	2.5	11	8	0.9162	0.7272	0.47922765	0.6841	0.3556	0.6005	6.4389	5.8999	3.8670	5.4060	2.3225	3.5433
	4.0	11	10	1.3862	0.9090	1.60773657	0.9460	0.1095	1.2703	2.5867	3.5860	3.2861	4.9713	4.1744	4.5555

Table 46 Second Working Table in the Cycle before Sufficient

(17)	(18)	(19)	(20)	(21)	(22)	(23)	(24)	(25)	(26)	(27)	(28)	(29)	(30)
Vaccine	Σw	Σwx	Σwy	Σwx^2	Σwy^2	Σwxy	S_{xx}	S_{xy}	S_{yy}	$\bar{x}$	$\bar{y}$	Common b	a
S	18.3707	14.7985	–2.1353	14.8519	17.8099	5.2783	2.9310	6.9984	17.5617	0.8055	–0.1162	2.4010	–2.0504
U	17.9623	12.6430	–0.5535	11.8612	18.3529	6.7618	2.9621	7.1515	18.3359	0.7038	–0.0308		–1.7208

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Table 47 First Working Table in the Sufficient Cycle

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)
Vaccine	Dose	n	r	x	p	Y	Φ	Z	y	w	wx	wy	wx^2	wy^2	wxy
S	1.0	12	0	0.0000	0.0000	–2.05041142	0.0201	0.0487	–2.4639	1.4436	0.0000	–3.5570	0.0000	8.7645	0.0000
	1.6	12	3	0.4700	0.2500	–0.92190250	0.1782	0.2608	–0.6469	5.5724	2.6190	–3.6052	1.2309	2.3324	–1.6944
	2.5	12	6	0.9162	0.5000	0.14966152	0.5594	0.3944	–0.0011	7.5774	6.9431	–0.0085	6.3619	0.0000	–0.0077
	4.0	11	10	1.3862	0.9090	1.27817044	0.8994	0.1762	1.3331	3.7771	5.2362	5.0354	7.2590	6.7128	6.9805
U	1.0	11	0	0.0000	0.0000	–1.72084529	0.0426	0.0907	–2.1906	2.2194	0.0000	–4.8621	0.0000	10.6512	0.0000
	1.6	12	4	0.4700	0.3333	–0.59233637	0.2768	0.3347	–0.4234	6.7171	3.1570	–2.8446	1.4838	1.2046	–1.3370
	2.5	11	8	0.9162	0.7272	0.47922765	0.6841	0.3556	0.6005	6.4389	5.8999	3.8670	5.4060	2.3225	3.5433
	4.0	11	10	1.3862	0.9090	1.60773657	0.9460	0.1095	1.2703	2.5867	3.5860	3.2861	4.9713	4.1744	4.5555

Table 48 Second Working Table in the Sufficient Cycle

(17)	(18)	(19)	(20)	(21)	(22)	(23)	(24)	(25)	(26)	(27)	(28)	(29)	(30)
Vaccine	Σw	Σwx	Σwy	Σwx^2	Σwy^2	Σwxy	S_{xx}	S_{xy}	S_{yy}	$\bar{x}$	$\bar{y}$	Common b	a
S	18.3707	14.7985	–2.1353	14.8519	17.8099	5.2783	2.9310	6.9984	17.5617	0.8055	–0.1162	2.4010	–2.0504
U	17.9623	12.6430	–0.5535	11.8612	18.3529	6.7618	2.9621	7.1515	18.3359	0.7038	–0.0308		–1.7208

Test of validity

Before calculating the potencies and confidence interval, test for linearity and parallelism of the assay must be assessed.

Test for linearity:

$$\chi^2 = \Sigma\left(S_{yy} - \frac{S_{xy}{}^2}{S_{xx}}\right) \text{ with } df = N - 2h = 8 - 2(2) = 4$$

$$\chi^2 = \left(17.5617 - \frac{(6.9984)^2}{2.9310}\right) + \left(18.3359 - \frac{(7.1515)^2}{2.9621}\right)$$

$$= 1.9214$$

The χ^2 with 4 degrees of freedom is 1.9214 representing a p-value of 0.7501 which is not significant deviations from linearity.

Test for parallelism:

$$\chi^2 = \Sigma\frac{S_{xy}^2}{S_{xx}} - \frac{(\Sigma S_{xy})^2}{\Sigma S_{xx}} \text{ with } df = h - 1 = 2 - 1 = 1$$

$$\chi^2 = \left(\frac{(6.9984)^2}{2.9310} + \frac{(7.1515)^2}{2.9621}\right) - \left(\frac{(6.9984 + 7.1515)^2}{2.9310 + 2.9621}\right)$$

$$= 0.0010$$

The χ^2 with 1 degree of freedom is 0.0010 representing a p-value of 0.9742 which is not significant.

Estimation of potency and confidence limits

When there are no indications for a significant departure from linearity and parallelism the ln (potency ratio), M'_U, is calculated as:

$$M'_U = \frac{a_U - a_S}{b}$$

$$= \frac{(-1.7208) - (-2.0504)}{2.4010}$$

$$= 0.1372$$

$$\text{Potency ratio} = \text{antiln } M'_U$$

$$= 1.1471$$

Given statistic values for the next steps:

From Table 73 with ∞ df of error, $\left(\frac{\alpha}{2} = 0.025\right)$, $t = 1.9600$

$s = 1$ (for Standard normal distribution)

$$C = \frac{b^2\Sigma S_{xx}}{b^2\Sigma S_{xx} - s^2t^2}$$

$$= \frac{(2.4010)^2(2.9310 + 2.9621)}{(2.4010)^2(2.9310 + 2.9621) - (1)^2(1.9600)^2}$$

$$= 1.1274$$

$$V = \frac{1}{\Sigma_S w} + \frac{1}{\Sigma_U w}$$

$$= \frac{1}{18.3707} + \frac{1}{17.9623}$$

$$= 0.1101$$

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$$
\begin{aligned}
D' &= (C-1)(\bar{x}_S - \bar{x}_U) \\
&= (1.1274-1)(0.8055-0.7038) \\
&= 0.0129 \\
CM'_U - D' &= \left((1.1274)(0.1372)\right) - 0.0129 \\
&= 0.1417 \\
C-1 &= 1.1274-1 \\
&= 0.1274 \\
V\Sigma S_{xx} + C(M'_U - \bar{x}_S + \bar{x}_U)^2 &= (0.1101)(2.9310+2.9621) + (1.1274)(0.1372-0.8055+0.7038)^2 \\
&= 0.6503
\end{aligned}
$$

Calculate and apply ln confidence limits to ln potency ratio (M_L, M_U):

$$
\begin{aligned}
M_L, M_U &= CM'_U - D' \pm \sqrt{(C-1)\left(V\Sigma S_{xx} + C(M'_U - \bar{x}_S + \bar{x}_U)^2\right)} \\
&= 0.1417 \pm \sqrt{(0.1274)(0.6503)} \\
&= 0.1417 \pm \sqrt{0.0829} \\
&= 0.1417 \pm 0.2879 \\
&= -0.1461, 0.4297
\end{aligned}
$$

Confidence limits are given by antiln M_L and M_U: antiln M_L = 0.8640 and antiln M_U = 1.5368

Thus, the potency is estimated to be 160.59 IU/vial with 95 per cent confidence limits from 120.97 IU/vial to 215.15 IU/vial.

If the precision of the assay is limited to be not less than 95.0 per cent and not more than 105.0 per cent of the estimated potency, the confidence limits of error will be 152.56 IU/vial and 168.62 IU/vial of the estimated potency.

Therefore, they do not imply such assay precision. The assay must be repeated and the combination of potency estimates is required.

3.2.3 The logit method

As indicated in Section 3.2.1 the logit method may sometimes be more appropriate. The range of these values varies from –∞ to +∞ which correspond to the cumulative standard normal distribution. The name of the method is derived from the logit function which is the inverse of the logistic distribution. The procedure is similar to that described for the probit method with the modifications as shown in Example 12.

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Example 12 Logit Analysis, An *In-vivo* Assay of a Diphtheria Vaccine

A diphtheria vaccine (assumed potency 140 IU/vial) is assayed against a standard (assigned potency 132/IU/vial). On the basis of this information, equivalent doses are prepared and randomly administered to groups of guinea-pigs. After a given period, the animals are challenged with diphtheria toxin and the number of surviving animals recorded as shown in Table 49.

From Tables 50 to 57, the results are computerized and displayed as four decimal digits, without rounding off, except column (7).

Table 49 Raw Data from a Diphtheria Assay in Guinea-pigs

Standard (S) Assigned Potency 132 IU/vial			Test Preparation (U) Assumed Potency 140 IU/vial		
Dose (IU/ml)	Challenged	Protected	Dose (IU/ml)	Challenged	Protected
1.0	12	0	1.0	11	0
1.6	12	3	1.6	12	4
2.5	12	6	2.5	11	8
4.0	11	10	4.0	11	10

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Table 50 First Working Table in the First Cycle

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)
Vaccine	Dose	n	r	x	p	Y	Φ	Z	y	w	wx	wy	wx^2	wy^2	wxy
S	1.0	12	0	0.0000	0.0000	0.00000000	0.5000	0.2500	–2.0000	3.0000	0.0000	–6.0000	0.0000	12.0000	0.0000
	1.6	12	3	0.4700	0.2500	0.00000000	0.5000	0.2500	–1.0000	3.0000	1.4100	–3.0000	0.6627	3.0000	–1.4100
	2.5	12	6	0.9162	0.5000	0.00000000	0.5000	0.2500	0.0000	3.0000	2.7488	0.0000	2.5187	0.0000	0.0000
	4.0	11	10	1.3862	0.9090	0.00000000	0.5000	0.2500	1.6363	2.7500	3.8123	4.5000	5.2849	7.3636	6.2383
U	1.0	11	0	0.0000	0.0000	0.00000000	0.5000	0.2500	–2.0000	2.7500	0.0000	–5.5000	0.0000	11.0000	0.0000
	1.6	12	4	0.4700	0.3333	0.00000000	0.5000	0.2500	–0.6666	3.0000	1.4100	–2.0000	0.6627	1.3333	–0.9400
	2.5	11	8	0.9162	0.7272	0.00000000	0.5000	0.2500	0.9090	2.7500	2.5197	2.5000	2.3088	2.2727	2.2907
	4.0	11	10	1.3862	0.9090	0.00000000	0.5000	0.2500	1.6363	2.7500	3.8123	4.5000	5.2849	7.3636	6.2383

Table 51 Second Working Table in the First Cycle

(17)	(18)	(19)	(20)	(21)	(22)	(23)	(24)	(25)	(26)	(27)	(28)	(29)	(30)
Vaccine	Σw	Σwx	Σwy	Σwx^2	Σwy^2	Σwxy	S_{xx}	S_{xy}	S_{yy}	$\bar{x}$	$\bar{y}$	Common b	a
S	11.7500	7.9711	–4.5000	8.4664	22.3636	4.8283	3.0588	7.8811	20.6402	0.6783	–0.3829	2.6412	–2.1748
U	11.2500	7.7421	–0.5000	8.2565	21.9696	7.5890	2.9285	7.9331	21.9474	0.6881	–0.0444		–1.8621

The data entered into the columns in the tables indicated by number:

(1) the vaccine label: S for standard, U for test preparation,
(2) the dose of the standard or the test preparations,
(3) the number n of replicates for each treatment,
(4) the number of positive responding units r per treatment group,
(5) the natural logarithm of the dose, x,
(6) the fraction of positive responses per group, $p = \frac{r}{n}$,

for Vaccine S dose 1.6, $p = \frac{3}{12}$

$= 0.25$

For the first cycle:

(7) column Y is filled with zeros,

(8) the cumulative standard normal distribution function, $\Phi = \frac{1}{1 + e^{-Y}}$,

for Vaccine S dose 1.6, $\Phi = 0.5000$

(9) the compute of Z, $Z = \frac{e^{-Y}}{\left(1 + e^{-Y}\right)^2}$,

for Vaccine S dose 1.6, $Z = \frac{e^{0}}{\left(1 + e^{0}\right)^2}$

$= 0.2500$

(10) the compute of y, $y = Y + \frac{p - \Phi}{Z}$,

for Vaccine S dose 1.6, $y = 0 + \frac{0.2500 - 0.5000}{0.2500}$

$= -1.0000$

(11) the compute of w, $w = \frac{nZ^2}{\Phi + \Phi^2}$,

for Vaccine S dose 1.6, $w = \frac{(12)(0.2500)^2}{0.5000 - (0.5000)^2}$

$= 3.0000$

The columns (12) to (16) in the first working table can be calculated from columns (5), (10) and (11) as wx, wy, wx^2, wy^2 and wxy, respectively.

The sums calculated from columns (11) to (16) are transferred to columns (18) to (23) respectively.

(24) the compute of S_{xx}, $S_{xx} = \Sigma wx^2 - \frac{(\Sigma wx)^2}{\Sigma w}$,

for Vaccine S, $S_{xx} = 8.4664 - \frac{(7.9711)^2}{11.7500}$

$= 3.0588$

(25) the compute of S_{xy}, $S_{xy} = \Sigma wxy - \frac{(\Sigma wx)(\Sigma wy)}{\Sigma w}$,

for Vaccine S, $S_{xy} = 4.8283 - \frac{(7.9711)(-4.5000)}{11.7500}$

$= 7.8811$

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(26) the compute of S_{yy}, $S_{yy} = \Sigma wy^2 - \frac{(\Sigma wy)^2}{\Sigma w}$,

for Vaccine S, $S_{yy} = 22.3636 - \frac{(-4.5000)^2}{11.7500}$

$= 20.6402$

(27) the compute of $\bar{x}$, $\bar{x} = \frac{\Sigma wx}{\Sigma w}$,

for Vaccine S, $\bar{x} = \frac{7.9711}{11.7500}$

$= 0.6783$

(28) the compute of $\bar{y}$, $\bar{y} = \frac{\Sigma wy}{\Sigma w}$,

for Vaccine S, $\bar{y} = \frac{-4.5000}{11.7500}$

$= -0.3829$

(29) the compute of common b, $b = \frac{\Sigma S_{xy}}{\Sigma S_{xx}}$,

$b = \frac{(7.8811 + 7.9331)}{(3.0588 + 2.9285)}$

$= 2.6412$

(30) the compute of a, $a = \bar{y} - b\bar{x}$,

for Vaccine S, $a = (-0.3829) - (2.6412)(0.6783)$

$= -2.1748$

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Table 52 First Working Table in the Second Cycle

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)
Vaccine	Dose	n	r	x	p	Y	Φ	Z	y	w	wx	wy	wx^2	wy^2	wxy
S	1.0	12	0	0.0000	0.0000	–2.17482456	0.1020	0.0916	–3.2884	1.0994	0.0000	–3.6155	0.0000	11.8896	0.0000
	1.6	12	3	0.4700	0.2500	–0.93341119	0.2822	0.2025	–1.0925	2.4309	1.1425	–2.6558	0.5370	2.9015	–1.2482
	2.5	12	6	0.9162	0.5000	0.24536008	0.5610	0.2462	–0.0024	2.9552	2.7079	–0.0072	2.4812	0.0000	–0.0066
	4.0	11	10	1.3862	0.9090	1.48677346	0.8155	0.1504	2.1084	1.6544	2.2934	3.4882	3.1794	7.3546	4.8356
U	1.0	11	0	0.0000	0.0000	–1.86214600	0.1344	0.1163	–3.0174	1.2801	0.0000	–3.8627	0.0000	11.6558	0.0000
	1.6	12	4	0.4700	0.3333	–0.62073262	0.3496	0.2273	–0.6923	2.7286	1.2824	–1.8891	0.6027	1.3079	–0.8878
	2.5	11	8	0.9162	0.7272	0.55803865	0.6359	0.2315	0.9523	2.5465	2.3333	2.4250	2.1380	2.3094	2.2220
	4.0	11	10	1.3862	0.9090	1.79945202	0.8580	0.1217	2.2183	1.3395	1.8570	2.9715	2.5743	6.5918	4.1194

Table 53 Second Working Table in the Second Cycle

(17)	(18)	(19)	(20)	(21)	(22)	(23)	(24)	(25)	(26)	(27)	(28)	(29)	(30)
Vaccine	Σw	Σwx	Σwy	Σwx^2	Σwy^2	Σwxy	S_{xx}	S_{xy}	S_{yy}	$\bar{x}$	$\bar{y}$	Common b	a
S	8.1401	6.1439	–2.7905	6.1976	22.1459	3.5807	1.5603	5.6869	21.1892	0.7547	–0.3428	3.6950	–3.1317
U	7.8948	5.4728	–0.3552	5.3151	21.8650	5.4536	1.5212	5.6999	21.8490	0.6932	–0.0449		–2.6064

For the second cycle:

the column (7) of the first working table in the second cycle (Table 52) can be replaced by Y = a + bx (using a and b from the first cycle),
for Vaccine S dose 1.6, Y = –2.1748 + (2.6412) (0.4700) = –0.93341119

The next cycles are not shown but they are repeated until the difference in Y between two consecutive cycles has become smaller than 10^{-8}.

Tables 54 and 55 show the cycle before sufficient and Tables 56 and 57 show the sufficient cycle.

The cycles shown in Tables 56 and 57 are sufficient for the estimation.

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Table 54 First Working Table in the Cycle before Sufficient

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)
Vaccine	Dose	n	r	x	p	Y	Φ	Z	y	w	wx	wy	wx^2	wy^2	wxy
S	1.0	12	0	0.0000	0.0000	–3.51218201	0.0289	0.0281	–4.5420	0.3375	0.0000	–1.5331	0.0000	6.9634	0.0000
	1.6	12	3	0.4700	0.2500	–1.58449417	0.1701	0.1412	–1.0190	1.6944	0.7964	–1.7267	0.3743	1.7597	–0.8115
	2.5	12	6	0.9162	0.5000	0.24592196	0.5611	0.2462	–0.0024	2.9550	2.7077	–0.0073	2.4810	0.0000	–0.0067
	4.0	11	10	1.3862	0.9090	2.17360980	0.8978	0.0917	2.2961	1.0088	1.3985	2.3164	1.9387	5.3187	3.2112
U	1.0	11	0	0.0000	0.0000	–2.89187227	0.0525	0.0497	–3.9473	0.5477	0.0000	–2.1621	0.0000	8.5346	0.0000
	1.6	12	4	0.4700	0.3333	–0.96418443	0.2760	0.1998	–0.6774	2.3981	1.1271	–1.6247	0.5297	1.1007	–0.7636
	2.5	11	8	0.9162	0.7272	0.86623170	0.7039	0.2083	0.9780	2.2923	2.1005	2.2421	1.9246	2.1930	2.0544
	4.0	11	10	1.3862	0.9090	2.79391954	0.9423	0.0543	2.1818	0.5976	0.8284	1.3039	1.1485	2.8448	1.8076

Table 55 Second Working Table in the Cycle before Sufficient

(17)	(18)	(19)	(20)	(21)	(22)	(23)	(24)	(25)	(26)	(27)	(28)	(29)	(30)
Vaccine	Σw	Σwx	Σwy	Σwx^2	Σwy^2	Σwxy	S_{xx}	S_{xy}	S_{yy}	$\bar{x}$	$\bar{y}$	Common b	a
S	5.9959	4.9026	–0.9508	4.7941	14.0419	2.3928	0.7854	3.1703	13.8911	0.8176	–0.1585	4.1014	–3.5121
U	5.8358	4.0561	–0.2407	3.6029	14.6733	3.0984	0.7838	3.2657	14.6633	0.6950	–0.0412		–2.8918

Table 56 First Working Table in the Sufficient Cycle

(1) Vaccine	(2) Dose	(3) n	(4) r	(5) x	(6) p	(7) Y	(8) Φ	(9) Z	(10) y	(11) w	(12) wx	(13) wy	(14) wx^2	(15) wy^2	(16) wxy
S	1.0	12	0	0.0000	0.0000	−3.51218201	0.0289	0.0281	−4.5420	0.3375	0.0000	−1.5331	0.0000	6.9634	0.0000
	1.6	12	3	0.4700	0.2500	−1.58449417	0.1701	0.1412	−1.0190	1.6944	0.7964	−1.7267	0.3743	1.7597	−0.8115
	2.5	12	6	0.9162	0.5000	0.24592196	0.5611	0.2462	−0.0024	2.9550	2.7077	−0.0073	2.4810	0.0000	−0.0067
	4.0	11	10	1.3862	0.9090	2.17360980	0.8978	0.0917	2.2961	1.0088	1.3985	2.3164	1.9387	5.3187	3.2112
U	1.0	11	0	0.0000	0.0000	−2.89187227	0.0525	0.0497	−3.9473	0.5477	0.0000	−2.1621	0.0000	8.5346	0.0000
	1.6	12	4	0.4700	0.3333	−0.96418443	0.2760	0.1998	−0.6774	2.3981	1.1271	−1.6247	0.5297	1.1007	−0.7636
	2.5	11	8	0.9162	0.7272	0.86623170	0.7039	0.2083	0.9780	2.2923	2.1005	2.2421	1.9246	2.1930	2.0544
	4.0	11	10	1.3862	0.9090	2.79391954	0.9423	0.0543	2.1818	0.5976	0.8284	1.3039	1.1485	2.8448	1.8076

Table 57 Second Working Table in the Sufficient Cycle

(17) Vaccine	(18) Σw	(19) Σwx	(20) Σwy	(21) Σwx^2	(22) Σwy^2	(23) Σwxy	(24) S_{xx}	(25) S_{xy}	(26) S_{yy}	(27) $\bar{x}$	(28) $\bar{y}$	(29) Common b	(30) a
S	5.9959	4.9026	−0.9508	4.7941	14.0419	2.3928	0.7854	3.1703	13.8911	0.8176	−0.1585	4.1014	−3.5121
U	5.8358	4.0561	−0.2407	3.6029	14.6733	3.0984	0.7838	3.2657	14.6633	0.6950	−0.0412		−2.8918

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Test of validity

Before calculating the potencies and confidence interval, test for linearity and parallelism of the assay must be assessed.

Test for linearity:

$$\chi^2 = \Sigma\left(S_{yy} - \frac{S_{xy}^2}{S_{xx}}\right) \text{ with } df = N - 2h = 8 - 2(2) = 4$$

$$\chi^2 = \left(13.8911 - \frac{(3.1703)^2}{0.7854}\right) + \left(14.6633 - \frac{(3.2657)^2}{0.7838}\right)$$

$$= 2.1504$$

The χ^2 with 4 degrees of freedom is 2.1504 representing a p-value of 0.7081 which is not significant deviations from linearity.

Test for parallelism:

$$\chi^2 = \Sigma\frac{S_{xy}^2}{S_{xx}} - \frac{(\Sigma S_{xy})^2}{\Sigma S_{xx}} \text{ with } df = h - 1 = 2 - 1 = 1$$

$$\chi^2 = \left(\frac{(3.1703)^2}{0.7854} + \frac{(3.2657)^2}{0.7838}\right) - \left(\frac{(3.1703 + 3.2657)^2}{0.7854 + 0.7838}\right)$$

$$= 0.0066$$

The χ^2 with 1 degree of freedom is 0.0066 representing a p-value of 0.9351 which is not significant.

Estimation of potency and confidence limits

When there are no indications for a significant departure from linearity and parallelism the ln (potency ratio), M'_U, is calculated as:

$$M'_U = \frac{a_U - a_S}{b}$$

$$= \frac{(-2.8918) - (-3.5121)}{4.1014}$$

$$= 0.1512$$

$$\text{Potency ratio} = \text{antiln } M'_U$$

$$= 1.1632$$

Given statistic values for the next steps:

From Table 73 with ∞ df of error, $\left(\frac{\alpha}{2} = 0.025\right)$, t = 1.9600

$$s = 1 \text{ (for Standard Normal Distribution)}$$

$$C = \frac{b^2\Sigma S_{xx}}{b^2\Sigma S_{xx} - s^2t^2}$$

$$= \frac{(4.1014)^2(0.7854 + 0.7838)}{(4.4014)^2(0.7854 + 0.7838) - (1)^2(1.9600)^2}$$

$$= 1.1703$$

$$V = \frac{1}{\Sigma w_S} + \frac{1}{\Sigma w_U}$$

$$= \frac{1}{5.9959} + \frac{1}{5.8358}$$

$$= 0.3381$$

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$$C - 1 = 1.1703 - 1$$
$$= 0.1703$$
$$D' = (C - 1)(\bar{x}_S - \bar{x}_U)$$
$$= (1.1703 - 1)(0.8176 - 0.6950)$$
$$= 0.0208$$
$$CM'_U - D' = \left((1.1703)(0.1512)\right) - 0.0208$$
$$= 0.1561$$
$$V\Sigma S_{xx} + C(M'_U - \bar{x}_S + \bar{x}_U)^2 = (0.3381)(0.7854 + 0.7838) + (1.1703)(0.1512 - 0.8176 + 0.6950)^2$$
$$= 0.5313$$

Calculate and apply ln confidence limits to ln potency ratio (M_L, M_U):

$$M_L, M_U = CM'_U - D' \pm \sqrt{(C - 1)\left(V\Sigma S_{xx} + C(M'_U - \bar{x}_S + \bar{x}_U)^2\right)}$$
$$= 0.1561 \pm \sqrt{(0.1703)(0.5313)}$$
$$= 0.1561 \pm \sqrt{0.0904}$$
$$= 0.1561 \pm 0.3008$$
$$= -0.1447, 0.4570$$

Confidence limits are given by antiln M_L and antiln M_U: antiln $M_L = 0.8652$ and antiln $M_U = 1.5793$

Thus, the potency is estimated to be 163.80 IU/vial with confidence limits from 122.22 IU/vial to 232.54 IU/vial.

If the precision of the assay is limited to be not less than 95.0 per cent and not more than 105.0 per cent of the estimated potency, the confidence limits of error will be 155.61 IU/vial and 171.99 IU/vial of the estimated potency.

Therefore, they do not imply such assay precision. The assay must be repeated and the combination of potency estimates is required.

3.2.4 The median effective dose

In some types of assay it is desirable to determine a median effective dose which is the dose that produces a response in 50 per cent of the units. The probit method can be used to determine this median effective dose (ED_{50}), but since there is no need to express this dose relative to a standard, the formulae are slightly different.

(**Note** A standard can optionally be included in order to validate the assay. Usually the assay is considered valid if the calculated ED_{50} of the standard is close enough to the assigned ED_{50}. What "close enough" in this context means depends on the requirements specified in the monograph).

The tabulation of the responses to the test samples, and optionally a standard, and the test for linearity are as described in Example 11. A test for parallelism is not necessary for this type of assay. The ED_{50} of test sample T, and similarly for the other samples, is obtained with the modifications as shown in Example 13.

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Example 13 The ED_{50} Determination of a Substance Using the Probit Method, An *in-vitro* Assay of Oral Poliomyelitis Vaccine

In an ED_{50} assay of oral poliomyelitis vaccine with 10 different dilutions in 8 replicates of 50 μl on an ELISA-plate, results were obtained as shown in Table 58.

From Tables 59 to 66, the results are computerized and displayed as four decimal digits, without rounding off, except column (7).

Table 58 Dilutions of the Undiluted Vaccine

Dilutions (μl)	Challenged	Protected
$10^{-3.5}$	8	0
$10^{-4.0}$	8	0
$10^{-4.5}$	8	1
$10^{-5.0}$	8	2
$10^{-5.5}$	8	6
$10^{-6.0}$	8	7
$10^{-6.5}$	8	7
$10^{-7.0}$	8	8
$10^{-7.5}$	8	8
$10^{-8.0}$	8	8

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Table 59 First Working Table in the First Cycle

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)
Vaccine	Dose	n	r	x	p	Y	Φ	Z	y	w	wx	wy	wx^2	wy^2	wxy
U	$10^{-3.5}$	8	0	–8.0590	0.0000	0.00000000	0.5000	0.3989	–1.2533	5.0929	–41.0443	–6.3830	330.7787	8.0000	51.4415
	$10^{-4.0}$	8	0	–9.2103	0.0000	0.00000000	0.5000	0.3989	–1.2533	5.0929	–46.9089	–6.3830	432.0375	8.0000	58.7903
	$10^{-4.5}$	8	1	–10.3616	0.1250	0.00000000	0.5000	0.3989	–0.9399	5.0929	–52.7713	–4.7873	546.7974	4.5000	49.6043
	$10^{-5.0}$	8	2	–11.5129	0.2500	0.00000000	0.5000	0.3989	–0.6266	5.0929	–58.6348	–3.1915	675.0586	2.0000	36.7439
	$10^{-5.5}$	8	6	–12.6642	0.7500	0.00000000	0.5000	0.3989	0.6266	5.0929	–64.4983	3.1915	816.8209	2.0000	–40.4183
	$10^{-6.0}$	8	7	–13.8155	0.8750	0.00000000	0.5000	0.3989	0.9399	5.0929	–70.3618	4.7873	972.0844	4.5000	–66.1390
	$10^{-7.0}$	8	7	–14.9668	0.8750	0.00000000	0.5000	0.3989	0.9399	5.0929	–76.2253	4.7873	1,140.8490	4.5000	–71.6506
	$10^{-7.5}$	8	8	–16.1180	1.0000	0.00000000	0.5000	0.3989	1.2533	5.0929	–82.0888	6.3830	1,323.1149	8.0000	–102.8830
	$10^{-7.5}$	8	8	–17.2693	1.0000	0.00000000	0.5000	0.3989	1.2533	5.0929	–87.9522	6.3830	1,518.8819	8.0000	–110.2318
	$10^{-8.0}$	8	8	–18.4206	1.0000	0.00000000	0.5000	0.3989	1.2533	5.0929	–93.8157	6.3830	1,728.1501	8.0000	–117.5806

Table 60 Second Working Table in the Second Cycle

(17)	(18)	(19)	(20)	(21)	(22)	(23)	(24)	(25)	(26)	(27)	(28)	(29)	(30)
Vaccine	Σw	Σwx	Σwy	Σwx^2	Σwy^2	Σwxy	S_{xx}	S_{xy}	S_{yy}	$\bar{x}$	$\bar{y}$	Common b	a
U	50.9295	–674.3007	11.1703	9,484.5738	57.5000	–312.3235	556.9233	–164.4291	55.0500	–13.2398	0.2193	–0.2952	–3.6896

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The data entered into the columns in the tables indicated by number:

(1) the vaccine label: U for test preparation,
(2) the dose of the standard or the test preparations,
(3) the number n of replicates for each treatment,
(4) the number of positive responding units r per treatment group,
(5) the natural logarithm of the dose, x,
(6) the fraction of positive responses per group, $p = \frac{r}{n}$,

for Vaccine U dose $10^{-5.0}$, $p = \frac{2}{8}$,

$= 0.2500$

For the first cycle:

(7) column Y is filled with zeros,
(8) the cumulative standard normal distribution function, $\Phi = \Phi(Y)$,

for Vaccine U dose $10^{-5.0}$, $\Phi = 0.5000$

(9) the compute of Z, $Z = \frac{e^{-\frac{Y^2}{2}}}{\sqrt{2\pi}}$,

for Vaccine U dose $10^{-5.0}$, $Z = \frac{e^0}{\sqrt{2\pi}}$

$= 0.3989$

(10) the compute of y, $y = Y + \frac{p - \Phi}{Z}$,

for Vaccine U dose $10^{-5.0}$, $y = 0 + \frac{0.2500 - 0.5000}{0.3989}$

$= -0.6266$

(11) the compute of w, $w = \frac{nZ^2}{\Phi - \Phi^2}$,

for Vaccine U dose $10^{-5.0}$, $w = \frac{(8)(0.3989)^2}{0.5000 - (0.5000)^2}$

$= 5.0929$

The columns (12) to (16) in the first working table can be calculated from columns (5), (10) and (11) as wx, wy, wx^2, wy^2 and wxy, respectively.

The sums calculated from columns (11) to (16) are transferred to columns (18) to (23) respectively.

(24) the compute of S_{xx}, $S_{xx} = \Sigma wx^2 - \frac{(\Sigma wx)^2}{\Sigma w}$,

for Vaccine U, $S_{xx} = 9484.5738 - \frac{(-674,3007)^2}{50.9295}$

$= 556.9233$

(25) the compute of S_{xy}, $S_{xy} = \Sigma wxy - \frac{(\Sigma wx)(\Sigma wy)}{\Sigma w}$,

for Vaccine U, $S_{xy} = -312.3235 - \frac{(-674.3007)(11.1703)}{50.9295}$

$= -164.4291$

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(26) the compute of S_{yy}, $S_{yy} = \Sigma wy^2 - \frac{(\Sigma wy)^2}{\Sigma w}$,

for Vaccine U, $S_{yy} = 57.5000 - \frac{(11.1703)^2}{50.9295}$

$= 55.0500$

(27) the compute of $\bar{x}$, $\bar{x} = \frac{\Sigma wx}{\Sigma w}$,

for Vaccine U, $\bar{x} = \frac{-674.3007}{50.9295}$

$= -13.2398$

(28) the compute of $\bar{y}$, $\bar{y} = \frac{\Sigma wy}{\Sigma w}$,

for Vaccine S, $\bar{y} = \frac{11.1703}{50.9295}$

$= 0.2193$

(29) the compute of common b, common b $= \frac{S_{xy}}{S_{xx}}$,

$b = \frac{-164.4291}{556.9233}$

$= -0.2952$

(30) the compute of a, $a = \bar{y} - b\bar{x}$,

for Vaccine U, $a = 0.2193 - (-0.2952)(-13.2398)$

$= -3.6896$

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Table 61 First Working Table in the Second Cycle

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)
Vaccine	Dose	n	r	x	p	Y	Φ	Z	y	w	wx	wy	wx^2	wy^2	wxy
U	$10^{-3.5}$	8	0	–8.0590	0.0000	–1.31028296	0.0950	0.1690	–1.8724	2.6590	–21.4290	–4.9787	172.6974	9.3224	40.1243
	$10^{-4.0}$	8	0	–9.2103	0.0000	–0.97036898	0.1659	0.2491	–1.6363	3.5879	–33.0458	–5.8712	304.3636	9.6075	54.0758
	$10^{-4.5}$	8	1	–10.3616	0.1250	–0.63045499	0.2641	0.3270	–1.0560	4.4014	–45.6065	–4.6483	472.5578	4.9090	48.1644
	$10^{-5.0}$	8	2	–11.5129	0.2500	–0.29054100	0.3857	0.3824	–0.6453	4.9387	–56.8597	–3.1872	654.6215	2.0569	36.6948
	$10^{-5.5}$	8	6	–12.6642	0.7500	0.04937298	0.5196	0.3984	0.6273	5.0884	–64.4412	3.1923	816.0976	2.0028	–40.4292
	$10^{-6.0}$	8	7	–13.8155	0.8750	0.38928697	0.6514	0.3698	0.9937	4.8190	–66.5773	4.7886	919.8000	4.7585	–66.1581
	$10^{-6.5}$	8	7	–14.9668	0.8750	0.72920095	0.7670	0.3058	1.0821	4.1870	–62.6667	4.5310	937.9214	4.9034	–67.8160
	$10^{-7.0}$	8	8	–16.1180	1.0000	1.06911494	0.8574	0.2252	1.7017	3.3222	–53.5489	5.6535	863.1064	9.6208	–91.1252
	$10^{-7.5}$	8	8	–17.2693	1.0000	1.40902892	0.9205	0.1478	1.9461	2.3917	–41.3044	4.6548	713.3030	9.0591	–80.3860
	$10^{-8.0}$	8	8	–18.4206	1.0000	1.74894291	0.9598	0.0864	2.2134	1.5509	–28.5694	3.4329	526.2685	7.5985	–63.2369

Table 62 Second Working Table in the Second Cycle

(17)	(18)	(19)	(20)	(21)	(22)	(23)	(24)	(25)	(26)	(27)	(28)	(29)	(30)
Vaccine	Σw	Σwx	Σwy	Σwx^2	Σwy^2	Σwxy	S_{xx}	S_{xy}	S_{yy}	$\bar{x}$	$\bar{y}$	Common b	a
U	36.9467	–474.0493	7.5679	6,380.7375	63.8394	–230.0922	298.3878	–132.9911	62.2892	–12.8306	0.2048	–0.4457	–5.5138

For the second cycle:

the column (7) of the first working table in the second cycle (Table 61) can be replaced by $Y = a + bx$ (using a and b from the first cycle),

for Vaccine U dose $10^{-5.0}$, $Y = -3.6896 + (-0.2952)(-11.5129) = -0.2905$

The next cycles are not show but they are repeated until the difference in Y between two consecutive cycles has become smaller than 10^{-8} .

Tables 63 and 64 show the cycle before sufficient and Tables 65 and 66 show the sufficient cycle.

The cycles shown in Tables 65 and 66 are sufficient for the estimation.

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Table 63 First Working Table in the Cycle before Sufficient

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)
Vaccine	Dose	n	r	x	p	Y	Φ	Z	y	w	wx	wy	wx^2	wy^2	wxy
U	$10^{-3.5}$	8	0	–8.0590	0.0000	–2.72328550	0.0032	0.0097	–3.0536	0.2376	–1.9155	–0.7258	15.4378	2.2164	5.8494
	$10^{-4.0}$	8	0	–9.2103	0.0000	–1.97930920	0.0238	0.0562	–2.4039	1.0858	–10.0009	–2.6103	92.1118	6.2750	24.0417
	$10^{-4.5}$	8	1	–10.3616	0.1250	–1.23533290	0.1083	0.1860	–1.1458	2.8649	–29.6858	–3.2828	307.5937	3.7615	34.0151
	$10^{-5.0}$	8	2	–11.5129	0.2500	–0.49135659	0.3115	0.3535	–0.6655	4.6626	–53.6803	–3.1031	618.0174	2.0652	35.7263
	$10^{-5.5}$	8	6	–12.6642	0.7500	0.25261971	0.5997	0.3864	0.6415	4.9760	–63.0173	3.1922	798.0660	2.0479	–40.4276
	$10^{-6.0}$	8	7	–13.8155	0.8750	0.99659601	0.8405	0.2427	1.1386	3.5181	–48.6048	4.0057	671.5002	4.5610	–55.3419
	$10^{-6.5}$	8	7	–14.9668	0.8750	1.74057232	0.9591	0.0877	0.7814	1.5696	–23.4924	1.2266	351.6062	0.9585	–18.3588
	$10^{-7.0}$	8	8	–16.1180	1.0000	2.48454862	0.9935	0.0182	2.8405	0.4119	–6.6404	1.1702	107.0307	3.3242	–18.8626
	$10^{-7.5}$	8	8	–17.2693	1.0000	3.22852492	0.9993	0.0021	3.5145	0.0608	–1.0513	0.2139	18.1561	0.7519	–3.6950
	$10^{-8.0}$	8	8	–18.4206	1.0000	3.97250122	0.9999	0.0001	4.2106	0.0050	–0.0924	0.0211	1.7024	0.0889	–0.3891

Table 64 Second Working Table in the Cycle before Sufficient

(17)	(18)	(19)	(20)	(21)	(22)	(23)	(24)	(25)	(26)	(27)	(28)	(29)	(30)
Vaccine	Σw	Σwx	Σwy	Σwx^2	Σwy^2	Σwxy	S_{xx}	S_{xy}	S_{yy}	$\bar{x}$	$\bar{y}$	Common b	a
U	19.3928	–238.1814	0.1079	2,981.2780	26.0510	–37.4424	55.8877	–36.1162	26.0504	–12.2819	0.0055	–0.6462	–7.9313

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Table 65 First Working Table in the Sufficient Cycle

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)
Vaccine	Dose	n	r	x	p	Y	Φ	Z	y	w	wx	wy	wx^2	wy^2	wxy
U	$10^{-3.5}$	8	0	−8.0590	0.0000	−2.72340177	0.0032	0.0097	−3.0537	0.2376	−1.9150	−0.7256	15.4334	2.2159	5.8480
	$10^{-4.0}$	8	0	−9.2103	0.0000	−1.97930920	0.0238	0.0562	−2.4039	1.0858	−10.0009	−2.6103	92.1118	6.2750	24.0417
	$10^{-4.5}$	8	1	−10.3616	0.1250	−1.23533290	0.1083	0.1860	−1.1458	2.8649	−29.6858	−3.2828	307.5937	3.7615	34.0151
	$10^{-5.0}$	8	2	−11.5129	0.2500	−0.49135659	0.3115	0.3535	−0.6655	4.6626	−53.6803	−3.1031	618.0174	2.0652	35.7263
	$10^{-5.5}$	8	6	−12.6642	0.7500	0.25261971	0.5997	0.3864	0.6415	4.9760	−63.0173	3.1922	798.0660	2.0479	−40.4276
	$10^{-6.0}$	8	7	−13.8155	0.8750	0.99659601	0.8405	0.2427	1.1386	3.5181	−48.6048	4.0057	671.5002	4.5610	−55.3419
	$10^{-6.5}$	8	7	−14.9668	0.8750	1.74057232	0.9591	0.0877	0.7814	1.5696	−23.4924	1.2266	351.6062	0.9585	−18.3588
	$10^{-7.0}$	8	8	−16.1180	1.0000	2.48454862	0.9935	0.0182	2.8405	0.4119	−6.6404	1.1702	107.0307	3.3242	−18.8626
	$10^{-7.5}$	8	8	−17.2693	1.0000	3.22852492	0.9993	0.0021	3.5145	0.0608	−1.0513	0.2139	18.1561	0.7519	−3.6950
	$10^{-8.0}$	8	8	−18.4206	1.0000	3.97250122	0.9999	0.0001	4.2106	0.0050	−0.0924	0.0211	1.7024	0.0889	−0.3891

Table 66 Second Working Table in the Sufficient Cycle

(17)	(18)	(19)	(20)	(21)	(22)	(23)	(24)	(25)	(26)	(27)	(28)	(29)	(30)
Vaccine	∑w	∑wx	∑wy	$\sum wx^2$	$\sum wy^2$	∑wxy	S_{xx}	S_{xy}	S_{yy}	$\bar{x}$	$\bar{y}$	Common b	a
U	19.3927	−238.1809	0.1081	2,981.2184	26.0506	−37.4439	55.8865	−36.1155	26.0500	−12.2819	0.0055	−0.6462	−7.9313

Test of validity

Before calculating the potencies and confidence interval, test for linearity and parallelism of the assay must be assessed.

Test for linearity:

$$\chi^2 = S_{yy} - \frac{S_{xy}^2}{S_{xx}} \text{ with } df = N - 2h = 10 - 2(1) = 8$$

$$\chi^2 = 26.0500 - \frac{(-36.1155)^2}{55.8865}$$

$$= 2.7110$$

The χ^2 with 8 degrees of freedom is 2.7110 representing a p-value of 0.9511 which is not significant deviations from linearity.

Estimation of potency and confidence limits

When there are no indications for a significant departure from linearity the ln (potency ratio), M'_U, is calculated as

$$M'_U = \frac{-a_U}{b}$$

$$= \frac{-(-7.9313)}{-0.6462}$$

$$= -12.2733$$

$$\text{Potency ratio} = \text{antiln } M'_U$$

$$= 4.67 \times 10^{-6}$$

Given statistic values for the next steps:

From Table 73 with ∞ df of residual error, $\left(\frac{\alpha}{2} = 0.025\right), t = 1.9600$

$$s = 1 \text{ (for Standard Normal Distribution)}$$

$$C = \frac{b^2 S_{xx}}{b^2 S_{xx} - s^2 t^2}$$

$$= \frac{(-0.6462)^2 (55.8865)}{(-0.6462)^2 (55.8865) - (1)^2 (1.9600)^2}$$

$$= 1.1970$$

$$V = \frac{1}{\sum w_U}$$

$$= \frac{1}{19.3927}$$

$$= 0.0515$$

$$D' = (C - 1)(\bar{x}_U)$$

$$= (1.1970 - 1)(-12.2819)$$

$$= -2.4199$$

$$CM'_U - D' = \left((1.1970)(-12.2733)\right) - (-2.4199)$$

$$= -12.2716$$

$$C - 1 = 1.1970 - 1$$

$$= 0.1970$$

$$VS_{xx} + C\left(M'_U - \bar{x}_U\right)^2 = (0.0515)(55.8865) + (1.1970)\left(-12.2733 - (-12.2819)\right)^2$$

$$= 2.8819$$

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Calculate and apply ln confidence limits to ln potency ratio (M_L, M_U):

$$M_L, M_U = CM'_U - D' \pm \sqrt{(C-1)\left(VS_{xx} + C(M'_U - \bar{x}_U)^2\right)}$$

$$= -12.2716 \pm \sqrt{(0.1970)(2.8819)}$$

$$= -12.2716 \pm 0.7535$$

$$= -11.5180, -13.0251$$

This estimate is still expressed in terms of the ln (dilutions). In order to obtain estimates expressed in ln (ED_{50})/ml the values are transformed to:

$$= -M'_U + \ln\left(\frac{1000}{50}\right)$$

$$= -(-12.2733) + 2.9957$$

$$= 15.2690$$

To express the potency of the vaccine in terms of $\log(ED_{50})$/ml, the results are divided by ln (10):

$$= \frac{15.2690}{2.3025}$$

$$= 6.6312$$

Confidence limits are given by M_L and M_U: $M_L = 6.3032$ $\log(ED_{50})$/ml and $M_U = 6.9577$ $\log(ED_{50})$/ml

Thus, the potency is estimated to be $10^{6.63}$ ED_{50}/ml with 95 per cent confidence limits from $10^{6.30}$ and $10^{6.96}$ ED_{50}/ml.

4. Combination of Assay Results

4.1 INTRODUCTION

Replication of independent assays and combination of their results is often needed to fulfil the requirements of the Pharmacopoeia. The question then arises as to whether it is appropriate to combine the results of such assays and if so in what way.

Two assays may be regarded as mutually independent when the execution of either does not affect the probabilities of the possible outcomes of the other. This implies that the random errors in all essential factors influencing the result (for example, dilutions of the standard and of the preparation to be examined, the sensitivity of the biological indicator) in one assay must be independent of the corresponding random errors in the other one. Assays on successive days using the original and retained dilutions of the standard therefore are not independent assays.

There are several methods for combining the results of independent assays, the most theoretically acceptable being the most difficult to apply. Three simple, approximate methods are described below; others may be used provided the necessary conditions are fulfilled.

Before potencies from assays based on the parallel-line or probit model are combined they must be expressed in logarithms; potencies derived from assays based on the slope-ratio model are used as such. As the former models are more common than those based on the slope-ratio model, the symbol *M* denoting ln potency is used in the formulae in this section; by reading *R* (slope-ratio) for *M*, the analyst may use the same formulae for potencies derived from assays based on the slope-ratio model. All estimates of potency must be corrected for the potency assigned to each preparation to be examined before they are combined.

4.2 WEIGHTED AND UNWEIGHTED COMBINATIONS OF ASSAY RESULTS

This method can be used provided the following conditions are fulfilled:

1) the potency estimates are derived from independent assays;
2) the number of degrees of freedom of the individual residual errors is not smaller than 6, but preferably larger than 15;
3) the individual potency estimates form a homogeneous set.

When the above conditions are met, the weighted mean potencies with confidence limits are calculated as described in Example 14.

In case a significant variability between the assays exists and the individual potency estimates form a heterogeneous set. Under this circumstance condition 3 is not fulfilled and the method described in Example 14 is no longer applicable. The method described in Example 15 may be used.

When these conditions are not fulfilled this method cannot be applied. The unweighted combination of assay results can be performed by the method described in Example 16 to obtain the best estimate of the mean potency to be adopted in further assays as an assumed potency.

Example 14 Weighted Combination of Assay Results, Homogeneous Set

Six independent potency estimates of the sample preparation are described in Table 67. In Table 68, the results are computerized but only four decimal digits, without rounding off, are displayed.

Table 67 Potency Estimates and Confidence Intervals of Six Independent Assays

Potency Estimate (IU/vial)	Lower Limit (L) (IU/vial)	Upper Limit (U) (IU/vial)	Degrees of Freedom
18,367	17,755	19,002	20
18,003	17,415	18,610	20
18,054	17,319	18,838	20
17,832	17,253	18,429	20
18,635	17,959	19,339	20
18,269	17,722	18,834	20

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Table 68 Weighted Mean of Individual Assay Results

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Potency Estimate	Lower Limit (L)	Upper Limit (U)	df	ln potency (M)	Weight (W)	WM	$W(M-\overline{M})^2$
18,367	17,755	19,002	20	9.8183	3,777.6976	37,090.6099	0.3716
18,003	17,415	18,610	20	9.7983	3,951.5439	38,718.3884	0.4030
18,054	17,319	18,838	20	9.8011	2,462.4715	24,134.9854	0.1301
17,832	17,253	18,429	20	9.7887	4,002.9713	39,184.0851	1.5444
18,635	17,959	19,339	20	9.8328	3,175.6286	31,225.3115	1.8913
18,269	17,722	18,834	20	9.8130	4,699.5160	46,116.1668	0.0980
				Total	22,069.8292	216,469.5473	

The data entered into the column in the tables indicated by number:

(1) the potency estimate,
(2) the lower limit of the estimated potency,
(3) the upper limit of the estimated potency,
(4) the number of degrees of freedom of their error variances,
(5) the natural logarithm of the potency, M,

Given statistic values for the next steps:

From Table 73 with 20 df of error, $\left(\frac{\alpha}{2} = 0.025\right)$, t = 2.0860

(6) the compute of W,

$$W = \frac{4t^2}{(\ln U - \ln L)^2}$$

for the potency estimate 18,367,

$$W = \frac{4(2.0860)^2}{(\ln 19{,}002 - \ln 17{,}755)^2}$$

$$= 3{,}777.6976$$

(7) the compute of WM for the potency estimates

for the ln potency 9.8183, $WM = (3{,}777.6976)(9.8183)$

$$= 37{,}090.6099$$

(8) the compute of $W(M - \overline{M})^2$ for the potency estimate 18,367

$$\overline{M} = \frac{\Sigma WM}{\Sigma W}$$

$$= \frac{21{,}6469.5473}{22{,}069.8292}$$

$$= 9.8083$$

$$W(M - \overline{M})^2 = (3{,}777.6976)(9.8183 - 9.8083)^2$$

$$= 0.3716$$

Test for homogeneity of potency estimate

$$\chi^2 = \Sigma W(M - \overline{M})^2$$

$$\chi^2 = 0.3716 + 0.4030 + 0.1301 + 1.5444 + 1.8913 + 0.0980$$

$$= 4.4386$$

The calculated is χ^2 smaller than the tabulated value corresponding to 5 degrees of freedom (11.070): the potencies are homogeneous.

Calculation of the weighted mean and confidence limits:

Given statistic values for the next steps:

From Table 73 with 120 df of error, $\left(\frac{\alpha}{2} = 0.025\right)$, t = 1.9799

$$S_{\overline{M}} = \sqrt{\frac{1}{\Sigma W}}$$

$$= \sqrt{\frac{1}{22{,}069.8292}}$$

$$= 0.0067$$

$$\ln M_L = \overline{M} - tS_{\overline{M}}$$

$$= 9.8083 - (1.9799)(6.7313 \times 10^{-3})$$

$$= 9.7950$$

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$$
\begin{aligned}
\ln M_U &= \overline{M} + tS_{\overline{M}} \\
&= 9.8083 + (1.9799)(0.0067) \\
&= 9.8217 \\
\text{Potency} &= \text{antiln}\,(\overline{M}) \\
&= \text{antiln}\,(9.8083) \\
&= 18{,}185.7301 \\
M_L &= \text{antiln}\,(M_L) \\
&= \text{antiln}\,(9.7950) \\
&= 17{,}944.9668 \\
M_U &= \text{antiln}\,(M_U) \\
&= \text{antiln}(9.8217) \\
&= 18{,}429.7237
\end{aligned}
$$

Thus, the potency is estimated to be 18,186 IU/vial with 95 per cent confidence limits from 17,945 to 18,430 IU/vial.

Example 15 Weighted Mean Confidence Limits based on the Intra-and Inter-assay Variation, Potency Estimates and Confidence Intervals of Six Independent Assays.

Six independent potency estimates of the same preparation are described in Table 69. In Table 70, the results are computerized but only four decimal digits, without rounding off, are displayed.

Table 69 Potency Estimates and Confidence Intervals of Six Independent Assays

Potency Estimate (IU/vial)	Lower Limit (L) (IU/vial)	Upper Limit (U) (IU/vial)	Degrees of Freedom
18,367	17,755	19,002	20
18,003	17,415	18,610	20
18,054	17,319	18,838	20
17,832	17,253	18,429	20
19,135	18,459	19,839	20
19,269	18,722	20,834	20

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Table 70 Weighted Mean of Individual Assay Results

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)
Potency Estimate	Lower Limit (L)	Upper Limit (U)	df	ln potency (M)	Weight (W)	WM	$(M-\overline{M})^2$	$W(M-\overline{M})^2$	S^2_M	$S^2_{\overline{M}}$	W′	W′M
18,367	17,755	19,002	20	9.8183	3,777.6977	37,090.6100	2.4035×10^{-6}	0.0091	2.6471×10^{-4}	1.4814×10^{-5}	3,777.4854	35,124.8639
18,003	17,415	18,610	20	9.7983	3,951.5440	38,718.3884	3.4102×10^{-4}	1.3476	2.5306×10^{-4}	1.4814×10^{-5}	3,733.0136	36,577.1630
18,054	17,319	18,838	20	9.8011	2,462.4715	24,134.9854	2.4454×10^{-4}	0.6021	4.0606×10^{-4}	1.4814×10^{-5}	2,375.8019	23,285.5256
17,832	17,253	18,429	20	9.7887	4,002.9713	39,184.0851	7.8459×10^{-4}	3.1407	2.4981×10^{-4}	1.4814×10^{-5}	3,778.8770	36,990.4821
19,135	18,459	19,839	20	9.8593	3,348.3532	33,012.3333	0.0018	6.0519	2.9865×10^{-4}	1.4814×10^{-5}	3,190.1109	31,452.1790
19,269	18,722	20,834	20	9.8663	1,523.4349	15,030.5935	0.0024	3.7317	6.5641×10^{-4}	1.4814×10^{-5}	1,489.8115	14,698.8572
				Total	19,066.4726	187,170.9957	0.0056	14.8831			18,145.1003	178,129.0708

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The data entered into the column in the tables indicated by number:

(1) the potency estimate,
(2) the lower limit of the estimated potency,
(3) the upper limit of the estimated potency,
(4) the number of degrees of freedom of their error variances,
(5) the natural logarithm of the potency, M,

Given statistic values for the next steps:

From Table 73 with 20 df of error, $\left(\frac{\alpha}{2} = 0.025\right)$, t = 2.0860

(6) the compute of W,

$$W = \frac{4t^2}{(\ln U - \ln L)^2}$$

for the potency estimate 18,054,

$$W = \frac{4(2.0860)^2}{(\ln 18{,}838 - \ln 17{,}319)^2}$$

$$= 2{,}462.4715$$

(7) the compute of WM for the potency estimate

for the ln potency 9.8011,

$$WM = (2{,}462.4715)(9.8011)$$

$$= 24{,}134.9854$$

(8) the compute of $W(M - \overline{M})^2$ for the potency estimate 18,054

$$\overline{M} = \frac{\Sigma WM}{\Sigma W}$$

$$= \frac{187{,}170.9957}{19{,}066.4726}$$

$$= 9.8168$$

$$W(M - \overline{M})^2 = (2{,}462.4715)(9.8011 - 9.8168)^2$$

$$= 0.6021$$

(9) the intra-assay variation S_M^2,

$$S_M^2 = \frac{1}{W}$$

for the potency estimate 18,054,

$$S_M^2 = \frac{1}{2{,}462.4715}$$

$$= 4.0606 \times 10^{-4}$$

Given statistic value for the next steps:

$$n' = 20$$

(10) the inter-assay variation $S_{\overline{M}}^2$

$$S_{\overline{M}}^2 = \frac{\Sigma(M - \overline{M})^2}{n'(n' - 1)}$$

$$= \frac{0.0056}{20(20 - 1)}$$

$$= 1.4814 \times 10^{-5}$$

(11) the compute of W',

$$W' = S\frac{1}{S_M^2 + S_{\overline{M}}^2}$$

$$= \frac{1}{(4.0606 \times 10^{-4})^2 + (1.4814 \times 10^{-5})^2}$$

$$= 2{,}375.8019$$

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(12) the compute of W′M for the potency estimate 18054,

$$W'M = (2{,}375.8019)(9.8011)$$
$$= 23{,}285.5256$$

Test for homogeneity of potency estimate

By squaring the deviation of each value of M from the weighted mean, multiplying by the appropriate weight and summing overall assays, a statistic is obtained which is approximately distributed as χ^2 and which may be used to test the homogeneity of a set of ln potency estimates.

$$\chi^2 = \Sigma W(M - \overline{M})^2$$
$$= 0.0091 + 1.3476 + 0.6021 + 3.1407 + 6.0519 + 3.7317$$
$$= 14.8831$$

The calculated χ^2 is greater than the tabulated value corresponding to 5 degrees of freedom (11.070): the potencies are heterogeneous.

$$\overline{M}' = \frac{\Sigma W'M}{\Sigma W'} = \frac{178{,}129.0708}{18{,}145.1003}$$
$$= 9.8169$$
$$S_{\overline{M}'} = \sqrt{\frac{1}{\Sigma W'}} = \sqrt{\frac{1}{18{,}145.1003}}$$
$$= 0.0074$$
$$\ln M_L = \overline{M}' - tS_{\overline{M}'}$$
$$= 9.8169 - \left((2.0860)(0.0074)\right)$$
$$= 9.8022$$
$$\ln M_U = \overline{M}' + tS_{\overline{M}'}$$
$$= 9.8169 + \left((2.0860)(0.0074)\right)$$
$$= 9.8316$$
$$\text{Potency} = \text{antiln}(\overline{M}')$$
$$= \text{antiln}(9.8169)$$
$$= 18{,}341.5450$$
$$M_L = \text{antiln}(M_L)$$
$$= \text{antiln}(9.8022)$$
$$= 18{,}073.9252$$
$$M_U = \text{antiln}(M_U)$$
$$= \text{antiln}(9.8316)$$
$$= 18{,}613.1275$$

Thus, the potency is estimated to be 18,341 IU/vial with 95 per cent confidence limits from 18,073 to 18,613 IU/vial.

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Example 16 Unweighted Combination of Assay Results

Six independent potency estimates of the sample preparation are described in Table 71. In Table 72, the results are computerized but only four decimal digits, without rounding off, are displayed.

Table 71 Potency Estimates and Confidence Intervals of Six Independent Assays

Potency Estimate (IU/vial)	Lower Limit (L) (IU/vial)	Upper Limit (U) (IU/vial)	Degrees of Freedom
18,367	17,755	19,002	20
18,003	17,415	18,610	20
18,054	17,319	18,838	20
17,832	17,253	18,429	20
18,635	17,959	19,339	20
18,269	17,722	18,834	20

Table 72 Unweighted Mean of Individual Assay Results

(1)	(2)	(3)	(4)	(5)	(6)
Potency Estimate	Lower Limit (L)	Upper Limit (U)	df	ln potency (M)	$(M - M)^2$
18,367	17,755	19,002	20	9.8183	9.2257×10^{-5}
18,003	17,415	18,610	20	9.7983	1.0841×10^{-4}
18,054	17,319	18,838	20	9.8011	5.7505×10^{-5}
17,832	17,253	18,429	20	9.7887	3.9823×10^{-4}
18,635	17,959	19,339	20	9.8328	5.8037×10^{-4}
18,269	17,722	18,834	20	9.8130	1.8106×10^{-5}

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The data entered into the column in the tables indicated by number:

(1) the potency estimate,
(2) the lower limit of the estimated potency,
(3) the upper limit of the estimated potency,
(4) the number of degrees of freedom of their error variances,
(5) the natural logarithm of the potency, M,

Given statistic values for the next steps:

$$n' = 6$$

(6) the compute of $(M - \overline{M})^2$ for the potency estimate 18,367,

$$\overline{M} = \frac{\Sigma M}{n'}$$

$$= \frac{9.8183 + 9.7983 + 9.8011 + 9.7887 + 9.8328 + 9.8130}{6}$$

$$= 9.8087$$

$$(M - \overline{M})^2 = (9.8183 - 9.8087)^2$$

$$= 9.2257 \times 10^{-5}$$

Calculation of the unweighted mean and confidence limits:

$$S_{\overline{M}} = \sqrt{\frac{\Sigma(M - \overline{M})^2}{n'(n' - 1)}}$$

$$= \sqrt{\frac{0.0012}{6(6 - 1)}}$$

$$S = 0.0064$$

Given statistic values for the next steps:

From Table 73 with 5 df of error, $\left(\frac{\alpha}{2} = 0.025\right)$, $t = 2.5706$

$$M_L = \overline{M} - tS_{\overline{M}}$$

$$= 9.8087 - (2.5706)(0.0064)$$

$$= 9.7920$$

$$M_U = \overline{M} + tS_{\overline{M}}$$

$$= 9.8087 + (2.5706)(0.0064)$$

$$= 9.8253$$

$$\text{Potency} = \text{antiln}(\overline{M})$$

$$= \text{antiln}(9.8087)$$

$$= 18{,}191.4282$$

$$M_L = \text{antiln}(M_L)$$

$$= \text{antiln}(9.7920)$$

$$= 17{,}891.4868$$

$$M_U = \text{antiln}(M_U)$$

$$= \text{antiln}(9.8253)$$

$$= 18{,}496.3979$$

Thus, the potency is estimated to be 18,191 IU/vial with 95 per cent confidence limits from 17,891 to 18,496 IU/vial.

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5. Table

The tables in this section list the critical values for the most frequently occurring numbers of degrees of freedom. If a critical value is not listed, reference should be made to more extensive tables. Many computer programs include statistical functions and their use is recommended instead of the tables in this section. Alternatively, the generating procedures given below each table can be used to compute the probability corresponding to a given statistic and number of degrees of freedom.

Table 73 Upper Percentiles of the t-Distribution

df	Area to the Right	
	0.05	0.025
1	6.3138	12.7062
2	2.9200	4.3027
3	2.3534	3.1824
4	2.1318	2.7764
5	2.0150	2.5706
6	1.9432	2.4469
7	1.8946	2.3646
8	1.8595	2.3060
9	1.8331	2.2622
10	1.8125	2.2281
11	1.7959	2.2010
12	1.7823	2.1788
13	1.7709	2.1604
14	1.7613	2.1448
15	1.7531	2.1314
16	1.7459	2.1199
17	1.7396	2.1098
18	1.7341	2.1009
19	1.7291	2.0930
20	1.7247	2.0860
21	1.7207	2.0796
22	1.7171	2.0739
23	1.7139	2.0687
24	1.7109	2.0639
25	1.7081	2.0595
26	1.7056	2.0555
27	1.7033	2.0518
28	1.7011	2.0484
29	1.6991	2.0452
30	1.6973	2.0423
31	1.6955	2.0395
32	1.6939	2.0369
33	1.6924	2.0345
34	1.6909	2.0322
35	1.6896	2.0301
36	1.6883	2.0281
37	1.6871	2.0262
38	1.6860	2.0244
39	1.6849	2.0227
40	1.6839	2.0211
41	1.6829	2.0195
42	1.6820	2.0181
43	1.6811	2.0167
44	1.6802	2.0154
45	1.6794	2.0141
46	1.6787	2.0129
47	1.6779	2.0117
48	1.6772	2.0106
49	1.6766	2.0096
50	1.6759	2.0086
51	1.6753	2.0076
52	1.6747	2.0066
53	1.6741	2.0057
54	1.6736	2.0049
55	1.6730	2.0040
56	1.6725	2.0032
57	1.6720	2.0025
58	1.6716	2.0017
59	1.6711	2.0010
60	1.6706	2.0003
120	1.6577	1.9799
∞	1.6450	1.9600

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Table 74 Percentage Points of the Chi-Square Distribution* (χ^2)

df	Probability (P)	
	0.01	0.05
1	6.63	3.84
2	9.21	5.99
3	11.34	7.81
4	13.28	9.49
5	15.09	11.07
6	16.81	12.59
7	18.48	14.07
8	20.09	15.51
9	21.67	16.92
10	23.21	18.31
11	24.72	19.68
12	26.22	21.03
13	27.69	22.36
14	29.14	23.68
15	30.58	25.00
16	32.00	26.30
17	33.41	27.59
18	34.81	28.87
19	36.19	30.14
20	37.57	31.41
21	38.93	32.67
22	40.29	33.92
23	41.64	35.17
24	42.98	36.42
25	44.31	37.65

*P. Armitage, *Statistical Methods in Medical Research* (1st ed.), Oxford: Blackwell Scientific Publications, 1971, pp. 460-461.

Table 75 Percentage Points of the F Distribution*

Degrees of Freedom. for Denominator (f_2)	Degrees of Freedom, for Number (f_1)										
	1	2	3	4	5	6	7	8	12	24	∞
12	4.75	3.89	3.49	3.26	3.11	3.00	2.91	2.85	2.69	2.51	2.30
	9.33	6.93	5.95	5.41	5.06	4.82	4.64	4.50	4.16	3.78	3.36
14	4.60	3.74	3.34	3.11	2.96	2.85	2.76	2.70	2.53	2.35	2.13
	8.86	6.51	5.56	5.04	4.69	4.46	4.28	4.14	3.80	3.43	3.00
16	4.49	3.63	3.24	3.01	2.85	2.74	2.66	2.59	2.42	2.24	2.01
	8.53	6.23	5.29	4.77	4.44	4.20	4.03	3.89	3.55	3.18	2.75
18	4.41	3.55	3.16	2.93	2.77	2.66	2.58	2.51	2.34	2.15	1.92
	8.29	6.01	5.09	4.58	4.25	4.01	3.84	3.71	3.37	3.00	2.57
20	4.35	3.49	3.10	2.87	2.71	2.60	2.51	2.45	2.28	2.08	1.84
	8.10	5.85	4.94	4.43	4.10	3.87	3.70	3.56	3.23	2.86	2.42
30	4.17	3.32	2.92	2.69	2.53	2.42	2.33	2.27	2.09	1.89	1.62
	7.56	5.39	4.51	4.02	3.70	3.47	3.30	3.17	2.84	2.47	2.01
40	4.08	3.23	2.84	2.61	2.45	2.34	2.25	2.18	2.00	1.79	1.51
	7.31	5.18	4.31	3.83	3.51	3.29	3.12	2.99	2.66	2.29	1.80
60	4.00	3.15	2.76	2.53	2.37	2.25	2.17	2.10	1.92	1.70	1.39
	7.08	4.98	4.13	3.65	3.34	3.12	2.95	2.82	2.50	2.12	1.60
120	3.92	3.07	2.68	2.45	2.29	2.17	2.09	2.02	1.83	1.61	1.25
	6.85	4.79	3.95	3.48	3.17	2.96	2.79	2.66	2.34	1.95	1.38
∞	3.84	3.00	2.60	2.37	2.21	2.10	2.01	1.94	1.75	1.52	1.00
	6.63	4.61	3.78	3.32	3.02	2.80	2.64	2.51	2.18	1.79	1.00

The upper and the lower values correspond to p = 0.05 and p = 0.01, respectively.

*P. Armitage, *Statistical Methods in Medical Research* (1st ed.), Oxford: Blackwell Scientific Publications, 1971, pp. 466-467.

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Table 76 The Φ – Distribution (the Cumulative Standard Normal Distribution)

x	Φ	x	Φ	x	Φ
0.00	0.500	1.00	0.841	2.00	0.977
0.05	0.520	1.05	0.853	2.05	0.980
0.10	0.540	1.10	0.864	2.10	0.982
0.15	0.560	1.15	0.875	2.15	0.984
0.20	0.579	1.20	0.885	2.20	0.986
0.25	0.599	1.25	0.894	2.25	0.988
0.30	0.618	1.30	0.903	2.30	0.989
0.35	0.637	1.35	0.911	2.35	0.991
0.40	0.655	1.40	0.919	2.40	0.992
0.45	0.674	1.45	0.926	2.45	0.993
0.50	0.691	1.50	0.933	2.50	0.994
0.55	0.709	1.55	0.939	2.55	0.995
0.60	0.726	1.60	0.945	2.60	0.995
0.65	0.742	1.65	0.951	2.65	0.996
0.70	0.758	1.70	0.955	2.70	0.997
0.75	0.773	1.75	0.960	2.75	0.997
0.80	0.788	1.80	0.964	2.80	0.997
0.85	0.802	1.85	0.968	2.85	0.998
0.90	0.816	1.90	0.971	2.90	0.998
0.95	0.829	1.95	0.974	2.95	0.998

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6. Glossary of Symbols

SYMBOL	DEFINITION
a	Intersection of linear regression of responses on dose or ln (dose)
b	Estimate of slope of regression line of responses on dose or on ln (dose) based on all preparations in the assay
d	Number of dose levels for each preparation (excluding the blank in slope ratio assay)
df	Degrees of freedom
e	Base of natural logarithms (= 2.71828182845905...)
h	Number of preparations in an assay, including the standard preparation
n	Number of replicates for each treatment or number of blocks
p-value	Probability of a given statistic being larger than the observed value. Also used as the ratio r/n in probit analysis
r	The number of responding units per treatment group in assays depending upon quantal responses
s	Estimate of standard deviation ($=\sqrt{s^2}$)
s^2	Estimate of variance given by mean square of residual error in analysis of variance
s_i	Dose level of i^{th} standard preparation (i = 1, ..., d)
s_M	Estimate of standard deviation of log potency (M)
$s_{\overline{M}}$	The standard error of the mean potency
t	Student's t-statistic
u	The ln (dose)-response function of four-parameter model
u_i, z_i	Dose level of i^{th} unknown preparations U and Z (i = 1, ..., d)
y	Individual response or transformed response
$F_{(cal)}$	Calculated variance ratio
$F_{(tab)}$	Percentage Points of the F Distribution
L	Sum of linear contrasts
M	Estimate of In potency ratio
$\overline{M}$	The logarithm of the weighted mean potency.
N	Total number of responses in an assay (= hdn)
Q	Quadratic curvature sum of squares from analysis of variance
R	Estimated potency ratio of a given test preparation
S	Total response to a standard preparation
U, Z	Total responses to test preparations *U* and *Z*
W	Weighting factor in combination of assay results
Y	Vector representing the (transformed) responses in general linear models
Z	The first derivative of Φ
α	Level of significance, the given probability of a false rejection of the true null hypothesis in a statistical test.
α′	Upper asymptote of the ln (dose)-response curve in four-parameter analysis
¶	3.14159265358979323 8...
Φ	Cumulative standard normal distribution function
χ^2	Percentage Points of the Chi-square Distribution

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APPENDIX 10 MICROBIOLOGICAL TESTS

10.1 STERILITY TEST

The test is designed to reveal the presence, if any, of contamination with viable micro-organisms in pharmaceutical or medical articles intended for parenteral administration or for other sterile applications, which, according to the Pharmacopoeia, are required to be sterile. A satisfactory negative result, however, only indicates that no contaminating micro-organism has been found in the sample examined in the conditions of the test. Nevertheless, because the sample to be tested is randomly selected from the particular batch it represents, it is thereafter assumed that the whole batch passes the sterility test, which is at present the only method available to the various authorities who have to examine a product for sterility.

Alternative procedures or procedural details may be employed to demonstrate that an article is sterile, provided the results obtained are at least of equivalent reliability. Where a difference appears, or in the event of a dispute, when evidence of microbial contamination is obtained by the procedure given in this Pharmacopoeia, the result so obtained is conclusive of failure of the article to meet the requirements of the test.

Test Conditions

Adventitious microbial growth that is transmitted to an article or to inoculated test culture media from the environment during the course of a sterility test invalidates the results of the test. Hence, it is necessary to demonstrate that the proper precautions have been taken to exclude extraneous micro-organisms throughout the test period.

The test should be carried out under aseptic conditions in an area as free from contamination as possible by the use of disinfecting agents, ultraviolet lamps and air filters. Ultraviolet lamps and disinfecting aerosols should not be used during actual testing operation. The test manipulations should be carried out in a clean room (class 10,000) under a laminar flow hood, with operators dressed in sterilized, static-free clothing, including head- and foot-wears. The air pressure in the testing room should be more than that of the exterior area. The performance of the laminar flow hood should be monitored by particulate count, settle plates, or slit-sampling devices, and the performance of the filters and ultraviolet lamps checked routinely. Regular, simultaneous control test with known sterile preparations are also advisable.

Culture Media

The culture media used for sterility tests for bacteria and fungi should be capable of supporting the growth of a wide variety of micro-organisms, with both aerobic and anaerobic growth characteristics, including the types found in the environment of the manufacturing operations. More than one culture medium will generally be needed to fulfil these criteria.

The following culture media have been found suitable for the test for Sterility. Fluid thioglycolate medium is intended primarily for the culture of anaerobic bacteria but will also sustain the growth of aerobic bacteria. Soybean-casein digest medium is intended primarily for the culture of aerobic bacteria but will also sustain the growth of fungi.

A. Preparation

Culture media for the tests may be prepared as described below, or dehydrated mixtures yielding similar formulations may be used, provided that, when reconstituted as directed by the manufacturer or distributor, they have growth-promoting properties equal or superior to those obtained from the formulae given herein. Media are sterilized in an autoclave using a validated process.

I. FLUID THIOGLYCOLATE MEDIUM (FLUID MERCAPTO ACETATE MEDIUM)

L-Cystine	0.5	g
Sodium chloride	2.5	g
Dextrose monohydrate	5.5	g
Agar, granulated (moisture content not in excess of 15 per cent)	0.75	g
Yeast extract (water-soluble)	5.0	g
Pancreatic digest of casein	15.0	g
Sodium thioglycolate (or thioglycolic acid 0.3 ml)	0.5	g
Resazurin sodium Solution (0.01 per cent w/v, freshly prepared)	1.0	ml
Water	1000	ml

Mix and heat until solution is effected. Adjust the pH of the solution with *sodium hydroxide TS* so that, after sterilization, it will have a pH of 7.1±0.2. Filter while hot through a filter paper, if necessary. Transfer the medium to suitable containers that provide a ratio surface to depth of medium such that not more than the upper half of the medium has undergone a colour change indicative of oxygen uptake at the end of the incubation period, and sterilize as directed above. If more than the upper one-third of the medium has a pink colour, the medium may be restored once by heating the containers until the pink colour disappears. When ready for use, not more than the upper one-third of the medium in a container should have a pink colour.

Use Fluid Thioglycolate Medium by incubating it under aerobic conditions.

II. ALTERNATIVE FLUID THIOGLYCOLATE MEDIUM (for devices having tubes with small lumina)

L-Cystine	0.5	g
Sodium chloride	2.5	g
Dextrose monohydrate	5.5	g
Yeast extract (water-soluble)	5.0	g
Pancreatic digest of casein	15.0	g
Sodium thioglycolate (or thioglycolic acid 0.3 ml)	0.5	g
Water	1000	ml

Mix and heat until solution is effected. Adjust the pH of the solution with *sodium hydroxide TS* so that, after sterilization, it will have a pH of 7.1±0.2. Filter, if necessary. Place in suitable vessels, and sterilize by steam under pressure (see Steam Sterilization under "Sterilization and Sterility Assurance" (Appendix 12). The medium is freshly prepared or heated on a steam-bath and allowed to cool just prior to use. Do not reheat.

Use Alternative Thioglycolate Medium in a manner that will assure anaerobic conditions for the duration of the incubation period.

III. SOYBEAN-CASEIN DIGEST MEDIUM

Pancreatic digest of casein	17.0	g
Papaic digest of soybean meal	3.0	g
Sodium chloride	5.0	g
Dipotassium hydrogenphosphate	2.5	g
Dextrose monohydrate	2.5	g
Water	1000	ml

Dissolve the solids with *water*, heating slightly to effect a solution. Cool the solution to room temperature, and adjust the pH with *sodium hydroxide VS* so that, after sterilization, it will have a pH of 7.3±0.2. Filter, if necessary, and dispense into suitable containers. Sterilize as directed above or by a validated filtration process. Incubate under aerobic conditions.

B. Properties and suitability

All the media to be used must comply with the following tests, carried out on each batch before or in parallel with the test on the article being examined.

(1) STERILITY Incubate portions of the media intended mainly for the detection of bacteria at 30° to 35° and those intended mainly for the detection of fungi at 20° to 25° for not less than 14 days or by incubating uninoculated containers as negative controls during a sterility test procedure. No growth of micro-organisms occurs.

(2) GROWTH PROMOTION Inoculate duplicate test containers of each medium with 10 to 100 viable micro-organisms listed in Table 1, and incubate according to the conditions specified for it. The test media are satisfactory if evidence of growth appears within 5 days. This test can be conducted simultaneously with the use of the media for sterility test purposes. However, the sterility test is considered invalid if the sterility of the media or this growth promotion test is not successful.

(3) VALIDATION TESTS FOR BACTERIOSTASIS AND FUNGISTASIS This validation is performed when the test for sterility has to be carried our on a new product or whenever there is a change in the experimental conditions of the test. The validation may be performed simultaneously with the test for sterility of the product to be examined, but before the results of this test are being interpreted. The procedures are as follows:

Membrane Filtration Method: Filter the test sample and rinse the membrane with minimum of three 100-ml portions of the appropriate rinsing fluid. Inoculate the final rinse with less than 100 colony-forming units, for each appropriate micro-organism specified in Table 1. Repeat the rinse procedure on another filter that has not been exposed to the specimen under test. This filter will serve as the positive control. Incubate these filters for not more than 7 days under the condition indicated in Table 1 and compare the growth.

If the growth of each test organism in the test containers is visually comparable to the growth in the positive control, use the same amounts of article, number and volume of rinses, and medium when conducting the sterility test. If the growth of the test organisms in the test containers is not visually comparable to that in the positive control, the amount of article used is bacteriostatic or fungistatic. Repeat the test, using a larger number of rinses. Changes in the type of membrane filter used and in the use of neutralizing agents, if

Table 1 Test Micro-organisms for Growth Promotion and the Validation Tests

Medium	Test Micro-organisms	Incubation Conditions	Incubation Temperature
Fluid Thioglycolate Medium	*Staphylococcus aureus* (ATCC[1] 6538, DMST[2] 8013)*	Aerobic	30° to 35°
	Pseudomonas aeruginosa (ATCC 9027, DMST 15501)**		
	Clostridium sporogenes (ATCC 11437, DMST 15536)†		
Alternative Thioglycolate Medium	*Clostridium sporogenes* (ATCC 11437, DMST 15536)†	Anaerobic	30° to 35°
Soybean-Casein Digest Medium	*Bacillus subtilis* (ATCC 6633, DMST 5871)	Aerobic	20° to 25°
	Candida albicans (ATCC 10231, DMST 5815)		
	Aspergillus niger (ATCC 16404, DMST 15538)		

*An alternative strain is *Bacillus subtilis* (ATCC 6633, DMST 5871).

**An alternative strain is *Micrococcus luteus* (ATCC 9341, DMST 15503).

†An alternative strain is *Bacteroides vulgatus* (ATCC 8482, DMST 15535).

[1]ATCC = American Type Culture Collection

[2]DMST = Department of Medical Sciences, Thailand

available, may reduce the antimicrobial effect of the article. If five rinses, each of about 500 ml, fail to neutralize the antimicrobial residue on the test filter membrane, proceed with sterility test.

Direct Inoculation Method: Inoculate two containers of each sterility test medium with less than 100 colony-forming units, using the volume of medium (see Tablet 3) for each appropriate micro-organism specified in Table 1. Add the specified portion of the article under test to one of the inoculated containers of each medium. The other inoculated container is the positive control. Repeat the procedure for each appropriate micro-organism, and incubate the containers at the appropriate temperature for not more than 7 days.

If the growth of the test organisms in the test container is not visually comparable to that of the inoculated control container, the article is bacteriostatic or fungistatic. The use of a sterile neutralizing agent, such as polysorbate 80, lecithin, azolectin, or β-lactamase, may be appropriate. If a neutralizing agent is not effective, establish suitable increased volumes of medium. Use the smallest volume of medium in which the growth of test micro-organisms in the presence of the article is not adversely affected. If the medium volume is increased to 2000 ml and antimicrobial activity is still present, proceed with the sterility test using the 2000 ml of medium. Volumes of medium more than 2000 ml may be needed for testing medical devices to permit complete immersion of the device.

Sampling of Test Samples

Unless otherwise directed in the individual monographs, test the number of articles specified in Table 2. If the contents of each article are of sufficient quantity (see Tables 3 and 4), they may be divided so that equal appropriate portions are added to each of the specified media. If each article does not contain sufficient quantities for each medium, use twice the number of articles indicated in Table 2.

Opening the containers

Cleanse the exterior surfaces of ampoules and closures of vials and bottles with a suitable decontaminating agent, and gain access to the contents in suitable aseptic manner. If the vial contents are packaged under vacuum, admit sterile air by means of a suitable sterile device, such as a needle attached to a syringe barrel filled with nonabsorbent cotton.

For purified cotton, gauze, surgical dressings, and related Pharmacopoeial articles, open the package or container aseptically.

Quantity of Article

When using the Membrane Filtration Method, unless otherwise specified elsewhere in this Appendix or in the individual monograph, use whenever possible the entire contents of each container, but not less than the quantities specified in Tables 3 and 4. When using the Direct Inoculation Method, use the quantities indicated in Tables 3 and 4.

Incubation

Unless otherwise directed in the individual monograph, incubate the test mixture for 14 days with Fluid thioglycolate medium or Alternative thioglycolate medium, where so indicated, at 30° to 35°, and with Soybean-casein digest medium at 20° to 25°. For products terminally sterilized by a validated moist heat process, incubate the test sample for not less than 7 days, if the Membrane Filtration Method is used.

Table 2 Minimum Number of Articles to Be Tested in Relation to the Number of Articles in the Batch

Number of Articles in the Batch	Number of Articles to Be Tested
Injections/for Injections	
Not more than 100 articles	10 per cent or 4 articles, whichever is greater
More than 100 but not more than 500 articles	10 articles
More than 500 articles	2 per cent or 20 articles, whichever is less
For large-volume parenterals	2 per cent or 10 containers, whichever is less
Antibiotic Solids	
• pharmacy bulk packages (<5 g)	20 containers
• pharmacy bulk packages (≥5 g)	6 containers
• bulks and blends	See *Solid Bulk Products*
Products Not Intended for Injection	
Not more than 200 articles	5 per cent or 2 articles, whichever is greater
More than 200 articles	10 articles
Devices	
Not more than 100 articles	10 per cent or 4 articles, whichever is greater
More than 100 but not more than 500 articles	10 articles
More than 500 articles	2 per cent or 20 articles, whichever is less
Solid Bulk Products	
Up to 4 containers	Each container
More than 4 but not more than 50 containers	20 per cent or 4 containers, whichever is greater
More than 50 containers	2 per cent or 10 containers, whichever is greater

Table 3 Quantities of Article for Liquid Products*

Container Content (ml)	Minimum Volume Taken from Each Product Container for Each Medium	Minimum Volume, in ml, of Each Medium	
		Used for Direct Inoculation of Volume Taken from Each Container**	Used for Membrane or Half Membrane Representing Total Required Volume from the Appropriate Number of Containers
Less than 10	1 ml, or entire contents if less than 1 ml	15	100
10 to less than 50	5 ml	40	100
50 to less than 100	10 ml	80	100
50 to less than 100, intended for intravenous administration	1/2 content	200	100
100 to 500	1/2 content	NA***	100
Over 500	500 ml	NA	100
Antibiotics (liquid)	1 ml	NA	100

*Constitute powder products according to the manufacturer's instructions, and then treat as liquid products.

**For products that cannot be tested by the membrane filtration test procedure.

***Not applicable.

Table 4 Quantities of Article for Solid Products

Container Content	Minimum Quantity Taken from Each Container for Each Medium	Minimum Volume, in ml, of Each Medium	
		Direct Inoculation*	Membrane Filtration
Less than 50 mg	Whole content	200	100
50 mg or more to less than 200 mg	Half the content	200	100
200 to less than 300 mg	100 mg	200	100
300 to 600 mg	200 mg	200	100
More than 600 mg	200 mg	200	100
Antibiotic solids			
for injection (<5 g)	150 mg	200	100
for injection,	500 mg	200	100
pharmacy bulk packages (≥5 g)		200	100
bulks and blends	See Table 2		
Surgical dressings, cotton, gauze (in packages)	100-mg portion	200	NA***
Sutures and other individually packaged single-use materials	Whole devices	Not more than 2000	NA
Other medical devices	Whole devices (Cut in pieces or disassembled)	Not more than 2000**	NA

*For products that cannot be tested by the membrane filtration test procedure.

**Unless the device is bulky and more than 2000 ml is needed to submerge the device in the medium.

***Not applicable

Test for Sterility of the Article

The test may be carried out using either Method I, Membrane Filtration, or Method II, Direct Inoculation, as described below, taking into account any modifications described in the appropriate section. Method I is to be preferred whenever the nature of the article to be examined permits, that is, for filterable liquids and for those miscible with, or soluble in, aqueous or oily solvents that do not have an antimicrobial effect under the conditions of the test.

Method I: Membrane Filtration The sterility testing of the articles should be performed when feasible by membrane filtration of the test substances. This procedure is applicable in the sterility testing of non-bacteriostatic or non-fungistatic liquids or soluble powders, and is particularly appropriate where the article is an oil, an ointment, or a cream that can be put into solution with non-bacteriostatic or non-fungistatic dilution fluids or solvents. The membrane filtration technique is suitable also in the sterility testing of liquids and soluble powders that possess inherent bacteriostatic or fungistatic properties. Certain devices also may be appropriately tested for sterility of the critical pathways by the membrane filtration technique.

Strict aseptic precautions are needed in the manipulations of the tests; the frequent use of negative controls is highly recommended.

Apparatus A suitable unit consists of a closed reservoir and a receptacle between which a properly supported membrane or membranes of appropriate porosity is (are) placed. A membrane generally suitable for sterility testing has a nominal porosity of not more than 0.45 µm, and a diameter of approximately 47 mm. Cellulose nitrate filters, for example, are used for aqueous, oily and weakly alcoholic solutions and cellulose acetate filters, for example, for strongly alcoholic solutions. The membranes having hydrophobic edges or low product binding characteristics that minimize inhibitory product residue may be needed for certain products, e.g. for antibiotics. The apparatus must be so designed that solution to be examined can be introduced and filtered under aseptic conditions and it must permit the removal of the membrane for transfer to the culture medium or be suitable for carrying out the incubation after adding the culture medium to the apparatus itself. The entire unit may be assembled and sterilized with the membrane(s) in place prior to use in the test, or the membranes may be sterilized separately by steam under pressure, or by any method that yields proper performance.

Where the article to be tested is an oil, sterilize the membrane separately, and after thorough drying, assemble the unit, using aseptic precautions.

Dissolving, dilution and rinsing fluids The following fluids are to be used for dissolving, diluting or rinsing articles under tests for sterility. They must be sterile and do not have antibacterial or antifungal properties.

FLUID A Dissolve 1 g of either peptic digest of animal tissue or dried meat peptone in *water* to make 1000 ml, filter or centrifuge to clarify, if necessary. Adjust to a pH of 7.1±0.2, dispense into containers, and sterilize in an autoclave using a validated process.

FLUID B Prepare Fluid B by adding 1 g of *polysorbate 80* to each litre of Fluid A. Adjust to pH 7.1±0.2, dispense into flasks, and sterilize in an autoclave using a validated process.

FLUID C Dissolve 5 g of either peptic digest of animal tissue or dried meat peptone, 3 g of beef extract and 10 g of *polysorbate 80* in *water* to make 1000 ml, filter or centrifuge to clarify, if necessary. Adjust to a pH of 6.9±0.2, dispense into flasks, and sterilize in an autoclave using a validated process.

FLUID D Adjust, if necessary, the pH of *isopropyl myristate* to be used as Fluid D to not less than 5.5, and sterilize by filtration through a 0.22-µm membrane filter. Fluid D must also be free from antimicrobial properties.

Generally, Fluid A is used for dissolving water-soluble solids, for dilution before filtration the liquid article miscible with aqueous vehicles, and for washing the membrane(s) by filtering through the latter thereafter. If the article under test contains lecithin or oil, substitute Fluid B for Fluid A.

Fluid C is used for rinsing or washing of the filtration membrane(s), in case the article under test contains petrolatum.

Fluid D is used to dissolve or dilute ointments and oils soluble in isopropyl myristate.

Procedure Either one or two filtering units may be used, and, after filtration, the membrane half, or the whole membrane, is transferred to each of the medium used.

(A) LIQUIDS Aseptically transfer a small quantity (sufficient to moisten the membrane) of a suitable, sterile diluting fluid (Fluid A or Fluid B) onto the membrane and filter. Remove liquids from test containers of the article being examined with a sterile pipette or with a sterile syringe and needle. For each medium to be used, transfer to a membrane not less than the quantity that is prescribed in Table 3, if necessary after diluting to about 100 ml with a suitable sterile diluting fluid. Filter immediately. (If the article is a viscous liquid or suspension not adaptable to rapid filtration, aseptically add a sufficient quantity of diluting fluid to the pooled sample to increase the flow rate.) In case the liquid being tested has antimicrobial properties, or contains a preservative, use *Fluid A*, or *Fluid B* and proceed as directed for Membrane Filtration Method under *Validation Tests for*

Bacteriostasis and Fungistasis, but exclude inoculation of the final rinse with challenge organisms.

Either transfer a membrane to each of the culture media used, or transfer each medium onto a membrane in the apparatus, and seal the apparatus so that the medium remains on the membrane.

Alternatively, transfer the combined quantity of the article being examined for both media to the membrane, diluting if necessary, filtering and washing as above. Aseptically remove and cut the membrane into two approximately equal parts and transfer one of them to each medium used.

Incubate the media for not less than 14 days unless otherwise prescribed in the monograph, at 30° to 35° in the test intended to detect bacteria and at 20° to 25° in the test intended to detect fungi. In some cases, where the liquid is highly viscous and not readily filterable through one or two membranes, more than two filter assemblies may be needed. In such cases, half the number of membranes used are incubated in each medium, provided that the volumes and requirements for numbers of containers per medium are complied with.

(B) OILS AND OILY SOLUTIONS For each medium, use not less than the quantity of the article being examined, that is prescribed in Table 3, if necessary after diluting to about 100 ml with Fluid D. Oils or oily solutions of sufficiently low viscosity may be filtered, with or without dilution, through a dry membrane. Viscous oils may be diluted as necessary with Fluid D. Allow the oil to penetrate the membrane and filter, applying pressure or suction gradually. Wash the membrane by filtering through it at least two successive quantities, each of approximately 200 ml, of Fluid B, and then wash with 100 ml of Fluid A. Complete the test described under Liquids, except that the sterility test medium to be used contains 1 g of *polysorbate 80* per litre.

(C) SOLUBLE SOLIDS For each medium, dissolve not less than the quantity of the article being examined that is prescribed in Table 4 in a suitable sterile fluid such as Fluid A and carry out the test described under Liquids using a membrane or membranes appropriate to the chosen fluid.

(D) OINTMENTS AND CREAMS For each medium, dissolve not less than 100 mg form each of not less than 20 containers in at least 100 ml of Fluid D, warming if necessary, to not more than 40°. In exceptional cases it may be necessary to heat to not more than 45°. Use warm solutions for washing. Filter as rapidly as possible and complete the test as described under Oils and Oily Solutions.

If the article under test contains petrolatum, use Fluid C in place of Fluid D for washing and moisten the membrane(s) with approximately 200 µl of Fluid C before the filtration operation begins, and keep the membrane(s) covered with liquid throughout the filtration operation for maximum efficiency of the filter. Following filtration of the specimen, wash the membrane(s) with three 100-ml portions of Fluid C. Treat the test membrane(s) as directed above.

(E) DEVICES Devices that are required to contain sterile pathways may be tested for sterility by the membrane filtration technique as follows.

Aseptically pass a sufficient volume of Fluid B through each device tested so that not less than 100 ml is recovered from each device. Collect the fluids in sterile containers, and filter the entire volume collected through membrane(s) as described under Liquids.

Method II: Direct Inoculation For each medium, use the quantity of the article being examined that is prescribed in Table 2. Eliminate any antimicrobial properties as previously described for *Direct Inoculation Method* under *Validation Tests for Bacteriostasis and Fungistasis*. Transfer the article directly into the culture medium so that volume of the product is not more than 10 per cent of the volume of the medium, unless otherwise prescribed. (A larger volume may be required if antimicrobial properties are eliminated by dilution.) For those liquid articles where it is necessary to use a large volume of the article being examined, it may be preferable to use a concentrated culture medium prepared in such a way that it takes account of the subsequent dilution. In appropriate cases the concentrated medium may be added directly to the article in its container.

(A) LIQUIDS Unless otherwise directed in the individual monograph, test 20 units of the article with each medium. Sterility tests are applied to individual discrete units or to composites of such units.

For liquid articles from each unit, use not less than the volumes of article and medium specified in Table 3. If the contents are of sufficient quantity, they may be divided so that portions are added to the two specified media.

Remove liquids from test containers with a sterile pipette or with a sterile syringe and needle. Aseptically transfer the specified volume of the material from each test container to a vessel of culture medium. Mix the liquid with the medium, but do not aerate excessively. Incubate in the specified media for not less than 14 days.

Where the material being tested renders the medium turbid, so that the presence or absence of microbial growth cannot be determined readily by visual examination, transfer suitable portions of the medium to fresh vessels of the same medium between the third and seventh days after the test is started. Continue incubation of the original and of the transfer vessels for not less than 7 additional days after the transfer and for a total of not less than 14 days.

(B) OILY LIQUIDS For oily liquids use media to which have been added 1 per cent w/v of polysorbate 80 or another suitable emulsifying agent in an appropriate concentration, shown not to have antimicrobial properties under the conditions of the test. Proceed as directed under Liquids.

Aerobic cultures containing oily liquids should be shaken gently each day during the incubation period.

(C) OINTMENTS AND OILS INSOLUBLE IN ISOPROPYL MYRISTATE Select 20 containers, assign them to two groups of 10 containers, and treat each group as follows. Aseptically transfer 100 mg from each of the 10 containers to a flask containing 200 ml of a sterile, aqueous vehicle capable of dispersing the test material homogeneously throughout the fluid mixture. (The choice of dispersing agent incorporated in the aqueous vehicle may differ according to the nature of the ointment or oil. Before use, test the dispersing agent to ascertain that in the concentration used it has no significant antimicrobial effect during the time interval for all transfers, using the test procedures set forth in *Direct Inoculation Method* under *Validation Tests for Bacteriostasis and Fungistasis*.) Mix 20 ml of the fluid mixture so obtained with 200 ml of medium, and proceed as directed under Liquids.

(D) INSOLUBLE SOLIDS Transfer a quantity of the product in the form of a dry solid (or prepare a suspension of the product by adding sterile diluent to the immediate container), corresponding to not less than the quantity indicated in Table 4. Transfer the material so obtained to 200 ml of Fluid thioglycolate medium, and mix. Similarly, transfer the same quantity to 200 ml of Soybean-casein digest medium, and mix. Proceed as directed under Liquids.

(E) COTTON, GAUZE, SURGICAL DRESSINGS, AND RELATED ARTICLES From each package of cotton, rolled gauze, or gauze bandage being tested, remove with sterile instruments two or more portions of 100 to 500 mg each from the innermost part of the sample. From individually packaged single-use materials such as gauze pads, remove a single portion of 250 to 500 mg or the entire article in the case of small, i.e. 25- by 75-mm or smaller, adhesive absorbent bandages.

Aseptically transfer these portions of the article to the similar number of containers of each medium, and incubate them as described above.

(F) SUTURES Place five containers of the sutures being examined in a suitable antimicrobial solution containing *crystal violet* or another suitable dye, for not less than 3 hours. Remove with sterile forceps, and if the containers show no evidence of leakage, hold under aseptic conditions prior to testing. Open the containers aseptically, and with sterile instruments transfer sutures to separate containers of appropriate media. Incubate them as described above for not less than 14 days.

Carry out also the test for the presence of antimicrobial activity in the suture being examined and ensure the neutralization of any inhibitory effects which, for catgut and other sutures, may be due, in part, to the methods of sterilization used or to the constituents of the tubing fluid.

(G) STERILIZED DEVICES Articles can be immersed intact or disassembled. To ensure that device pathways are also in contact with the media, immerse the appropriate number of units per medium in a volume of medium sufficient to immerse the device completely, and incubate them as described above for not less than 14 days.

For catheters where the inside lumen and outside are required to be sterile, either cut them into pieces such that the medium is in contact with the entire lumen or fill the lumen with medium, and then immerse the intact unit.

For extremely large devices, immerse those portions of the device that are to come into contact with the patient in a volume of medium sufficient to achieve complete immersion of those portions.

Observation and Interpretation of Results

At intervals during the incubation period and at its conclusion, examine the contents of all of the vessels for macroscopic evidence of microbial growth, such as the development of turbidity. If no evidence of growth is found, the material tested meets the requirements of the test for sterility. If evidence of microbial growth is found, and confirmed microscopically, the material test fails to meet the requirements of the test for sterility, unless it can be demonstreated that microbial growth observed in the test was due to inadequate aseptic sampling and testing technique rather than to intrinsic contamination of the article, the test is invalid and must be repeated. If microbial growth is not observed, the material tested meets the requirements of the sterility test. If microbial growth is observed and confimed microscopically, the material tested does not meet the requirements of the sterility test.

10.2 MICROBIAL LIMIT TESTS

Introduction

The hazard of microbiological contamination in non-sterile pharmaceuticals has been well realized, especially in those products of vegetable, animal and mineral origins and in those which lack good manufacturing practices (GMP). Microbiological attributes of non-sterile pharmaceutical products are described in Appendix 10.4.

This appendix, therefore, comprises two parts of tests. Part I allows quantitative enumeration of mesophilic bacteria and fungi that may grow under aerobic condition and Part II allows determination of the absence or limited occurrence of specified micro-organisms that may be detected under the conditions described.

The tests are designed primarily to determine whether a substance or preparation complies with an established specification for microbiological quality.

When used for such purposes, follow the instructions given below, including the number of samples to be taken, and interpret the results as stated below.

The methods are not applicable to products containing viable micro-organisms as active ingredients.

Alternative microbiological procedures, including automated methods, may be used, provided that their equivalence to the Pharmacopoeial method has been demonstrated.

Acceptance criteria for microbiological quality of non-sterile pharmaceuticals are given in the table under "Limits for Microbial Contamination" (Appendix 10.5).

In the following, the term "micro-organisms" is covering bacteria and fungi only; the term "pharmaceuticals" means pharmaceutical products of any kind, from raw materials to the finished forms; the term "growth" is used to designate the presence and presumed proliferation of micro-organisms.

Part I Microbial Enumeration Tests

PROCEDURE

In preparing for and in applying the tests, precautions are taken so as to avoid the accidental microbial contamination of the product to be examined, as well as the inadvertent suppression of the growth of any micro-organisms that are to be revealed in the test.

If the product to be examined has antimicrobial activity, this is insofar as possible removed or neutralized. If inactivators are used for this purpose, their efficacy and their absence of toxicity for micro-organisms must be demonstrated.

If surface-active substances are used for sample preparation, their absence of toxicity for micro-organisms and their compatibility with inactivators used must be demonstrated.

ENUMERATION METHODS

Use the membrane filtration method or the plate-count method, as prescribed. The most probable number (MPN) method is generally the least accurate method for microbial counts; however, for certain product groups with a very low bioburden, it may be the most appropriate method. The choice of method is based on factors such as the nature of the product and the required limit of micro-organisms. The chosen method must allow testing of a sufficient sample size to judge compliance with the specification. The suitability of the method chosen must be established.

GROWTH PROMOTION TEST, SUITABILITY OF THE COUNTING METHOD AND NEGATIVE CONTROLS

The ability of the test to detect micro-organisms in the presence of product to be tested must be established. Suitability must be confirmed if a change in testing performance, or the product, which may affect the outcome of the test is introduced.

Preparation of test strains

Use standardized stable suspensions of test strains or prepare them as stated below. Seed lot culture maintenance techniques (seed-lot systems) are used so that the viable micro-organisms used for inoculation are not more than five passages removed from the original master seed-lot. Grow each of the bacterial and fungal test strains separately as described in Table 1.

Use Buffered sodium chloride-peptone solution pH 7.0 or Phosphate buffer pH 7.2 to make test suspensions; to suspend *Aspergillus niger* spores, 0.05 per cent of *polysorbate 80* may be added to the buffer. Use the suspensions within 2 hours or within 24 hours if stored at 2° to 8°. As an alternative to preparing and then diluting a fresh suspension of vegetative cells of *A. niger* or *Bacillus subtilis*, a stable spore suspension is prepared and then an appropriate volume of the spore suspension is used for test inoculation. The stable spore suspension may be maintained at 2° to 8° for a validated period of time.

Negative control

To verify testing conditions, a negative control is performed using the chosen diluent in place of the test preparation. There must be no growth of micro-organisms. A negative control is also performed when testing the products as described in Testing of Products. A failed negative control requires an investigation.

Growth promotion of the media

Test each batch of ready-prepared medium and each batch of medium, prepared either from dehydrated medium or from the ingredients described.

Inoculate portions/plates of Soybean-casein digest broth and Soybean-casein digest agar with a small number (not more than 100 CFU) of the micro-organisms indicated in Table 1, using a separate portion/plate of medium for each. Inoculate plates of Sabouraud dextrose agar with a small number (not more than 100 CFU) of the micro-organisms indicated in Table 1, using a separate plate of medium for each. Incubate in the conditions described in Table 1.

For solid media, growth obtained must not differ by a factor greater than 2 from the calculated value for a standardized inoculum. For a freshly prepared inoculum, growth of the micro-organisms comparable to that previously obtained with a previously tested and approved batch of medium occurs. Liquid media are suitable if clearly visible growth of the micro-organisms comparable to that previously obtained with a previously tested and approved batch of medium occurs.

Suitability of the counting method in the presence of product

PREPARATION OF THE SAMPLE The method for sample preparation depends upon the physical characteristics of the product to be tested. If none of the procedures described below can be demonstrated to be satisfactory, an alternative procedure must be developed.

Water-soluble products Dissolve or dilute (usually a 1 in 10 dilution is prepared) the product to be examined in Buffered sodium chloride-peptone solution pH 7.0, Phosphate buffer solution pH 7.2 or Soybean-casein digest broth. If necessary, adjust to pH 6 to 8. Further dilutions, where necessary, are prepared with the same diluent.

Table 1 Preparation and Use of Test Micro-organisms

Micro-organism*	Preparation of Test Strain	Growth Promotion		Suitability of Counting Method in the Presence of the Product	
		Total Aerobic Microbial Count (TAMC)	Total Yeasts and Moulds Count (TYMC)	Total Aerobic Microbial Count (TAMC)	Total Yeasts and Moulds Count(TYMC)
Staphylococcus aureus such as: ATCC 6538 DMST 8013 NCIMB 9518 C.I.P. 4.83 NBRC 13276	Soybean-casein digest agar or Soybean-casein digest broth 30° to 35° 18 to 24 hours	Soybean-casein digest agar or Soybean-casein digest broth ≤100 CFU 30° to 35° ≤3 days		Soybean-casein digest agar / MPN Soybean-casein digest broth ≤100 CFU 30° to 35° ≤3 days	
Pseudomonas aeruginosa such as: ATCC 9027 DMST 15501 NCIMB 8626 C.I.P. 82.118 NBRC 13275	Soybean-casein digest agar or Soybean-casein digest broth 30° to 35° 18 to 24 hours	Soybean-casein digest agar or Soybean-casein digest broth ≤100 CFU 30° to 35° ≤3 days		Soybean-casein digest agar / MPN Soybean-casein digest broth ≤100 CFU 30° to 35° ≤3 days	
Bacillus subtilis such as: ATCC 6633 DMST 15896 NCIMB 8054 C.I.P. 52.62 NBRC 3134	Soybean-casein digest agar or Soybean-casein digest broth 30° to 35° 18 to 24 hours	Soybean-casein digest agar or Soybean-casein digest broth ≤100 CFU 30° to 35° ≤3 days		Soybean-casein digest agar / MPN Soybean-casein digest broth ≤100 CFU 30° to 35° ≤3 days	
Candida albicans such as: ATCC 10231 DMST 5815 NCPF 3179 I.P. 48.72 NBRC 1594	Sabouraud dextrose agar or Sabouraud dextrose broth 20° to 25° 2 to 3 days	Soybean-casein digest agar ≤100 CFU 30° to 35° ≤5 days	Sabouraud dextrose agar ≤100 CFU 20° to 25° ≤5 days	Soybean-casein digest agar ≤100 CFU 30° to 35° ≤5 days MPN: not applicable	Sabouraud dextrose agar ≤100 CFU 20° to 25° ≤5 days
Aspergillus niger such as: ATCC 16404 DMST 15538 IMI 149007 I.P. 1431.83 NBRC 9455	Sabouraud dextrose agar or Potato dextrose agar 20° to 25° 5 to 7 days, or until good sporulation is achieved	Soybean-casein digest agar ≤100 CFU 30° to 35° ≤5 days	Sabouraud dextrose agar ≤100 CFU 20° to 25° ≤5 days	Soybean-casein digest agar ≤100 CFU 30° to 35° ≤5 days MPN: not applicable	Sabouraud dextrose agar ≤100 CFU 20° to 25° ≤5 days

*ATCC = American Type Culture Collection, USA; DMST = Department of Medical Sciences, Thailand; NCIMB = National Collection of Industrial and Marine Bactéria Ltd., Great Britain; C.I.P. = Collection de Bactéries de l'Institut Pasteur, France; NBRC = Biological Resource Center, Japan; NCPF = National Collection of Pathogenic Fungi, London School of Hygiene and Tropical Medicine, Great Britain; I.P. = Collection Nationale de Culture de Micro-organismes (C.N.C.M.) Institut Pasteur, France; IMI = International Mycological Institute, Great Britain

ลิขสิทธิ์ กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข
สำเนาหน้านี้จัดทำเพื่อการศึกษาเท่านั้น ห้ามใช้ แก้ไข ดัดแปลง โดยไม่ได้รับอนุญาต

Non-fatty products insoluble in water Suspend the product to be examined (usually a 1 in 10 dilution is prepared) in Buffered sodium chloride-peptone solution pH 7.0, Phosphate buffer solution pH 7.2 or Soybean-casein digest broth. A surface-active agent such as 0.1 per cent w/v of *polysorbate 80* may be added to assist the suspension of poorly wettable substances. If necessary, adjust to pH 6 to 8. Further dilutions, where necessary, are prepared with the same diluent.

Fatty products Dissolve in *isopropyl myristate*, sterilized by filtration or mix the product to be examined with the minimum necessary quantity of sterile polysorbate 80 or another non-inhibitory sterile surface-active agent, heated if necessary to not more than 40°, or in exceptional cases to not more than 45°. Mix carefully and if necessary maintain the temperature in a water-bath. Add sufficient of the pre-warmed chosen diluent to make a 1 in 10 dilution of the original sample. Mix carefully while maintaining the temperature for the shortest time necessary for the formation of an emulsion. Further serial tenfold dilutions may be prepared using the chosen diluent containing a suitable concentration of sterile polysorbate 80 or another non-inhibitory sterile surface-active agent.

Fluids or solids in aerosol form Chill the container(s) for approximately 1 hour, cut open the container(s), and allow to reach room temperature, permitting the propellant to escape, or warming to drive off the propellant if feasible. Aseptically transfer the product into a membrane filter apparatus or a sterile container for further sampling. Use either the total contents or a defined number of metered doses from each of the containers tested.

Transdermal patches Remove the protective cover sheets ("release liners") of the transdermal patches and place them, adhesive side upwards, on sterile glass or plastic trays. Cover the adhesive surface with a sterile porous material, for example sterile gauze, to prevent the patches from sticking together, and transfer the patches to a suitable volume of the chosen diluent containing inactivators such as polysorbate 80 and/or lecithin. Shake the preparation vigorously for at least 30 minutes.

INOCULATION AND DILUTION Add to the sample prepared as described in Preparation of the Sample and to a control (with no test material included) a sufficient volume of the microbial suspension to obtain an inoculum of not more than 100 CFU. The volume of the suspension of the inoculum should not exceed 1 per cent of the volume of diluted product.

To demonstrate acceptable microbial recovery from the product, the lowest possible dilution factor of the prepared sample must be used for the test. Where this is not possible due to antimicrobial activity or poor solubility, further appropriate protocols must be developed. If inhibition of growth by the sample cannot otherwise be avoided, the aliquot of the microbial suspension may be added after neutralization, dilution or filtration.

NEUTRALIZATION/REMOVAL OF ANTIMICROBIAL ACTIVITY The number of micro-organisms recovered from the prepared sample diluted as described in Inoculation and Dilution and incubated following the procedure described in Recovery of Micro-organism in the Presence of Product, is compared to the number of micro-organisms recovered from the control preparation.

If growth is inhibited (reduction by a factor greater than 2), then modify the procedure for the particular enumeration test to ensure the validity of the results. Modification of the procedure may include, for example, (1) an increase in the volume of the diluent or culture medium, (2) incorporation of specific or general neutralizing agents into the diluent such as Casein digest-soy lecithin polysorbate 20 broth, (3) membrane filtration, or (4) a combination of the above measures.

Neutralizing agents Neutralizing agents may be used to neutralize the activity of antimicrobial agents (Table 2). They may be added to the chosen diluent or the medium preferably before sterilization. If used, their efficacy and their absence of toxicity for micro-organisms must be demonstrated by carrying out a blank with the neutralizer and without the product.

Table 2 Common Neutralizing Agents for Interfering Substances

Interfering Substance	Potential Neutralizing Method
Glutaraldehyde, mercurials	Sodium hydrogensulfite (sodium bisulfite)
Phenolics, ethanol, aldehydes, sorbate	Dilution
Aldehydes	Glycine
Quaternary Ammonium Compounds (QACs), parahydroxy benzoates (parabens), bisbiguanides	Lecithin
QACs, iodine, parabens	Polysorbate
Mercurials	Thioglycolate
Mercurials, halogens, aldehydes	Thiosulfate
EDTA (edetate)	Mg^{2+} or Ca^{2+} ions

If no suitable neutralizing method can be found, it can be assumed that the failure to isolate the inoculated organism is attributable to the microbicidal activity of the product. This information serves to indicate that the product is not likely to be contaminated with the given species of the micro-organism. However, it is possible that the product only inhibits some of the micro-organisms specified herein, but does not inhibit others not included amongst the test strains or for which the latter are not representative. Then, perform the test with the highest dilution factor compatible with microbial growth and the specific acceptance criterion.

RECOVERY OF MICRO-ORGANISMS IN THE PRESENCE OF PRODUCT For each of the micro-organisms listed, separate tests are performed. Only micro-organisms of the added test strain are counted.

Membrane filtration Use membrane filters having a nominal pore size not greater than 0.45 μm. The type of filter material is chosen such that the bacteria-retaining efficiency is not affected by the components of the sample to be investigated. For each of the micro-organisms listed, one membrane filter is used.

Transfer a suitable amount of the sample prepared as described under Preparation of the Sample, under Inoculation and Dilution, and under Neutralization/Removal of Antimicrobial Activity (preferably representing 1 g of the sample, or less if large numbers of CFU are expected) to the membrane filter, filter immediately and rinse the membrane filter with an appropriate volume of diluent.

For the determination of total aerobic microbial count (TAMC), transfer the membrane filter to the surface of Soybean-casein digest agar. For the determination of total combined yeasts and moulds count (TYMC), transfer the membrane to the surface of Sabouraud dextrose agar. Incubate the plates as indicated in Table 1. Perform the counting.

Plate-count methods Perform plate-count methods at least in duplicate for each medium and use the mean count of the result.

Pour-plate method For Petri dishes 9 cm in diameter, add to the dish 1 ml of the sample prepared as described under Preparation of the Sample, under Inoculation and Dilution, and under Neutralization Removal of Antimicrobial Activity and 15 to 20 ml of Soybean-casein digest agar or Sabouraud dextrose agar, both media being at not more than 45°. If larger Petri dishes are used, the amount of agar medium is increased accordingly. For each of the micro-organisms listed in Table 1, at least two Petri dishes are used. Incubate the plates as indicated in Table 1. Take the arithmetic mean of the counts per medium and calculate the number of CFU in the original inoculum.

Surface-spread method For Petri dishes 9 cm in diameter, add 15 to 20 ml of Soybean-casein digest agar or Sabouraud dextrose agar at about 45° to each Petri dish and allow to solidify. If larger Petri dishes are used, the volume of the agar is increased accordingly. Dry the plates, for example in a laminar-air-flow cabinet or an incubator. For each of the micro-organisms listed in Table 1, at least two Petri dishes are used. Spread a measured volume of not less than 0.1 ml of the sample prepared as described under Preparation of the Sample, under Inoculation and Dilution, and under Neutralization/Removal of Antimicrobial Activity over the surface of the medium. Incubate and count as prescribed under Pour-plate Method.

Most probable number (MPN) method The precision and accuracy of the MPN method is less than that of the membrane filtration method or the plate-count method. Unreliable results are obtained particularly for the enumeration of moulds. For these reason the MPN method is reserved for the enumeration of TAMC in situations where no other method is available. If the use of the method is justified, proceed as follows.

Prepare a series of at least three serial tenfold dilutions of the product as described under Preparation of the Sample, under Inoculation and Dilution, and under Neutralization/Removal of Antimicrobial Activity. From each level of dilution, three aliquots of 1 g or 1 ml are used to inoculate three tubes with 9 to 10 ml of Soybean-casein digest broth. If necessary, a surface-active agent such as polysorbate 80 or an inactivator of antimicrobial agents may be added to the medium. Thus, if three levels of dilution are prepared, nine tubes are inoculated.

Incubate all tubes at 30° to 35° for not more than 3 days. If reading of the results is difficult or uncertain owing to the nature of the product to be examined, subculture in the same broth, or in Soybean-casein digest agar, for 1 to 2 days at the same temperature and use these results. Determine the most probable number of micro-organisms per g or per ml of the product to be examined from Table 3.

Results and interpretation

When verifying the suitability of the membrane filtration method or the plate-count method, a mean count of any of the test organisms not differing by a factor greater than 2 from the value of the control defined in Inoculation and Dilution in the absence of the product must be obtained. When verifying the suitability of the MPN method the calculated value from the inoculum must be within 95 per cent confidence limits of the results obtained with the control.

If the above criteria cannot be met for one or more of the organisms tested with any of the described methods, the method and test conditions that come closest to the criteria are used to test the product.

TESTING OF PRODUCTS

Amount used for the test

Unless otherwise prescribed, use 10 g or 10 ml of the product to be examined taken with the precautions referred to above. For fluids or solids in aerosol form, sample 10 containers. For transdermal patches, sample 10 patches.

The amount to be tested may be reduced for active substances that will be formulated in the following conditions: the amount per dosage unit (e.g., tablet, capsule, injection) is less than or equal to 1 mg or the amount per g or per ml (for preparations not presented in dosage units) is less than 1 mg. In these cases, the amount to be tested is not less than the amount present in 10 dosage units or 10 g or 10 ml of the product.

For materials used as active substances where sample quantity is limited or batch size is extremely small (i.e., less than 1000 ml or 1000 g), the amount tested shall be 1 per cent of the batch unless a lesser amount is prescribed or justified and authorized.

For products where the total number of entities in a batch is less than 200 (e.g., samples used in clinical trials), the sample size may be reduced to 2 units, or 1 unit if the size is less than 100.

Table 3 Most Probable Number (MPN) Values of Micro-organisms

Observed Combinations of Numbers of Tubes Showing Growth in Each Set			MPN per g or per ml of Product	95 Per Cent Confidence Limits
Number of Grams or Millilitres of Product per Tube				
0.1	0.01	0.001		
0	0	0	<3	0-9.4
0	0	1	3	0.1-9.5
0	1	0	3	0.1-10
0	1	1	6.1	1.2-17
0	2	0	6.2	1.2-17
0	3	0	9.4	3.5-35
1	0	0	3.6	0.2-17
1	0	1	7.2	1.2-17
1	0	2	11	4-35
1	1	0	7.4	1.3-20
1	1	1	11	4-35
1	2	0	11	4-35
1	2	1	15	5-38
1	3	0	16	5-38
2	0	0	9.2	1.5-35
2	0	1	14	4-35
2	0	2	20	5-38
2	1	0	15	4-38
2	1	1	20	5-38
2	1	2	27	9-94
2	2	0	21	5-40
2	2	1	28	9-94
2	2	2	35	9-94
2	3	0	29	9-94
2	3	1	36	9-94
3	0	0	23	5-94
3	0	1	38	9-104
3	0	2	64	16-181
3	1	0	43	9-181
3	1	1	75	17-199
3	1	2	120	30-360
3	1	3	160	30-380
3	2	0	93	18-360
3	2	1	150	30-380
3	2	2	210	30-400
3	2	3	290	90-990
3	3	0	240	40-990
3	3	1	460	90-1980
3	3	2	1100	200-4000
3	3	3	>1100	

ลิขสิทธิ์ กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข
ห้ามทำซ้ำ ดัดแปลง แก้ไข โดยไม่ได้รับอนุญาต

Select the sample(s) at random from the bulk material or from the available containers of the preparation. To obtain the required quantity, mix the contents of a sufficient number of containers to provide the sample

Examination of the product

MEMBRANE FILTRATION Use a filtration apparatus designed to allow the transfer of the filter to the medium. Prepare the sample using a method that has been shown suitable as described in Growth Promotion Test, Suitability of the Counting Method and Negative Controls. Transfer the appropriate amount to each of two membrane filters and filter immediately. Wash each filter following the procedure shown to be suitable.

For the determination of TAMC, transfer one of the membrane filters to the surface of Soybean-casein digest agar. For the determination of TYMC, transfer the other membrane to the surface of Sabouraud dextrose agar. Incubate the plate of Soybean-casein digest agar at 30° to 35° for 3 to 5 days and the plate of Sabouraud dextrose agar at 20° to 25° for 5 to 7 days. Calculate the number or CFU per g or per ml of product.

When examining transdermal patches, filter 10 per cent of the volume of the preparation described under Preparation of the Sample separately through each of two sterile filter membranes. Transfer one membrane to Soybean-casein digest agar for TAMC and the other membrane to Sabouraud dextrose agar for TYMC.

PLATE-COUNT METHODS

Pour-plate method Prepare the sample using a method that has been shown to be suitable as described in Growth Promotion Test, Suitability of the Counting Method and Negative Controls. Prepare for each medium at least two Petri dishes for each level of dilution. Incubate the plates of Soybean-casein digest agar at 30° to 35° for 3 to 5 days and the plates of Sabouraud dextrose agar at 20° to 25° for 5 to 7 days. Select the plates corresponding to a given dilution and showing the highest number of colonies less than 250 for TAMC and 50 for TYMC. Take the arithmetic mean per culture medium of the counts and calculate the number of CFU per g or per ml of product.

Surface-spread method Prepare the sample using a method that has been shown to be suitable as described in Growth Promotion Test, Suitability of the Counting Method and Negative Controls. Prepare at least two Petri dishes for each medium and each level of dilution. For incubation and calculation of the number of CFU proceed as described for the pour-plate method.

MOST PROBABLE NUMBER METHOD

Prepare and dilute the sample using a method that has been shown to be suitable as described in Growth Promotion Test, Suitability of the Counting Method and Negative Controls. Incubate all tubes at 30° to 35° for 3 to 5 days. Subculture if necessary, using the procedure shown to be suitable. Record for each level of dilution the number of tubes showing microbial growth. Determine the most probable number of micro-organisms per g or per ml of the product to be examined from Table 3.

Interpretation of the results

The total aerobic microbial count (TAMC) is considered to be equal to the number of CFU found using Soybean-casein digest agar; if colonies of fungi are detected on this medium, they are counted as part of the TAMC. The total combined yeasts and moulds count (TYMC) is considered to be equal to the number of CFU found using Sabouraud dextrose agar; if colonies of bacteria are detected on this medium, they are counted as part of the TYMC. When the TYMC is expected to exceed the acceptance criterion due to the bacterial growth, Sabouraud dextrose agar with antibiotics may be used. If the count is carried out by the MPN method the calculated value is the TAMC.

The recommended solutions and media are described in Part II.

The limits prescribed in the "Limits for Microbial Contamination" (Appendix 10.5) are the maximum acceptable limits.

Part II Test for Specified Micro-organisms

PROCEDURE

The preparation of the samples is carried out as described in Part I.

If the product to be examined has antimicrobial activity, this is insofar as possible removed or neutralized as described in Part I.

If surface-active substances are used for sample preparation, their absence of toxicity for micro-organisms and their compatibility with inactivators used must be demonstrated as described in Part I.

GROWTH-PROMOTING AND INHIBITORY PROPERTIES OF THE MEDIA, SUITABILITY OF THE TEST AND NEGATIVE CONTROLS

The ability of the test to detect micro-organisms in the presence of the product to be tested must be established. Suitability must be confirmed if a change in testing performance, or the product, which may affect the outcome of the test is introduced.

Preparation of test strains

Use standardized stable suspensions of test strains or prepare them as stated below. Seed lot culture maintenance techniques (seed-lot systems) are used so that the viable micro-organisms used for inoculation are not more than five passages removed from the original master seed-lot.

AEROBIC MICRO-ORGANISMS Grow each of the bacterial test strains separately in Soybean-casein digest broth or on Soybean-casein digest agar at 30° to 35° for 18 to 24 hours. Grow the test strain for *Candida albicans* separately on Sabouraud dextrose agar or in Sabouraud dextrose broth at 20° to 25° for 2 to 3 days.

– *Staphylococcus aureus* such as ATCC 6538, DMST 8013, NCIMB 9518, C.I.P. 4.83 or NBRC 13276;

– *Pseudomonas aeruginosa* such as ATCC 9027, DMST 15501, NCIMB 8626, C.I.P. 82.118 or NBRC 13275;

– *Escherichia coli* such as ATCC 8739, DMST 15537, NCIMB 8545, C.I.P. 53.126 or NBRC 3972;

– *Salmonella enterica* subsp. *enterica* serovar Typhimurium, such as ATCC 14028, DMST 13311, or, as an alternative, *Salmonella enterica* subsp. *enterica* serovar Abony such as NCTC 6017, DMST 21863, C.I.P. 80.39, or NBRC 100797;

– *Candida albicans* such as ATCC 10231, DMST 5815, NCPF 3179, I.P. 48.72 or NBRC 1594.

Use Buffered sodium chloride-peptone solution pH 7.0 or Phosphate buffer pH 7.2 to make test suspensions. Use the suspensions within 2 hours or within 24 hours if stored at 2° to 8°.

CLOSTRIDIUM SPP. Use *Clostridium sporogenes* such as ATCC 11437 (DMST 15536, NCIMB 12343, C.I.P. 100651, NBRC 14293) or ATCC 19404 (DMST 15282, NCTC 532, C.I.P. 79.03). Grow the clostridial test strain under anaerobic conditions in Reinforced medium for clostridia at 30° to 35° for 24 to 48 hours. As an alternative to preparing and then diluting down a fresh suspension of vegetative cells of *Cl. sporogenes*, a stable spore suspension is used for test inoculation. The stable spore suspension may be maintained at 2° to 8° for a validated period.

Negative control

To verify testing conditions, a negative control is performed using the chosen diluent in place of the test preparation. There must be no growth of micro-organisms. A negative control is also performed when testing the products as described in Testing of Products. A failed negative control requires an investigation.

Growth promotion and inhibitory properties of the media

Test each batch of ready-prepared medium and each batch of medium prepared either from dehydrated medium or from ingredients.

Verify suitable properties of relevant media as described in Table 4.

TEST FOR GROWTH PROMOTING PROPERTIES, LIQUID MEDIA: inoculate a portion of the appropriate medium with a small number (not more than 100 CFU) of the appropriate micro-organism. Incubate at the specified temperature for not more than the shortest period of time specified in the test. Clearly visible growth of the micro-organism comparable to that previously obtained with a previously tested and approved batch of medium occurs.

TEST FOR GROWTH PROMOTING PROPERTIES, SOLID MEDIA: perform the surface-spread method, inoculating each plate with a small number (not more than 100 CFU) of the appropriate micro-organism. Incubate at the specified temperature for not more than the shortest period of time specified in the test. Growth of the micro-organism comparable to that previously obtained with a previously tested and approved batch of medium occurs.

TEST FOR INHIBITORY PROPERTIES, LIQUID OR SOLID MEDIA: inoculate the appropriate medium with at least 100 CFU of the appropriate micro-organism. Incubate at the specified temperature for not less than the longest period of time specified in the test. No growth of the test micro-organism occurs.

TEST FOR INDICATIVE PROPERTIES: perform the surface-spread method, inoculating each plate with a small number (not more than 100 CFU) of the appropriate micro-organism. Incubate at the specified temperature for a period of time within the range specified in the test. Colonies are comparable in appearance and indication reactions to those previously obtained with a previously tested and approved batch of medium.

Suitability of the test method

For each product to be tested, perform the sample preparation as described in the following paragraph in Testing of Products. Add each test strain at the time of mixing, in the prescribed growth medium. Inoculate the test strains individually. Use a number of micro-organisms equivalent to not more than 100 CFU in the inoculated test preparation. Perform the test as described in the following paragraph in Testing of Products using the shortest incubation period prescribed.

The specified micro-organisms must be detected with the indication reactions as described in Testing of Products.

Any antimicrobial activity of the sample necessitates a modification of the test procedure described in Neutralization/Removal of Antimicrobial Activity under Part I.

If for a given product the antimicrobial activity with respect to a micro-organism for which testing is prescribed cannot be neutralized, then it is to be assumed that the inhibited micro-organism will not be present in the product.

TESTING OF PRODUCTS

Bile-tolerant gram-negative bacteria

SAMPLE PREPARATION AND PRE-INCUBATION Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in Part I, but using Soybean-casein digest broth as the chosen diluent, mix and incubate at 20° to 25° for a time sufficient to resuscitate the bacteria but not sufficient to encourage multiplication of the organisms (usually 2 hours but not more than 5 hours).

TEST FOR ABSENCE Use the volume corresponding to 1 g of the product, as prepared in Sample Preparation and Pre-incubation, to inoculate Enterobacteria enrichment broth-Mossel. Incubate at 30° to 35° for 24 to 48 hours. Subculture on plates of Violet red bile dextrose agar. Incubate at 30° to 35° for 18 to 24 hours. The product passes the test if there is no growth of colonies of Gram-negative bacteria on any plate.

QUANTITATIVE TEST

Selection and subculture Inoculate suitable quantities of Enterobacteria enrichment broth-Mossel with the preparation as described under Sample Preparation and Pre-incubation and/or dilutions of it containing respectively 0.1 g (or 0.1 ml), 0.01 g (or 0.01 ml), and 0.001 g

Table 4 Growth Promoting, Inhibitory and Indicative Properties of Media

Test	Medium	Property	Test Strains
Test for bile-tolerant gram-negative bacteria	Enterobacteria enrichment broth-Mossel	Growth promoting Inhibitory	*E. coli* *P. aeruginosa* *S. aureus*
	Violet red bile dextrose agar	Growth promoting + indicative	*E. coli* *P. aeruginosa*
Test for *Escherichia coli*	MacConkey broth	Growth promoting Inhibitory	*E. coli* *S. aureus*
	MacConkey agar	Growth promoting + indicative	*E. coli*
Test for *Salmonella*	Rappaport-Vassiliadis broth Tetrathionate bile brilliant green broth	Growth promoting Inhibitory	*Salmonella enterica* subsp. *enterica* serovar Typhimurium or *Salmonella enterica* subsp. *enterica* serovar Abony *S. aureus*
	Xylose-lysine-deoxycholate agar Brilliant green agar Bismuth sulfite agar	Growth promoting + indicative	*Salmonella enterica* subsp. *enterica* serovar Typhimurium or *Salmonella enterica* subsp. *enterica* serovar Abony
Test for *Pseudomonas aeruginosa*	Cetrimide agar Pseudomonas for detection fluorescin Pseudomonas for detection pyocyanin	Growth promoting Inhibitory	*P. aeruginosa* *E. coli*
Test for *Staphylococcus aureus*	Mannitol salt agar Baird-Parker agar Vogel-Johnson agar	Growth promoting + indicative Inhibitory	*S. aureus* *E. coli*
Test for *Clostridium* spp.	Reinforced medium for Clostridia	Growth promoting	*Cl. sporogenes*
	Columbia agar Defibrinated sheep blood agar	Growth promoting	*Cl. sporogenes*
Test for *Candida albicans*	Sabouraud dextrose broth	Growth promoting	*C. albicans*
	Sabouraud dextrose agar	Growth promoting + indicative	*C. albicans*

ลิขสิทธิ์ กรมวิทยาศาสตรการแพทย์ กระทรวงสาธารณสุข
ห้ามทำซ้ำ ดัดแปลง แก้ไข โดยไม่ได้รับอนุญาต

(or 0.001 ml) of the sample to be examined. Incubate at 30° to 35° for 24 to 48 hours. Subculture each of the cultures on a plate of Violet red bile dextrose agar to obtain selective isolation. Incubate at 30° to 35° for 18 to 24 hours.

Interpretation Growth of well-developed colonies, generally red or reddish, of Gram-negative bacteria constitutes a positive result. Note the smallest quantity of the product that gives a positive result and the largest quantity that gives a negative result. Determine from Table 5 the probable number of bacteria.

Salmonella species

SAMPLE PREPARATION AND PRE-INCUBATION Prepare the product to be examined as described in Part I, and use the portion corresponding to not less than 10 g or 10 ml to inoculate a suitable amount (determined as described under Suitability of the Test Method) of Soybean-casein digest broth, mix and incubate at 30° to 35° for 18 to 24 hours.

SELECTION AND SUBCULTURE Separately transfer 0.1 ml and 1 ml of the enrichment culture to 10 ml of Rappaport-Vassiliadis broth and Tetrathionate bile brilliant green broth, respectively, mix and incubate at 30° to 35° for 18 to 24 hours. Subculture on plates of Xylose-lysine-deoxycholate agar, Brilliant green agar, and Bismuth sulfite agar. Cover and invert the dishes, and incubate at 30° to 35° for 18 to 48 hours.

INTERPRETATION Upon examination, if none of the colonies conforms to the description given in Table 6, the product meets the requirements of the test for absence of the genus Salmonella. If colonies of Gram-negative rods matching the description in Table 6 are found, proceed with further identification.

IDENTIFICATION Transfer representative suspect colonies individually, by means of an inoculating wire, to a butt-slant tube of Triple sugar-iron agar by first streaking the surface of the slant and then stabbing the wire well beneath the surface, and incubate. If the examination discloses no evidence of tubes having alkaline (red) slants and acid (yellow) butts (with or without concomitant blackening of the butt from hydrogen sulfide production), the product meets the requirements of the test for absence of the genus Salmonella. The presence of Salmonella may be confirmed by other suitable cultural or biochemical and serological tests, if necessary.

Table 5 Probable Number of Bacteria

Results for Each Quantity of Product			Probable Number of Bacteria per g or per ml of Product
0.1 g (or 0.1 ml)	0.01 g (or 0.01 ml)	0.001 g (or 0.001 ml)	
+	+	+	More than 10^3
+	+	–	Less than 10^3 and more than 10^2
+	–	–	Less than 10^2 and more than 10
–	–	–	Less than 10

Table 6 Morphology Characteristics of *Salmonella* Species on Selective Agar Media

Selective Medium	Characteristic Colonial Morphology
Xylose-lysine-deoxycholate agar	Red, with or without black centres
Brilliant green agar	Small, transparent, colourless or pink to white opaque (frequently surrounded by pink to red zone)
Bismuth sulfite agar	Black or green

Escherichia coli

SAMPLE PREPARATION AND PRE-INCUBATION Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in Part I, and use 10 ml or the portion corresponding to 1 g or 1 ml to inoculate a suitable amount (determined as described under Suitability of the Test Method) of Soybean-casein digest broth, mix and incubate at 30° to 35° for 18 to 24 hours.

SELECTION AND SUBCULTURE Transfer 1 ml of the enrichment culture to 100 ml of MacConkey broth and incubate at 42° to 44° for 24 to 48 hours. Subculture on plates of MacConkey agar and incubate at 30° to 35° for 18 to 72 hours.

INTERPRETATION Upon examination, if none of the colonies conforms to the description given in Table 7, the product meets the requirements of the test for absence of *Escherichia coli*. If colonies matching the description in Table 7 are found, proceed with further identification.

IDENTIFICATION Transfer the suspect colonies individually, making subculture the suspect colonies individually on plates of Levine eosin-methylene blue agar, and incubate at 30° to 35° for 18 to 24 hours.

Upon examination, if none of the colonies exhibits both a characteristic metallic sheen under reflected light and a blue-black appearance under transmitted light, the product meets the requirements of the test for absence of *Escherichia coli*. The presence of *Escherichia coli*

may be confirmed by suitable cultural and, if necessary, biochemical tests. Further serological test may be performed.

Staphylococcus aureus* and *Pseudomonas aeruginosa

SAMPLE PREPARATION AND PRE-INCUBATION Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in Part I, and use 10 ml or the portion corresponding to 1 g or 1 ml to inoculate a suitable amount (determined as described under Suitability of the Test Method) of Soybean-casein digest broth and mix. When testing transdermal patches, filter the volume of sample corresponding to one patch of the preparation described under Preparation of the Sample in Part I through a sterile filter membrane and place in 100 ml of Soybean-casein digest broth. Incubate at 30° to 35° for 18 to 24 hours.

SELECTION AND SUBCULTURE If growth is present, use an inoculating loop to streak a portion of the culture medium on the surface of Mannitol-salt agar, or Baird-Parker agar, or Vogel-Johnson agar and of Cetrimide agar, and incubate at 30° to 35° for 18 to 72 hours.

INTERPRETATION Upon examination, if none of the plates contains colonies having the characteristics listed in Tables 8 and 9 for the media used, the product meets the requirements for the absence of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. If colonies matching the description in Tables 8 and 9 are found, proceed with further identification.

IDENTIFICATION

Coagulase test (for *Staphylococcus aureus*) With the aid of an inoculating loop, transfer representative suspect colonies from the agar surfaces of the Mannitol-salt agar (or Baird-Parker agar or Vogel-Johnson agar) to individual tubes, each containing 0.5 ml of mammalian, preferably rabbit or horse, plasma with or without suitable additives. Incubate in a water-bath at 37°, examining the tubes at 3 hours and subsequently at suitable intervals up to 24 hours. Test positive and negative controls simultaneously with the unknown products. If no coagulation in any degree is observed, the product meets the requirements of the test for absence of *Staphylococcus aureus*.

Oxidase and pigment tests (for *Pseudomonas aeruginosa*) With the aid of an inoculating loop, streak representative suspect colonies from the agar surfaces of Cetrimide agar on the agar surface of Pseudomonas agar for detection of fluorescin and Pseudomonas agar for detection of pyocyanin contained in Petri dishes. Cover and invert the inoculated media, and incubate at

Table 7 Morphology Characteristics of *Escherichia coli* on MacConkey Agar

Gram Stain	Characteristic Colonial Morphology
Negative rods (cocco-bacilli)	Brick-red; may have surrounding zone of precipitated bile

Table 8 Morphology Characteristics of *Staphylococcus aureus* on Selective Agar Media

Selective Medium	Characteristic Colonial Morphology	Gram Stain
Mannitol-salt agar	Yellow colonies surrounded by yellow zone	Positive cocci (in clusters)
Baird-Parker agar	Black, shiny colonies surrounded by clear zones of 2 to 5 mm	Positive cocci (in clusters)
Vogel-Johnson agar	Black surrounded by yellow zones	Positive cocci (in clusters)

Table 9 Morphology and Diagnostic Characteristics of *Pseudomonas aeruginosa* on Selective Agar Media

Selective Medium	Characteristic Colonial Morphology	Fluorescence in UV Light	Oxidase Test	Gram Stain
Cetrimide agar	Generally greenish	Greenish	Positive	Negative rods
Pseudomonas agar for detection of fluorescin	Generally colourless to yellowish	Yellowish	Positive	Negative rods
Pseudomonas agar for detection of pyocyanin	Generally greenish	Blue	Positive	Negative rods

30° to 35° for not less than 3 days. Examine the streaked surfaces under UV light. Examine the plates to determine whether colonies having the characteristics listed in Table 9 are present.

Confirm any suspect colonial growth on one or more of the media as *Pseudomonas aeruginosa* by means of the oxidase test. Upon the colonial growth, place or transfer colonies to strips or discs of filter paper that previously has been impregnated with *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride. If there is no development of a pink colour, changing to purple, the product meets the requirements of the test for the absence of *Pseudomonas aeruginosa*. The presence of *Pseudomonas aeruginosa* may be confirmed by suitable cultural and, if necessary, biochemical tests.

Candida albicans

SAMPLE PREPARATION AND PRE-INCUBATION Prepare the product to be examined as described under Preparation of the Sample and use 10 ml or the portion corresponding to 1 g or 1 ml to inoculate a suitable amount (determined as described under Suitability of the Test Method) of Sabouraud dextrose broth and mix. Incubate at 30° to 35° for 3 to 5 days.

SELECTION AND SUBCULTURE Subculture on a plate of Sabouraud dextrose agar and incubate at 30° to 35° for 24 to 48 hours.

INTERPRETATION When growth of white colonies may indicate the presence of *Candida albicans* occurs, proceed with further identification.

IDENTIFICATION Transfer the suspect colonies individually, making subculture the suspect colonies individually on plates of a suitable selective medium[1].

Upon examination, the product passes the test if there is no growth of colonies of *Candida albicans* on any plate.

***Clostridium* spp.**

SAMPLE PREPARATION AND HEAT TREATMENT Prepare the product to be examined as described under Preparation of the Sample in Part I. Use two 10-ml portions each corresponding to 1 g or 1 ml of the product to be examined to inoculate a suitable amount (determined as described under Suitability of the Test Method) of Reinforced medium for clostridia. Heat one portion at 80° for 10 minutes and cool rapidly. Do not heat the other portion. Incubate both containers under anaerobic conditions at 30° to 35° for 48 hours.

SELECTION AND SUBCULTURE After incubation, make subcultures from each container on plates of Columbia agar to which gentamicin has been added and incubate under anaerobic conditions at 30° to 35° for 48 to 72 hours.

INTERPRETATION If no growth occurs, the product passes the test for absence of *Clostridium* spp. When growth of rods (with or without endospores) giving a negative catalase reaction occurs, subculture each distinct colony from on plates of Columbia agar, without gentamicin, and incubate at 30° to 35° for 48 to 72 hours, one plate anaerobically and the other aerobically, to check that the organism will not grow under aerobic condition.

Examine the appearance of only anaerobic growth of Gram-positive bacilli giving a negative catalase reaction together with the extent of hemolysis, by making subculture on a plate of Defibrinated sheep blood agar, and also examine microscopically for spore formation, using Gram stain or spore stain technique and confirmed by further suitable biochemical and biological tests. The description in Table 10 gives the characteristics of some *Clostridium* species on Defibrinated sheep blood agar.

Table 10 Characteristics of *Clostridium* Species on Defibrinated Sheep Blood Agar

Selective Species	Colonies	Hemolysis	Spores (staining)
Clostridium botulinum	Irregular, translucent with a granular surface and indefinited fimbriated spreading edge.	+	Oval, central, subterminal distend bacilli
Clostridium perfringens	Large, circular, convex, semitranslucent, smooth with an entire edge.	Double zone	Oval and subterminal (very rare)
Clostridium tetani	Transparent with long feathery spreading projections.	+	Spherical and terminal (drum stick)

Buffer Solution and Media

Culture media may be prepared as follows, or dehydrated culture media may be used if they have similar or comparable nutritive and selective properties for the micro-organisms to be tested for.

In preparing the media according to the formulae set forth herein, dissolve the soluble solids in the water, using heat, if necessary, to effect complete solution, and add other ingredients. Add, if necessary, a solution of hydrochloric acid or sodium hydroxide in quantities sufficient to yield the desired pH in the medium when it is ready for use. Determine the pH at 25°±2°.

Where agar is called for in a formula, use agar that has a moisture content of not more than 15 per cent.

Unless otherwise indicated, the buffer solution and media should be dispensed and sterilized by heating in an autoclave at 121°±2° for not less than 15 minutes, depending on the volume to be sterilized. Store under refrigeration.

[1]Biggy agar, CHROMagar Candida, or Candida isolation agar is recommended.

BUFFER SOLUTION

Stock buffer solution

Place 34 g of *potassium dihydrogenphosphate* in a 1000-ml volumetric flask, dissolve in 500 ml of *water*, adjust to pH 7.2±0.2 with *sodium hydroxide*, dilute to 1000.0 ml with *water* and mix. Dispense into containers and sterilize. Store at 2° to 8°.

Phosphate buffer pH 7.2

Prepare a mixture of 1 volume of stock buffer solution and 800 volumes of *water* and sterilize.

Buffered sodium chloride-peptone solution pH 7.0

Potassium dihydrogenphosphate	3.6	g
Disodium hydrogenphosphate dihydrate	7.2	g
Sodium chloride	4.30	g
Peptone, dried	1.0	g
Water	1000	ml

Polysorbate 20 or *80* may be added to obtain a 0.1 to 1.0 per cent w/v solution.

pH after sterilization: 7.0±0.1.

MEDIA

Baird-Parker agar

Pancreatic digest of casein	10.0	g
Beef extract	5.0	g
Yeast extract	1.0	g
Lithium chloride	5.0	g
Agar	20.0	g
Glycine	12.0	g
Sodium pyruvate	10.0	g
Water	950	ml

Heat with frequent agitation, and boil for 1 minute. Sterilize, cool to between 45° and 50°, and add 10 ml of a sterile, 1 per cent w/v solution of *potassium tellurate*(IV) and 50 ml of egg-yolk emulsion. Mix intimately but gently, and pour into plates.

pH after sterilization: 6.8±0.2.

Preparation of the egg-yolk emulsion: Disinfect the surface of whole shell eggs, aseptically crack the eggs, and separate out intact yolks into a sterile graduated cylinder. Add *saline TS* to obtain a 3 to 7 ratio of egg-yolk to saline. Add to a sterile blender cup, and mix at high speed for 5 seconds.

Bismuth sulfite agar

Beef extract	5.0	g
Pancreatic digest of casein	5.0	g
Peptic digest of animal tissue	5.0	g
Dextrose monohydrate	5.0	g
Disodium hydrogenphosphate heptahydrate	4.0	g
Iron(II) sulfate	0.3	g
Bismuth sulfite indicator	8.0	g
Agar	20.0	g
Brilliant green	25.0	mg
Water	1000	ml

Heat the mixture of solids and *water*, with swirling, just to the boiling point. *Do not overheat or sterilize*. Transfer at once to a water-bath maintained at about 50°, and pour into plates as soon as the medium has cooled.

Final pH: 7.6±0.2.

Brilliant green agar

Yeast extract	3.0	g
Peptic digest of animal tissue	5.0	g
Pancreatic digest of casein	5.0	g
Lactose	10.0	g
Sodium chloride	5.0	g
Sucrose	10.0	g
Phenol red	80.0	mg
Agar	20.0	g
Brilliant green	12.5	mg
Water	1000	ml

Boil the solution of solids for 1 minute. Sterilize just prior to use. Melt the medium, pour into Petri dishes, and allow to cool.

pH after sterilization: 6.9±0.2.

Casein digest-soy lecithin polysorbate 20 broth

Pancreatic digest of casein	20.0	g
Soy lecithin	5.0	g
Polysorbate 20	40	ml
Water	960	ml

Dissolve pancreatic digest of casein and soy lecithin in 960 ml of *water*, heating in a water-bath at 48° to 50° for about 30 minutes to effect solution. Add 40 ml of *polysorbate 20*. Mix and dispense as desired.

pH after sterilization: 7.3±0.2.

Cetrimide agar

Pancreatic digest of casein	20.0	g
Magnesium chloride	1.4	g
Potassium sulfate	10.0	g
Agar	13.6	g
Cetrimide	0.3	g
Glycerol	10.0	ml
Water	1000	ml

Dissolve all solid components in *water*, and add *glycerol*. Heat, with frequent agitation, and boil for 1 minute to effect solution.

pH after sterilization: 7.2±0.2.

Columbia agar

Pancreatic digest of casein	10.0	g
Peptic digest of animal tissue	5.0	g
Heart pancreatic digest	3.0	g
Yeast extract	5.0	g
Maize starch	1.0	g
Sodium chloride	5.0	g

Agar, according to gelling power	10.0 to 15.0	g
Water	1000	ml

Hydrate the agar, and dissolve by heating to boiling with continuous stirring. Sterilize, cool to between 45° and 50° and add, where necessary, gentamicin sulfate corresponding to 20 mg of gentamicin base. Pour into Petri dishes.

pH after sterilization: 7.3±0.2.

Defibrinated sheep blood agar (Blood agar)

Heat Soybean-casein digest agar and cool to 45° to 50° in a water-bath. Add sufficient amount of defibrinated sheep blood to make 5 per cent and mix.

Enterobacteria enrichment broth-Mossel

Pancreatic digest of gelatin	10.0	g
Dextrose monohydrate	5.0	g
Dehydrated ox bile	20.0	g
Potassium dihydrogenphosphate	2.0	g
Disodium hydrogenphosphate dihydrate	8.0	g
Brilliant green	15.0	mg
Water	1000	ml

Mix and heat at 100° for 30 minutes to sterilize and cool immediately. *Do not autoclave*.

Final pH: 7.2±0.2.

Lactose broth

Beef extract	3.0	g
Pancreatic digest of gelatin	5.0	g
Lactose	5.0	g
Water	1000	ml

Cool as quickly as possible after sterilization.

pH after sterilization: 6.9±0.2.

Levine eosin-methylene blue agar

Pancreatic digest of gelatin	10.0	g
Dipotassium hydrogenphosphate	2.0	g
Agar	15.0	g
Lactose	10.0	g
Eosin Y	0.4	g
Methylene blue	65.0	mg
Water	1000	ml

Dissolve pancreatic digest of gelatin, *dipotassium hydrogenphosphate* and *agar* in *water*, with warming, and allow to cool. Just prior to use, liquefy the gelled agar solution, add the remaining ingredients, as solutions, in the following amounts, and mix: for each 100 ml of the liquefied agar solution 5 ml of a 20 per cent w/v solution of *lactose*, 2 ml of a 2 per cent w/v solution of *eosin Y*, and 2 ml of a 0.33 per cent w/v solution of *methylene blue*. The finished medium may not be clear.

pH after sterilization: 7.1±0.2.

MacConkey agar

Pancreatic digest of gelatin	17.0	g
Pancreatic digest of casein	1.5	g
Peptic digest of animal tissue	1.5	g
Lactose	10.0	g
Bile salts mixture	1.5	g
Sodium chloride	5.0	g
Agar	13.5	g
Neutral red	30.0	mg
Crystal violet	1.0	mg
Water	1000	ml

Boil the mixture of solids and *water* for 1 minute to effect solution.

pH after sterilization: 7.1±0.2.

MacConkey broth

Pancreatic digest of gelatin	20.0	g
Lactose	10.0	g
Dehydrated ox bile	5.0	g
Bromocresol purple	10.0	mg
Water	1000	ml

Prepare as directed under *Buffer Solution and Media*.

pH after sterilization: 7.3±0.2.

Mannitol-salt agar

Pancreatic digest of casein	5.0	g
Papaic digest of animal tissue	5.0	g
Beef extract	1.0	g
Mannitol	10.0	g
Sodium chloride	75.0	g
Agar	15.0	g
Phenol red	25.0	mg
Water	1000	ml

Mix, then heat with frequent agitation, and boil for 1 minute to effect solution.

pH after sterilization: 7.4±0.2.

Potato dextrose agar

Cook 300 g of peeled and diced potatoes in 500 ml of *water* prepared by distillation, filter through cheesecloth, add *water* prepared by distillation to make 1000 ml, and add the following:

Agar	15.0	g
Dextrose monohydrate	20.0	g

Dissolve by heating and sterilize.

pH after sterilization: 5.6±0.2.

For use, just prior to pouring the plates, adjust the melted and cooled to 45° medium with a sterile 10 per cent w/v solution of *tartaric acid* to a pH of 3.5±0.1. *Do not reheat the pH 3.5 medium*.

Pseudomonas agar for detection of fluorescin

Pancreatic digest of casein	10.0	g
Peptic digest of animal tissue	10.0	g
Dipotassium hydrogenphosphate	1.5	g
Magnesium sulfate	1.5	g
Agar	15.0	g
Glycerol	10.0	ml
Water	1000	ml

Dissolve the solid components in *water* before adding *glycerol*. Heat, with frequent agitation, and boil for 1 minute to effect solution.

pH after sterilization: 7.2±0.2.

Pseudomonas agar for detection of pyocyanin

Pancreatic digest of gelatin	20.0	g
Magnesium chloride	3.0	g
Potassium sulfate	10.0	g
Agar	15.0	g
Glycerol	10.0	ml
Water	1000	ml

Dissolve the solid components in *water* before adding *glycerol*. Heat, with frequent agitation, and boil for 1 minute to effect solution.

pH after sterilization: 7.2±0.2.

Rappaport-Vassiliadis broth

Soya peptone	4.5	g
Sodium chloride	8.0	g
Dipotassium phosphate	0.4	g
Potassium dihydrogenphosphate	0.6	g
Magnesium chloride	29.0	g
Malachite green	36.0	mg
Water	1000	ml

Mix and heat to effect solution.

pH after sterilization: 5.2±0.2.

Reinforced medium for clostridia

Beef extract	10.0	g
Peptone	10.0	g
Yeast extract	3.0	g
Soluble starch	1.0	g
Dextrose monohydrate	5.0	g
Cysteine hydrochloride	0.5	g
Sodium chloride	5.0	g
Sodium acetate	3.0	g
Agar	0.5	g
Water	1000	ml

Hydrate the agar, and dissolve by heating to boiling with continuous stirring.

pH after sterilization: 6.8±0.2.

Sabouraud dextrose agar

Dextrose monohydrate	40.0	g
Mixture of equal parts of peptic digest of animal tissue and pancreatic digest of casein	10.0	g
Agar	15.0	g
Water	1000	ml

Mix and boil to effect solution.

pH after sterilization: 5.6±0.2.

Sabouraud dextrose agar with antibiotics

Dextrose monohydrate	40.0	g
Mixture of equal parts of peptic digest of animal tissue and pancreatic digest of casein	10.0	g
Agar	15.0	g
Water	1000	ml

Mix and boil to effect solution. Immediately before use, add 0.10 g of *benzylpenicillin sodium* and 0.10 g of *tetracycline* per litre of medium as sterile solutions or alternatively, add 50 mg of *chloramphenicol* per litre of medium before sterilization.

pH after sterilization: 5.6±0.2.

(Note Other antibiotics can all be used, individually or in combination.)

Sabouraud dextrose broth

Dextrose monohydrate	20.0	g
Mixture of equal parts of peptic digest of animal tissue and pancreatic digest of casein	10.0	g
Water	1000	ml

Prepare as directed under *Buffer Solution and Media*.

pH after sterilization: 5.6±0.2.

Soybean-casein digest agar

Pancreatic digest of casein	15.0	g
Papaic digest of soybean meal	5.0	g
Sodium chloride	5.0	g
Agar	15.0	g
Water	1000	ml

Prepare as directed under *Buffer Solution and Media*.

pH after sterilization: 7.3±0.2.

Soybean-casein digest broth

Pancreatic digest of casein	17.0	g
Papaic digest of soybean meal	3.0	g
Sodium chloride	5.0	g
Dipotassium hydrogenphosphate	2.5	g
Dextrose monohydrate	2.5	g
Water	1000	ml

Prepare as directed under *Buffer Solution and Media*.

pH after sterilization: 7.3±0.2.

Tetrathionate bile brilliant green broth

Peptone	8.6	g
Ox bile, dried	8.0	g
Sodium chloride	6.4	g
Calcium carbonate	20.0	g
Potassium tetrathionate	20.0	g
Brilliant green	70.0	mg
Water	1000	ml

Heat the solution of solids to boiling. *Do not reheat*.

Final pH: 7.0±0.2.

Triple sugar-iron-agar

Pancreatic digest of casein	10.0	g
Pancreatic digest of animal tissue	10.0	g
Lactose	10.0	g
Sucrose	10.0	g
Dextrose monohydrate	1.0	g
Ammonium iron(II) sulfate	0.2	g
Sodium chloride	5.0	g

Sodium thiosulfate	0.2	g
Agar	13.0	g
Phenol red	25.0	mg
Water	1000	ml

Prepare as directed under *Buffer Solution and Media*.

pH after sterilization: 7.3±0.2.

Violet red bile dextrose agar

Yeast extract	3.0	g
Pancreatic digest of gelatin	7.0	g
Bile salts mixture	1.5	g
Lactose	10.0	g
Sodium chloride	5.0	g
Dextrose monohydrate	10.0	g
Agar	15.0	g
Neutral red	30.0	mg
Crystal violet	2.0	mg
Water	1000	ml

Mix and heat to boiling. *Do not overheat or sterilize*. Transfer at once to a water-bath maintained at about 50°, and pour into plates as soon as the medium has cooled.

Final pH: 7.4±0.2.

Vogel-Johnson agar

Pancreatic digest of casein	10.0	g
Yeast extract	5.0	g
Mannitol	10.0	g
Dipotassium hydrogenphosphate	5.0	g
Lithium chloride	5.0	g
Glycine	10.0	g
Agar	16.0	g
Phenol red	25.0	mg
Water	1000	ml

Boil the solution of solids for 1 minute. Sterilize, cool to between 45° and 50°, and add 20 ml of a sterile 1 per cent w/v solution of *potassium tellurate(IV)*.

pH after sterilization: 7.2±0.2.

Xylose-lysine-deoxycholate agar

Xylose	3.5	g
L-Lysine	5.0	g
Lactose	7.5	g
Sucrose	7.5	g
Sodium chloride	5.0	g
Yeast extract	3.0	g
Agar	13.5	g
Sodium desoxycholate	2.5	g
Sodium thiosulfate	6.8	g
Ammonium iron(III) citrate	0.8	g
Phenol red	80.0	mg
Water	1000	ml

Heat the mixture of solids and *water*, with swirling, just to the boiling point. *Do not overheat or sterilize*. Transfer at once to a water-bath maintained at about 50°, and pour into plates as soon as the medium has cooled.

Final pH: 7.4±0.2.

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10.4 MICROBIOLOGICAL ATTRIBUTES OF NON-STERILE PHARMACEUTICAL PRODUCTS

Few raw materials used in producing pharmaceutical products are sterile as received, and special treatment may be required to render them microbiologically acceptable for use. Strict adherence to effective environmental control and sanitation, equipment cleaning practices, and good personal hygiene practices in pharmaceutical manufacture is vital in minimizing both the type and the number of micro-organisms.

Monitoring, in the form of regular surveillance, should include an examination of the microbiological attributes of Pharmacopoeial articles and a determination of compliance with such microbiological standards as are set forth in the individual monographs. It may be necessary also to monitor the early and intermediate stages of production, with emphasis being placed on raw materials, especially those of animal or botanical origin, or from natural mineral sources which may harbour objectionable micro-organisms not destroyed during subsequent processing. It is essential that ingredients and components be stored under conditions designed to deter microbial proliferation. Microbiological purity of the raw materials as well as manufacturing conditions, including water used for the production, should be such a degree that the microbiological purity requirements for the final product i.e. the pharmaceutical preparation, are observed to fulfill the requirements for good manufacturing practices (GMP).

The nature and frequency of testing vary according to the product. Monographs for some articles require freedom from one or more species of selected indicator micro-organisms such as *Salmonella* spp., *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. For some articles, a specific limit on the total aerobic microbial count (TAMC) and/or the total combined yeasts and moulds count (TYMC) is set forth in the individual monograph. In these cases a requirement for freedom from specified indicator micro-organisms is also included. The significance of micro-organisms in a non-sterile pharmaceutical product should be evaluated in terms of the use of the product, the nature of the product, and the potential hazard to the user. Also taken into account is the processing of the product in relation to an acceptable quality for pharmaceutical purposes.

It is suggested that certain categories of products be tested routinely for total microbial count and for specified indicator microbial contaminants, e.g. natural plant, animal, and some mineral products for *Salmonella* species; oral solutions and suspensions for *E. coli*; articles applied topically for *P. aeruginosa* and *S. aureus*; and articles intended for rectal, urethral, or vaginal administration for yeasts and moulds.

Definitive microbial limits (stipulated micro-organisms and/or counts) are incorporated into specific monographs on the basis of a major criterion, i.e. the potential of the stipulated micro-organisms and/or counts, and of any others that they may reflect, to constitute a hazard in the end product. Such considerations also take into account the processing to which the product components are subjected, the current technology for testing, and the availability of desired quality material. Any of these may preclude the items from specific requirements in the "Microbial Limit Tests" (Appendix 10.2). Regardless of such preclusion, it remains essential to apply strict good manufacturing practices to assure a lowest possible load of micro-organisms. "Limits for Microbial Contamination" (Appendix 10.5) is also set forth to control microbial purity in non-sterile pharmaceutical products. For herbal remedies or herbal drug preparation described in Table 2, the same criteria are applicable to both crude drugs and their preparations.

The relevant tests for determining the total aerobic microbial count and the total combined yeasts and moulds count, and for detection and identification of designated species are given in the "Microbial Limit Tests" (Appendix 10.2). For reliable results, the personnel responsible for the conduct of the test should have specialized training in microbiology and in the interpretation of microbiological data.

10.5 LIMITS FOR MICROBIAL CONTAMINATION

In the manufacture, packaging, storage and distribution of pharmaceutical preparations, suitable means must be taken to ensure their microbiological quality. Unless otherwise specified in the individual monograph, the non-sterile pharmaceutical preparations should comply with the acceptance criteria given in Table 1 and the herbal drug preparations should comply with the acceptance criteria given in Table 2.

Table 1 Acceptance Criteria for Microbiological Quality of Non-sterile Pharmaceutical Preparations

Category	Types	Requirements*
1	Topical preparations for broken skins, abscess, lesions, and mucous membranes excluding for vaginal and rectal routes.	– Total aerobic microbial count: not more than 2×10^1 CFU per g or per ml and total combined yeasts and moulds count: not more than 2×10^1 CFU per g or per ml. – Absence of *Pseudomonas aeruginosa* and *Staphylococcus aureus* per g or per ml.
2	A. Preparations for inhalation use except where required to be sterile.	– Total aerobic microbial count: not more than 2×10^2 CFU per g or per ml and total combined yeasts and moulds count: not more than 2×10^1 CFU per g or per ml. – Absence of bile-tolerant gram-negative bacteria, *Pseudomonas aeruginosa* and *Staphylococcus aureus* per g or per ml.
	B. Preparations for vaginal use.	– Total aerobic microbial count: not more than 2×10^2 CFU per g or per ml and total combined yeasts and moulds count: not more than 2×10^1 CFU per g or per ml. – Absence of *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans* per g or per ml.
	C. Preparations for rectal use.	– Total aerobic microbial count: not more than 2×10^3 CFU per g or per ml and total combined yeasts and moulds count: not more than 2×10^2 CFU per g or per ml.
3	Transdermal patches and topical preparations for intact skin, e.g. creams, lotions, ointments, solutions, powders, etc.	– Total aerobic microbial count: not more than 2×10^2 CFU per g or per ml or per patch and total combined yeasts and moulds count: not more than 2×10^1 CFU per g or per ml or per patch. – Absence of *Pseudomonas aeruginosa* and *Staphylococcus aureus* per g or per ml or per patch.
4	A. Aqueous preparations for oral use.	– Total aerobic microbial count: not more than 2×10^2 CFU per g or per ml and total combined yeasts and moulds count: not more than 2×10^1 CFU per g or per ml. – Absence of *Escherichia coli* per g or per ml.
	B. Non-aqueous preparations for oral use.	– Total aerobic microbial count: not more than 2×10^3 CFU per g or per ml and total combined yeasts and moulds count: not more than 2×10^2 CFU per g or per ml. – Absence of *Escherichia coli* per g or per ml.
5	Preparations for oral administration containing raw materials of natural origin (animal, vegetable or mineral) which cannot be treated with a process for reduction of microbial count.**	– Total aerobic microbial count: not more than 2×10^4 CFU per g or per ml and total combined yeasts and moulds count: not more than 2×10^2 CFU per g or per ml. – Bile-tolerant gram-negative bacteria: not more than 10^2 probable number of bacteria per g or per ml. – Absence of *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli* per g or per ml. – Absence of *Salmonella* spp. per 10 g or per 10 ml.

*Carry out the tests as described in the "Microbial Limit Tests" (Appendix 10.2).

**Specified for raw materials of natural origin that are not obtainable with the required purity including those for manufacturing drugs where an antimicrobial treatment (e.g., with ethylene oxide or ionizing radiations) is not feasible or permissible. The examples are arabic gum, tragacanth, pancreas powder, pepsin, and trypsin.

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Table 2 Acceptance Criteria for Microbiological Quality of Herbal Drug Preparations

Category	Types	Requirements*
1	Preparations from crude drug extracts which underwent a process for reduction of micro-organisms.	
	A. Preparations for oral use.	– Total aerobic microbial count: not more than 5×10^3 CFU per g or per ml and total combined yeasts and moulds count: not more than 5×10^2 CFU per g or per ml. – Bile-tolerant gram-negative bacteria: not more than 10^2 probable number of bacteria per g or per ml. – Absence of *Escherichia coli* per g or per ml. – Absence of *Salmonella* spp. per 10 g or per 10 ml.
	B. Topical preparations for intact skin.	– Total aerobic microbial count: not more than 5×10^2 CFU per g or per ml and total combined yeasts and moulds count: not more than 5×10^1 CFU per g or per ml. – Absence of *Pseudomonas aeruginosa* and *Staphylococcus aureus* per g or per ml.
2	Preparations of crude drugs and mixtures of crude drugs for internal use which will undergo a process for reduction of microbial count before use (e.g., by pouring boiling water over them).	– Total aerobic microbial count: not more than 5×10^7 CFU per g or per ml and total combined yeasts and moulds count: not more than 5×10^5 CFU per g or per ml. – Absence of *Escherichia coli* and *Clostridium* spp. per g or per ml. – Absence of *Salmonella* spp. per 10 g or per 10 ml.
3	Preparations of crude drugs and mixtures of crude drugs for external use.**	– Total aerobic microbial count: not more than 5×10^7 CFU per g or per ml and total combined yeasts and moulds count: not more than 5×10^5 CFU per g or per ml. – Absence of *Pseudomonas aeruginosa, Staphylococcus aureus* and *Clostridium* spp. per g or per ml.
4	Other preparations for internal use containing whole or ground crude drugs.	– Total aerobic microbial count: not more than 5×10^5 CFU per g or per ml and total combined yeasts and moulds count: not more than 5×10^4 CFU per g or per ml. – Bile-tolerant gram-negative bacteria: not more than 10^3 probable number of bacteria per g or per ml. – Absence of *Escherichia coli* and *Clostridium* spp. per g or per ml. – Absence of *Salmonella* spp. per 10 g or per 10 ml.

*Carry out the tests as described in the "Microbial Limit Tests" (Appendix 10.2).

**The examples are LUKPRAKHOP (herbal compress), YAPHOK (herbal poultice).

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10.6 EFFICACY OF ANTIMICROBIAL PRESERVATION

Antimicrobial preservatives are substances added to pharmaceutical preparations to prevent microbial proliferation or to limit microbial contamination that may occur during normal conditions of storage and use. They are used primarily in multiple-dose parenteral, oral, nasal, topical, ear, and eye preparations made with aqueous bases or vehicles. Antimicrobial preservatives should not be used as a substitute for good manufacturing practices or solely to reduce the viable microbial population of a nonsterile product or control the presterilization bioburden of multidose formulations during manufacturing.

The efficacy of an antimicrobial preservative may be enhanced or diminished by many factors. Those include the active ingredient, the formulation, and the container or closure used for that product. The test for efficacy of antimicrobial preservation should therefore be carried out on the product as presented, wherever possible in its original, unopened container in which it was distributed by the manufacturer.

During development of a pharmaceutical preparation, it should be demonstrated that the antimicrobial activity of the preparation provides adequate protection from microbial contamination during storage and use. The test described below is therefore designed to determine the efficacy of antimicrobial activity of the product. The test is not intended to be performed on a routine control basis.

The test for efficacy of antimicrobial preservation in the preparation consists of challenging the preparation with a prescribed inoculum of suitable micro-organisms, storing the inoculated preparation at a prescribed condition, and withdrawing samples from the container at prescribed time intervals to count the remaining viable organisms.

The preservative properties of the preparation are adequate if, in the conditions of the test, there is a significant decrease or no increase in the number of micro-organisms in the inoculated preparation after the times and at the temperatures prescribed. The criteria of acceptance vary for different types of preparation according to the degree of protection intended.

Product Categories

For the purpose of testing, products have been divided into four categories (see Table 1). The criteria of antimicrobial effectiveness for these products are a function of the route of administration.

Table 1 Product Categories

Category	Product Description
1	Injections, other parenterals including emulsions, ear preparations, sterile nasal preparations, and eye preparations made with aqueous bases or vehicles.
2	Topically used preparations made with aqueous bases or vehicles, nonsterile nasal preparations, and emulsions, including those applied to mucous membranes.
3	Oral preparations other than antacids, made with aqueous bases or vehicles.
4	Antacids made with an aqueous base.

Test Organisms

Aspergillus niger	ATCC[1] 16404, DMST[2] 15538
Candida albicans	ATCC 10231, DMST 5815
Escherichia coli	ATCC 8739, DMST 15537
Pseudomonas aeruginosa	ATCC 9027, DMST 15501
Staphylococcus aureus	ATCC 6538, DMST 8013

Single-strain challenges, either ATCC or DMST, should be used throughout the test.

Media

For the initial cultivation of the test organisms, select an agar medium that promotes vigorous growth of the respective stock culture, such as Soybean-casein digest agar medium for bacteria and Sabouraud dextrose agar medium for fungi (Appendix 10.2).

Preparation of Inoculum

Begin the test by inoculating the surface of a suitable solid agar medium from the recently grown stock culture of each of the specified micro-organisms. Incubate the bacterial cultures at 30° to 35° for 18 to 24 hours, the culture of *Candida albicans* at 20° to 25° for 48 hours, and the culture of *Aspergillus niger* at 20° to 25° for 1 week or until good sporulation is obtained.

To harvest the bacterial and *Candida albicans* cultures, use a sterile 0.9 per cent w/v solution of *sodium chloride* for dispersal and transfer of the surface growth into a suitable vessel. Add sufficient suspending fluid to reduce the microbial count to about 1×10^8 colony-forming units (CFU) per ml. To harvest the *Aspergillus niger* culture, use a sterile 0.9 per cent w/v solution of *sodium chloride* containing 0.05 per cent w/v of *polysorbate 80* and adjust the spore count to about 1×10^8 CFU per ml by adding the same solution.

Alternatively, the stock culture organisms may be grown in a suitable liquid medium, and the micro-organisms may be harvested by centrifugation, washed, and dispersed in a sterile 0.9 per cent w/v solution of *sodium chloride* to give the required microbial or spore count.

Determine immediately the number of colony-forming units per ml in each suspension by means of Plate Method (Appendix 10.2). This value serves to calibrate the size of inoculum used in the test. The

[1]ATCC = American Type Culture Collection

[2]DMST = Department of Medical Sciences, Thailand

bacterial and yeast suspensions are to be used within 24 hours of harvest, but the fungal preparation may be stored under refrigeration for up to seven days.

Procedure

To count the viable micro-organisms in the inoculated preparations, use the agar medium corresponding to that used for the initial cultivation of the respective micro-organisms. Ensure that any residual antimicrobial activity of the products is eliminated either by dilution, by filtration or by the use of a specific inactivator.

If the product container can be entered aseptically, with needle and syringe through stopper, conduct the test in five original containers. If not, transfer a 20-ml portion of preparation from five containers to each of five sterile capped tubes. Inoculate each container with one of the prepared and standardized inoculum, and mix. The volume of the suspension inoculum used is between 0.5 per cent and 1.0 per cent of the volume of the product. The concentration of test micro-organisms that is added to the product (Categories 1, 2, and 3) are such that the final concentration of the test preparation after inoculation is between 1×10^5 and 1×10^6 CFU per ml of the product. For Category 4 products (antacids) the final concentration of the test preparation after inoculation is between 1×10^3 and 1×10^4 CFU per ml of the product. Determine the number of viable micro-organisms in each inoculated suspension and calculate the initial concentration of micro-organisms per ml of preparation.

Incubate the inoculated containers or tubes at 20° to 25°. Sample each container at the appropriate intervals specified in Table 2. Record any changes observed in appearance at these intervals. Determine by the Plate Method the number of CFU present in each test preparation for the applicable intervals. Using the calculated concentrations of CFU per ml present at the start of the test, calculate the change in $\log_{10}$ values of the concentration of CFU per ml for each micro-organism at the applicable test internals, and express the changes in terms of log reductions.

Evaluation

The criteria for evaluation of antimicrobial activity are given in Table 2 in terms of the log reduction in the number of viable micro-organisms. No increase (NI) is defined as not more than 0.5 $\log_{10}$ unit higher than the previous value measured.

Table 2 Criteria for the Evaluation of Preservative Efficacy

For Category 1 Products			
Test Organisms	Log Reduction		
	7 days	14 days	28 days
Bacteria	≥1.0	≥3.0	NI
Yeasts and Molds	NI	NI	NI

For Category 2 Products		
Test Organisms	Log Reduction	
	14 days	28 days
Bacteria	≥2.0	NI
Yeasts and Molds	NI	NI

For Category 3 Products		
Test Organisms	Log Reduction	
	14 days	28 days
Bacteria	≥1.0	NI
Yeasts and Molds	NI	NI

For Category 4 Products		
Test Organisms	Log Reduction	
	14 days	28 days
Bacteria	NI	NI
Yeasts and Molds	NI	NI

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APPENDIX 11 CONTAINERS

11.1 GLASS CONTAINERS

Glass containers for pharmaceutical products principally consist of silicon dioxide together with varying amounts of other oxides such as those of sodium, potassium, calcium, magnesium, aluminium, boron, and iron. Glass containers should not impart any potential hazards to the pharmaceutical products contained therein and should meet the requirements set up for each type of glass containers.

Glass Types

Glass containers suitable for packaging pharmaceutical products may be classified as in Table 1.

Containers of Type I borosilicate glass or neutral glass are generally used for preparations that are intended for parenteral administration, either acidic, neutral, or alkaline preparations. Those of Type II glass, that is soda-lime glass suitably de-alkalized, are usually used for packaging acidic and neutral parenteral preparations and where stability data demonstrate their suitability, for alkaline parenteral preparations also. Type III soda-lime glass containers usually are not used for parenteral preparations, except where suitable stability test data indicate that Type III glass is satisfactory for the parenteral preparations that are packaged therein. Type NP glass containers are intended for packaging non-parenteral articles; i.e., those intended for oral or topical use.

Containers for parenteral preparations are made from uncoloured glass except that, for substances known to be extremely light-sensitive, coloured glass may be used; such preparations should not show any change of colour during storage. Containers fabricated of Type II or Type III glass and intended for parenteral preparations should be used once only.

Light Transmission

A container intended to provide protection from light or offered as a "light-resistant" container meets the requirements for Light Transmission, where such protection or resistance is by virtue of the specific properties of the material of which the container is composed, including any coating applied thereto. A clear and colourless or a translucent container that is made light-resistant by means of an opaque enclosure is exempt from the requirements for Light Transmission.

Apparatus Use a spectrophotometer of suitable sensitivity and accuracy, adapted for measuring the amount of light transmitted by either transparent or translucent glass materials used for pharmaceutical containers. For transparent glass pharmaceutical containers, use a spectrophotometer of suitable sensitivity and accuracy for measuring and recording the amount of light transmitted. For translucent glass pharmaceutical containers, use a spectrophotometer as described above that, in addition, is capable of measuring and recording light transmitted in diffused as well as parallel rays.

Preparation of sample Break the container or cut it with a circular saw fitted with a wet abrasive wheel, such as a carborundum or a bonded diamond wheel. Select sections to represent the average wall thickness in the case of blown glass containers, and trim them as necessary to give segments of a size convenient for mounting in the spectrophotometer. After cutting, wash and dry each sample, taking care to avoid scratching the surfaces. If the sample is too small to cover the opening in the sample holder, mask the uncovered portion of the opening with opaque paper or masking tape, provided that the length of the sample is greater than that of the slit in the spectrophotometer. Immediately before mounting in the sample holder, wipe the sample with lens tissue. Mount the sample with the aid of a tackywax, or by other convenient means, taking care to avoid leaving fingerprints or other marks on the surfaces through which light must pass.

Procedure Place the section in the spectrophotometer with its cylindrical axis parallel to the plane of the slit and approximately centred with respect to the slit. When properly placed, the light beam is normal to the surface of the section and reflection losses are at a minimum.

Table 1 Glass Types and Test Limits

Type	General Description	Type of Test	Limits	
			Size*, ml	ml of 0.01 M *sulfuric acid VS*
I	Highly resistant glass (borosilicate glass, neutral glass)	Powdered Glass	All	1.0
II	Treated soda-lime glass	Water Attack	100 or less	0.7
			Over 100	0.2
III	Soda-lime glass	Powered Glass	All	8.5
NP	General-purpose soda-lime glass	Powered Glass	All	15.0

*Size indicates the overflow capacity of the container.

Measure the transmittance of the section with reference to air in the spectral region of interest, continuously with a recording instrument or at intervals of about 20 nm with a manual instrument, in the region of interest, continuously with a recording instrument or at intervals of about 20 nm with a manual instrument, in the region of 290 to 450 nm.

Limits The observed light transmission does not exceed the limits given in Table 2 for containers intended for parenteral use.

The observed light transmission for containers of Type NP glass for products intended for oral or topical administration does not exceed 10 per cent at any wavelength in the range from 290 to 450 nm.

Table 2 Limits for Glass Types I, II, and III

Nominal Size (in ml)	Maximum Percentage of Light Transmission at Any Wavelength Between 290 and 450 nm	
	Flame-sealed Containers	Closure-sealed Containers
1	50	25
2	45	20
5	40	15
10	35	13
20	30	12
50	15	10

Note Any container of a size intermediate to those listed above exhibits a transmission not greater than that of the next larger size container listed in the table. For containers larger than 50 ml, the limits for 50 ml apply.

Tests

The following tests are designed to determine the degree of alkali released from the new (not previously used) glass containers under the influence of the attacking medium and the conditions specified. The tests should be conducted in an area relatively free from fumes and excesssive dust.

Apparatus

AUTOCLAVE An autoclave capable of maintaining a temperature of 121°±2°, equipped with a thermometer, a pressure gauge, a vent cock, and a rack adequate to accommodate at least 12 test containers above the water level.

MORTAR AND PESTLE A hardened-steel mortar and pestle, made according to the specifications in the accompanying illustration (Fig. 1).

GLASSWARE Laboratory glassware of highly resistant borosilicate glass.

OTHER EQUIPMENT Stainless steel sieves, 20.3 cm (8 inches) in diameter, including the No. 850, No. 425 and No. 280 sieves along with the pan and cover, a 900-g (2-Ib) hammer and a permanent magnet.

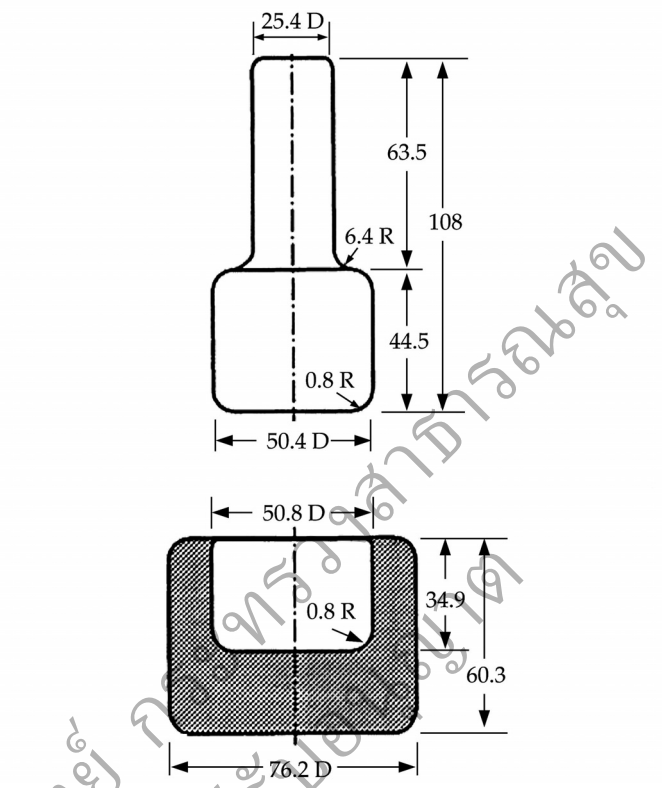

Fig. 1 Special Mortar and Pestle for Pulverizing Glass
Dimensions in mm

Reagents

HIGH-PURITY WATER[1] The water used in these tests is copper-free water which has a conductivity at 25° of not greater than 0.15 μmho/cm and meets the requirements of the tests under Purified Water.

METHYL RED SOLUTION Dissolve 24 mg of *methyl red sodium* in *water* to make 100 ml. If necessary, neutralize the solution with 0.02 M *sodium hydroxide* or acidify it with 0.01 M *sulfuric acid* so that the titration of 100 ml of high-purity water, containing 5 drops of the indicator, does not require more than 0.02 ml of 0.020 M *sodium hydroxide* to effect the colour change of the indicator, which should occur at a pH of 5.6.

I. Powdered Glass Test

Procedure Digest (age) two 250-ml conical flasks and two glass beakers that are of such size that, when inverted, the inner sides of their bottoms fit snugly down on the top rims of the flasks, with high-purity water in a bath at 90° for at least 24 hours or at 121° for 1 hour.

[1]There must be an assurance that this water is not contaminated by copper or its products (e.g., copper pipes, stills, or receivers). The water may be prepared by passing distilled water through a deionizer cartridge packed with a mixed bed of nuclear-grade resin, then through a cellulose ester membrane having openings not exceeding 0.45 μm. Do not use copper tubing. Flush the discharge lines before water is dispensed into test vessels. When the low conductivity specification can no longer be met, replace the deionizer cartridge.

Rinse thoroughly with *water* six or more containers selected at random, and dry them with a stream of clean, dry air. Coarsely break the containers into fragments about 25 mm in size. Divide about 100 g of the crushed glass into three approximately equal portions and transfer one portion of the sample to the mortar. Insert the pestle and strike 3 or 4 blows with the hammer. Nest the sieves and transfer the contents of the mortar to the No. 850 sieve. Repeat the operation on each of the two remaining portions until the whole sample has been treated. Rapidly sift the glass, and remove the portion retained on the No. 850 and No. 425 sieves, and again crush and sieve as before. Repeat again this crushing and sieving operation. Empty the receiving pan, reassemble the nest of sieves, and shake mechanically or manually for 5 minutes. Reserve the portion of the sample passing through the No. 425 sieve but retained by the No. 280 sieve, which should weigh in the excess of 10 g. Spread the reserved sample on a piece of glazed paper and pass a magnet over it to remove any metal particles that may be introduced during the crushing. Transfer the sample to a 250-ml conical flask of resistant glass, and wash it with six 30-ml portions of *acetone*, swirling each time for about 30 seconds to suspend all fine particles and rapidly decanting the supernatant liquid. Spread the glass powder in an evaporating dish, evaporate the acetone at room temperature and dry in an oven at 140° for 20 minutes. Transfer the glass powder to a weighing bottle and cool in a desiccator. Use the test sample within 48 hours after drying.

Transfer 10.00 g of the prepared sample, accurately weighed, to one of the two 250-ml conical flasks previously digested (aged) with high-purity water as described above and add 50.0 ml of high-purity water. Ensure that the glass powder is uniformly spread over the bottom of the flask. In the second aged flask place 50.0 ml of high-purity water to serve as a blank. Close the flasks with the inverted glass beakers that previously have been similarly treated. Place the closed flasks in the autoclave, and close it securely, leaving the vent cock open. Heat until steam issues vigorously from the vent cock, and continue heating for 10 minutes. Close the vent cock, and raise the temperature from 100° to 121° over 20 minutes. Maintain the temperature at 121°±2° for 30 minutes, counting from the time when this temperature is reached. Lower the temperature from 121° to 100° over 40 minutes, being vented as needed to prevent the formation of a vacuum. Remove the flasks from the autoclave and cool them at once in running water. Decant the supernatant water from the sample flask into a suitably cleansed conical flask, and wash the residual powdered glass with four 15-ml portions of high-purity water, adding the decanted washings to the main portion. Discard the flask containing the washed glass powder. To each flask add 5 drops of methyl red solution, and titrate immediately with 0.01 M *sulfuric acid VS*. Record the volume of 0.01 M *sulfuric acid VS* used to neutralize the extract from the prepared sample of glass, corrected for a blank. The volume does not exceed that in the table for the type of glass concerned.

II. Water Attack Test

The following test is designed to determine the hydrolytic resistance of glass containers. The hydrolytic resistance is the resistance offered by the inner surface of the glass to the release of soluble mineral substances into water under conditions specified. It is evaluated by titrating the alkalinity of the solution.

Procedure Rinse thoroughly with high-purity water at room temperature three or more randomly selected containers twice. Digest (age) a similar number of glass beakers that are of such size that, when inverted, the inner sides of their bottoms fit snugly down on the top rims of the selected containers, with high-purity water in a bath of 90° for at least 24 hours or at 121° for 1 hour. Fill each container to 90 per cent of its overflow capacity with high-purity water, and close the containers with the inverted glass beakers previously treated as described above. Place the closed containers in the autoclave, and close it securely, leaving the vent cock open. Heat until steam issues vigorously from the vent cock, and continue heating for 10 minutes. Close the vent cock, and raise the temperature from 100° to 121° over 20 minutes. Hold the temperature at 121°±2° for 1 hour, counting from the time when this temperature is reached. Lower the temperature from 121° to 100° over 40 minutes, being vented as needed to prevent the formation of a vacuum. Remove the containers from the autoclave.

Empty the contents from one or more containers to obtain a volume of 100 ml. Place the pooled sample in a 250-ml conical flask of resistant glass, add 5 drops of methyl red solution and titrate, while warm, with 0.01 M *sulfuric acid VS*. Complete the titration within 1 hour after removing the containers from the autoclave. Record the volume of 0.01 M *sulfuric acid VS* used, corrected for a blank obtained by titrating 100 ml of high-purity water in the same manner. The volume does not exceed that indicated in the table for the type of glass concerned.

III. Arsenic

Test solution Prepare the test solution as directed for Procedure under Water Attack Test, using 35 ml of the water from one Type I glass container or in the case of smaller containers, 35 ml of the combined contents of several Type I glass containers.

Procedure Perform the test according to the "Limit Test for Arsenic" (Appendix 5.2), the limit is 0.1 ppm.

11.2 PLASTIC CONTAINERS

Plastic containers for pharmaceutical products are made from plastics based on the following polymers: polyethylene, polypropylene, polyvinyl chloride, polystyrene and, to a lesser extent, polymethyl methacrylate, polyethylene terephthalate, polytetrafluoroethylene, the amino formaldehydes and polyamides, to which certain additives are added such as antioxidants, antistatic agents, colours, impact modifiers, lubricants, plasticizers and stabilizers. For pharmaceutical purposes antistatic agents are not normally used, and colours are also not normally used in containers for injections. These containers should not impart any potential hazards to the pharmaceutical products contained therein and should meet the requirements set up for each type of those products.

Plastic Containers for Pharmaceutical Products and Their Requirements

1. Containers for solid dosage forms
   Comply with the physico-chemical tests 1.2, 1.3.1 and biological test 2.6.
2. Containers for semisolid dosage forms
   Comply with the physico-chemical tests 1.2, 1.3.2 and biological test 2.6.
3. Containers for oral liquid dosage forms
   Comply with the physico-chemical tests 1.2, 1.3.3 and biological test 2.6.
4. Containers for ophthalmic preparations
   Comply with the physico-chemical tests 1.2 and either 1.3.2 or 1.3.4, and biological test 2.6.
5. Containers for parenteral preparations
   Comply with the physico-chemical tests 1.1, 1.2, 1.3.4 and biological tests 2.
6. Containers for irrigations
   Comply with the test under parenteral preparations.
7. Containers for human blood and blood components
   Comply with the physico-chemical tests 1.1, 1.2, 1.3.5 and biological tests 2 and tests under Appendix 11.3.

1. Physico-chemical Tests

The following tests, designed to determine physical and chemical properties of plastics and their extracts, are based on the extraction of the plastic material, and it is essential that the designated amount of the plastic be used. Also, the specified surface area must be available for extraction at the designated temperature.

1.1 Transparency and Appearance The containers have a transparency which does not practically interfere with the test according to "Particulate Matter in Injections" (Appendix 4.27). For the test of observed light transmission for the plastic containers intended for parenteral use, perform the test according to "Light Transmission" under Glass Containers (Appendix 11.1). The containers do not have scratches, cracks, bubbles, or other faults which make them difficult to be used.

1.2 Tests of Extractive Substances

Extracting medium Unless otherwise directed in a specific test below, use Purified Water as the extracting medium, maintained at a temperature of 70° during the extraction of the prepared sample.

Apparatus

– WATER-BATH

– EXTRACTION CONTAINERS Use only containers, such as ampoules or screw-capped culture test-tubes, of Type I glass. If used, culture test tubes are closed with screw caps having suitable elastomeric liners. The exposed surface of the elastomeric liner is completely protected with an inert solid disc 0.05 mm to 0.075 mm in thickness. A suitable disc may be fabricated from a polytef resin.

Preparation of apparatus Cleanse all glassware thoroughly with *chromic acid cleansing mixture*, or if necessary with hot *nitric acid*, followed by prolonged rinsing with *water*. Clean cutting utensils by an appropriate method (e.g., successive cleaning with *acetone* and *dichloromethane*) prior to use in subdividing a sample. Clean all other equipment by thorough scrubbing with a suitable detergent and prolonged rinsing with *water*.

Preparation of sample From a homogeneous sample, use a portion, for each 20.0 ml of extracting medium, equivalent to 120 cm^2 in total surface area (both sides combined), and subdivide into strips approximately 3 mm in width and as near to 5 cm in length as is practical. Transfer the subdivided sample to a glass-stoppered, 250-ml graduated cylinder of Type I glass, and add about 150 ml of *water*. Agitate for about 30 seconds, drain off and discard the liquid, and repeat with a second washing.

Transfer the prepared sample to a suitable extraction flask, and add the required amount of extracting medium. Extract by heating in a water-bath at the temperature specified for the extracting medium for 24 hours. Cool, but not below 20°, and use this solution as the test solution. Pipette 20 ml of test solution into a suitable container. Use this portion in the test for Buffering Capacity. Immediately decant the remaining extract into a suitably cleansed container, and seal.

Blank Use Purified Water where a blank is specified in the following tests.

1.2.1 Nonvolatile residue Transfer, in suitable portions, 50.0 ml of the test solution to a suitable, tared crucible (preferably a fused-silica crucible that has been acid-cleaned), and evaporate the volatile matter on a steam bath. Similarly evaporate 50.0 ml of the blank in a second crucible. (**Note** If an oily residue is expected, inspect the crucible repeatedly during the evaporation and drying period, and reduce the amount of heat if the oil tends to creep along the walls of the crucible.) Dry at 105° for 1 hour: the difference between the amounts obtained from the sample and the blank does not exceed 15 mg.

1.2.2 Sulfated ash (**Note** It is not necessary to perform this test when the nonvolatile residue test result does not exceed 5 mg.) Proceed with the nonvolatile residue obtained from the sample and from the blank, using, if necessary, additional *sulfuric acid* but adding the same amount of *sulfuric acid* to each crucible and perform the test according to "Sulfated Ash (Method II, Appendix 5.3): the difference between the amounts of residue on ignition obtained from the sample and the blank does not exceed 5 mg.

1.2.3 Heavy metals Pipette 20 ml of the test solution, filtered if necessary, into one of two matched 50-ml comparison tubes. Adjust with 1 M *acetic acid* or 6 M *ammonium hydroxide* to a pH between 3.0 and 4.0, using short-range pH paper as an external indicator, dilute with *water* to about 35 ml, and mix. Into the second comparison tube pipette 2 ml of *lead standard solution (1 ppm Pb)*, and add 20 ml of the blank. Adjust with 1 M *acetic acid* or 6 M *ammonium hydroxide* to a pH between 3.0 and 4.0, using short-range pH paper as an external indicator, dilute with *water* to about 35 ml, and mix. To each tube add 1.2 ml of *thioacetamide reagent* and 2 ml of *acetate buffer pH 3.5*, dilute with *water* to 50 ml, and mix: any brown colour produced within 10 minutes in the tube containing the extract of the prepared sample does not exceed that in the tube containing the Lead Standard Solution (1 ppm Pb), both tubes being viewed downward over a white surface (1 ppm in extract).

1.2.4 Buffering capacity Titrate the previously collected 20-ml portion of the test solution potentiometrically to a pH of 7.0, using either 0.010 M *hydrochloric acid* or 0.010 M *sodium hydroxide*, as required. Treat a 20.0-ml portion of the blank similarly: if the same titrant was required for both sample and blank, the difference between the two volumes is not greater than 10.0 ml; and if acid was required for either the sample or the blank and alkali for the other, the total of the two volumes required is not greater than 10.0 ml.

1.3 Water Vapour Permeability

1.3.1 Containers for solid dosage forms

PROCEDURE Prepare a quantity of granular *anhydrous calcium chloride* (nominal width of aperture of test sieves between 1.20 mm and 0.71 mm, and free from fine powder) by drying for 1 hour at 110° and cooling subsequently in a desiccator.

Select a sample of 13 containers at random from the containers to be tested, fill 10 of the containers with the calcium chloride to within 13 mm of the closure, and close the containers tightly.

Fill the remaining three containers with a sufficient amount of glass beads, or other suitable inert material, so that when the closure has been applied, the weights of the closed containers are approximately equal to the mean weight of the 10 filled test containers; two of these containers serve as controls and the third is used as a counterpoise. Weigh each of the test containers and the control containers separately, using the counterpoise container and appropriate weights, and immediately place the test and control containers in a chamber in which the air is controlled at a temperature of 30°±1° and a relative humidity of 80±3 per cent, keep the counterpoise container under ordinary laboratory conditions.

After an interval of 7 days, measured to the nearest hour, remove the test and control containers from the chamber, and immediately reweigh each test and control container against the counterpoise container.

From the gross increase in weight in each test container subtract the mean increase in weight of the two control containers, and from the net increases in weight, calculate the rate of passage of water vapour into each test container, in milligrams per 24 hours (mg/d).

ASSESSMENT OF RESULTS The containers shall be deemed to pass the test if in no case the rate of passage of water vapour into the containers in the sample exceeds the appropriate value indicated in Table 1.

If more than one container in the sample gives a value exceeding the appropriate figure, the containers shall be deemed to fail the test. If only one container in the sample gives a figure exceeding this value, then a further 10 containers shall be tested and if the appropriate figure is not exceeded with any of these further containers, the containers shall be deemed to pass the test.

Table 1 Water-Vapour Permeability of Solid Dosage Form Containers

Capacity of Container (ml)	Maximum Rate of Passage of Water Vapour (mg/d)
Up to 35	15
36 to 60	20
61 to 120	25

1.3.2 Containers for semisolid dosage forms

PROCEDURE Select a sample of 10 containers at random from the containers to be tested and weigh each separately with its closure. Fill each container to approximately 90 per cent of its capacity with cream having the following formula: *cetrimide* 5 g; *cetostearyl alcohol* 50 g; Purified Water, freshly boiled and cooled 445 g; *liquid paraffin* 500 g. Apply the closure to each container and weigh the closed containers separately. Place them in a chamber in which the air is controlled at a temperature of 30°±1° and at a relative humidity of 65±3 per cent. After 14 days, reweigh each closed container.

ASSESSMENT OF RESULTS The containers shall be deemed to pass the test if none of the contents of the closed containers shows a loss in weight during the test greater than 1.5 per cent of its weight.

If more than one container in the sample gives a figure exceeding this value, the containers shall be

deemed to fail the test. If only one container in the sample gives a figure exceeding the value, then a further 10 containers shall be tested and if this value is not exceeded with any of these further containers, the containers shall be deemed to pass the test.

1.3.3 Containers for oral liquid dosage forms

PROCEDURE Select a sample of 10 containers at random from the containers to be tested and weigh each separately with its closure. Fill each container with *water* equivalent to the labelled volume. Close the containers tightly and weigh each container separately. Immediately place the test containers in a chamber in which the air is controlled at a temperature of 30°±1° and at a relative humidity of 65±3 per cent. Allow to stand for 14 days and reweigh each test container. Calculate the net decrease in weight.

ASSESSMENT OF THE RESULTS The loss in weight should not exceed 0.2 per cent of the contents in 14 days.

If more than one container in the sample gives a figure exceeding this value, the containers shall be deemed to fail the test. If only one container in the sample gives a figure exceeding the value, then a further 10 containers shall be tested and if this value is not exceeded with any of these further containers, the containers shall be deemed to pass the test.

1.3.4 Containers for parenteral preparations

PROCEDURE Select a sample of 10 containers at random from the containers to be tested and weigh each separately with its closure. Fill each container with *water* equivalent to the labelled volume. Close the containers tightly and weigh each container separately. Heat the test containers for 30 minutes at 110° and place them in a chamber in which the air is controlled at a temperature of 30°±1° and at relative humidity of 65±3 per cent. Allow to stand for 14 days and reweigh each test container. Calculate the net decrease in weight.

ASSESSMENT OF THE RESULTS The loss in weight should not exceed 0.2 per cent of the contents in 14 days.

If more than one container in the sample gives a figure exceeding this value, the containers shall be deemed to fail the test. If only one container in the sample gives a figure exceeding the value, then a further 20 containers shall be tested and if this value is not exceeded with any of these further containers, the containers shall be deemed to pass the test.

1.3.5 Containers for human blood and blood components

PROCEDURE Select a sample of 10 containers at random from the containers to be tested and weigh each separately. Fill each container with the labelled amount of anticoagulant and a volume of *saline TS* equal to the volume of blood to be collected. Seal the containers as for issue, weigh separately, and place them in a chamber at a temperature of 10° in still air conditions at approximately zero humidity, using *self-indicating silica gel* as a desiccant. Allow to stand for 28 days and reweigh each test container. Calculate the net decrease in weight.

ASSESSMENT OF THE RESULTS The loss from the specified combined volume should not exceed 5 per cent w/w of the contents in 28 days.

If more than one container in the sample gives a figure exceeding this value, the containers shall be deemed to fail the test. If only one container in the sample gives a figure exceeding the value, then a further 20 containers shall be tested and if this value is not exceeded with any of these further containers, the containers shall be deemed to pass the test.

Note

1. Circulating the air in a closed chamber over a saturated solution of ammonium sulfate will give the required relative humidity of 80±3 per cent at 30°.

2. Circulating the air in a closed chamber over a saturated solution of ammonium chloride and potassium nitrate, as by dissolving 365 g of each substance in 600 ml of *water*, will give the required relative humidity of 65±3 per cent at 30°.

2. Biological Tests

Perform the *in vitro* biological tests according to the procedures set forth under *In vitro* biological reactivity tests. Materials that meet the requirements of the *in vitro* tests are not required to undergo further testing. No plastic class designation is assigned to these materials. Materials that do not meet the requirements of the *in vitro* tests are not suitable for containers for pharmaceutical products.

The tests are designed for plastics in condition in which they are received. If, however, a plastic is to be exposed to any sterilization process prior to its use, then the tests are conducted on plastics preconditioned by the appropriate sterilization procedure.

The *in vivo* biological reactivity tests are designed to determine the biological response of animals to elastomerics, plastics and other polymeric materials with direct or indirect patient contact, or by the injection of specific extracts prepared from the material under test. The Acute Systemic Toxicity Test and the Intracutaneous Reactivity Test are used for elastomeric materials, especially elastomeric closures for which the appropriate *in vitro* biological reactivity tests, have indicated significant biological reactivity. These two tests are used for plastics and other polymers in addition to a third test, the Acute Intramuscular Tissue Toxicity Test, to test the suitability of these materials intended for use in fabricating containers and accessories thereto, for use in parenteral preparations, and for use in medical device, implants and other systems.

The *in vivo* biological reactivity tests do not apply to plastics that are intended for use as containers for oral or topical products, or that may be used as an integral part of a drug formulation.

Extracting media

PYROGEN-FREE WATER (Diluent A, Appendix 8)
PYROGEN-FREE SALINE TS
ETHANOL IN PYROGEN-FREE SALINE TS (1 in 20)
POLYETHYLENE GLYCOL 400

VEGETABLE OIL Use freshly refined *sesame oil* or *cottonseed oil*. The oil meets the following additional requirements. Obtain, if possible, freshly refined oil. Use three properly prepared animals (see under Intracutaneous Reactivity Test) and inject the oil intracutaneously in a dose of 200 µl into each of 10 sites per animal, and observe the animals at 24, 48 and 72 hours following injection. No site shows a tissue reaction, such as edema and erythema, that is larger than 5 mm in overall diameter.

Apparatus The apparatus for the test includes the following.

AUTOCLAVE Use an autoclave capable of maintaining a temperature of 121°±2°, equipped with a thermometer, a pressure gauge, a vent cock, a rack adequate to accommodate the test containers above the water level, and a water cooling system that will allow for cooling of the test containers to about, but not below, 20° immediately following the heating cycle.

OVEN Use an oven, preferably of a forced-circulation model, that will maintain operating temperatures of 50°±2°.

EXTRACTION CONTAINERS Use containers, such as ampoules or screw-capped culture test-tubes, of borosilicate glass. Culture test-tubes used are closed with screw caps having suitable rubber liners. The exposed surface of the rubber liner is completely protected with an inert solid disc 50 to 75 µm in thickness. A suitable disc may be fabricated from a polytetrafluoroethylene resin.

Preparation of apparatus Clean all glassware thoroughly with *chromic acid cleansing mixture*, or if necessary with hot *nitric acid*, followed by prolonged rinsing with *water*. Clean cutting devices by an appropriate method (e.g., successive cleaning with *acetone* and *dichloromethane*) prior to use in subdividing a specimen. Clean all other equipment by thorough scrubbing with a suitable detergent and prolonged rinsing with *water*.

Render containers and devices used for extraction, and in transfer and administration of test material, sterile and dry by a suitable process. If ethylene oxide is used as the sterilizing agent, allow adequate time for complete degassing.

Preparation of sample From a homogeneous plastic sample use a portion equivalent to 120 cm^2 when the thickness is 500 µm or less, or 60 cm^2 when the thickness is greater than 500 µm, and subdivide into strips of approximately 0.3 × 5 cm. Remove particulate matter, such as lint and free particles, by treating each subdivided sample as follows: transfer the subdivided sample to a clean, glass-stoppered 100-ml graduate cylinder of Type I glass and add about 70 ml of *pyrogen-free water*. Agitate for about 30 seconds, and drain off the water; repeat this step. Dry those pieces prepared for the extraction with vegetable oil in an oven at a temperature not exceeding 50°.
(**Note** Do not clean the plastic with a dry or wet cloth or by rinsing or washing with an organic solvent or surfactant.)

Extracts Place two properly prepared samples of the plastic to be tested in separate extraction flasks, and add to each flask 20 ml of the appropriate extracting medium (i.e., the fluid nearest in properties and composition to the products intended to be held). Repeat these directions for each extracting medium required for testing. Also prepare one 20-ml blank of each medium for parallel injections and comparisons. Extract by heating in an autoclave at 121°, or in an oven at 50° (Table 2). Allow adequate time for the liquid within the container to reach the extraction temperature.
(**Note** The extraction conditions should not in any instance cause physical changes such as fusion or melting of the plastic pieces which result in a decrease in the available surface area. A slight adherence of the pieces can be tolerated. Always add the cleaned pieces individually to the extracting medium. If culture tubes are used for autoclave extractions with sesame oil, seal their screw caps adequately with pressure-sensitive tape.)

Cool all containers at the end of the extraction period to about, but not below, 20°. Agitate vigorously for several minutes, transfer each extract immediately to a dry, sterile container, and test within the following 24 hours. No extract should be stored at any time at temperatures below 20°

Table 2 Test Conditions

	Test Solutions				
	Pyrogen-free Water	Pyrogen-free Saline	1 in 20 Solution of Ethanol in Pyrogen-free Saline TS	Polyethylene Glycol 400	Vegetable Oil
Injection: ready-for-use syringes transfusion, blood	121° × 1 h	121° × 1 h	–	–	–
Single-use syringes and administration devices	50° × 24 h	50° × 24 h	–	–	50° × 24 h
Prosthesis	121° × 1 h	121° × 30 min	–	–	–

2.1 Acute Systemic Toxicity Test

Test animal Use healthy, not previously used, white mice weighing between 17 and 23 g. For each test group use only mice of the same source. Allow water and food *ad libitum*.

Procedure Inject each extract of the sample, and the corresponding blank, into groups of five mice each in the amount and by the route set forth in Table 3, except to dilute the extracts of sample prepared with *polyethylene glycol 400*, and the corresponding blank with 4.6 volumes of *a pyrogen-free saline TS* to obtain a solution having a concentration of 200 mg of polyethylene glycol per ml. The extract prepared with *pyrogen-free water* should be rendered isotonic prior to injection by the addition of 900 mg of *pyrogen-free sodium chloride* for each 100 ml.

Table 3 Test to Be Conducted

Test	Test Material	Test Animal	Dose	Route	Injection Rate µl/second
Acute systemic toxicity	Extract of sample in:				
	1. *Pyrogen-free Water*	Mouse	50 ml/kg	Intravenous	100
	2. *Pyrogen-free Saline TS*	Mouse	50 ml/kg	Intravenous	100
	3. *Ethanol in Pyrogen-free Saline TS* (1 in 20)	Mouse	50 ml/kg	Intravenous	100
	4. *Polyethylene Glycol 400*	Mouse	50 ml/kg	Intraperitoneal	–
	5. Vegetable oil	Mouse	50 ml/kg	Intraperitoneal	–
Intracutaneous reactivity	Extract of sample in:				
	1. *Pyrogen-free Water*	Rabbit	200 µl/site	Intracutaneous	–
	2. *Pyrogen-free Saline TS*	Rabbit	200 µl/site	Intracutaneous	–
	3. *Ethanol in Pyrogen-free Saline TS* (1 in 20)	Rabbit	200 µl/site	Intracutaneous	–
	4. *Polyethylene Glycol 400*	Rabbit	200 µl/site	Intracutaneous	–
	5. Vegetable oil	Rabbit	200 µl/site	Intracutaneous	–
Acute intramuscular tissue toxicity	Implantation strips of sample	Rabbit	Four strips of 10 × 1 mm	Intramuscular	–
Pyrogen	Extract of sample in *Pyrogen-free Saline TS*	Rabbit	10 ml/kg	Intravenous	–

Evaluation Observe the animals immediately after injection, and at least at 24, 48 and 72 hours. If during the observation period all five of the animals treated with the extract of the sample show no significantly greater reaction than the animals treated with the blank, the sample meets the requirements of this test.

If any animals treated with the sample show only slight signs of toxicity and not more than one animal shows gross symptoms of toxicity or death, repeat the test using groups of 10 mice each. On the repeated test, the requirements of the test are met if none of the animals treated with the sample shows a significantly greater reaction than that observed in the animals treated with the blank.

2.2 Intracutaneous Reactivity Test

Test animal Select healthy, thin-skinned albino rabbits, not previously used for any test, whose fur can be clipped closely and whose skin is free from mechanical irritation or trauma. In handling the animals during observation periods, avoid touching the injection sites.

Procedure On the day of the test, closely clip the fur on the animal's back on both sides of the spinal column over a sufficiently large test area. Avoid mechanical

irritation and trauma. Remove loose hair by means of vacuum. If necessary, swab the skin lightly with *ethanol (70 per cent)*, and dry the skin prior to injection.

For each sample use two rabbits. Inject intracutaneously into each of five sites on one side of each animal 200 µl of the sample. Similarly, inject into each of five sites on the other side 200 µl of the corresponding blank (Table 3). The extract prepared with polyethylene glycol 400 and the corresponding blank should be diluted prior to injection with 8.3 volumes of *pyrogen-free saline TS* to obtain a concentration of 120 mg of polyethylene glycol per ml.

Evaluation Examine the injection sites for evidence of any tissue reaction such as erythema, edema and necrosis. Swab the skin lightly, if necessary, with *ethanol (70 per cent)* to facilitate reading of the injection sites. Observe animals at 24, 48 and 72 hours after the injection. Clip the fur as necessary during the observation period. Rate the observations on a numerical scale for the extract of the sample and for the blank, respectively, using Table 4.

If each animal at any observation period shows an average reaction to the sample which is not significantly greater than that to the blank, the sample meets the requirements of the test.

If at any observation period the average reaction to the sample is questionably greater than the average reaction to the blank, repeat the test using three additional rabbits. On the repeated test, the average reaction to the sample in any of the three animals should not be significantly greater than that to the blank.

Table 4 Evaluation of Skin Reactions

Erythema and Necrosis Formation	Value
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Moderate to severe (beet-redness) to slight necrosis formation (injuries in depth)	4
Edema Formation	**Value**
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well defined by definite raising)	2
Moderate edema (raised approximately 1 mm)	3
Severe edema (raised more than 1 mm and extending beyond the area of exposure)	4

2.3 Acute Intramuscular Tissue Toxicity Test (Implantation Test)

The acute intramuscular tissue toxicity test is designed for the evaluation of a plastic material in direct contact with living tissue. Of importance are the proper preparation of the implant strips and their proper implantation under aseptic conditions. Prepare for implantation eight strips of the sample and four strips of Negative Control Plastic. (**Note** A Negative Control Plastic is a plastic sample that gives no reaction under the conditions of the test.) Each strip should be not less than 10 × 1 mm. The edges of the strips should be as smooth as possible to avoid additional mechanical trauma upon implantation. Strips of the specified minimum size are implanted by means of a hypodermic needle such as a 15-gauge needle with intravenous point and of 19-mm cannula length, and a sterile trocar. Use either pre-sterilized needles into which the sterile plastic strips are aseptically inserted, or insert each clean strip into a needle, the cannula and hub of which are protected with an appropriate cover, and then subjected to the appropriate sterilization procedure. Allow for proper degassing if agents such as ethylene oxide are used.

Test animal Select healthy, adult rabbits weighing not less than 2.5 kg, and whose paravertebral muscles are sufficiently large in size to allow for implantation of the test strips. Do not use any muscular tissue other than the paravertebral site. The animal may be anesthetized with a commonly used anesthetic agent to a degree deep enough to prevent muscular movements, such as twitching.

Procedure Perform the test in a clean area. On the day of the test or up to 20 hours before testing, clip the fur of the animals on both sides of the spinal column. Remove loose hair by means of vacuum.

Implant four strips of the sample into the paravertebral muscle on one side of the spine of each of two rabbits, 2.5 to 5 cm from the midline and parallel to the spinal column, and about 2.5 cm apart from each other. In a similar fashion implant two strips of Negative Control Plastic in the opposite muscle of each animal. Insert a sterile stylet into the needle to hold the plastic strip in the tissue while withdrawing the needle. If excessive bleeding is observed after implantation of a strip, place a duplicate strip at another site. Close the incision after implantation is complete.

Keep the animals for a period of not less than 72 hours, and sacrifice them at the end of the observation period by administering an overdose of an anesthetic agent. Allow sufficient time to elapse for the tissue to be cut without bleeding. Examine macroscopically the area of the tissue surrounding the centre

portion of each implant strip. Use a magnifying lens if necessary.

Evaluation The tissue immediately surrounding the Negative Control Plastic strips appears normal and entirely free from hemorrhage, film or encapsulation. The requirements of the test are met if, in each rabbit, the reaction to not more than one of the four sample strips is significantly greater than that to the strips of Negative Control Plastic.

2.4 Pyrogen Test

Perform the test according to the "Pyrogen Test" (Appendix 8.2) as specified in Table 3.

2.5 Hemolysis Test

Preparation of sample Proceed as described in the general method of Preparation of sample but using homogeneous plastic material 200 cm^2 in total surface area as a sample.

Extracts Proceed as described in the general procedure for Extracts, using 50 ml of *pyrogen-free water* as the extracting medium.

Procedure Dilute *sodium phosphate-sodium chloride buffer TS* as follows:

Designation of Intermediate Solution	Sodium Phosphate-Sodium Chloride Buffer TS (ml)	Water (ml)
a_0	30.0	10.0
b_0	30.0	20.0
c_0	15.0	85.0

Dilute further the intermediate solution a_0 and b_0 as follows:

Diluted Solution	Intermediate Solution	Water (ml)
a_1	3.0 ml a_0	12.0
b_1	4.0 ml b_0	11.0
c_1	4.75 ml c_0	10.25

To each of three centrifuge tubes add 1.40 ml of the extract of the sample, and to a fourth centrifuge tube add 1.40 ml of the corresponding blank. To the first tube add 1.0 ml of a_0, to the second and the fourth tubes add 0.10 ml of b_0, and to the third tube add 0.01 ml of c_0.

(**Note** The osmotic effects of the solutions correspond to those of sodium chloride solutions having a concentration of 0.5 per cent w/v, 0.4 per cent w/v, and 0.1 per cent w/v for the first tube, the second and the fourth tubes, and the third tube, respectively.) To each tube add 0.02 ml of freshly prepared, well mixed, *heparinized human blood TS* and place the tubes in a water-bath at 30°±1° for exactly 40 minutes. Immediately to the first tube add 1.50 ml of a_1, to the second and the fourth tubes add 1.50 ml of b_1, and to the third tube add 1.50 ml of c_1. Centrifuge the tubes for 5 minutes, preferably in a horizontal centrifuge.

Measure the absorbance at the maximum at about 540 nm, using *sodium phosphate-sodium chloride buffer TS* as a blank (Appendix 2.2).

Calculate the degree of hemolysis (h_n) from the formula:

$$h_n = \frac{A_n}{A_{100}} \times 100,$$

where A_{100} is the absorbance of the solution in the third tube,
n is the tube number, and
A_n is the absorbance of the solutions in the first, the second and the fourth tubes, respectively.

Evaluation The degree of hemolysis (h_1) in the first tube should not exceed 10, and the difference between the degrees of hemolysis in the second and the fourth tubes should not exceed 10.

The degree of hemolysis (h_4) for the fourth tube should be between 60 and 75.

2.6 *In Vitro* Biological Reactivity Tests

The following tests are designed to determine the biological reactivity of mammalian cell cultures following contact with the elastomeric plastics and other polymeric materials with direct or indirect patient contact or of specific extracts prepared from the materials under test. It is essential that the tests be performed on the specified surface area. When the surface area of the sample cannot be determined, use 0.1 g of elastomer or 0.2 g of plastic or other material for every ml of extraction fluid. Exercise care in the preparation of the materials to prevent contamination with micro-organisms and other foreign matter.

Three tests are described (i.e., the Agar diffusion test, the Direct contact test, and the Elution test)[1]. The decision as to which type of test or the number of tests to be performed to assess the potential biological response of a specific sample or extract depends upon the material, the final product, and its intended use. Other factors that may also affect the suitability of sample for a specific use are the polymeric composition; processing and cleaning procedures; contacting media; inks; adhesives; absorption, adsorption, and permeability of preservatives; and conditions of storage. Evaluation of such factors should be made by appropriate additional specific tests before determining that a product made from a specific material is suitable for its intended use.

Reference substances High-Density Polyethylene RS and Positive Bioreaction RS.

Cell culture preparation Prepare multiple cultures

[1]Further details are given in the following publications of the American Society of Testing and Materials, 1916 Race St., Philadelphia, PA 19103: "Standard Test Method for Agar Diffusion Cell Culture Screening for Cytotoxicity," ASTM Designation F 895-84; "Standard Practice for Direct Contact Cell Culture Evaluation of Materials for Medical Devices," ASTM Designation F 813-83.

of L-929 (ATCC cell line CCL 1, NCTC clone 929) mammalian fibroblast cells in serum-supplemented minimum essential medium having a seeding density of about 10^5 cells per ml. Incubate the cultures at 37°±1° in a humidified incubator for not less than 24 hours in a 5±1 per cent carbon dioxide atmosphere until a monolayer, with greater than 80 per cent confluence, is obtained. Examine the prepared cultures under a microscope to ensure uniform, near-confluent monolayers. (**Note** The reproducibility of the *in vitro* biological reactivity tests depends upon obtaining uniform cell culture density.)

Extraction solvents Sodium Chloride Injection (use Sodium Chloride Injection containing 0.9 per cent NaCl). Alternatively, serum-free mammalian cell culture media or serum-supplemented mammalian cell culture media may be used. Serum supplementation is used when extraction is done at 37° for 24 hours.

Apparatus

AUTOCLAVE Use an autoclave capable of maintaining a temperature of 121°±2°, equipped with a thermometer, a pressure gauge, a vent cock, a rack adequate to accommodate the test containers above the water level, and a water cooling system that will allow for cooling of the test containers to about 20°, but not below 20°, immediately following the heating cycle.

OVEN Use an oven, preferably of a mechanical convection model, that will maintain operation temperatures in the range of 50° to 70° within ±2°.

INCUBATOR Use an incubator capable of maintaining a temperature of 37°±1° and a humidified atmosphere of 5±1 per cent carbon dioxide in air.

EXTRACTION CONTAINERS Use only containers, such as ampoules or screw-capped culture test-tubes, or their equivalent, of Type I glass. If used, culture test tubes, or their equivalent, are closed with a screw cap having a suitable elastomeric liner. The exposed surface of the elastomeric liner is completely protected with an inert solid disc 50 to 75 µm in thickness. A suitable disc can be fabricated from polytef.

PREPARATION OF APPARATUS Cleanse all glassware thoroughly with *chromic acid cleansing mixture* and, if necessary, with hot *nitric acid* followed by prolonged rinsing with Sterile Water for Injection. Sterilize and dry by a suitable process containers and devices used for extraction, transfer, or administration of test material. If ethylene oxide is used as the sterilizing agent, allow not less than 48 hours for complete degassing.

Procedure

PREPARATION OF SAMPLE FOR EXTRACTS Prepare as directed in Preparation of sample.

PREPATATION OF EXTRACTS Prepare as directed in Extracts using either Sodium Chloride Injection (0.9 per cent NaCl) or serum-free mammalian cell culture media as an extracting medium.
(**Note** If extraction is done at 37° for 24 hours in an incubator, use cell culture media supplemented by serum. The extraction conditions should not in any instance cause physical changes, such as fusion or melting of the material pieces, other than a slight adherence.)

2.6.1 Agar diffusion test

This test is designed for elastomeric closures in a variety of shapes. The agar layer acts as a cushion to protect the cells from mechanical damage while allowing the diffusion of leachable chemicals from the polymeric samples. Extracts of materials that are to be tested are applied to a piece of filter paper.

Sample preparation Use extracts prepared as directed or use portions of the test specimens having flat surfaces not less than 100 mm^2 in surface area.

Positive control preparation Proceed as directed for Sample preparation.

Negative control preparation Proceed as directed for Sample preparation.

Procedure Using 7 ml of cell suspensio prepared as directed under Cell culture preparation, prepare the monolayers in plates having a 60-mm diameter. Following incubation, aspirate the culture medium from the monolayers, and replace it with serum-supplemented culture medium containing not more than 2 per cent for agar.
(**Note** The quality of the agar must be adequate to support cell growth. The agar layer must be thin enough to permit diffusion of leached chemicals.) Place the flat surfaces of sample preparation, negative control preparation, and positive control preparation or their extracts in an appropriate extracting medium, in duplicate cultures in contact with the solidified agar surface. Use no more than three samples per prepared plate. Incubate all cultures for not less than 24 hours at 37°±1°, preferably in a humidified incubator containing 5±1 per cent carbon dioxide. Examine each culture around each-sample. Negative control, and positive control, under a microscope, using a suitable stain, if desired.

Evaluation The biological reactivity (cellular degeneration and malformation) is described and rated on a scale of 0 to 4 (Table 5). Measure the responses of the cell cultures to the sample preparation, the negative control preparation, and the positive control preparation. The cell culture test system is suitable if the observed responses to the positive control preparation is grade 0 (no reactivity) and to the positive control preparation is at least grade 3 (moderate). The requirements of the test are met if the response to the sample preparation is not greater than grade 2 (mildly reactive). Repeat the procedure if the suitability of the system is not confirmed.

Table 5 Reactivity Grades for Agar Diffusion Test and Direct Contact Test

Grade	Reactivity	Description of Reactivity Zone
0	None	No detectable zone around or under sample.
1	Slight	Some malformed or degenerated cells under sample.
2	Mild	Zone limited or area under sample.
3	Moderate	Zone extends 0.5 to 1.0 cm beyond sample.
4	Severe	Zone extends greater than 1.0 cm beyond sample.

2.6.2 Direct contact test

This test is designed for materials in a variety of shapes. The procedure allows for simultaneous extraction and testing of leachable chemicals from the sample with a serum-supplemented medium. The procedure is not appropriate for very low- or high-density materials that could cause mechanical damage to the cells.

Sample preparation Use portions of the test sample having flat surfaces not less than 100 mm^2 in surface area.

Positive control preparation Proceed as directed for Sample preparation.

Negative control preparation Proceed as directed for Sample preparation.

Procedure Using 2 ml of cell suspension prepared as directed under Cell culture preparation, prepare the monolayers in plates having a 35-mm diameter. Following incubation, aspirate the culture medium from the cultures, and replace it with 0.5 ml of fresh culture medium. Place a single sample preparation, negative control preparation, and a positive control preparation in each of duplicate cultures. Incubate all cultures for not less than 24 hours at 37°±1° in a humidified incubator containing 5±1 per cent carbon dioxide. Examine each culture around each sample, negative control, and positive control preparation, either visually or under a microscope, using a suitable stain, if desired.

Evaluation Proceed as directed for Evaluation under Agar diffusion test. The requirements of the test are met if the response to the sample preparation is not greater than grade 2 (mildly reactive). Repeat the procedure if the suitability of the system is not confirmed.

2.6.3 Elution test

This test is designed for the evaluation of extracts of polymeric materials. The procedure allows for extraction of the sample at physiological or non-physiological temperatures for varying time intervals. It is appropriate for high-density materials and for dose response evaluations.

Sample preparation Prepare as directed in Preparation of Extracts, using either Sodium Chloride Injection (0.9 per cent NaCl) or serum-free mammalian cell culture media as extracting medium. If the size of the sample cannot be readily measured, a mass of not less than 0.1 g of elastomeric material or 0.2 g of plastic or polymeric material per ml of extraction medium may be used. Alternatively, use serum-supplemented mammalian cell culture media as the extracting medium to simulate more closely physiological conditions. Prepare the extracts by heating for 24 hours in an incubator containing 5±1 per cent carbon dioxide. Maintain the extraction temperature at 37°±1° because higher temperatures may cause denaturation of serum proteins.

Positive control preparation Proceed as directed for Sample preparation.

Negative control preparation Proceed as directed for Sample preparation.

Procedure Using 2 ml of cell suspension prepared as directed under Cell culture preparation, prepare the monolayers in plates having a 35-mm diameter. Following incubation, aspirate the culture medium from the monolayers, and replace it with extracts of the sample preparation, negative control preparation, or positive control preparation. The serum-supplemented and serum-free cell culture media extracts are tested in duplicate without dilution (100 per cent). The Sodium Chloride Injection extract is diluted with serum-supplemented cell culture medium and tested in duplicate at 25 per cent extract concentration. Incubate all cultures for 48 hours at 37°±1° in a humidified incubator preferably containing 5±1 per cent carbon dioxide. Examine each culture at 48 hours, under a microscope, using a suitable stain, if desired.

Evaluation Proceed as directed for Evaluation under Agar diffusion test but using Table 6. The requirements of the test are met if the response to the sample preparation is not greater than grade 2 (mildly reactive). Repeat the procedure if the suitability of the system is not confirmed. For dose response evaluations, repeat the procedure, using quantitative denaturation of the sample extract.

Table 6 Reactivity Grades for Elution Test

Grade	Reactivity	Conditions of All Cultures
0	None	Discrete intracytoplasmic granules; no cell lysis.
1	Slight	Not more than 20 per cent of the cells are round, loosely attached, and without intracytoplasmic granules; occasional lysed cells are present.
2	Mild	Not more than 50 per cent of the cells are round and devoid of intracytoplasmic granules; no extensive cell lysis and empty areas between cells.
3	Moderate	Not more than 70 per cent of the cell layers contain rounded cells or are lysed.
4	Severe	Nearly complete destruction of the cell layers.

11.3 CONTAINERS FOR HUMAN BLOOD AND BLOOD COMPONENTS

Appendix 11.3 should be read in conjunction with Appendix 11.2.

1. Sterile Plastic Containers for Human Blood and Blood Components

Plastic containers for the collection, storage, processing, and administration of blood and blood components are supplied sterile. In normal conditions of use, the materials of the different parts of the containers do not release monomers or other substances in amounts likely to be harmful and do not lead to any abnormal modifications of the blood. The containers may contain anticoagulant solutions depending on their intended use.

Each container is fitted with attachments suitable for its intended use. The container may be in the form of a single unit or the collecting container may be connected by one or more tubes to one or more secondary containers to allow separation of the blood components to be effected within a closed system. The outlets are of a shape and size allowing for adequate connection of the container with the blood transfusion equipment. The protective coverings on the blood-taking needle and on the appendages are designed to ensure that sterility is maintained. They are easily removable but are tamper-evident. The containers are fitted with a suitable device for suspending or fixing that does not hinder the collection, storage, processing, or administration of blood.

The capacity of the containers is related to the nominal capacity, that is, to the volume of blood to be collected in the container and to the appropriate volume of anticoagulant solution. The containers are shaped in such a manner that when filled they may be centrifuged. They are enclosed in sealed, protective envelopes.

Description The container is sufficiently transparent to allow adequate visual examination of its contents before and after taking the blood. It is also sufficiently flexible to offer minimal resistance during filling and emptying under normal conditions of use. The container contains not more than 5 ml of air.

Resistance to centrifugation Introduce into the container a sufficient volume of *water*, previously acidified with 1 ml of 2 M *hydrochloric acid*, to fill it to its nominal capacity. Envelop the container with absorbent paper that has been impregnated with a fivefold dilution of *aqueous bromophenol blue TS* or other suitable indicator and then dried. Centrifuge at 5000 × *g* for 10 minutes. No leakage is detectable on the indicator paper and no permanent distortion occurs.

Resistance to stretch Introduce into the container a sufficient volume of *water* previously acidified with 1 ml of 2 M *hydrochloric acid* to fill it to its nominal capacity. Suspend the container by the suspending device at the opposite end from the blood-taking tube, apply an immediate force of 20 N along the axis of the tube and maintain the traction for 5 seconds. Repeat the test with the force applied to each of the parts for filling and emptying. No break and no deterioration occur.

Leakage Place the container that has been used in the test for Resistance to stretch between two plates covered with absorbent paper that has been impregnated with a fivefold dilution of *aqueous bromophenol blue TS* or other suitable indicator and then dried. Apply a force progressively to the plates to press the container so that its internal pressure (that is, the difference between the applied pressure and atmospheric pressure) reaches 67 kPa (about 502 Torr) within 1 minute. Maintain the pressure for 10 minutes. No signs of leakage are detectable on the indicator paper or at any point of attachment (seals, joints, etc.).

Emptying under pressure Fill the container with a volume of *water*, at 4° to 6°, equal to the nominal capacity. Attach a transfusion set without an intravenous cannula to one of the connectors. Compress the container so as to maintain an internal pressure of 40 kPa (about 300 Torr) throughout the emptying. The container empties in less than 2 minutes.

Speed of filling Attach the container by means of the blood-taking tube fitted with the needle to a reservoir containing a suitable solution having a viscosity equal to that of blood (a 33.5 per cent w/v solution of *sucrose* at 37° is suitable). Maintain the internal pressure

of the reservoir at 9.3 kPa (about 70 Torr) with the base of the reservoir and the upper part of the container at the same level. The volume of liquid that flows into the container in 8 minutes is not less than the nominal capacity of the container.

Resistance to temperature variations Place the container in a suitable chamber having an initial temperature of 20° to 23°. Cool rapidly to –80° and maintain at this temperature for 24 hours. Raise the temperature to 50° and maintain for 12 hours. Allow to cool to room temperature. The container complies with the test for Water Vapour Permeability described in 1.3.5 (Appendix 11.2) and the tests for Resistance to centrifugation, Resistance to stretch, Leakage, Emptying under pressure, and Speed of filling.

Transparency Prepare a 16-fold dilution of the suspension prepared for Standard of opalescence (Method I, Appendix 4.1), so as to give an absorbance at 640 nm of 0.37 to 0.43 (Appendix 2.2). Fill the empty container to its nominal capacity with the diluted suspension. The cloudiness of the diluted suspension is detectable when viewed through the container, as compared with a similar container filled with *water*.

Sterility Complies with the "Sterility Test" (Method I, Appendix 10.1) with the following modifications. Introduce aseptically into the container 100 ml of *saline TS* and shake the container to ensure that the internal surfaces have been entirely wetted. Filter the contents through a membrane filter and place the membrane in the appropriate culture medium.

Pyrogens If the container being examined contains an anticoagulant solution, empty it, rinse the container with 250 ml of Sterile Water for Injection at 19° to 21° and discard the rinsings. Fill the container with 100 ml of *pyrogen-free sodium chloride TS*. Close the container and heat it in an autoclave so that the contents are maintained at 110° for 30 minutes (solution A). Solution A complies with the "Pyrogen Test" (Appendix 8.2), using 10 ml of the solution per kg of the rabbit's weight.

Abnormal toxicity Solution A complies with the "Abnormal Toxicity Test for Biological Products" (Appendix 8.1), using 0.5 ml of the solution.

Packaging and storage Sterile plastic containers for human blood and blood components are packed in protective tamper-evident envelopes and stored at a temperature not exceeding 30°, protected from light. The protective envelopes are sufficiently robust to withstand normal handling.

On removal from its protective envelope the container shows no signs of leakage and no growth of micro-organisms.

Labelling The label states (1) the date after which the container is not intended to be used; (2) that, once withdrawn from its protective envelope, the container must be used within 10 days.

A part of the label is reserved for the information required concerning the blood or blood component for which the container is intended to be used.

The ink or other substance used to print the labels or the writing does not diffuse into the plastic material of the container and it remains legible up to the time of use.

2. Empty Sterile Containers of Plasticized Polyvinyl Chloride for Human Blood and Blood Components

Unless otherwise authorized as described in Appendix 11.2; the nature and composition of the material from which the containers are made should contain not less than 55 per cent of polyvinylchloride. No colouring matter is added.

Empty sterile containers of plasticized polyvinyl chloride for human blood and blood components comply with the tests stated under Sterile Plastic Containers for Human Blood and Blood Components, and with the following tests.

Acidity or alkalinity Introduce into the container a volume of Sterile Water for Injection corresponding to the intended volume of anticoagulant solution. Close the container and heat in an autoclave so that the contents are maintained at 110° for 30 minutes. Cool and add sufficient Sterile Water for Injection to fill the container to its nominal capacity (solution A). To a volume of solution A corresponding to 4 per cent of the nominal capacity of the container add 0.1 ml of *dilute phenolphthalein TS*: the solution remains colourless. Add 0.4 ml of 0.010 M *sodium hydroxide*: the solution is pink. Add 0.8 ml of 0.010 M *hydrochloric acid* and 0.1 ml of *methyl red TS*: the solution is orange-red or red.

Light absorption Heat Sterile Water for Injection in a borosilicate-glass flask in an autoclave at 110° for 30 minutes (solution B). Measure the light absorption of solution A (Appendix 2.2) in the range 230 to 360 nm using solution B in the reference cell. The absorbance is not more than 0.30 at any wavelength from 230 to 250 nm and not more than 0.10 at any wavelength from 251 to 360 nm.

Ammonium Place 10.0 ml of solution A in a comparison tube, add sufficient *water* to make 50.0 ml, add 2.0 ml of *sodium hydroxide* solution (1 in 3) and 1.0 ml of *alkaline mercuric potassium iodide TS*, and shake well. The solution has no more colour than the following control solution.

Control solution: Instead of solution A, use the solution prepared by the addition of *water* to 0.50 ml of *ammonium standard solution* (*10 ppm* NH_4) to make 10.0 ml, and perform in the same manner.

Extractable di(2-ethylhexyl) phthalate Prepare five standard solutions containing 0.020 per cent w/v, 0.010 per cent w/v, 0.0050 per cent w/v, 0.0020 per cent w/v and 0.0010 per cent w/v of *di(2-ethylhexyl) phthalate* in Extraction Solvent ES and measure the absorbances at

the maximum at 272 nm (Appendix 2.2). Plot a calibration curve of absorbance against concentration of di(2-ethylhexyl) phthalate.

Using the donor tubing and the needle or adaptor, fill the empty container with a volume equivalent to half the nominal capacity with Extraction Solvent ES previously heated to 37° in a well-stoppered flask. Remove the air completely from the container and seal the donor tube. Immerse the filled container and seal the donor tube. Immerse the filled container in a horizontal position in a water-bath maintained at 36° to 38° for 59 to 61 minutes without shaking. Remove the container from the water-bath, invert it gently 10 times and transfer the contents to a glass flask. Immediately measure the absorbance at the maximum at 272 nm. The percentage of di(2-ethylhexyl) phthalate, calculated from the calibration curve, is not more than 0.010 per cent w/v.

Reagent

EXTRACTION SOLVENT ES Ethanol diluted to have a relative density (Appendix 4.9), of 0.9373 to 0.9378, verified with a pycnometer.

3. Sterile Containers of Plasticized Polyvinyl Chloride for Human Blood Containing an Anticoagulant Solution

Unless otherwise authorized as described under Appendix 11.2, the nature and composition of the material from which the containers are made should meet the type specification as described under Empty Sterile Containers of Plasticized Polyvinyl Chloride for Human Blood and Blood Components.

Sterile plastic containers containing an anticoagulant solution that complies with the monograph for Anticoagulant and Preservative Solutions for Blood are used for the collection, storage and administration of blood. Before filling they comply with the description and characteristics stated under Empty Sterile Containers of Plasticized Polyvinyl Chloride for Human Blood and Blood Components.

After addition of the anticoagulant solution the containers comply with the tests stated under Sterile Plastic Containers for Human Blood and Blood Components and with the following tests.

Light absorption Measure the light absorption (Appendix 2.2), of the anticoagulant solution from the container in the range 250 to 350 nm using in the reference cell an anticoagulant solution of the same composition that has not been in contact with a plastic material. The absorbance at the maximum at 280 nm is not more than 0.5.

Extractable di(2-ethylhexyl) phthalate Carefully remove the anticoagulant solution by means of the flexible transfer tube. Using a funnel fitted to the tube, completely fill the container with *water*, leave in contact for 1 minute squeezing the container gently and empty completely. Repeat the rinsing. The container then complies with the test described under Empty Sterile Containers of Plasticized Polyvinyl Chloride for Human Blood and Blood Components.

Volume of anticoagulant solution The volume does not differ by more than ±10 per cent from the stated volume when determined by emptying the container and collecting the anticoagulant solution in a graduated cylinder.

11.4 SETS FOR THE TRANSFUSION OF HUMAN BLOOD AND BLOOD COMPONENTS

Sets for the transfusion of human blood and blood components consist principally of plastic tubing to which are fitted the parts necessary to enable the set to be used for transfusion in the appropriate manner. The sets include a closure-piercing device, a blood filter, a drip chamber, a flow regulator, and a Luer connector. Provision to allow an injection to be made into the transfusion line during use is also usually included. When the sets are to be used with containers requiring an air filter, this may be incorporated in the closure-piercing device or a separate air-inlet device may be used. The chamber enclosing the blood filter, the drip chamber and the main tubing are transparent.

All parts of the set that may be in contact with blood and blood components are sterile and pyrogen-free. The sets are not to be resterilized or reused. Each set is presented in an individual package that maintains the sterility of the contents.

Sets for the transfusion of human blood and blood components are manufactured in accordance with the requirements of good manufacturing practice for medical devices. The materials chosen and the design of the set are such as to ensure the absence of hemolytic effects. Carry out the following test on sterilized sets.

Acidity or alkalinity Make a closed circulation system from three sets and a 300-ml borosilicate-glass vessel. Fit a thermostat device to the vessel so that the temperature of the liquid in the vessel is maintained at 37°±1°. Circulate 250 ml of Sterile Water for Injection through the system in the direction used for transfusion for 2 hours at a rate of 1 litre per hour using for example a peristaltic pump applied to a piece of suitable silicone tubing as short as possible. Collect the whole of the solution and allow to cool (solution A). To 25.0 ml of solution A add 0.15 ml of a solution containing 0.1 per cent w/v of *bromothymol blue*, 0.02 per cent w/v of *methyl red* and 0.2 per cent w/v of *phenolphthalein* in *ethanol*. Not more than 0.5 ml of 0.01 M *sodium hydroxide VS* is required to change the colour of the solution to blue. To a further 25.0 ml of solution A add 0.2 ml of *methyl orange TS*. Not more than 0.5 ml of 0.01 M *hydrochloric acid VS* is required to begin the change in colour of the indicator.

Clarity and colour of solution Solution A is clear (Appendix 4.1) and colourless (Appendix 4.2).

Flow rate Using a complete set with the flow regulator fully open, pass 50 ml of a solution having a viscosity of 3 mPa.s (a 3.3 per cent w/v solution of *polyethylene glycol 4000* at 20° is suitable) under a static head of 1 m. The time taken for the passage of the solution is not more than 90 seconds.

Light absorption The absorbance of solution A is not more than 0.30 at any wavelength in the range 230 to 250 nm and not more than 0.15 at any wavelength in the range 251 to 360 nm (Appendix 2.2).

Resistance to pressure Make tight the extremities of the set and any air-inlet device. Connect the set to a compressed air outlet fitted with a pressure regulator. Immerse the set in a tank of water at 20° to 23°. Apply progressively an excess pressure of 100 kPa (750 Torr) and maintain for 1 minute. No air bubble escapes from the set.

Transparency Prepare an eightfold dilution of the suspension prepared for Standard of opalescence (Method I, Appendix 4.1) for sets having tubing with an external diameter of less than 5 mm and a 16-fold dilution of the suspension for sets having tubing with an external diameter equal to or greater than 5 mm. The opalescence and presence of bubbles of the diluted suspension are discernible when it is circulated through the set, as compared with a set from the same batch filled with *water*.

Ethylene oxide Not more than 10 ppm if the label states that ethylene oxide has been used for sterilization, using the following method. Carry out the determination as described in the "Gas Chromatography" (Appendix 3.4) using the following gaseous solutions. For solution A, remove the set from the packaging and weigh. Cut the set into pieces with a maximum dimension of 1 cm and place the pieces in a 250- to 500-ml vial containing 150 ml of *dimethylacetamide*. Close the vial with a suitable stopper and secure the stopper. Place the vial in an oven at 70°±1° for 16 hours. Remove 1 ml of the hot gas from the vial and inject it onto the column. Prepare solution B under a ventilated hood as follows. Place 50 ml of *dimethylacetamide* in a 50-ml vial, stopper, secure the stopper, and weigh to the nearest 0.1 mg. Fill a 50-ml polyethylene or polypropylene syringe with gaseous *ethylene oxide*, and allow the gas to remain in contact with the syringe for about 3 minutes, empty the syringe, and fill again with 50 ml of gaseous *ethylene oxide*. Fit a hypodermic needle to the syringe and reduce the volume of gas in the syringe from 50 ml to 25 ml. Inject the remaining 25 ml of gas slowly into the vial, shaking gently and avoiding contact between the needle and the dimethylacetamide. Weigh the vial again. The increase in weight is 45 to 60 mg. Using this increase in weight calculate the exact concentration of the solution (about 1 g per litre).

Prepare a calibration curve using a series of seven vials of the same type as that used in the preparation of solution A, each containing 150 ml of *dimethylacetamide*. Introduce respectively 0, 0.05, 0.10, 0.20, 0.50, 1.00, and 2.00 ml of solution B, that is, about 0, 50, 100, 200, 500, 1000, and 2000 µg of ethylene oxide. Stopper the vials, secure the stoppers and place the vials in an oven at 70°±1° for 16 hours. Inject 1 ml of the hot gas from each vial onto the column and prepare a calibration curve from the heights of the peaks and the weight of ethylene oxide in each flask.

The chromatographic procedure may be carried out using (a) a stainless steel column (1.5 m × 6.4 mm) packed with *silanized diatomaceous support* coated with 30 per cent w/w of polyethylene glycol 1500 and maintained at 40°; (b) *helium* as the carrier gas with a flow rate of 20 ml per minute and (c) an inlet port temperature of 100° and a detector temperature of 150°. Verify the absence of peaks interfering with the ethylene oxide peak by carrying out the test using an unsterilized set or using the chromatographic procedure prescribed above but using a column such as a stainless steel column (3 m × 3.2 mm) packed with *silanized diatomaceous support* coated with 20 per cent w/w of triscyanoethoxypropane and maintained at 60°.

Calculate the weight of ethylene oxide in the vial used in the preparation of solution A from the calibration curve prepared as described above and from the height of the peak obtained in the chromatogram obtained with solution A.

Extraneous particles Using the normal inlet, fill the set with a 0.01 per cent w/v solution of *sodium dodecyl sulfate* previously filtered through a sintered-glass filter (pore size 10 to 16 µm) and heated to 37°. Collect the liquid through the normal outlet. When examined under suitable conditions of visibility, the liquid is clear and practically free from visible particles and filaments. (It is assumed that particles and filaments with a diameter equal to or greater than 50 µm are visible to the naked eye.)

Reducing substances Carry out the test within 4 hours of preparing solution A. To 20.0 ml of solution A, add 1 ml of 1 M *sulfuric acid* and 20.0 ml of 0.002 M *potassium permanganate VS* and boil for 3 minutes. Cool immediately, add 1 g of *potassium iodide* and titrate with 0.01 M *sodium thiosulfate VS* using 0.25 ml of *starch TS* as indicator. Repeat the operation using 20 ml of Sterile Water for Injection. The difference between the titration volumes is not more than 2.0 ml.

Residue on evaporation Evaporate 50.0 ml of solution A to dryness on a water-bath and dry to constant weight at 100° to 105°. Repeat the operation using 50.0 ml of Sterile Water for Injection. The difference between the weights of the residues is not more than 1.5 mg.

Sterility Comply with the "Sterility Test" (Appendix 10.1) with the following modifications.

If the sets are stated to be sterile internally only, pass 50 ml of *buffered sodium chloride-peptone solution pH 7.0* through the set and use to carry out the test by

Method I: Membrane filtration.

If the sets are stated to be sterile both internally and externally, open the packaging using aseptic precautions. When carrying out the test by Method I: Membrane filtration, place the set or its components in a suitable container containing a sufficient quantity of *buffered sodium chloride-peptone solution pH 7.0* to allow total rinsing for 10 minutes. When carrying out the test by Method II: Direct inoculation, place the set or its components in a suitable container containing a sufficient quantity of the culture medium to ensure complete immersion.

Pyrogens Connect together five sets and pass through the assembly 250 ml of *pyrogen-free sodium chloride TS* with a flow rate not exceeding 10 ml per minute. Collect the solution aseptically in a pyrogen-free container. The solution complies with the "Pyrogen Test" (Appendix 8.2), using 10 ml of the solution per kg of the rabbit's weight.

Labelling The label states, where appropriate, that the set has been sterilized using ethylene oxide.

APPENDIX 12 STERILIZATION AND STERILITY ASSURANCE

Sterilization is the process of rendering an article, or product, free from viable micro-organisms. It may be effected by killing the micro-organisms by physical or chemical methods or by removing them by filtration. Wherever possible, a process in which the product is sterilized in its final container (terminal sterilization) is chosen. If terminal sterilization is not possible, filtration through a bacteria-retentative filter or aseptic processing is used.

The method of attaining sterility in an article is determined by the nature of the product, the extent and type of any contamination present and the conditions under which the product has been prepared; it is assumed that the principles of good manufacturing practice will have been observed. Materials to be sterilized should be as free as possible from microbial contamination. The effect of the chosen sterilization process on the product (including its final container or package) should be validated before that procedure is applied in practice. Failure to follow a process meticulously involves the risk of a non-sterile or deteriorated product. Proper validation of the sterilization process or the aseptic process requires, however, a high level of knowledge of the field of sterilization and clean room technology. In order to comply with currently acceptable and achievable limits in sterilization parameters, it is necessary to employ appropriate instrumentation and equipment to control the critical parameters such as temperature and time, humidity, and sterilizing gas concentration, or absorbed radiation. An important aspect of the validation programme in many sterilization procedures involves the employment of biological indicators. The validated and certified process should be revalidated periodically; however, the revalidation programme need not necessarily be as extensive as the original programme.

Within the strictest definition of sterility, an article would be deemed sterile only when there is complete absence of viable micro-organisms from it. However, this absolute definition cannot currently be applied to an entire lot of finished compendial articles because of limitations in testing. Absolute sterility cannot be practically demonstrated without complete destruction of every finished article. The sterility of a lot purported to be sterile is therefore defined in probabilistic terms, where the likelihood of a contaminated unit or article is acceptably remote. Such a state of sterility assurance can be established only through the use of adequate sterilization cycles and subsequent aseptic processing, if any, under appropriate current good manufacturing practice, and not by relying solely on sterility testing.

Sterility assurance level (SAL) The SAL of a sterilizing process is the degree of assurance with which the process in question renders a population of items sterile. The SAL for a given process is expressed as the probability of a non-sterile item in that population. An

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SAL of 10^{-6}, for example, denotes a probability of not more than one viable micro-organism in 1×10^6 sterilized items of the final product. The SAL of a process for a given product is established by appropriate validation studies.

Methods of Sterilization

Methods of terminal sterilization, including removal of micro-organism by filtration, and aseptic processing are described. Modern technological developments, however, have led to the use of additional procedures. The choice of the appropriate process for a given dosage form or component requires a high level of knowledge of sterilization techniques and information concerning any effects of the process on the material being sterilized.

Steam sterilization The process of thermal sterilization employing saturated steam under pressure is carried out in a chamber called an autoclave. It is probably the most widely employed sterilization process, especially for aqueous preparation. The basic principle of operation is that the air in the sterilizing chamber is displaced by the saturated steam, achieved by employing vents or traps. In order to displace air more effectively from the chamber and from within articles, the sterilization cycle may include air and steam evacuation stages.

For this method of terminal sterilization, the reference conditions for aqueous preparations are heating at a minimum of 121° for 15 minutes. Other combinations of time and temperature may be used provided that it has been satisfactorily demonstrated that the process chosen delivers an adequate and reproducible level of lethality when operating routinely within the established tolerances. The procedures and precautions employed are such as to give an SAL of 10^{-6} or better. A biological assessment of the process may be obtained by including a suitable biological indicator.

With large batch sizes of aqueous preparations, it is essential to have knowledge of the physical conditions within the autoclave chamber during the sterilization procedure. To obtain this information, recording temperature-sensitive elements inserted into representative containers may be used together with additional elements at the previously-established coolest part of the loaded chamber. It is desirable that each sterilization cycle be recorded on a temperature-time chart. Other types of temperature indicator may be inserted at appropriate positions in the load but total reliance should not be placed on chemical indicators except when they suggest failure to attain sterilizing conditions.

When surgical dressings are sterilized by Steam sterilization, the steam used should not contain more than 5 per cent of entrained moisture. Most dressings are sterilized by maintaining at a temperature of 134° to 138° for 3 minutes, but other suitable combinations of temperature and time may be used, the conditions being chosen with regard to the stability of the dressings.

Apart from that description of sterilization cycle parameters, using a temperature of 121°, the F_0 concept may be appropriate. The F_0, at a particular temperature other than 121°, is the time (in minutes) required to provide the lethality equivalent to that provided at 121° for a stated time.

The total F_0 of a process takes account of the heating up and cooling down phases of the cycle and can be calculated by integration of lethal rates with respect to time at discrete temperature intervals.

When a steam sterilization cycle is chosen on the basis of the F_0 concept, great care must be taken to ensure that an adequate assurance of sterility is consistently achieved. In addition to validating the process, it may also be necessary to perform continuous, rigorous microbiological monitoring during routine production to demonstrate that the microbiological parameters are within the established tolerances so as to given as SAL of 10^{-6} or better.

In connection with sterilization by steam, the Z-value relates the heat resistance of a micro-organism to changes in temperature. The Z-value is the change in temperature required to alter the D-value by a factor of 10.

The D-value (or decimal reduction value) is the value of a parameter of sterilization (duration or absorbed dose) required to reduce the number of viable organisms to 10 per cent of the original number. It is only of significance under precisely defined experimental conditions.

The following mathematical relationships apply:

$$F_0 = D_{121}(\log N_0 - \log N) = D_{121} \log IF$$

where D_{121} = D-value of the reference spores at 121°,
N_0 = initial number of viable micro-organisms,
N = final number of viable micro-organisms,
IF = inactivation factor.

$$Z = (T_2 - T_1)/(\log D_1 - \log D_2)$$

where D_1 = D-value of the micro-organism at temperature T_1,
D_2 = D-value of the micro-organism at temperature T_2.

$$IF = N_0/N = 10^{t/D}$$

where t = exposure time,
D = D-value of micro-organism in the exposure conditions.

Dry-heat sterilization Dry-heat sterilization may be used for heat stable non-aqueous preparations, powders and certain impregnated dressings. For this method of terminal sterilization the reference conditions are a minimum of 160° for at least 2 hours. Other combinations of time and temperature may be used provided that it has been satisfactorily demonstrated that the process chosen delivers an adequate and reproducible level of lethality when operated routinely within the established tolerances. The procedures and precautions employed are such as to give an SAL of 10^{-6} or better. A

modern oven is supplied with heated, filtered air, distributed uniformly throughout the chamber by convection or radiation and employing a blower system with devices for sensing, monitoring, and controlling the critical parameters.

Appropriate biological indicators may be employed to demonstrate the effectiveness of the sterilization process. An example of a biological indicator for validating and monitoring dry-heat sterilization is a preparation of *Bacillus subtilis* spores. For heat-stable articles or components, the conditions of sterilization are not less than 250°. A microbial survival probability of 10^{-12} is considered achievable for heat-stable articles or components. Since dryheat at 250° is frequently employed to render glassware or containers free from pyrogens as well as viable microbes, a pyrogen challenge, where necessary, should be an integral part of the validation program, e.g., by inoculating one or more of the articles to be treated with 1000 or more EU of bacterial endotoxin. The test with *Limulus* lysate could be used to demonstrate that the endotoxic substance has been inactivated to not more than 1/1000 of the original amount (3 log cycle reduction). For the test to be valid, both the original amount and, after acceptable inactivation, the remaining amount of endotoxin should be measured. For additional information on the endotoxin assay, see under the "Test for Bacterial Endotoxins" (Appendix 8.5).

Gas sterilization This method of sterilization is only to be used where there is no suitable alternative. It is essential that penetration by gas and moisture into the material to be sterilized is ensured and that it is followed by a process of elimination of the gas under conditions that have been previously established to ensure that any residue of gas or its transformation products in the sterilized product is below the concentration that could give rise to toxic effects during use of the product. The active agent generally employed in gaseous sterilization is ethylene oxide of acceptable sterilizing quality or a mixture of ethylene oxide with a suitable inert gas.

Wherever possible, the gas concentration, relative humidity, temperature and duration of the process are measured and recorded. Measurements are made where sterilization conditions are least likely to be achieved, as determined at validation.

The effectiveness of the process applied to each sterilization load is checked using a suitable biological indicator.

Ionizing radiation sterilization Sterilization by this method is achieved by exposure of the product to ionizing radiation in the form of gamma radiation from a suitable radioisotopic source, such as cobalt-60 (^{60}Co) or of a beam of electrons energized by a suitable electron accelerator.

For this method of terminal sterilization the reference absorbed dose is 25 kGy. Other doses may be used provided that it has satisfactorily been demonstrated that the dose chosen delivers an adequate and reproducible level of lethality when the process is operated routinely within the established tolerances. The procedures and precautions employed are such as to give an SAL of 10^{-6} or better.

During the sterilization procedure the radiation absorbed by the product is monitored regularly by means of established dosimetry procedures that are independent of dose rate. Dosimeters are calibrated against a standard source at a reference radiation plant on receipt from the supplier and at suitable intervals of not longer than one year thereafter.

Where a biological assessment is carried out, this is obtained using a suitable biological indicator.

Filtration Sterilization by Filtration may be used for certain medicaments and preparations which are not sufficiently stable to heat to allow sterilization by steam sterilization. Solutions or liquids may be sterilized by passage through a sterile bacteria-retaining filter of a type that has been demonstrated to be satisfactory by means of a microbial challenge test using a suitable test micro-organism. A suspension of *Pseudomonas diminuta* (ATCC 19146, NCIMB 11091 or CIP 103020) may be suitable. It is recommended that a challenge of at least 10^7 CFU per cm^2 of active filter surface is used and that the suspension is prepared in Soybean-casein digest medium which, after passage through the filter, is collected aseptically and incubated aerobically at 30° to 35°. Such products need special precautions. The production process and environment are regularly subjected to appropriate monitoring procedures. The equipment, containers and closures and, wherever possible, the ingredients are subjected to an appropriate sterilization process. It is recommended that the filtration process be carried out as close as possible to the filling point. The operations following filtration are carried out under aseptic conditions.

Filtration for sterilization is usually carried out with assemblies having membranes of porosity not greater than 0.22 μm; however, membranes of smaller porosities are also used and may be needed for some products. The types of membrane filter which are now available include cellulose acetate, cellulose nitrate, fluorocarbonate, acrylic polymers, polycarbonate, polyester, polyvinyl chloride, and even metal membranes, and they may be reinforced or supported by an internal fabric. A membrane filter assembly should be tested for integrity of the membrane and its effectiveness confirmed before and after use. A typical test is the bubble-point test, whereby it is determined that a prescribed pressure is necessary to force air bubbles through the intact membrane wetted with either product, water or hydrocarbon liquid.

Biological Indicators

Biological indicators are standardized preparations of selected micro-organisms used to assess the effectiveness of a sterilization procedure. They usually consist of a population of bacterial spores placed on an inert carrier, for example a strip of filter paper, a glass slide or a plastic tube. The inoculated carrier is covered in such

a way that it is protected from any deterioration or contamination, while allowing the sterilizing agent to enter into contact with the micro-organisms. Spore suspensions may be presented in sealed ampoules. Biological indicators are prepared in such a way that they can be stored under defined conditions; an expiry date is set. Micro-organisms of the same bacterial species as the bacteria used to manufacture the biological indicators may be inoculated directly into a liquid product to be sterilized or into a liquid product similar to that to be sterilized. In this case, it must be demonstrated that the liquid product has no inhibiting effect on the spores used, especially as regards their germination.

A biological indicator is characterized by the name of the species of bacterium used as the reference micro-organism, the number of the strain in the original collection, the number of viable spores per carrier and the D-value. The D-value is the value of a parameter of sterilization (duration or absorbed dose) required to reduce the number of viable organisms to 10 per cent of the original number. It is of significance only under precisely defined experimental conditions. Only the stated micro-organisms are present. Biological indicators consisting of more than one species of bacteria on the same carrier may be used. Information on the culture medium and the incubation conditions is supplied. It is recommended that the indicator organisms be placed at the locations presumed, or wherever possible, found by previous physical measurement to be least accessible to the sterilizing agent. After exposure to the sterilizing agent, aseptic technique is used to transfer carriers of spores to the culture media, so that no contamination is present at the time of examination. Biological indicators that include an ampoule of culture medium placed directly in the packaging protecting the inoculated carrier may be used.

A choice of indicator organisms is made such that:

(a) the resistance of the test strain to the particular sterilization method is great compared to the resistance of all pathogenic micro-organisms and to that of micro-organisms potentially contaminating the product;

(b) the test strain is non-pathogenic;

(c) the test strain is easy to culture.

After incubation, growth of the reference micro-organisms subjected to the sterilization procedure demonstrates that this procedure is unsatisfactory.

Steam sterilization The use of biological indicators intended for steam sterilization is recommended for the validation of sterilization cycles. Spores of *Bacillus stearothermophilus* (for example, ATCC 7953, NCTC 10007, NCIMB 8157 or CIP 52.81) are recommended. The number of viable spores exceeds 5×10^5 per carrier. The D-value at 121° exceeds 1.5 minutes. It is verified that exposing the biological indicators to steam at 121°±1° for 6 minutes leaves revivable spores, and that there is no growth of the reference micro-organisms after the biological indicators have been exposed to steam at 121°±1° for 15 minutes.

Dry-heat sterilization Spores of *Bacillus subtilis* (for example, var. *niger* ATCC 9372, NCIMB 8058 or CIP 77.18) are recommended for the preparation of biological indicators. The number of viable spores exceeds 1×10^5 per carrier and the D-value at 160° is approximately 5 to 10 minutes. Dry heat at 250° is frequently used for sterilization and depyrogenation of glassware. In this case, demonstration of a 3 log reduction in heat resistant bacterial endotoxin can be used as a replacement for biological indicators.

Gas sterilization The use of biological indicators is necessary for all gas sterilization procedures, both for the validation of the cycles and for routine operations. The number of viable spores exceeds 5×10^5 per carrier. For hydrogen peroxide and peracetic acid spores of *Bacillus stearothermophilus* (for example ATCC 7953, NCTC 10007, NCIMB 8157 or CIP 52.81), for ethylene oxide and formaldehyde spores of *Bacillus subtilis* (for example, var. *niger* ATCC 9372, NCIMB 8058 or CIP 77.18) are recommended. The parameters of resistance are known for the procedure used: for example, for ethylene oxide, the D-value exceeds 2.5 minutes for a test cycle involving 600 mg per litre of ethylene oxide, at 54° and at 60 per cent relative humidity. It is verified that there is no growth of the reference micro-organisms after the biological indicators have been exposed to the test cycle described above for 60 minutes and that exposing the indicators to a reduced temperature cycle (600 mg per litre at 30° and 60 per cent relative humidity) for 15 minutes leaves revivable spores. It is essential that the biological indicator be able to reveal insufficient humidification in the sterilizer and the product to ensure dehydrated micro-organisms are inactivated. Exposing the indicators to 600 mg per litre of ethylene oxide at 54° for 60 minutes without humidification must leave revivable spores.

Ionizing radiation sterilization Biological indicators may be used to monitor routine operations, as an additional possibility to assess the effectiveness of the set dose of radiation energy, especially in the case of accelerated electron sterilization. The spores of *Bacillus pumilus* (for example, ATCC 27.142, NCTC 10327, NCIMB 10692 or CIP 77.25) are recommended. The number of viable spores exceeds 1×10^7 per carrier. The D-value exceeds 1.9 kGy. It is verified that there is no growth of the reference micro-organisms after the biological indicators have been exposed to 25 kGy (minimum absorbed dose).

Aseptic Processing

While there is general agreement that sterilization of the final filled container as a dosage form or final packaged device is the preferred process for assuring the minimal risk of microbial contamination in a lot, there is a substantial class of products that are not terminally sterilized but are prepared by a series of aseptic steps. These are designed to prevent the introduction of viable micro-organisms into components, where sterile, or once an intermediate process has rendered the bulk

product or its components free from viable micro-organisms. A review of the principles involved in producing aseptically processed products with a minimal risk of microbial contamination in the finished lot of final dosage forms is hereby provided.

A product defined as aseptically processed is likely to consist of components that have been sterilized by one of the processes described above. For example, the bulk product, if filterable, may have been sterilized by filtration. The final empty container components would probably be sterilized by heat, dry heat being employed for glass vials and an autoclave being employed for rubber closures. The areas of critical concern are the immediate microbial environment where these pre-sterilized components are exposed during assembly to produce the finished dosage form and the aseptic filling operation.

The requirements for a properly designed, validated and maintained filling or other aseptic processing facility are mainly directed to (1) an air environment free from viable micro-organisms, of a proper design to permit effective maintenance of air supply units, and (2) the provision of trained operating personnel who are adequately equipped and gowned. The desired environment may be achieved through the high level of air filtration technology, which contributes to the delivery of air of the requisite microbiological quality. The facilities include both primary (in the vicinity of the exposed article) and secondary (where the aseptic processing is carried out) barrier systems.

For a properly designed aseptic processing facility or aseptic filling area, consideration should be given to such features as nonporous and smooth surfaces, including walls and ceilings that can be sanitized frequently; gowning rooms with adequate space for personnel and storage of sterile garments; adequate separation of preparatory rooms for personnel from final aseptic processing rooms, with the availability where necessary of such devices as airlocks and/or air showers; proper pressure differentials between rooms, the most positive pressure being in the aseptic processing rooms or areas; the employment of laminar (unidirectional) air flow in the immediate vicinity of exposed product or components, and filtered air exposure there to, with adequate air change frequency; appropriate humidity and temperature environmental controls; and a documented sanitization programme. Proper training of personnel in hygienic and gowning techniques should be undertaken so that, for example, gowns, gloves, and other body coverings substantially cover exposed skin surfaces.

Certification and validation of the aseptic process and facility is achieved by establishing the efficiency of the filtration systems, by employing microbiological environmental monitoring procedures, and by processing of sterile culture medium as simulated product.

Monitoring of the aseptic facility should include periodic environmental filter examination as well as routine particulate and microbiological environmental monitoring, and may include periodic sterile culture medium processing.

Sterility Testing of Lots

It should be recognized that the referee sterility test might not detect microbial contamination if present in only a small percentage of the finished articles in the lot because the specified number of units to be taken imposes a significant statistical limitation on the utility of the test results. This inherent limitation, however has to be accepted since current knowledge offers no non-destructive alternatives for ascertaining the microbiological quality of every finished article in the lot, and it is not a feasible option to increase the number of sample s significantly.

The primary means of supporting the claims that a lot of finished articles purporting to be sterile meets the specifications consist of the documentation of the actual production and sterilization record of the lot and of the additional validation records that the sterilization process possesses the capability of totally inactivating the established product microbial burden or a more resistant challenge. Further, it should be demonstrated that any processing steps involving exposed product following the sterilization procedure are performed in an aseptic manner, to prevent contamination. If data derived from the manufacturing process sterility assurance validation studies and from inprocess controls are judged to provide greater assurance that the lot meets the required low probability of containing a contaminated unit (compared to sterility testing results from finished units drawn from that lot), any sterility test procedures adopted may be minimal, or dispensed with on a routine basis. However, assuming that all of the above production criteria have been met, it may still be desirable to perform sterility testing on samples of the lot of finished articles. Such sterility testing is usually carried out directly after the lot is manufactured as a final product quality control test. Sterility tests employed in this way in manufacturing control should not be confused with those described under the other section of "Sterility Test" (Appendix 10.1). The procedural details may be the same with regard to media, inocula and handling of samples, but the number of units and/or incubation time(s) selected for testing may differ. The number should be chosen relative to the purpose to be served, i.e., according to whether greater or lesser reliance is placed on sterility testing in the context of all the measures for sterility assurance in manufacture. Also, longer times of incubation would make the test more sensitive to slow-growing micro-organisms. In the growth promotion tests for media, such slow growers, particularly if isolated from the product microbial burden, should be included with the other test strains.

Negative or satisfactory sterility test results serve only as further support of the existing evidence concerning the quality of the lot if all of the pertinent production records of the lot are in order and the sterilizing or aseptic process is known to be effective. Unsatisfactory test results, however, in manufacturing quality

control indicate a need for further action.

Interpretation of quality control tests Quality control sterility tests (either according to the official referee test or modified tests) may be carried out in two separate stages in order to rule out false positive results.

First stage Regardless of the sampling plan used, if no evidence of microbial growth is found, the results of the test may be taken as indicative of absence of intrinsic contamination of the lot.

If microbial growth is found, proceed to the Second stage (unless the First stage test can be invalidated). Evidence for invalidating a First stage test in order to repeat it as a First stage test may be obtained from a review of the testing environment and the relevant records there to. Finding of microbial growth in negative controls need not be considered the sole grounds for invalidating a First stage test. When proceeding to the Second stage, particularly where depending on the results of the test for lot release, concurrently initiate and document a complete review of all applicable production and control records. In this review consideration should be paid to the following:

a. A check on monitoring records of the validated sterilization cycle applicable to the product.

b. Sterility test history relating to the particular product for both finished and in-process samples, as well as sterilization records of supporting equipment, con-tainers/closures, and sterile components, if any.

c. Environmental control data, including those obtained from media fills, exposure plates, filtering records, any sanitization records and microbial monitoring records of operators, gowns, gloves, and garbing practices.

Failing any lead from the above review, the current microbial profile of the product should be checked against the known historical profile for possible change. Records should be checked concurrently for any changes in source of product components and/or inprocessing procedures that might be contributory. Depending on the findings, and in extreme cases, consideration may have to be given to revalidation of the total manufacturing process.

Second stage For the Second stage it is not possible to specify a particular number of sample to be taken for testing. It is usual to select double the number specified for the First stage under "Sterility Tests" (Appendix 10.1), or other reasonable number. The minimum volumes tested from each sample, the media, and the incubation periods are the same as those indicated for the First stage.

If no microbial growth is found in the Second stage, and the documented review of appropriate records and the indicated product investigation does not support the possibility of intrinsic contamination, the lot may be considered to meet the requirements of a test for sterility. If growth is found, the lot fails to meet the requirements of the test. As was indicated for the First stage test, the Second stage test may similarly be invalidated with appropriate evidence, and, if so done, repeated as a Second stage test.

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APPENDIX 13 INFRARED REFERENCE SPECTRA

Introduction

This section of infrared absorption spectra consists of a collection of reference spectra for use in conjunction with the analytical specifications that are contained in the monographs.

The infrared spectroscopy is useful as a means of verifying identity of the substances in the monographs, which can be performed by comparing with the TP infrared spectra or spectra prepared from reference substances similarly treated. The advantage of publishing these spectra is principally that substantial economies of both time and expense will be effected by allowing a reduction in the number of Reference Substances to be collected by the drug manufacturers and the laboratories concerned.

Reference Spectra

The differences of a spectrum obtained from one laboratory to another may be associated with the use of different types of instruments. A major contributory cause to such differences would be the nature of monochromator whether prism or grating. Spectra of TP substances were recorded on a nondispersive multiplex instrument employing the Fourier transform. As an aid to comparison, a Reference Spectrum of polystyrene film is presented with the recommendation that a similar spectrum be prepared on the instrument being used to carry out the test.

Preparation of Spectra

All reference spectra were prepared by Department of Medical Sciences, Bureau of Drug and Narcotic and recorded on a Jasco model 4100 fourier transform infrared spectrophotometer.

The substances used for the preparation of reference spectra were USP Reference Standards, BP Chemical Reference Substances, Ph. Eur. Chemical Reference Substances, WHO International Chemical Reference Substances, WHO International Biological Standards and DMSc Reference Standards. In cases the mentioned substances were not available, the high purity substances were obtained from pharmaceutical manufacturers.

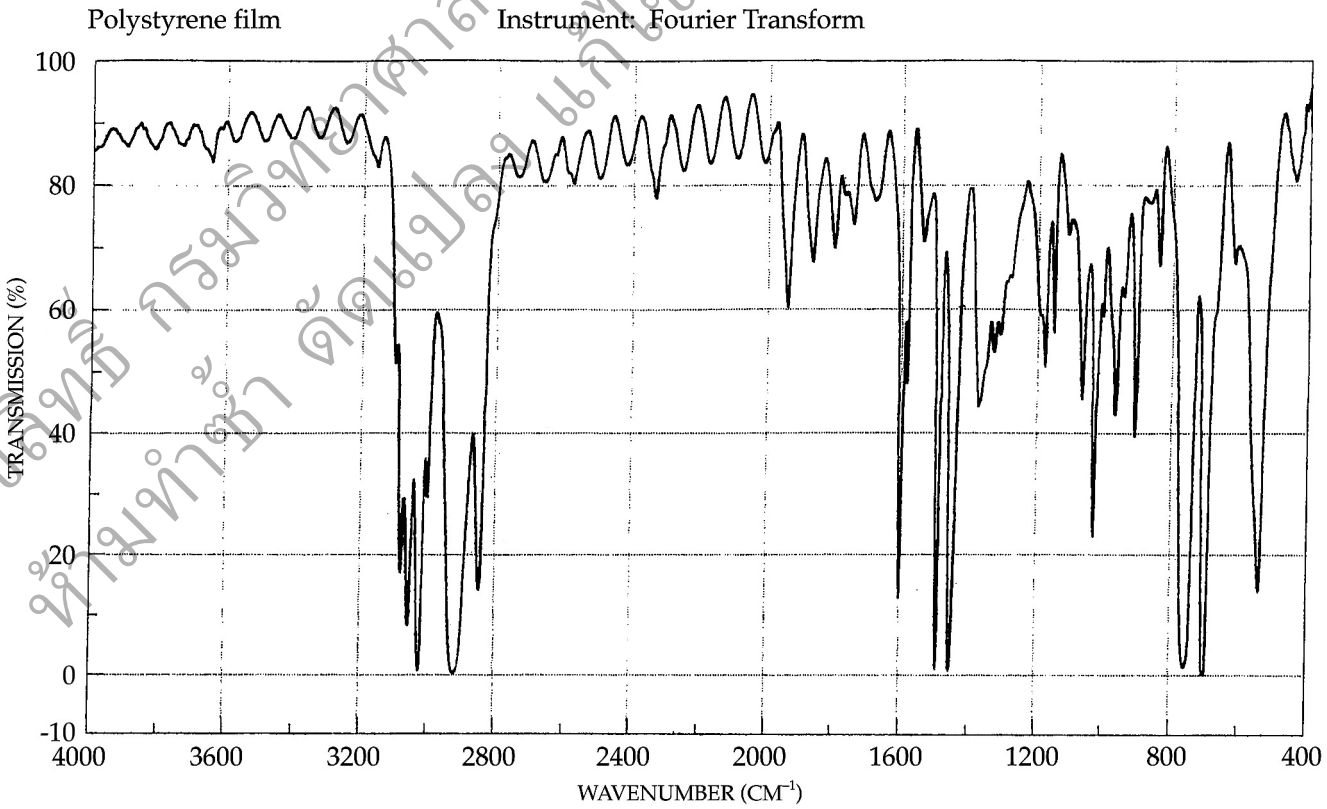
The method for preparation of sample being examined: an approximate 5 per cent of the substance to be examined was triturated and mixed with finely powdered and dried potassium bromide. Place the mixture in a sample cup and examine the reflectance spectrum. The prepared spectra were carefully compared with the approved appropriate reference spectra, using the maxima of a polystyrene spectrum to account for possible differences in wavelength calibration.

The spectra were labelled with the following information:

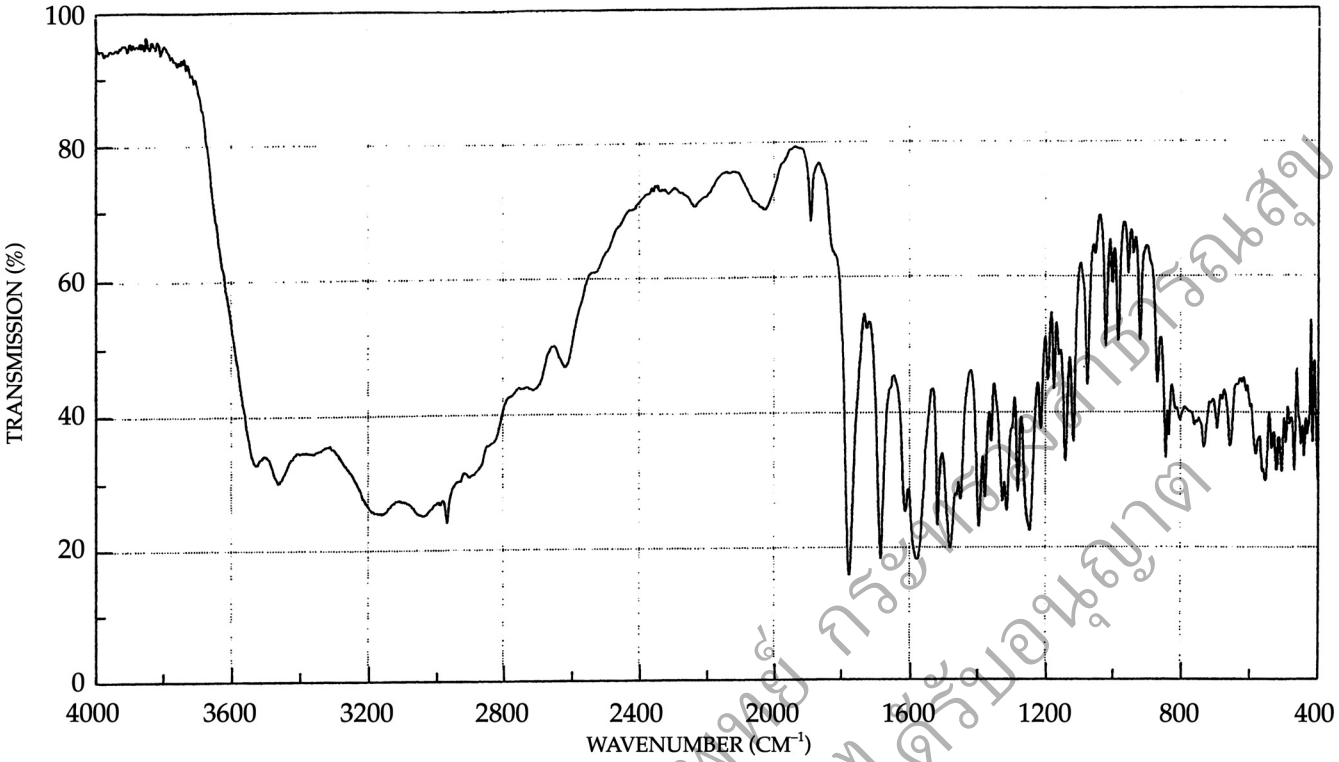
Generic Name

Phase of the Sample Preparation.

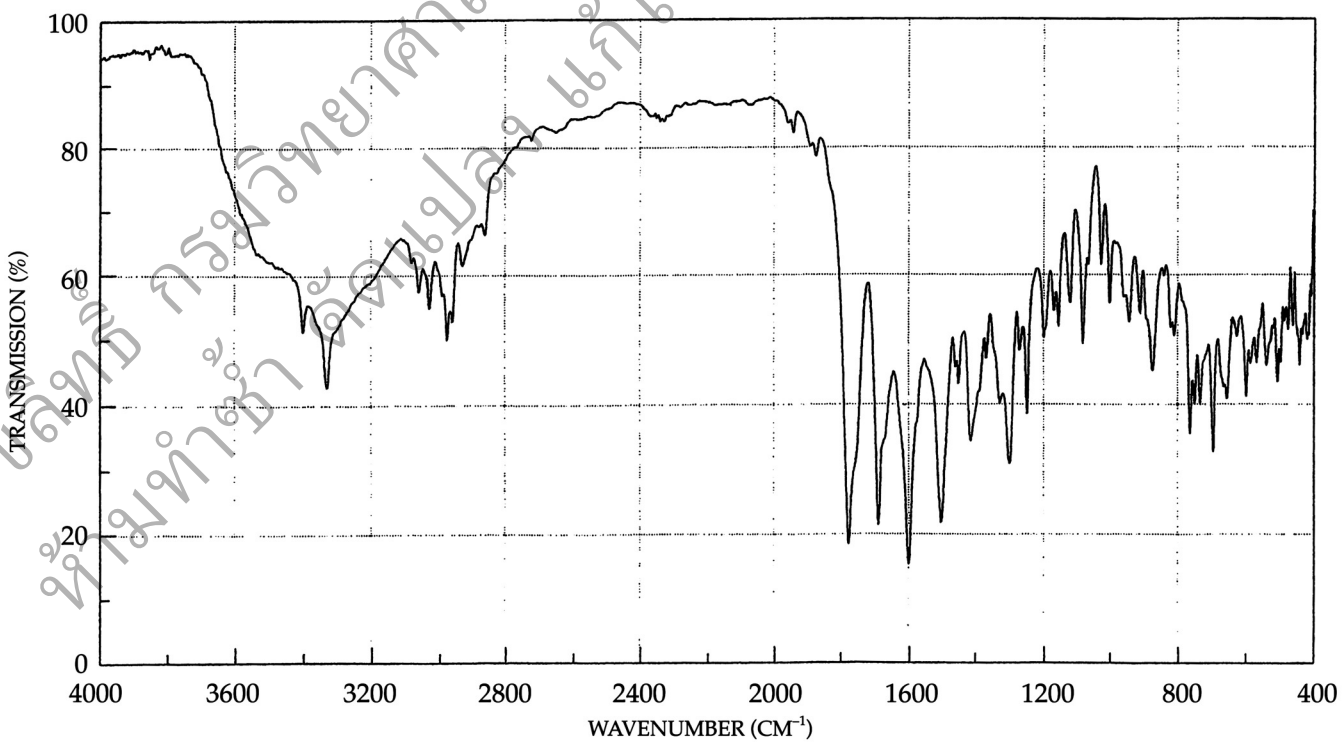
The spectra were determined in the region from 400 cm^{-1} to 4000 cm^{-1} and were represented on a transmittance (%) *vs* linear wavenumber (cm^{-1}) format. The spectra were labelled in accordance with the final form of the substance and not necessarily with the name of the monograph in question.



Amoxicillin Trihydrate Instrument: Fourier Transform Phase: Potassium Bromide mixture



Ampicillin Sodium Instrument: Fourier Transform Phase: Potassium Bromide mixture



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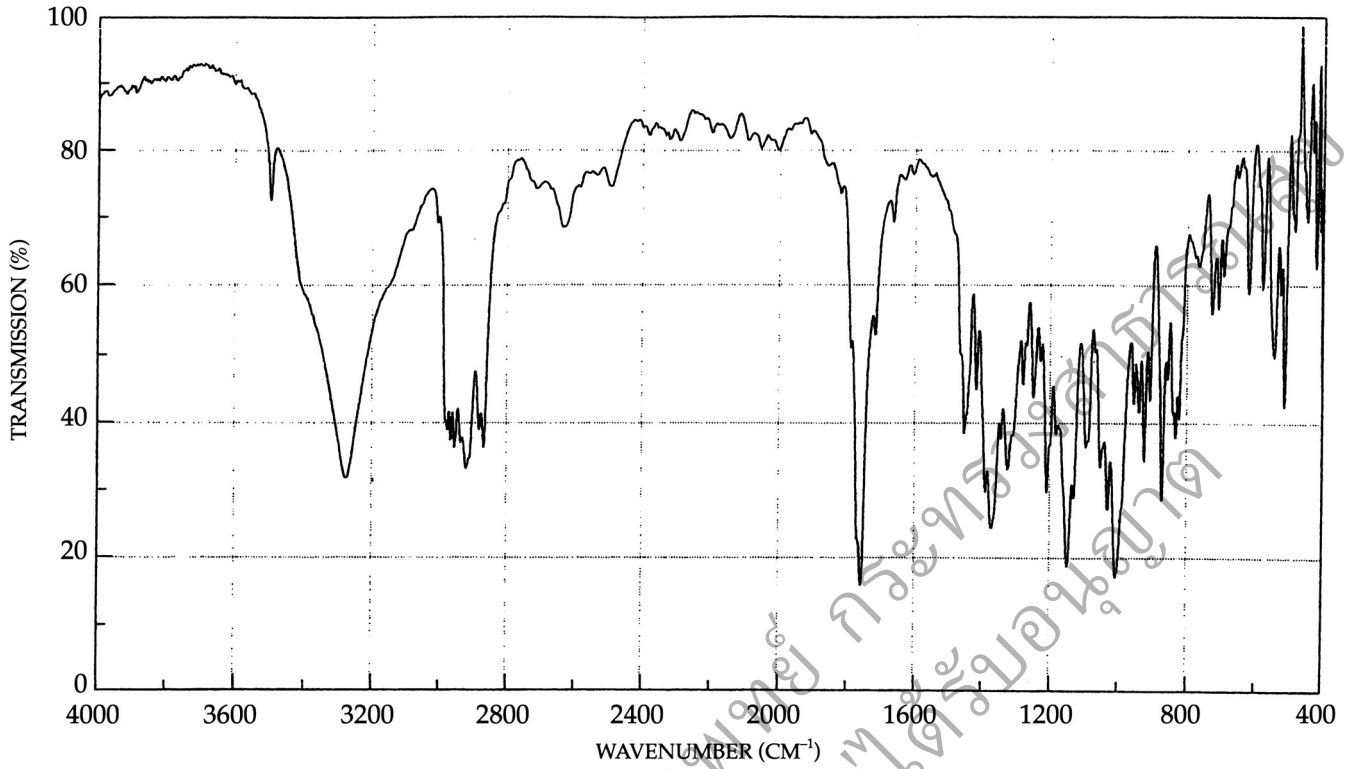
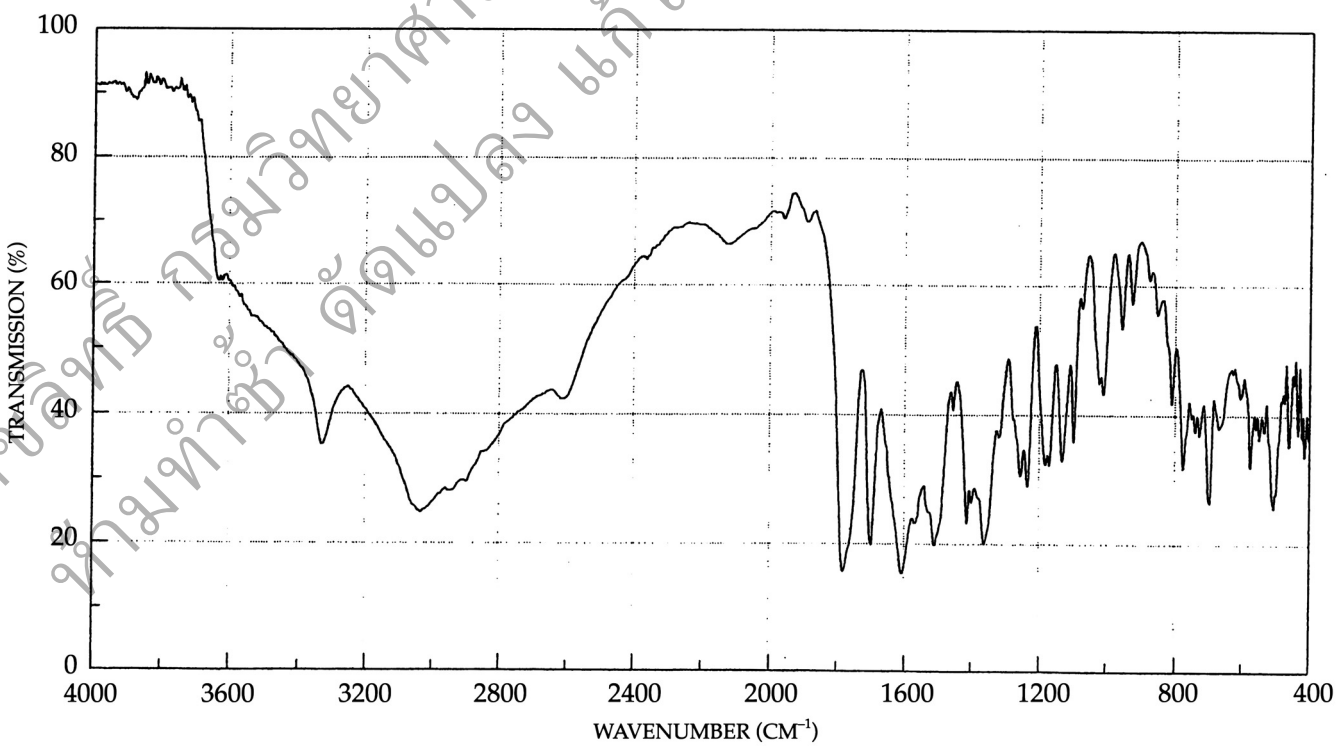
Artesunate Instrument: Fourier Transform Phase: Potassium Bromide mixture



Cefaclor Instrument: Fourier Transform Phase: Potassium Bromide mixture



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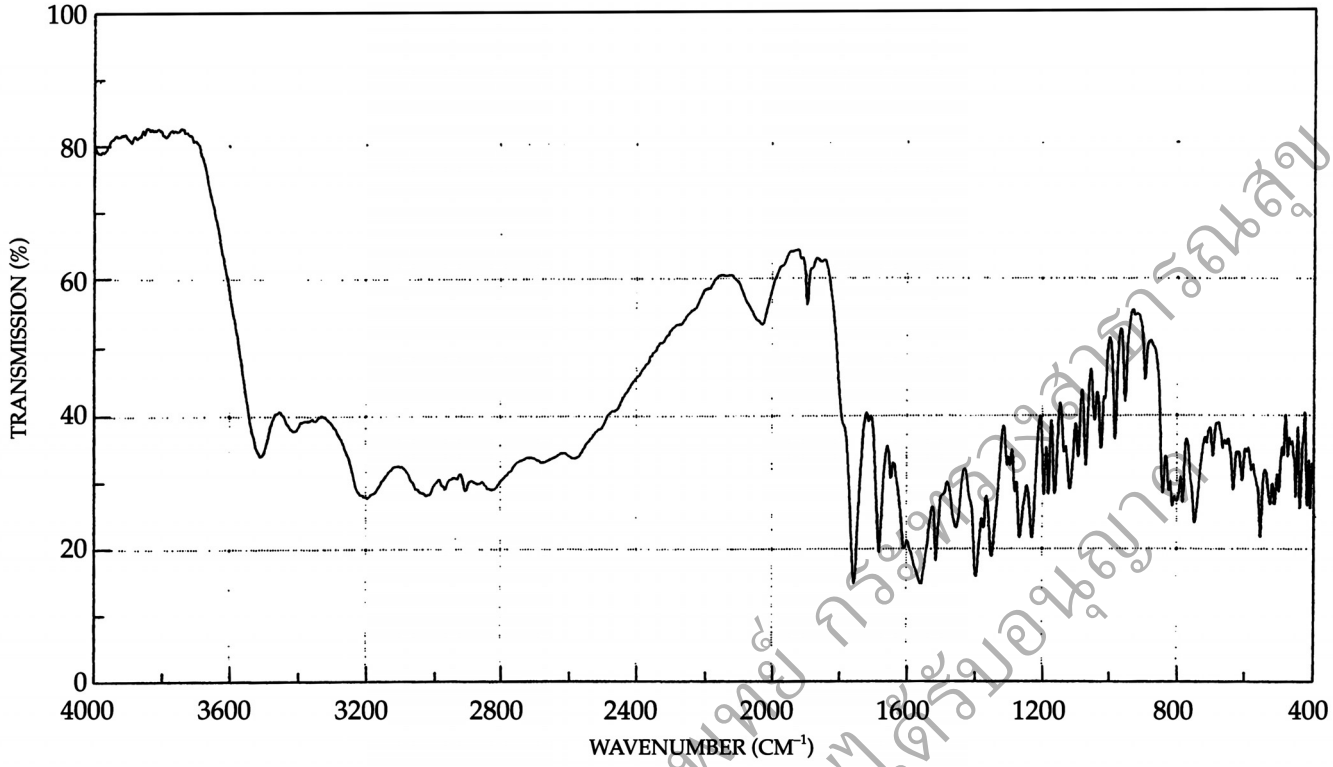
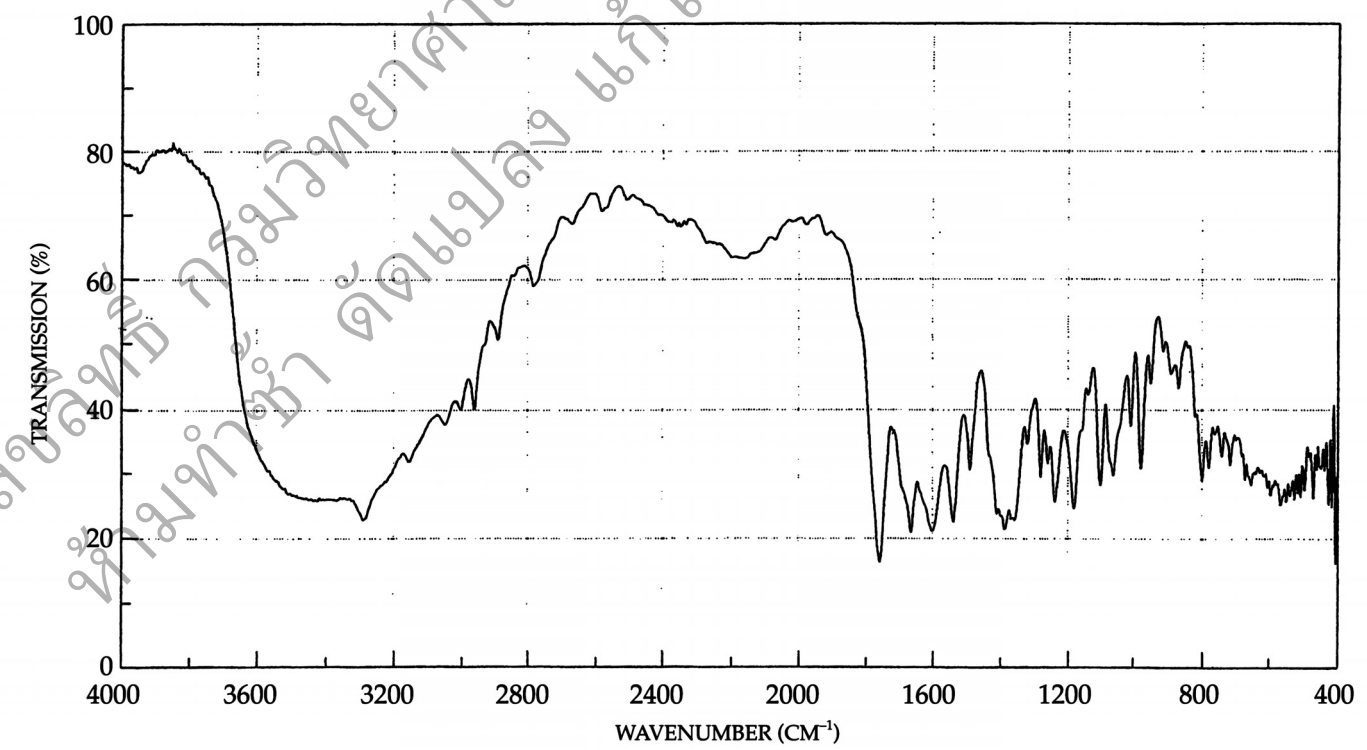
Cefadroxil Instrument: Fourier Transform Phase: Potassium Bromide mixture

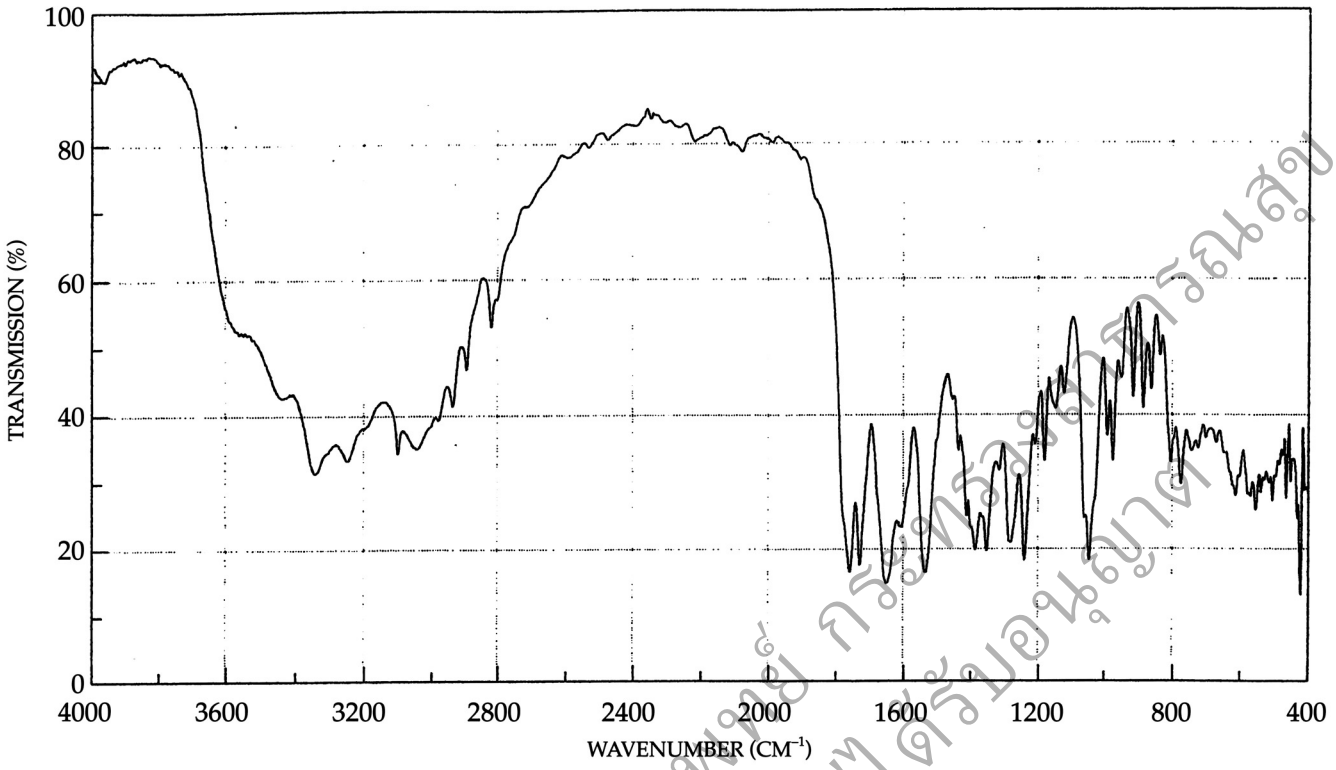


Cefazolin Sodium Instrument: Fourier Transform Phase: Potassium Bromide mixture

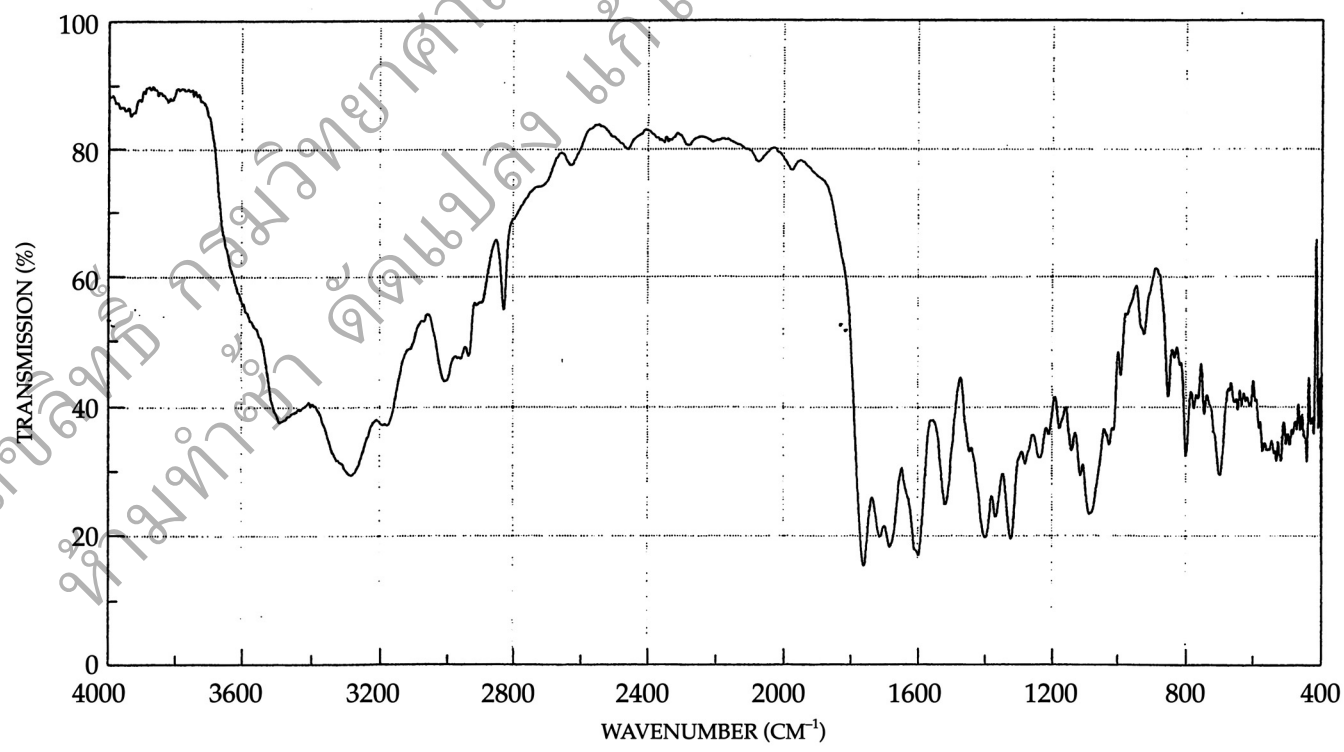


ลิขสิทธิ์ กลุ่มวิทยาศาสตร์การแพทย์ กรมวิทยาศาสตร์การแพทย์ ห้ามทำซ้ำ ดัดแปลง แก้ไข โดยไม่ได้รับอนุญาต

Cefotaxime Sodium Instrument: Fourier Transform Phase: Potassium Bromide mixture



Cefoxitin Sodium Instrument: Fourier Transform Phase: Potassium Bromide mixture



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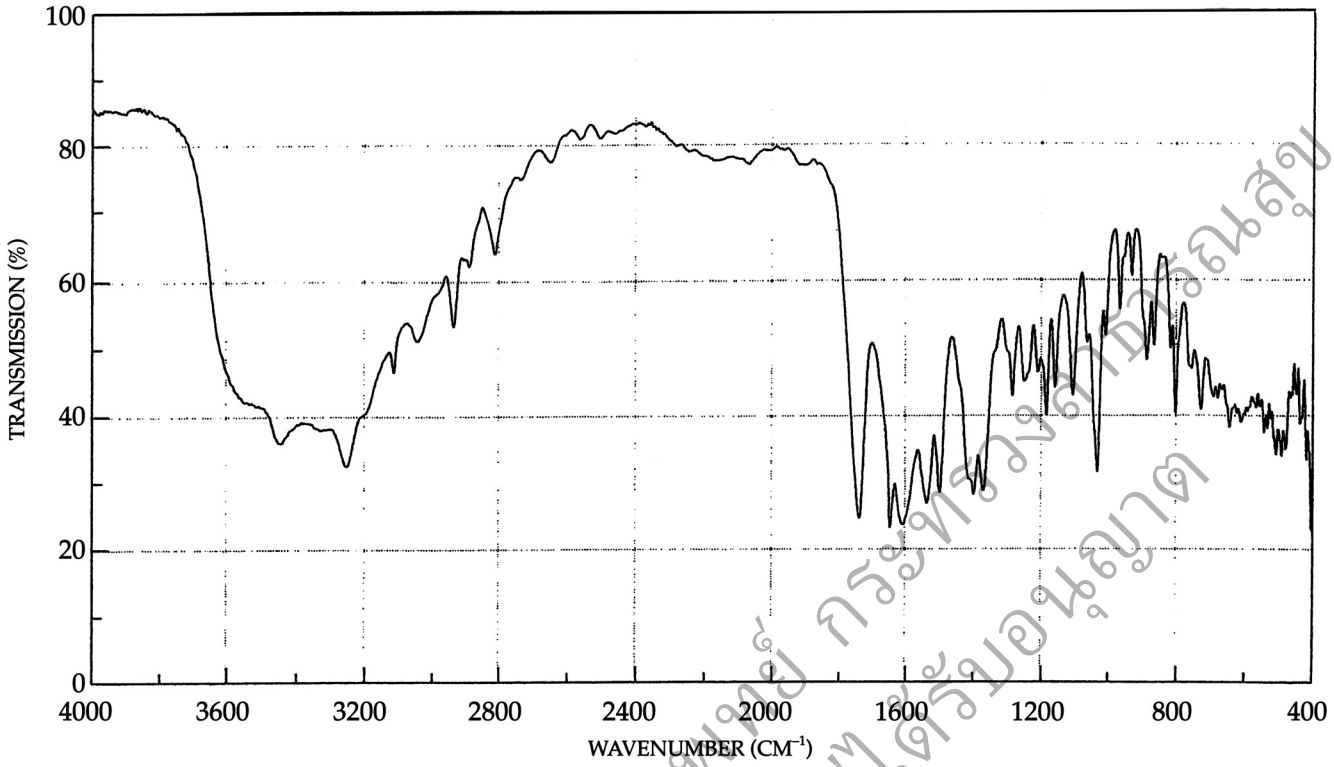
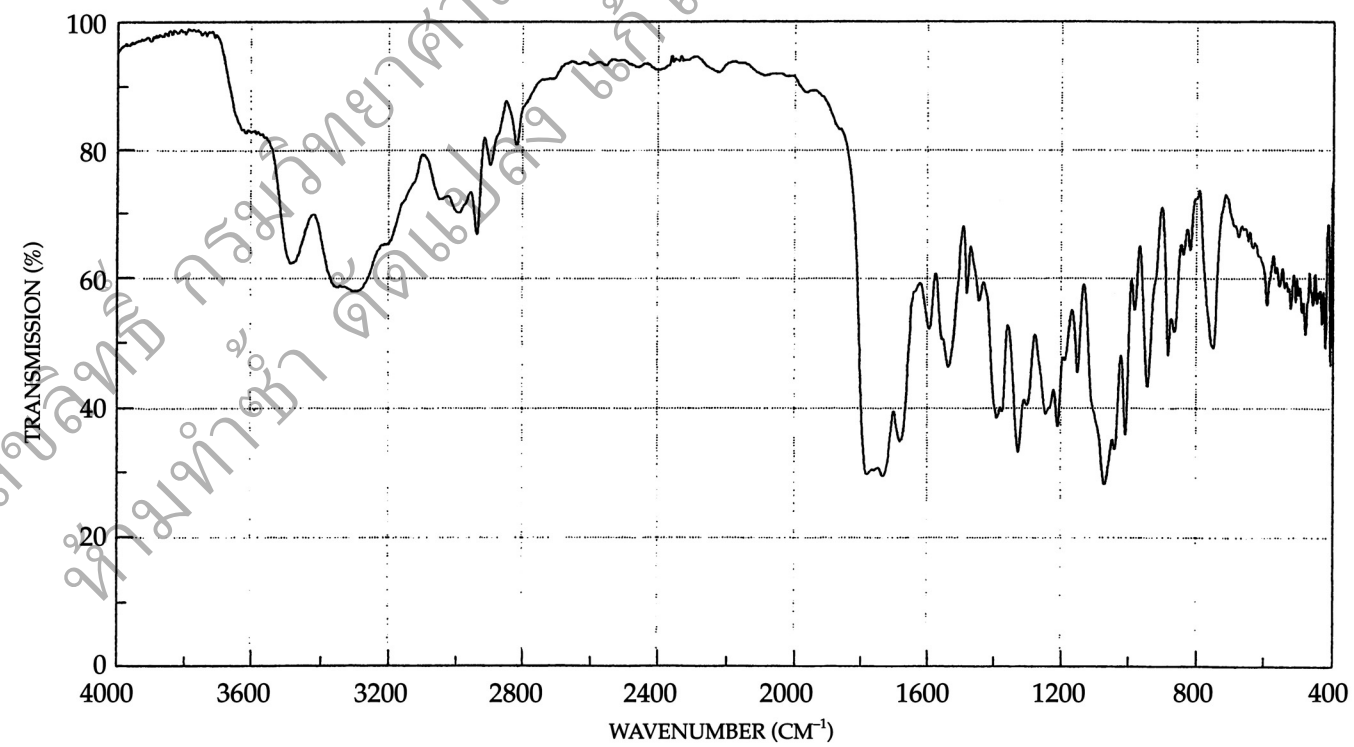
Ceftriaxone Sodium Instrument: Fourier Transform Phase: Potassium Bromide mixture



Cefuroxime Axetil Instrument: Fourier Transform Phase: Potassium Bromide mixture



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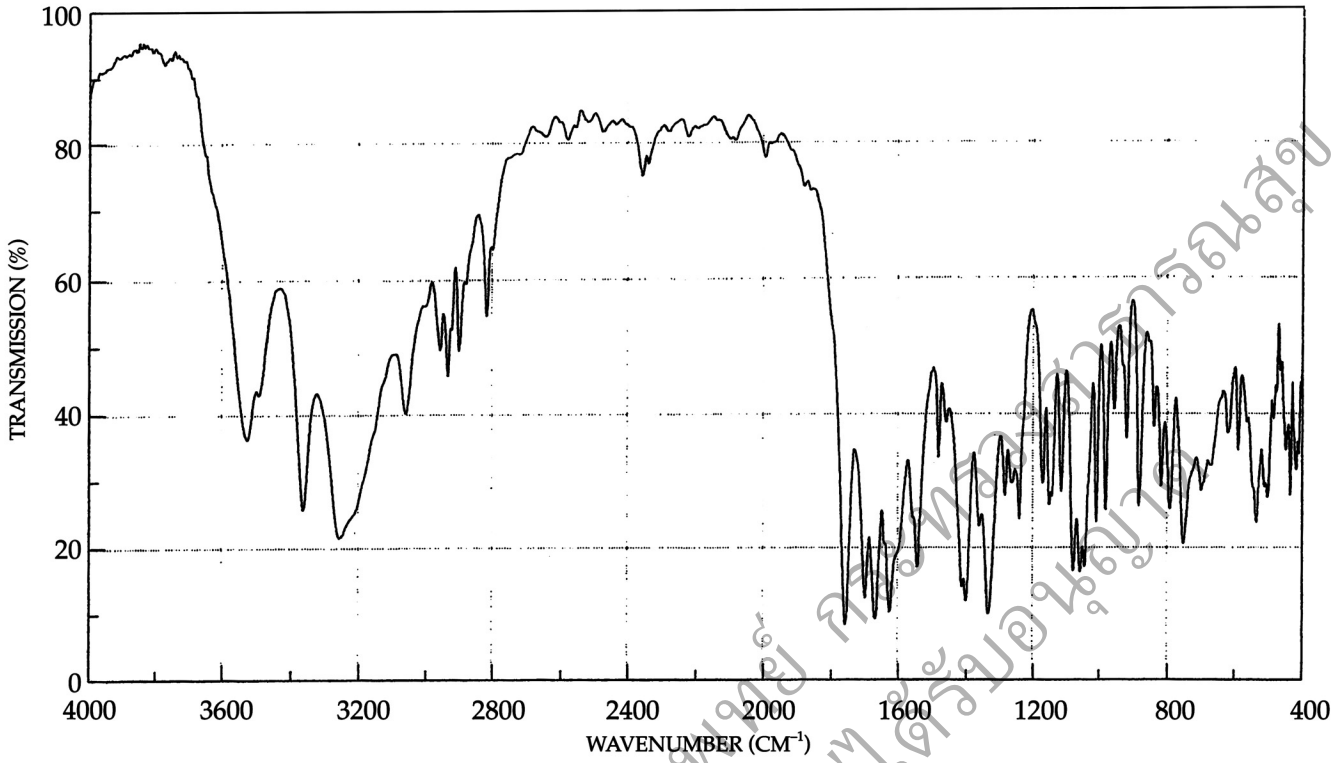
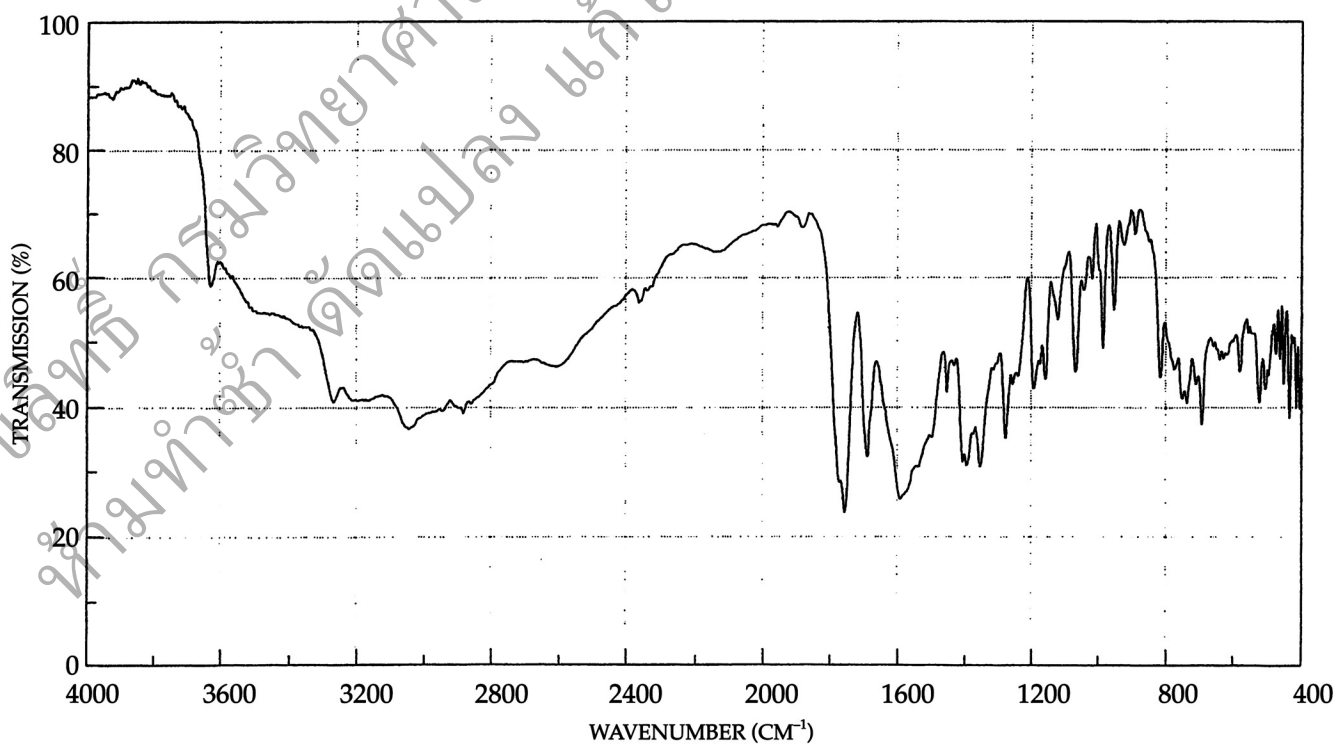
Cefuroxime Sodium Instrument: Fourier Transform Phase: Potassium Bromide mixture



Cephalexin Instrument: Fourier Transform Phase: Potassium Bromide mixture



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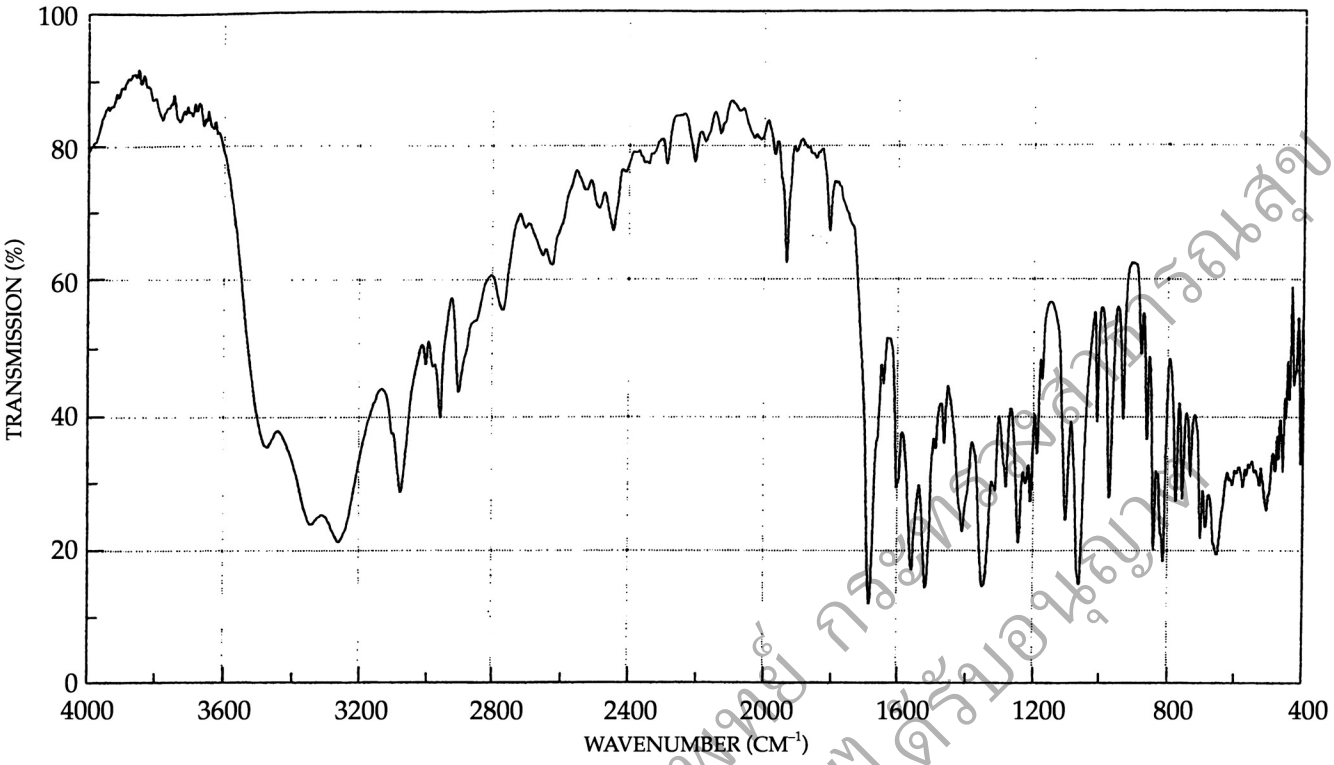
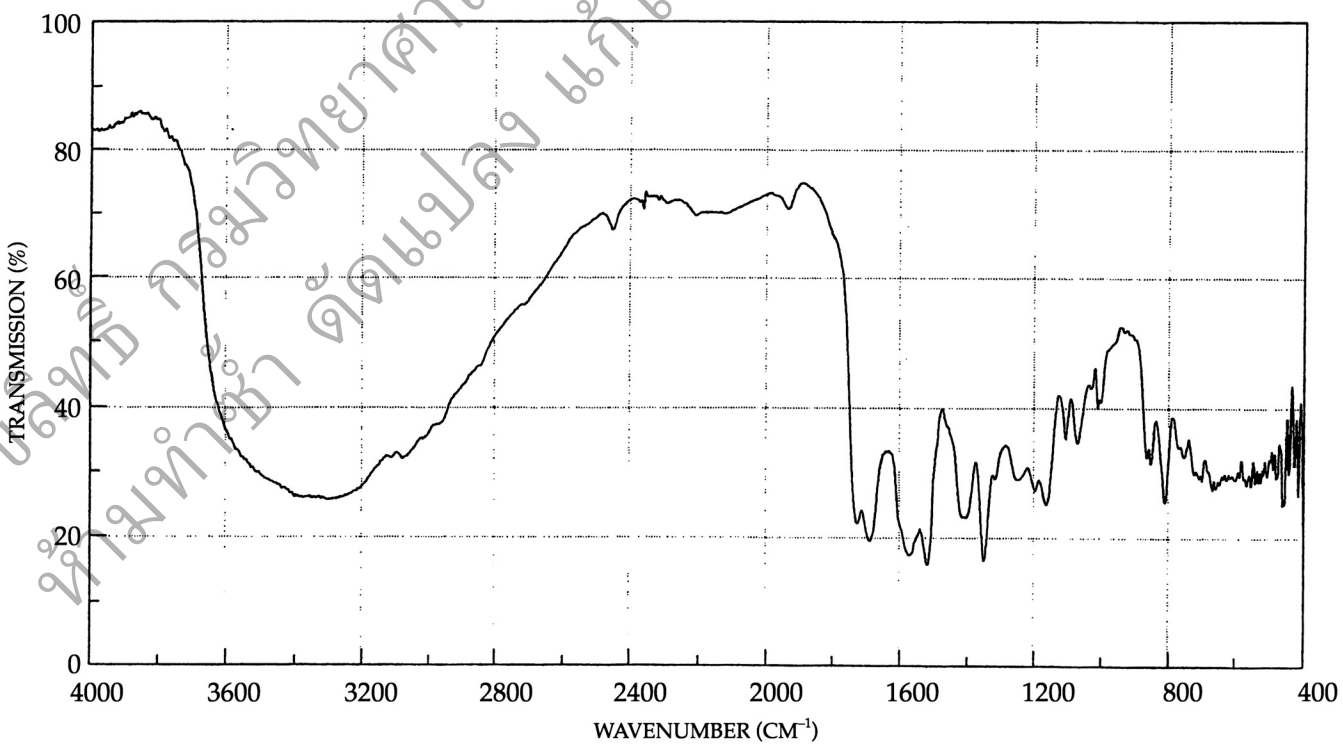
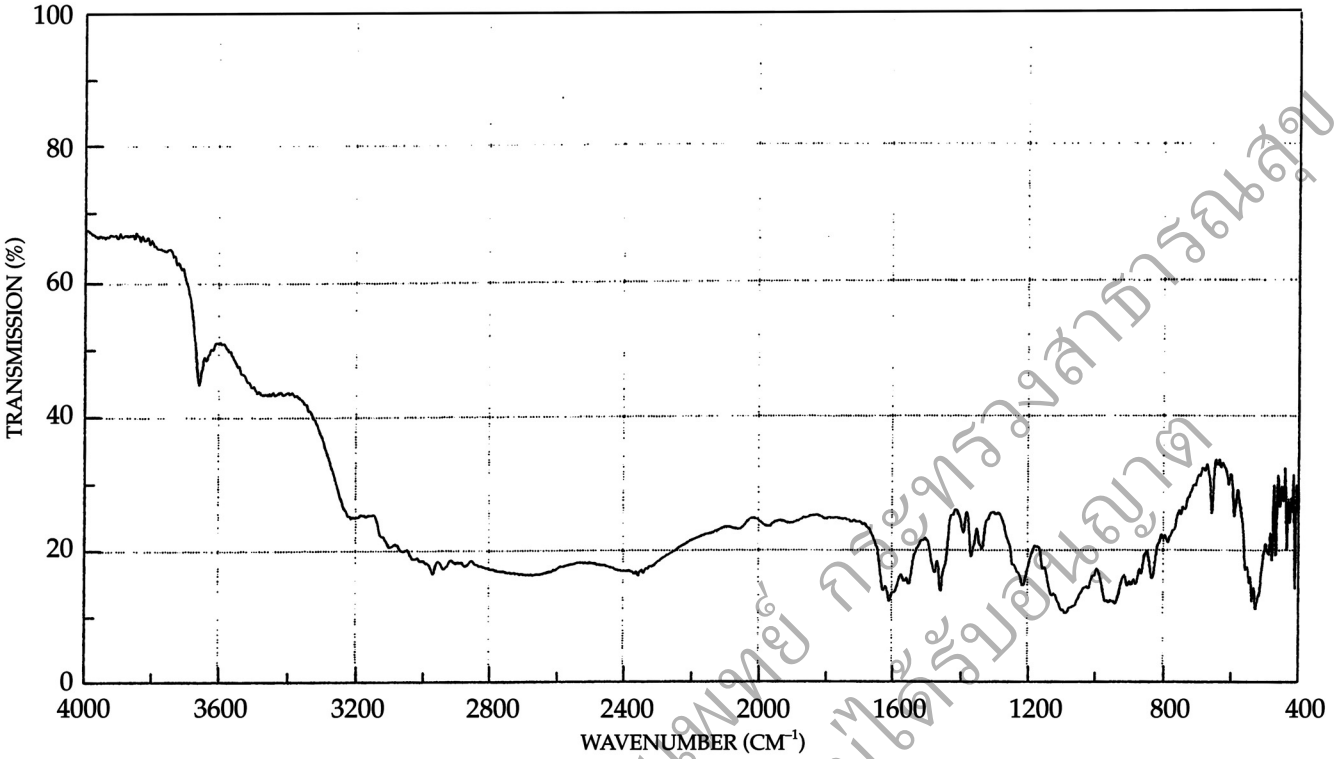
Chloramphenicol Instrument: Fourier Transform Phase: Potassium Bromide mixture



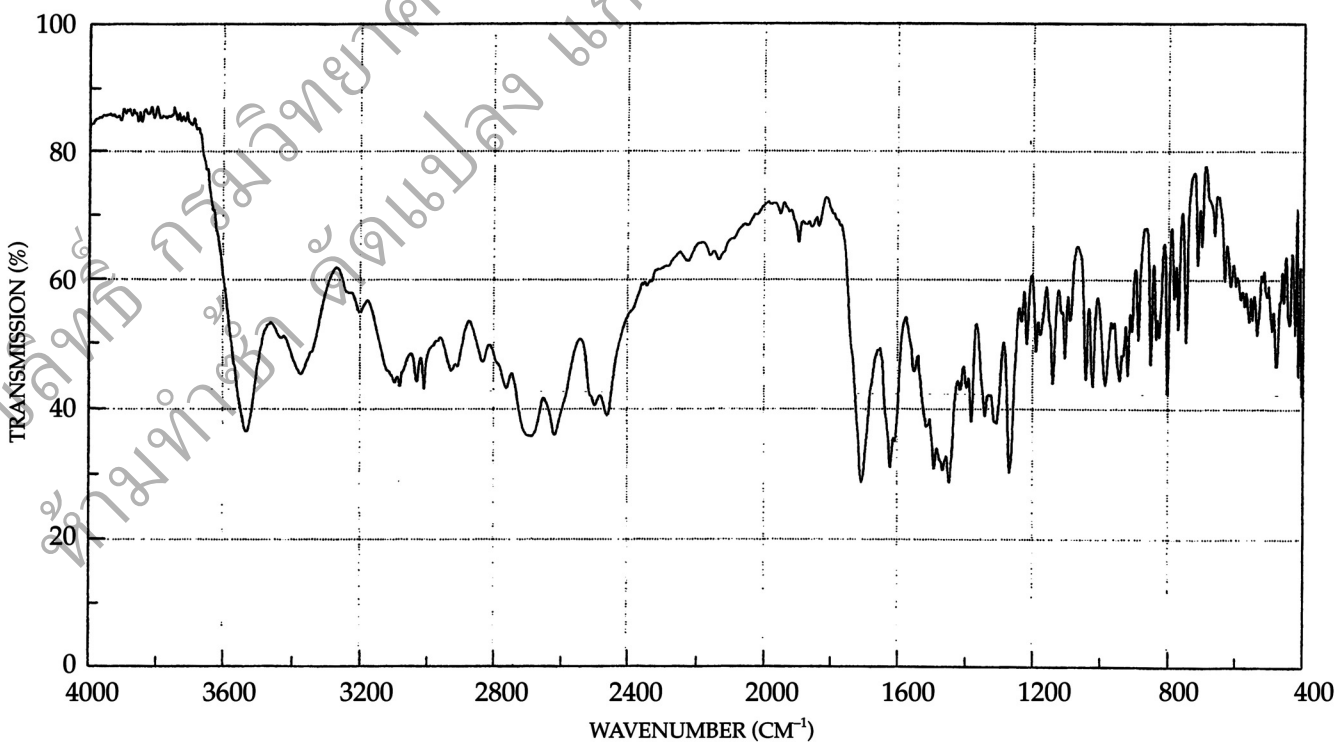
Chloramphenicol Sodium Succinate Instrument: Fourier Transform Phase: Potassium Bromide mixture



Chloroquine Phosphate Instrument: Fourier Transform Phase: Potassium Bromide mixture



Ciprofloxacin Hydrochloride Instrument: Fourier Transform Phase: Potassium Bromide mixture



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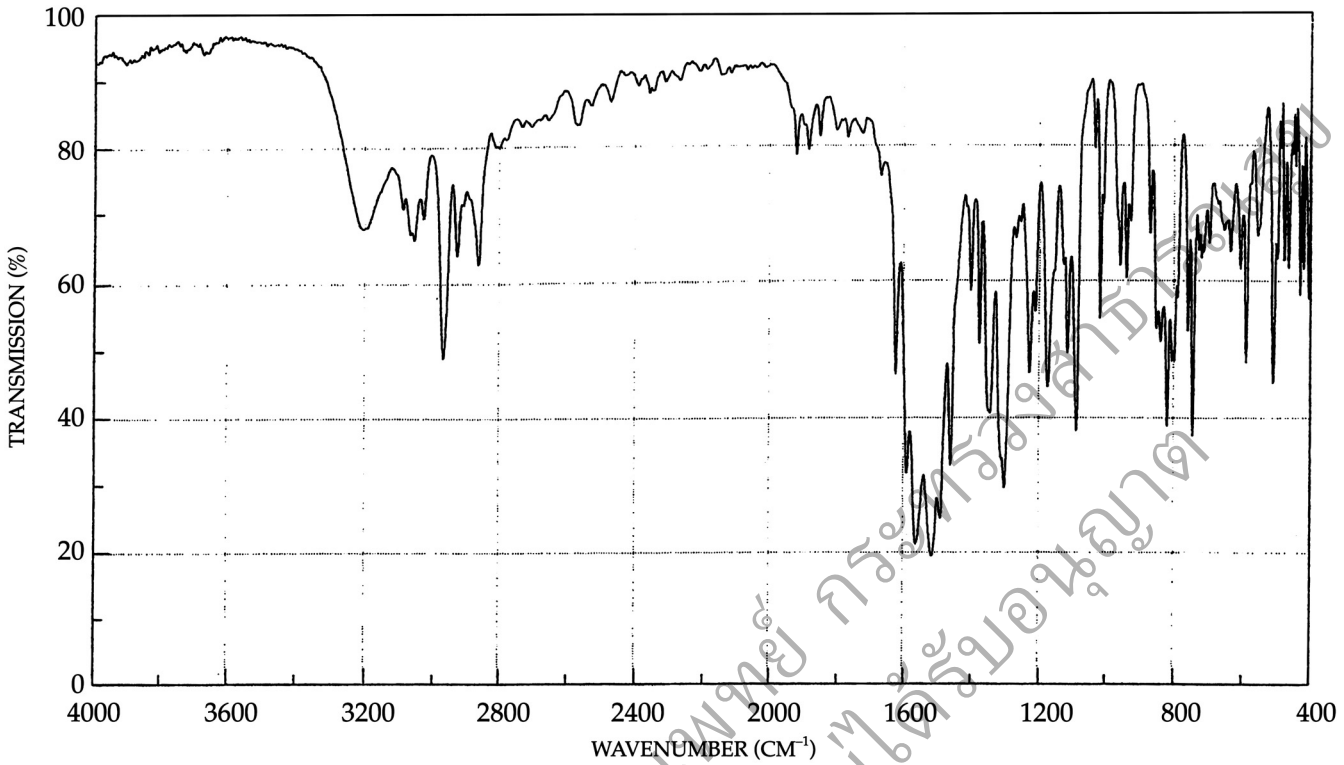
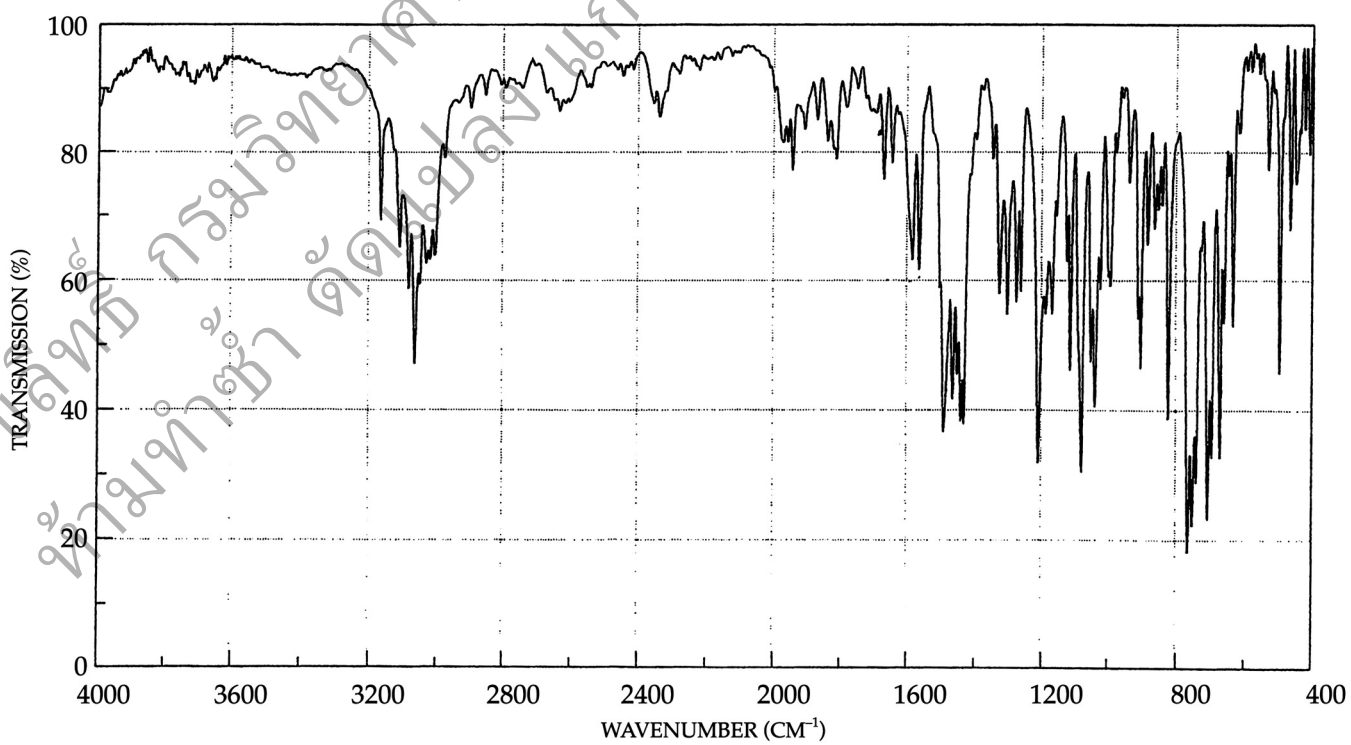
Clofazimine Instrument: Fourier Transform Phase: Potassium Bromide mixture



Clotrimazole Instrument: Fourier Transform Phase: Potassium Bromide mixture



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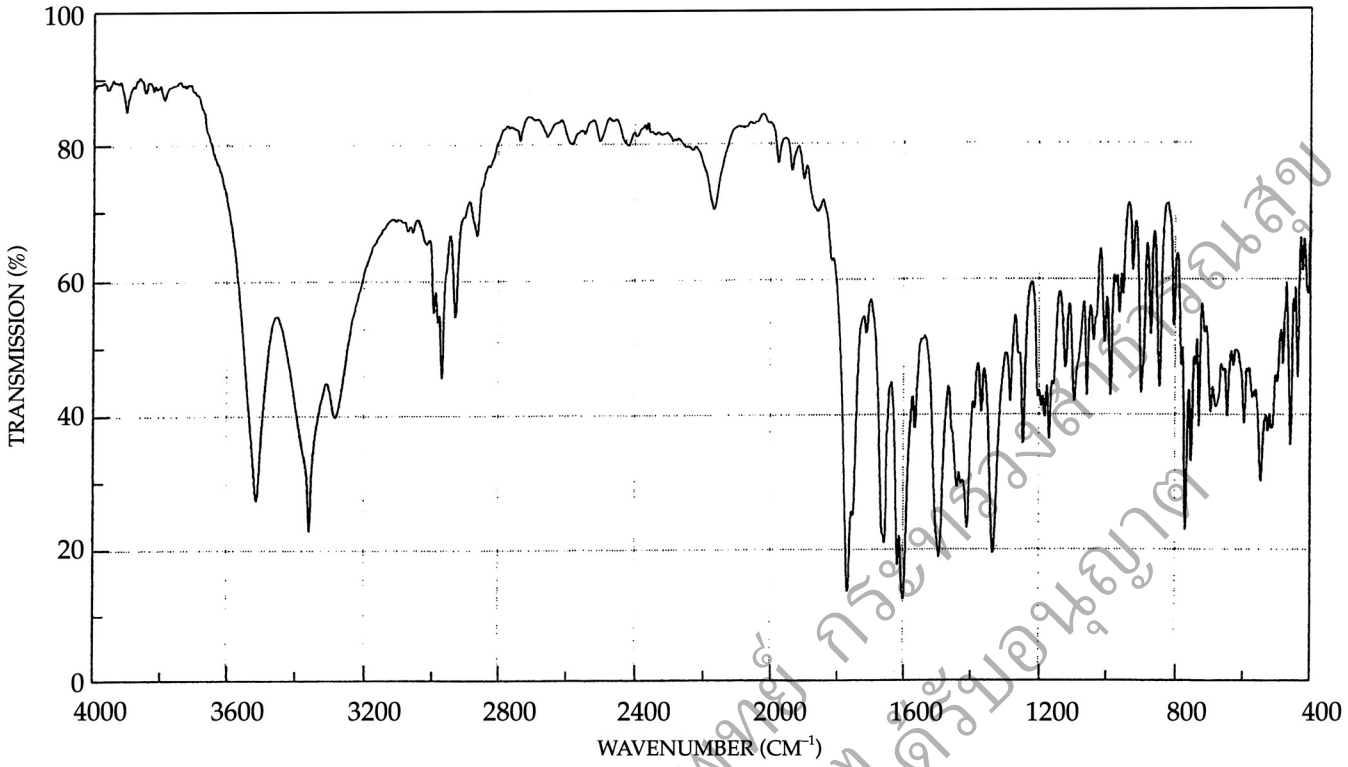
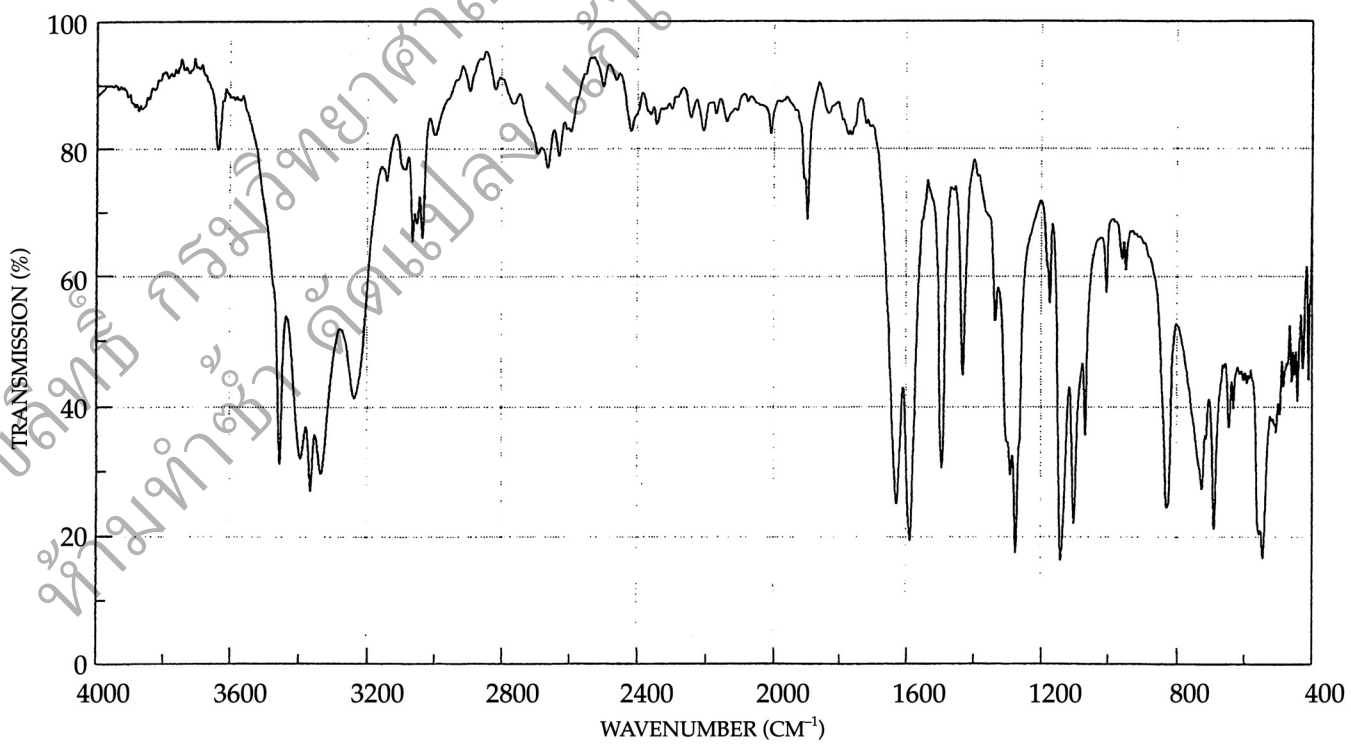
Cloxacillin Sodium Instrument: Fourier Transform Phase: Potassium Bromide mixture



Dapsone Instrument: Fourier Transform Phase: Potassium Bromide mixture



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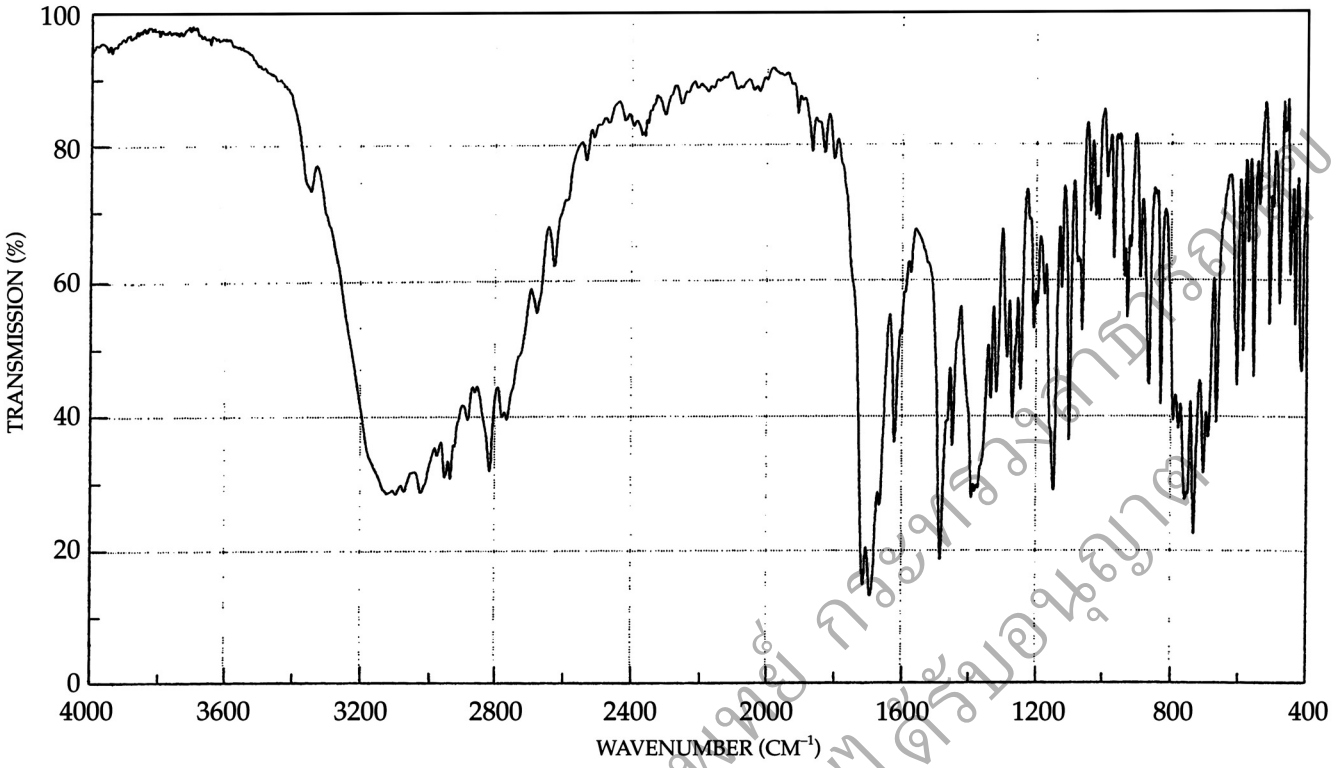
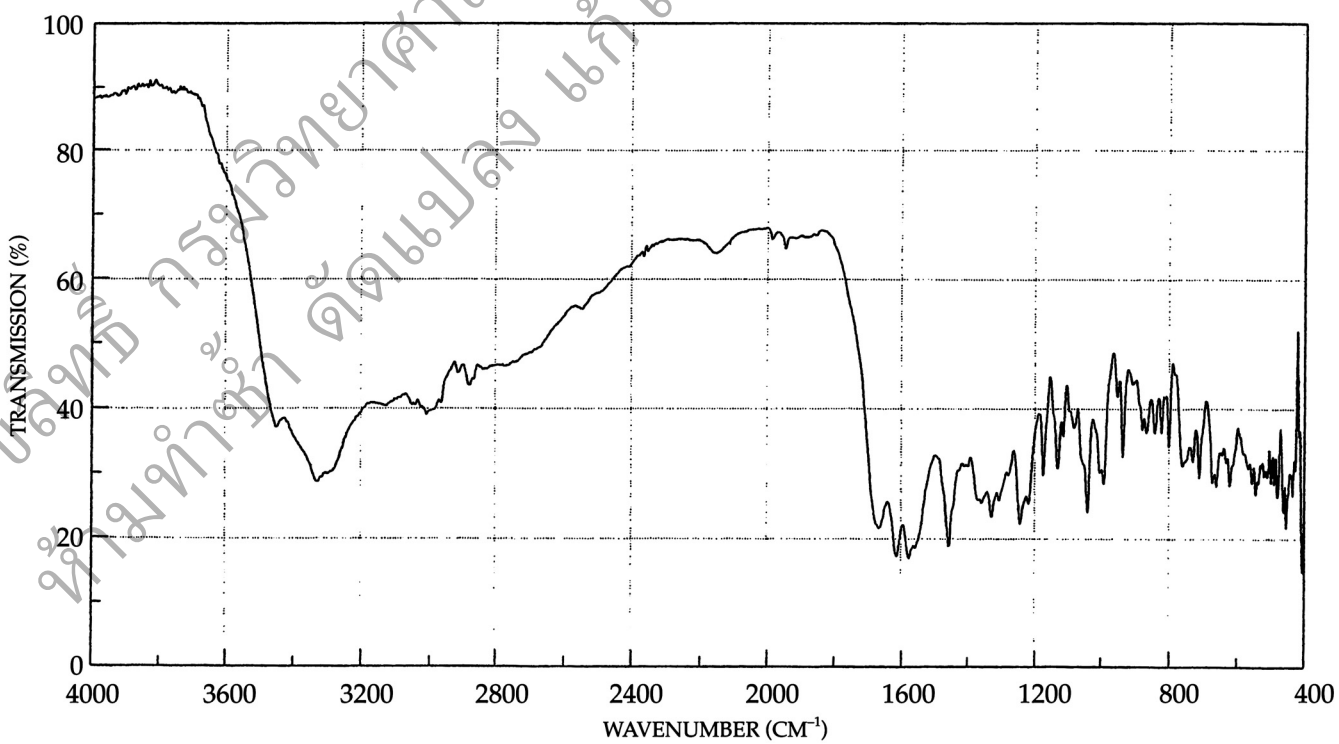
Domperidone Instrument: Fourier Transform Phase: Potassium Bromide mixture



Doxycycline Hyclate Instrument: Fourier Transform Phase: Potassium Bromide mixture



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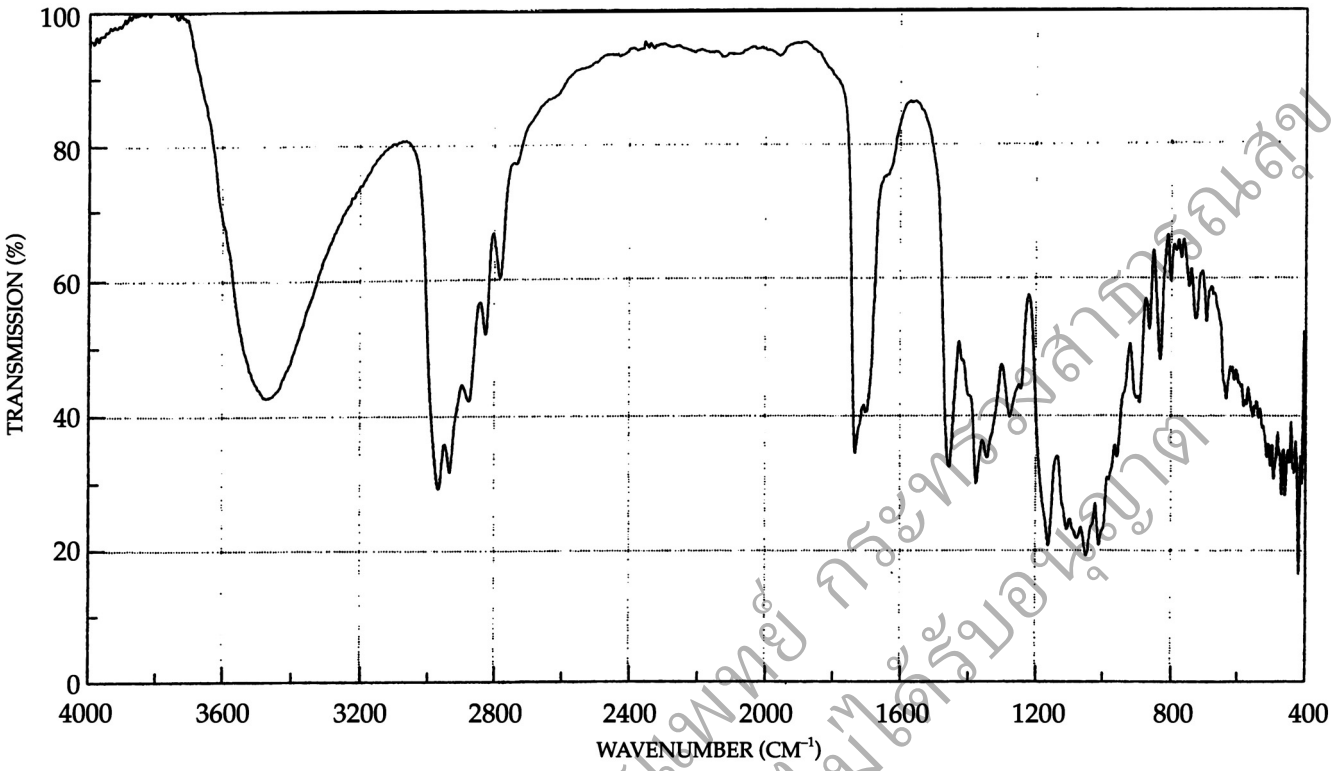
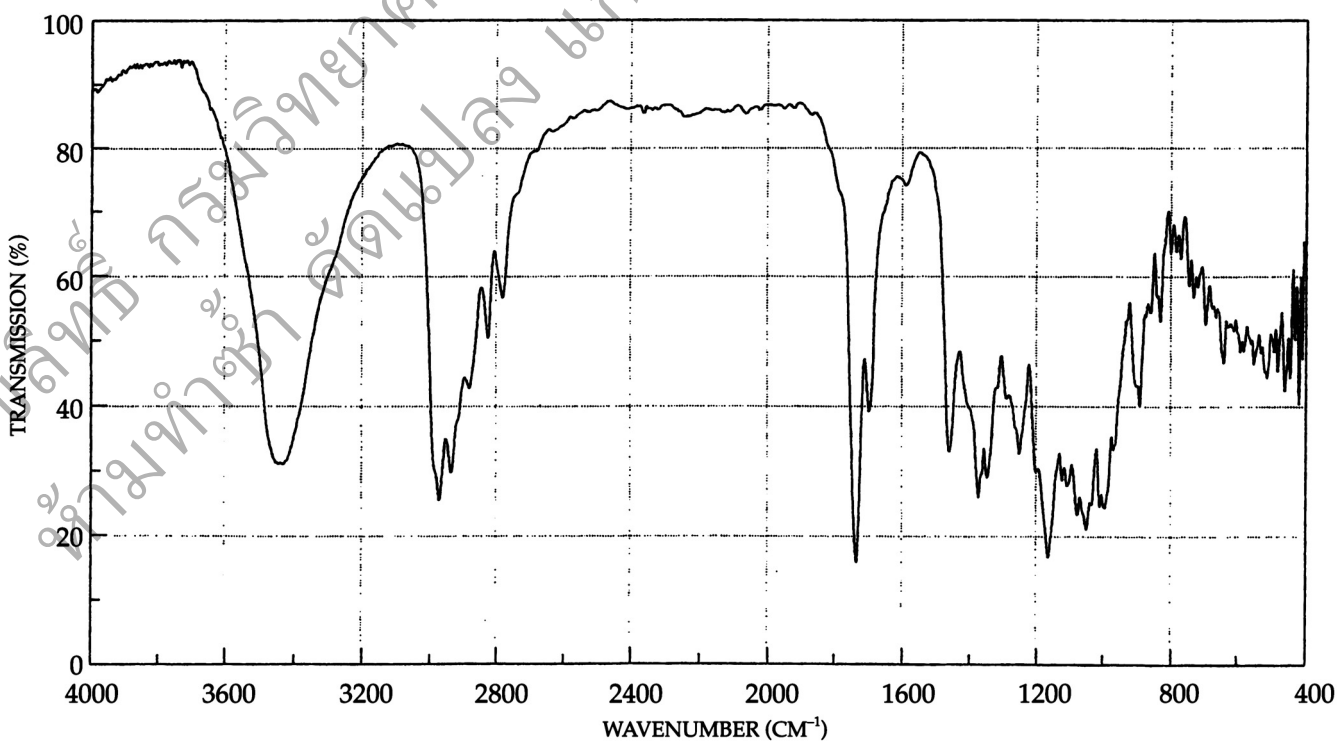
Erythromycin Instrument: Fourier Transform Phase: Potassium Bromide mixture



Erythromycin Ethylsuccinate Instrument: Fourier Transform Phase: Potassium Bromide mixture



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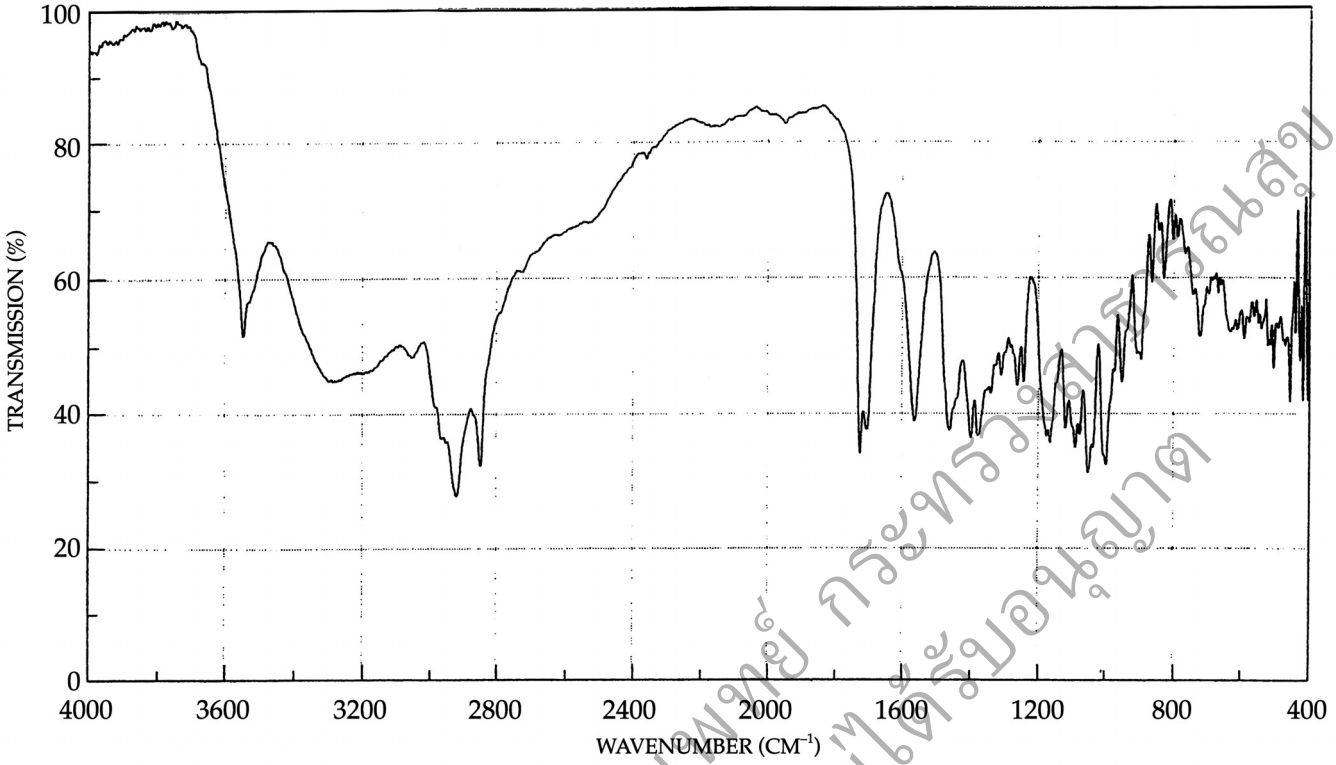
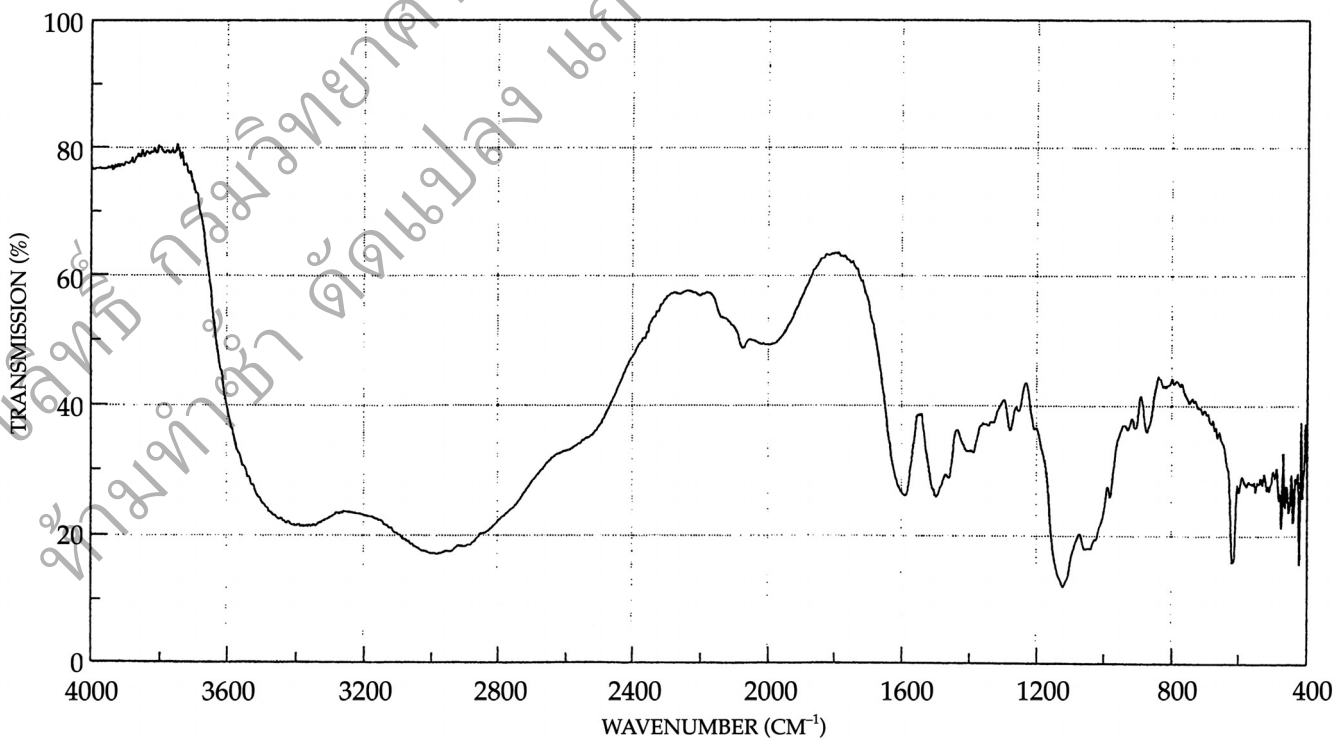
Erythromycin Stearate Instrument: Fourier Transform Phase: Potassium Bromide mixture



Gentamicin Sulfate Instrument: Fourier Transform Phase: Potassium Bromide mixture



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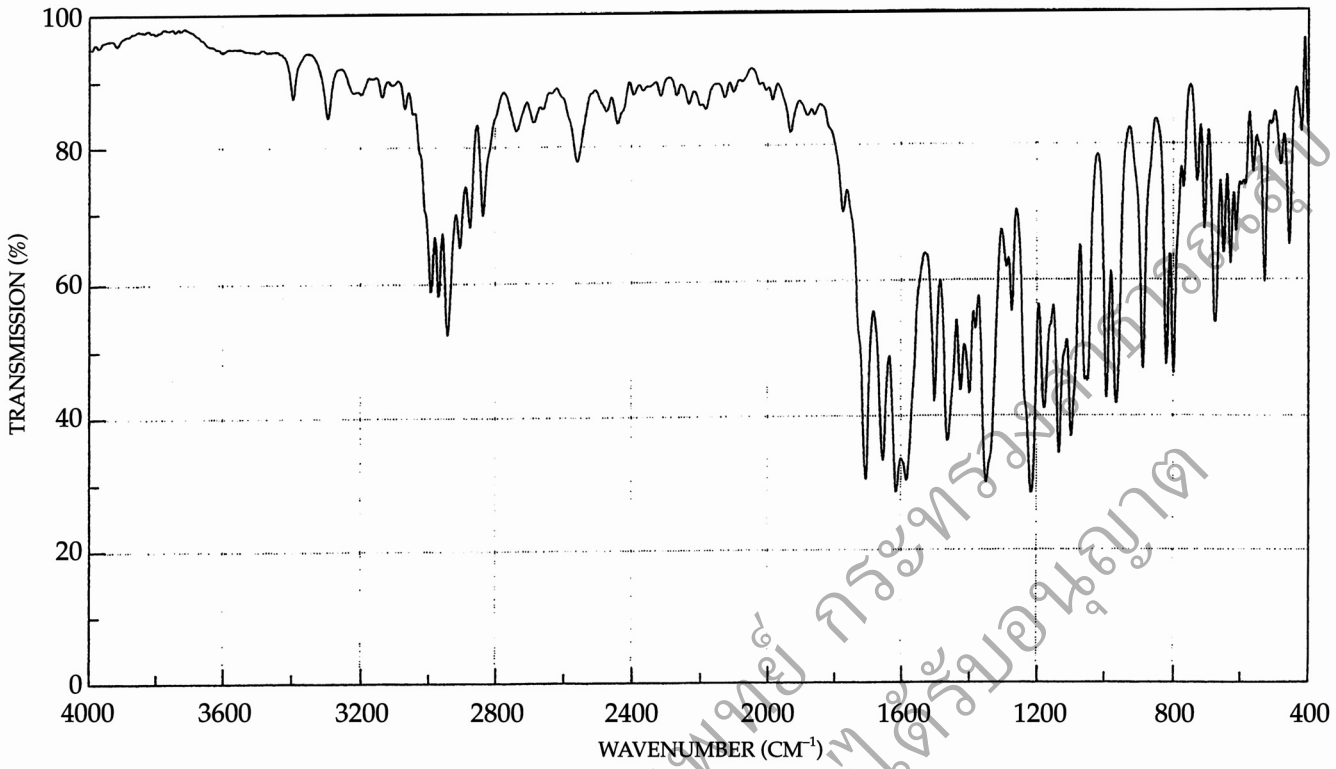
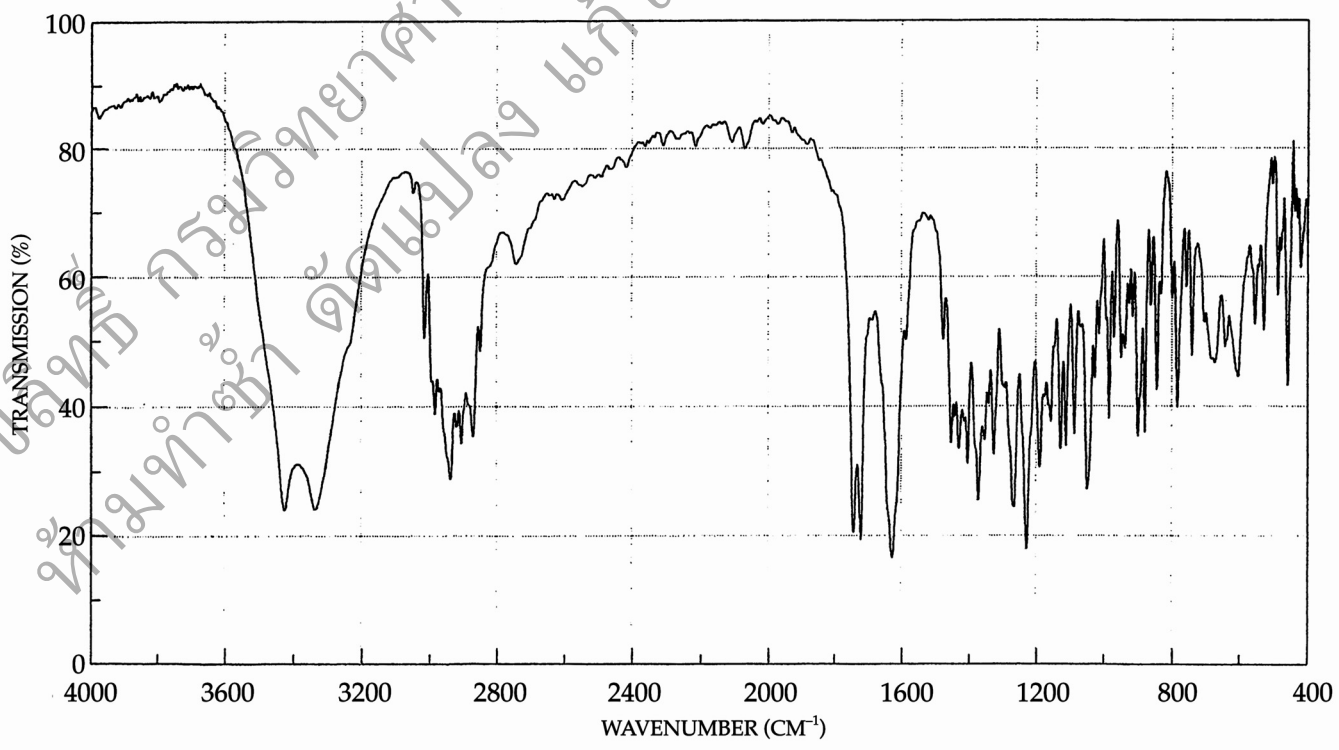
Griseofulvin Instrument: Fourier Transform Phase: Potassium Bromide mixture



Hydrocortisone Acetate Instrument: Fourier Transform Phase: Potassium Bromide mixture



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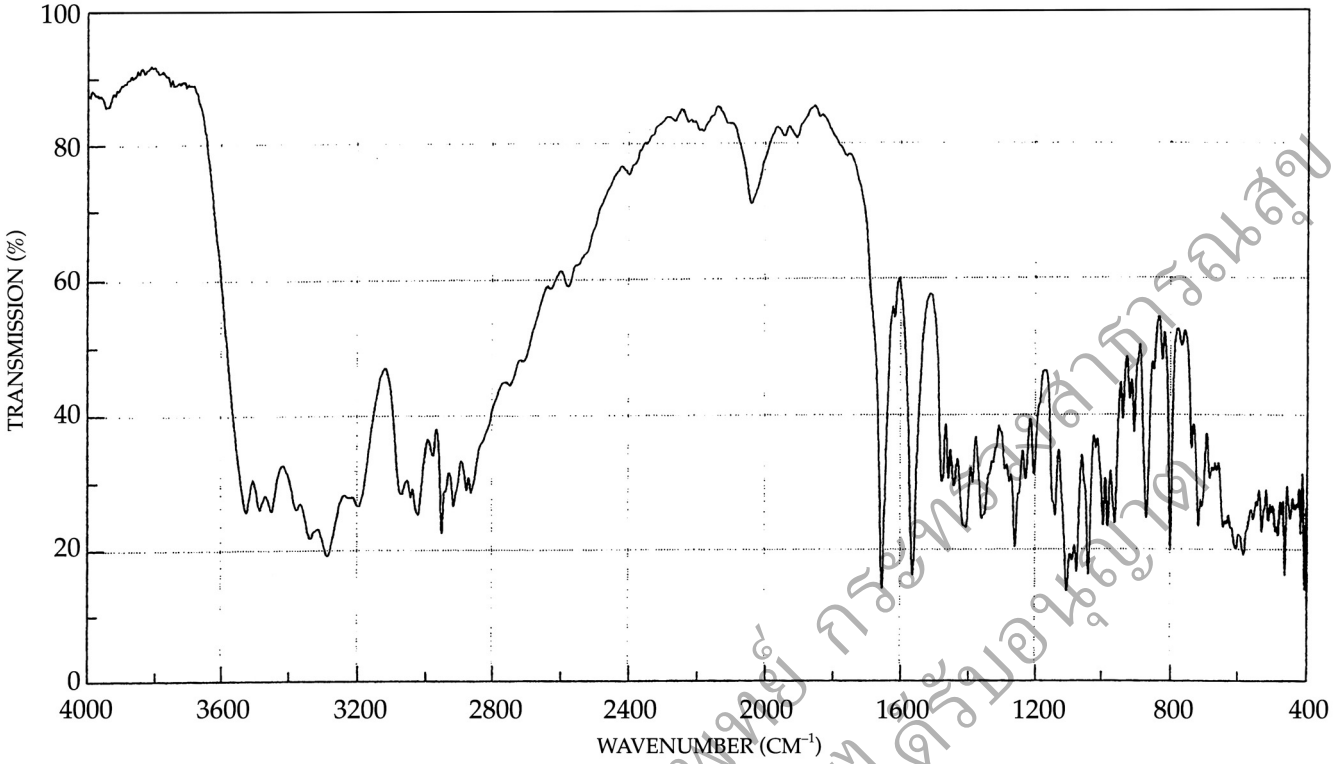
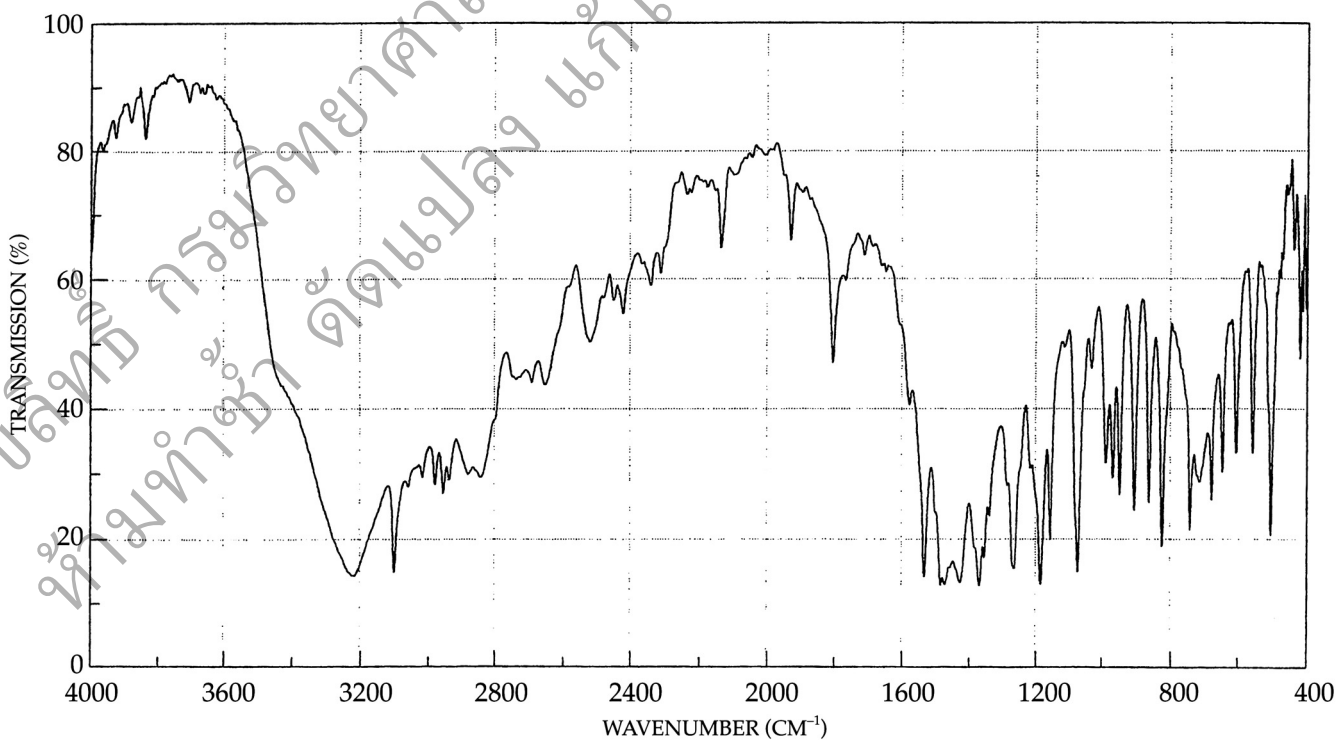
Lincomycin Hydrochloride Instrument: Fourier Transform Phase: Potassium Bromide mixture



Metronidazole Instrument: Fourier Transform Phase: Potassium Bromide mixture



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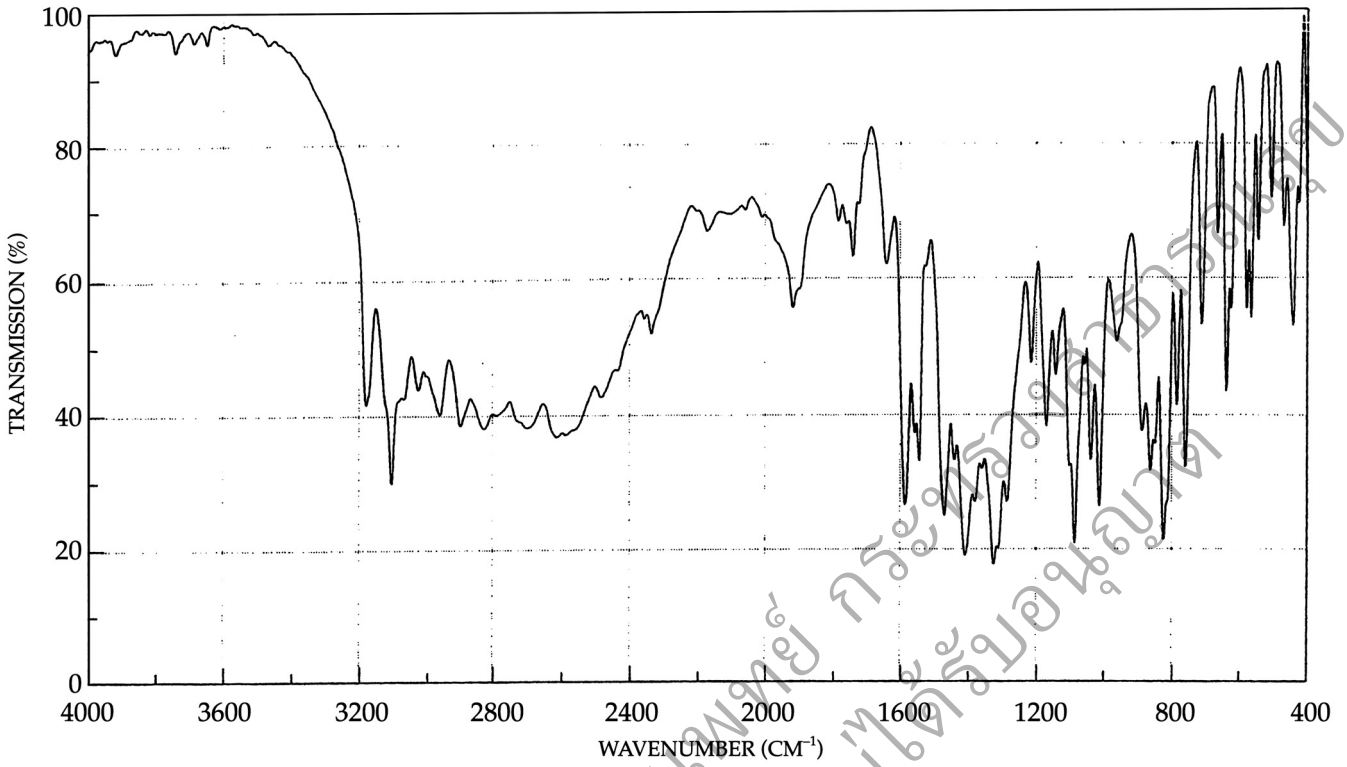
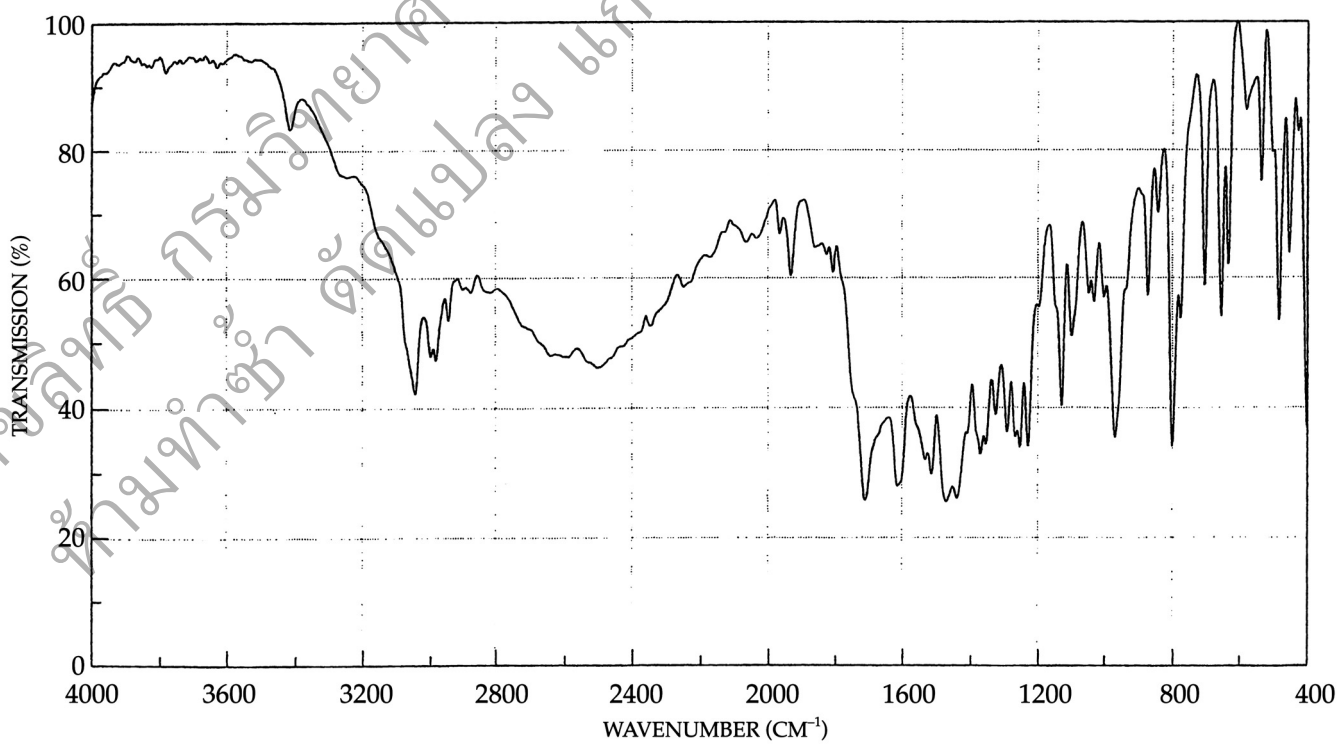
Miconazole Nitrate Instrument: Fourier Transform Phase: Potassium Bromide mixture



Nalidixic Acid Instrument: Fourier Transform Phase: Potassium Bromide mixture



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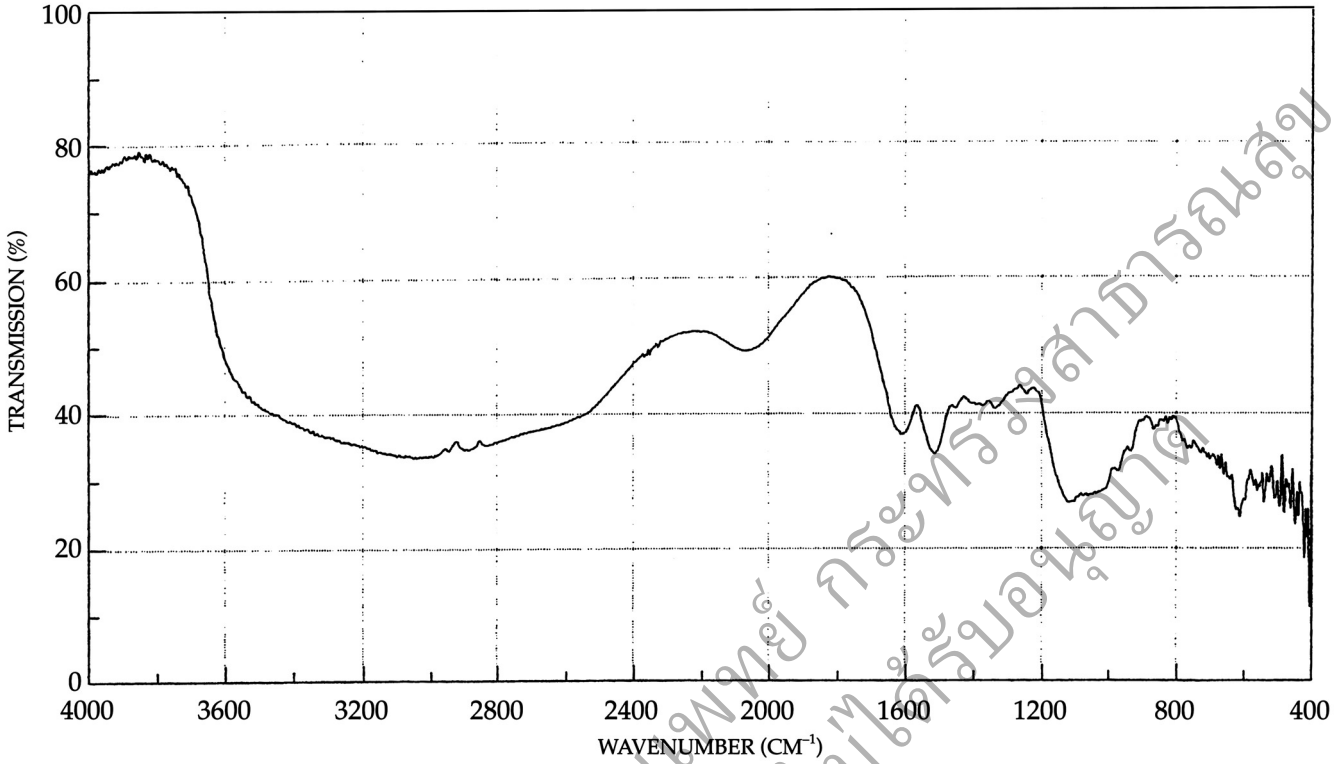
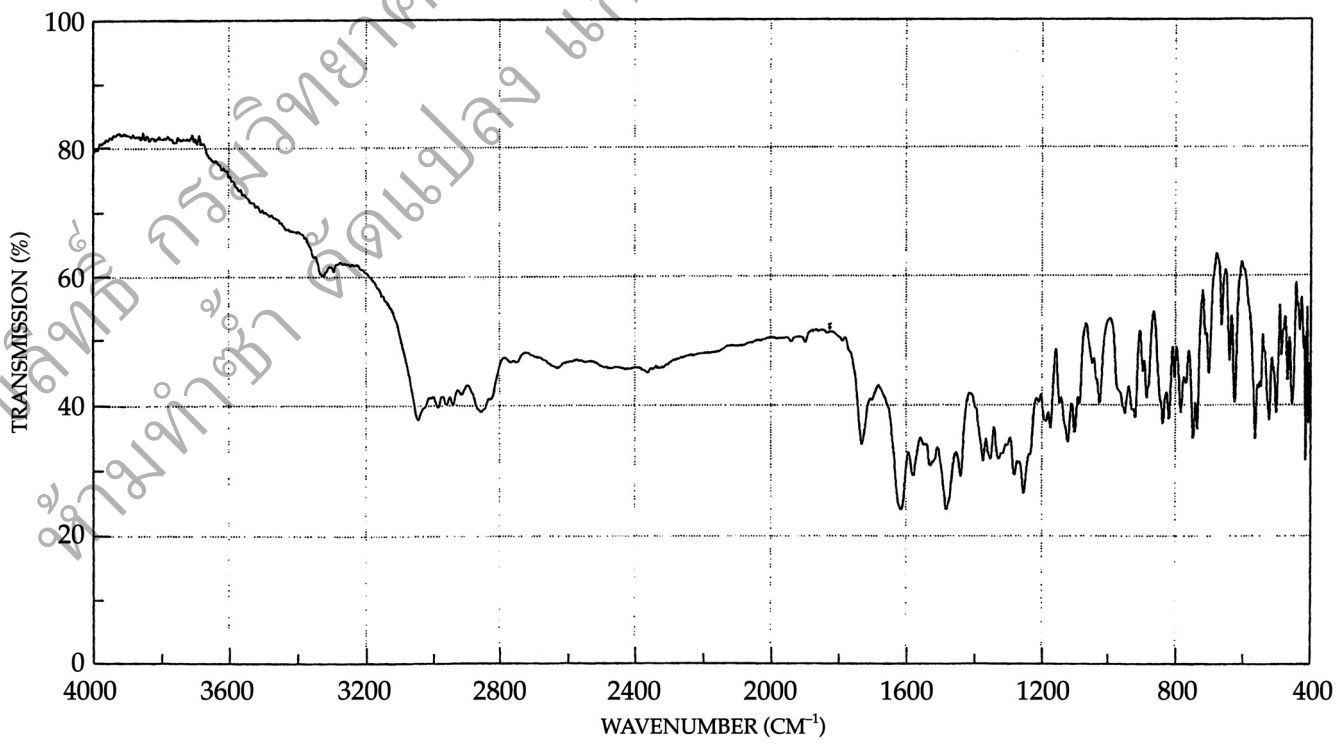
Neomycin Sulfate Instrument: Fourier Transform Phase: Potassium Bromide mixture



Norfloxacin Instrument: Fourier Transform Phase: Potassium Bromide mixture



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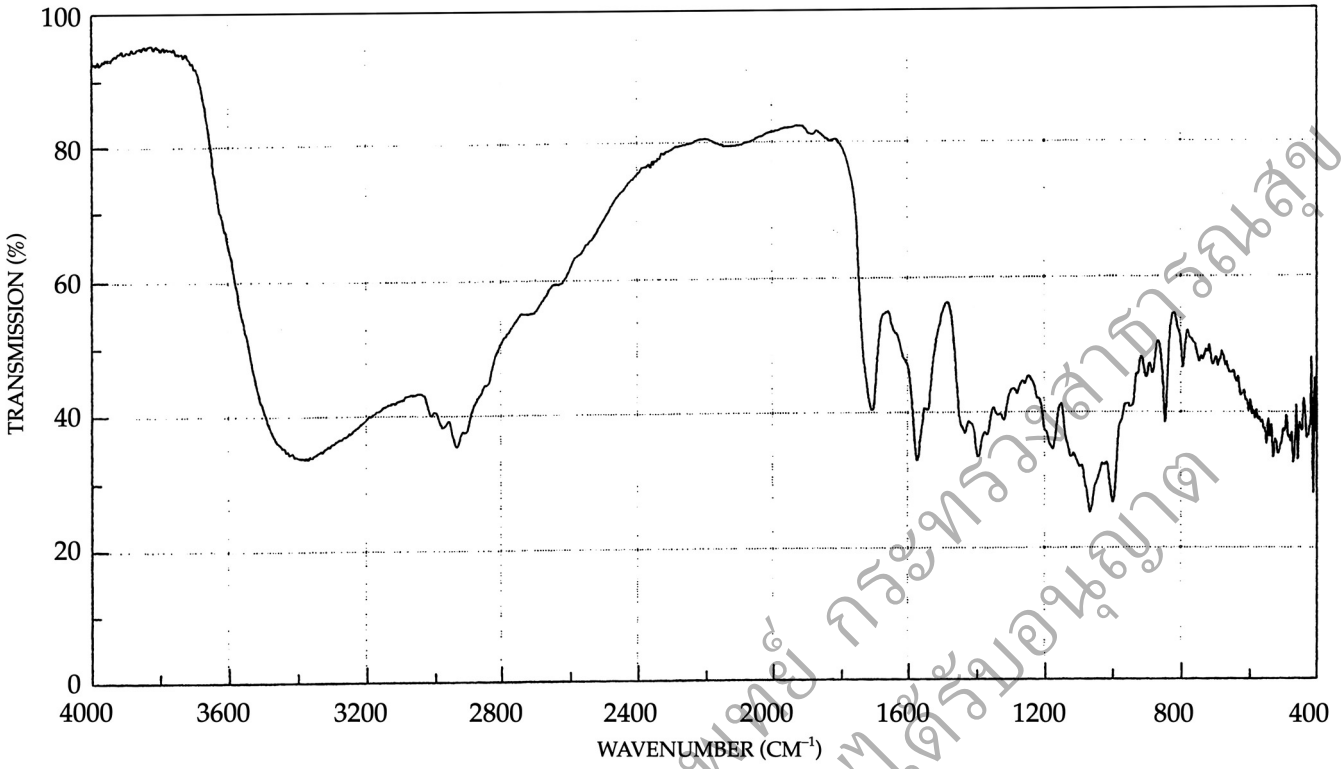
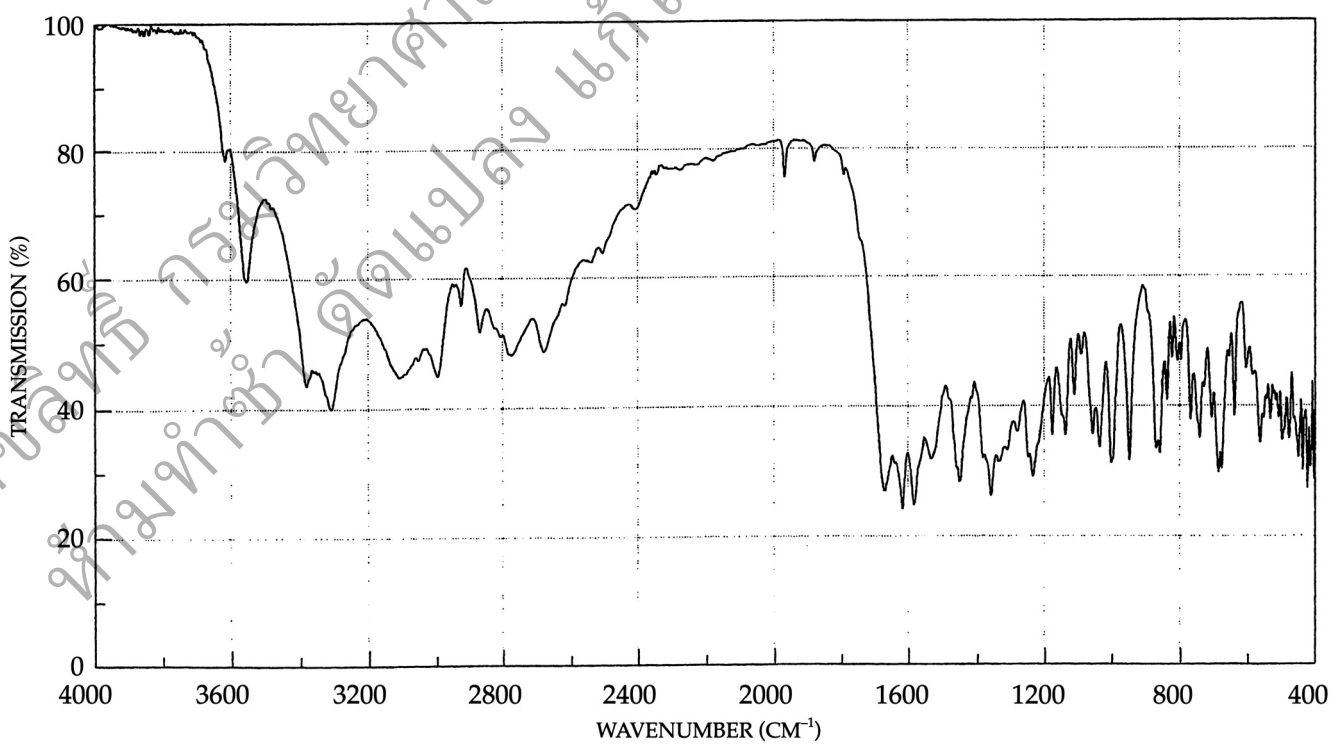
Nystatin Instrument: Fourier Transform Phase: Potassium Bromide mixture



Oxytetracycline Hydrochloride Instrument: Fourier Transform Phase: Potassium Bromide mixture



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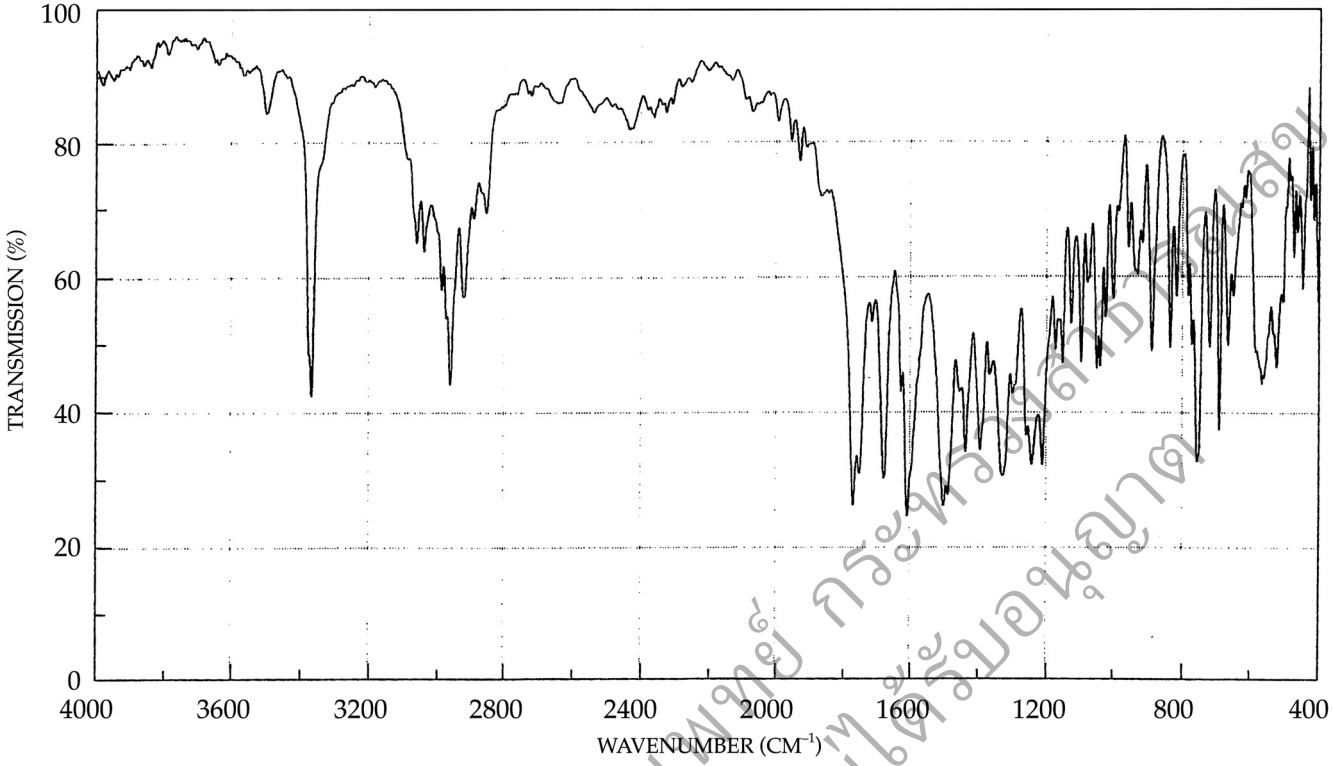
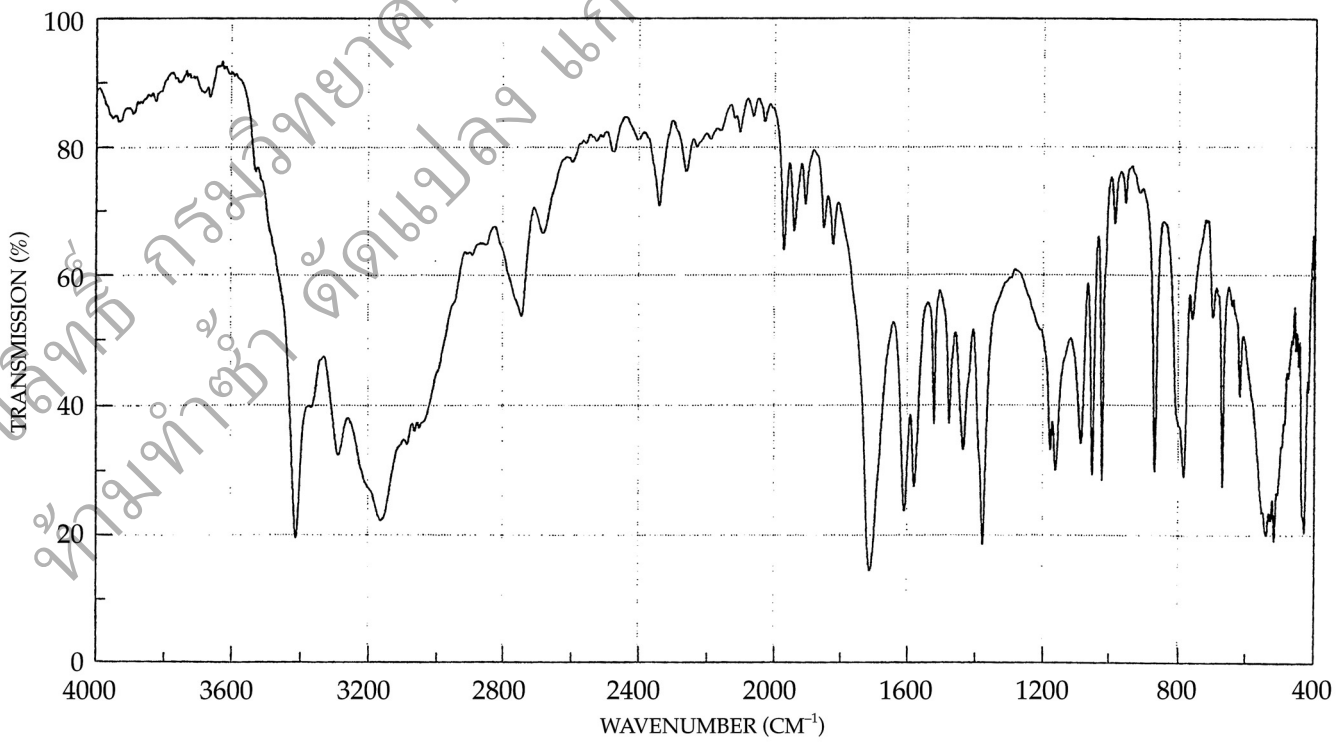
Penicillin V Potassium Instrument: Fourier Transform Phase: Potassium Bromide mixture



Pyrazinamide Instrument: Fourier Transform Phase: Potassium Bromide mixture



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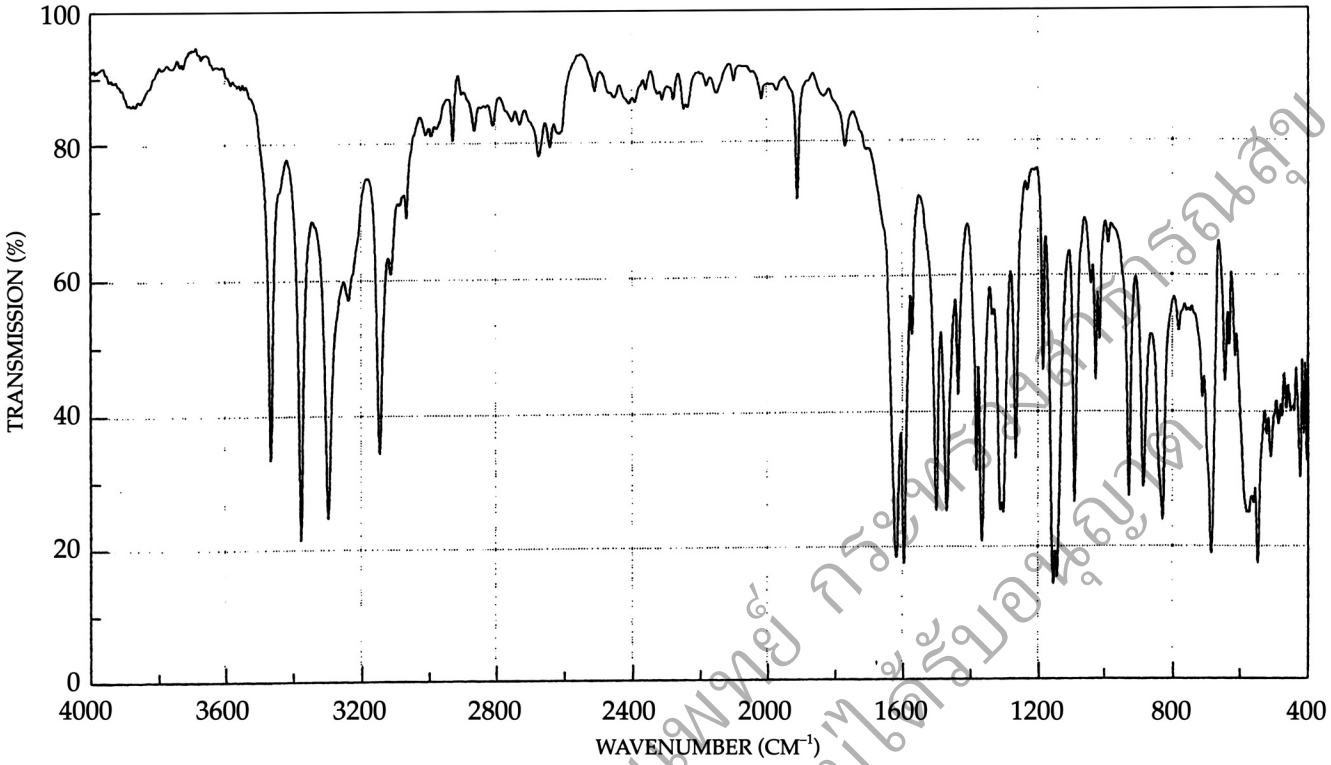
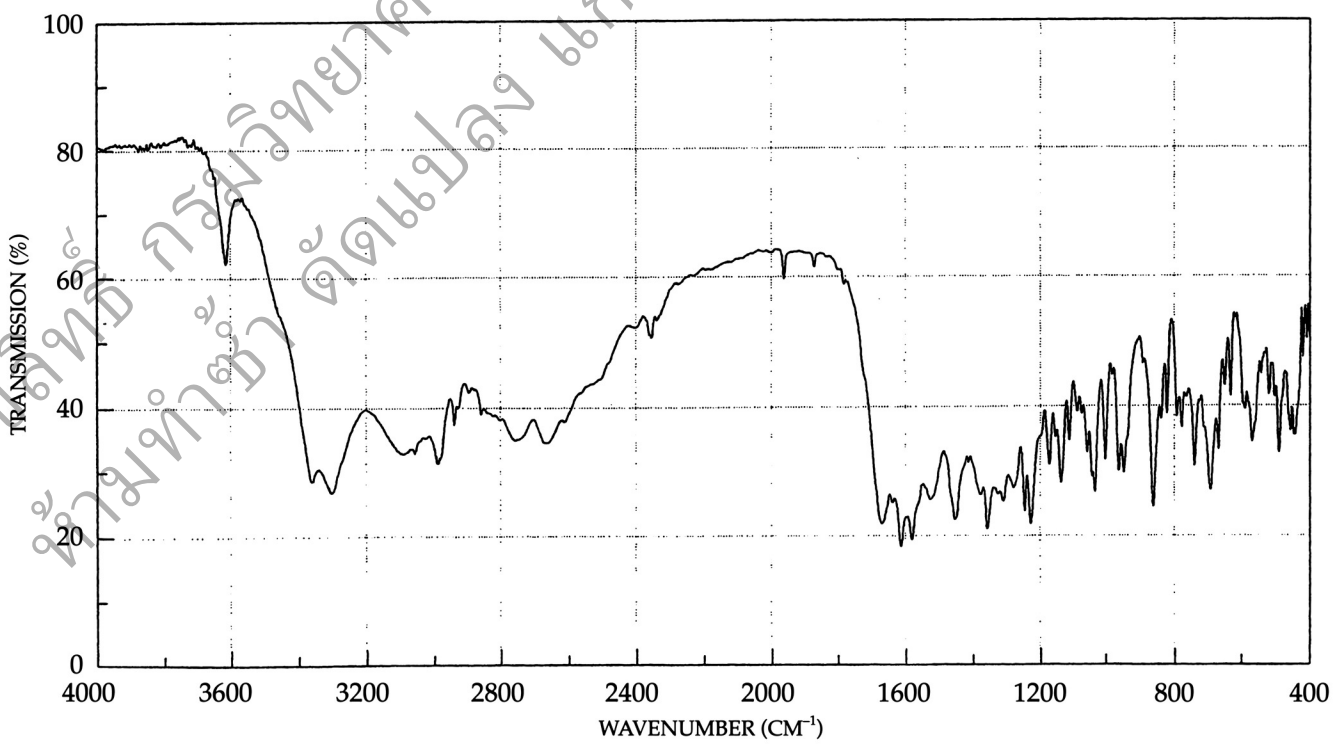
Sulfamethoxazole Instrument: Fourier Transform Phase: Potassium Bromide mixture



Tetracycline Hydrochloride Instrument: Fourier Transform Phase: Potassium Bromide mixture



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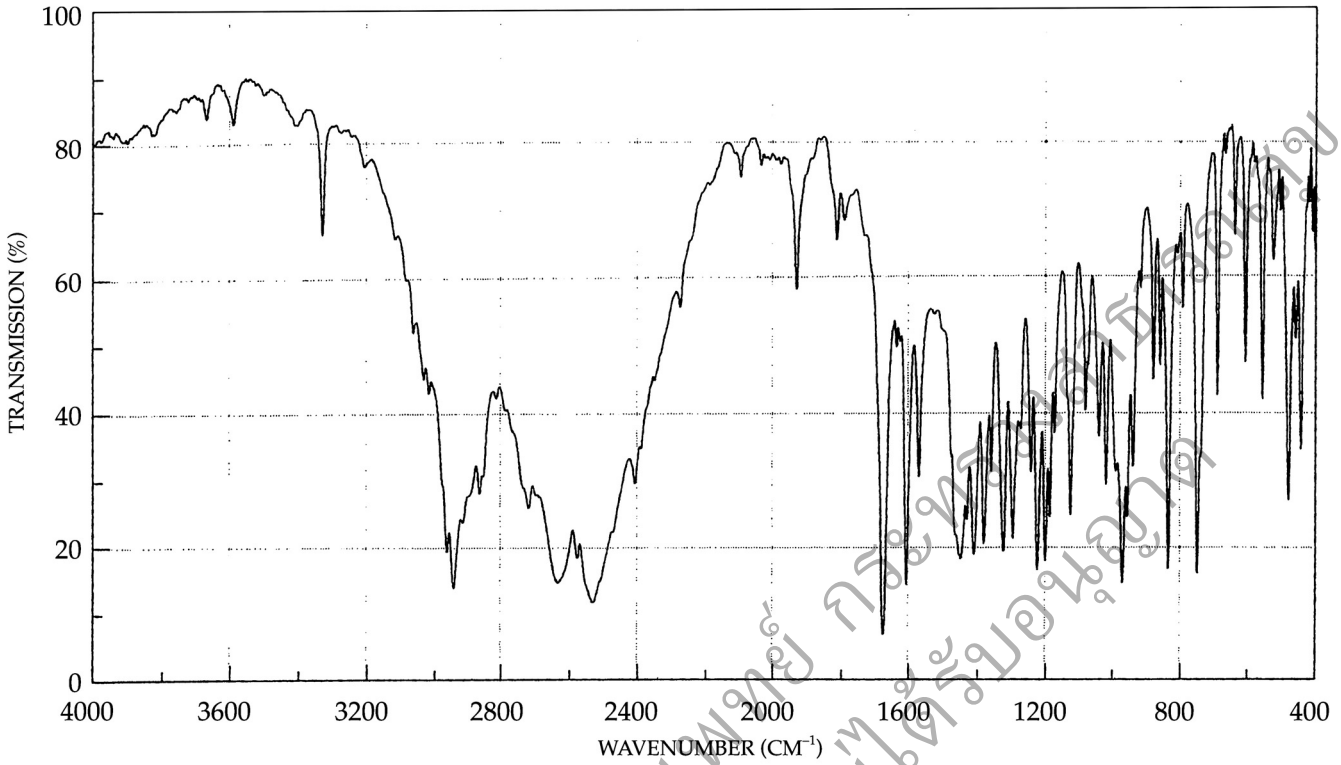
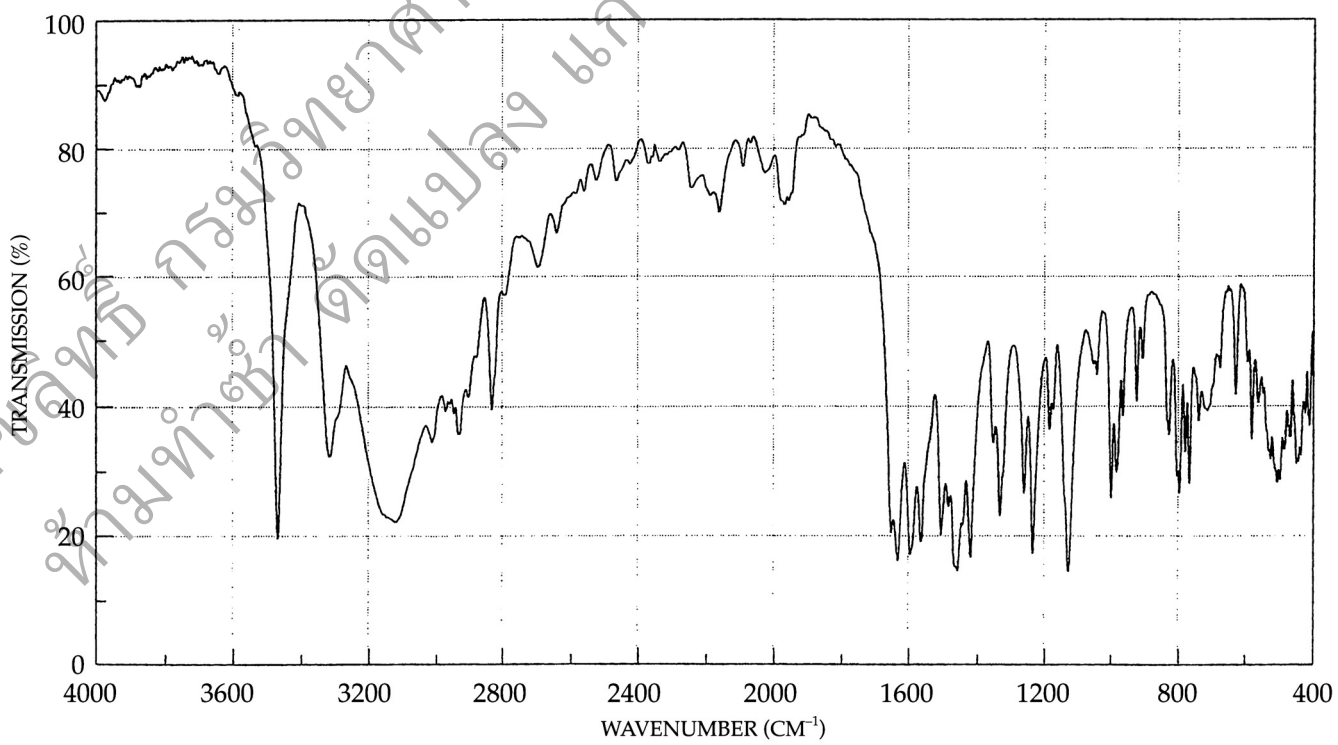
Tolperisone Hydrochloride Instrument: Fourier Transform Phase: Potassium Bromide mixture



Trimethoprim Instrument: Fourier Transform Phase: Potassium Bromide mixture



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APPENDIX 14 BIOLOGICAL AND BIOCHEMICAL ASSAYS

14.2 BLOOD AND RELATED PRODUCTS

14.2.1 Biological Assay of Heparin in Coagulation Factors

Heparin is assayed as a complex with antithrombin III (AT) via its inhibition of coagulation factor Xa (anti-Xa activity). An excess of AT is maintained in the reaction mixture to ensure a constant concentration of the heparin-AT complex. Factor Xa is neutralized by the heparin-AT complex and the residual factor Xa hydrolyses, a specific chromogenic peptide substrate to release a chromophore. The quantity of chromophore is inversely proportional to the activity of the heparin.

Factor Xa chromogenic substrate Specific chromogenic substrate for factor Xa such as: *N*-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-4-nitroanilide hydrochloride. Reconstitute according to the manufacturer's instructions.

Dilution buffer A 0.605 per cent w/v solution of *tris(hydroxymethyl)aminomethane*. Adjust to pH 8.4 if necessary using *hydrochloric acid*.

Test solution Dilute the preparation to be examined with dilution buffer to obtain a solution expected to contain 0.1 IU of heparin per ml.

Reference solution Dilute the heparin reference preparation with dilution buffer to obtain a solution containing 0.1 IU of heparin per ml.

The following working conditions apply to microtitre plates. If the assay is carried out in tubes, the volumes are adjusted while maintaining the proportions in the mixture.

Warm all solutions to 37° in a water-bath shortly before the test.

Distribute in a series of wells, 20 µl of normal human plasma and 20 µl of *antithrombin III solution 1*. Add to the wells a series of volumes (20 µl, 60 µl, 100 µl and 140 µl) of the test solution or the reference solution and make up the volume in each well to 200 µl using dilution buffer (0.02 to 0.08 IU of heparin per ml in the final reaction mixture).

End-point method Transfer 40 µl from each well to a second series of wells, add 20 µl of *bovine factor Xa solution* and incubate at 37° for 30 seconds. Add 40 µl of a 1 mmol per litre solution of factor Xa chromogenic substrate and incubate at 37° for 3 minutes. Terminate the reaction by lowering the pH by the addition of a suitable reagent, such as a 20 per cent v/v solution of *glacial acetic acid* and measure the absorbance at 405 nm (Appendix 2.2). Appropriate reaction times are usually between 3 and 15 minutes, but deviations are permissible if better linearity of the dose-response relationship is thus obtained.

Kinetic method Transfer 40 µl from each well to a second series of wells, add 20 µl of *bovine factor Xa solution* and incubate at 37° for 30 seconds. Add 40 µl of a 2 mmol per litre solution of factor Xa chromogenic substrate, incubate at 37° and measure the rate of substrate cleavage by continuous measurement of the absorbance change at 405 nm (Appendix 2.2), thus allowing the initial rate of substrate cleavage to be calculated. This rate must be linear with the concentration of residual factor Xa.

Check the validity of the assay and calculate the heparin activity of the test preparation as described in the "Statistical Analysis of Results of Biological Assay and Tests" (Appendix 9, slope-ratio assay).

14.2.2 Test for Prekallikrein Activator

Prekallikrein activator (PKA) activates prekallikrein to kallikrein and may be assayed by its ability to cleave a chromophore from a synthetic peptide substrate so that the rate of cleavage can be measured spectrophotometrically and the concentration of PKA calculated by comparison with a standard preparation calibrated in International Units.

PREPARATION OF PREKALLIKREIN SUBSTRATE

(**Note** To avoid coagulation activation, blood or plasma used for the preparation of prekallikrein must come into contact only with plastics or silicone-treated glass surfaces.)

Draw 9 volumes of human blood into 1 volume of anticoagulant solution (ACD, CPD or 3.8 per cent w/v solution of *sodium citrate*) to which 1 mg per ml of *hexadimethrine bromide* has been added. Centrifuge the mixture at 3600 × *g* for 5 minutes. Separate the plasma and centrifuge again at 6000 × *g* for 20 minutes to sediment platelets. Separate the platelet-poor plasma and dialyse against 10 volumes of buffer A for 20 hours. Apply the dialyzed plasma to a chromatography column containing *agarose-DEAE for ion exchange chromatography* which has been equilibrated in buffer A and is equal to twice the volume of the plasma. Elute from the column with buffer A at about 20 ml per cm^2 per hour. Collect the eluate in fractions and record the absorbance at 280 nm (Appendix 2.2). Pool the fractions containing the first protein peak so that the volume of the pool is about 1.2 times the volume of the platelet-poor plasma.

Test the substrate pool for absence of kallikrein activity by mixing 1 part with 20 parts of the prewarmed chromogenic substrate solution to be used in the assay and incubate at 37° for 2 minutes. The substrate is suitable if the increase in absorption is less than 0.001 per minute. Add 0.7 per cent w/v solution of *sodium chloride* to the pooled and filter using a membrane filter (porosity 0.45 µm). Freeze the filtrate in portions and store at –25°; the substrate may be freeze-dried before storage.

Carry out all procedures from the beginning of the chromatography to freezing in portions during a single working day.

METHOD

The assay may be carried out using an automated enzyme analyzer or a suitable microtitre plate system allowing kinetic measurements, with appropriate software for calculation of results. Standards, samples and prekallikrein substrate may be diluted as necessary using buffer B.

Incubate diluted standards or samples with prekallikrein substrate for 10 minutes such that the volume of the undiluted sample does not exceed 1/10 of the total volume of the incubation mixture to avoid errors caused by variation in ionic strength and pH in the incubation mixture. Incubate the mixture or a part thereof with at least an equal volume of a solution of a suitable synthetic chromogenic substrate, known to be specific for kallikrein (for example, *N*-benzoyl-L-prolyl-L-phenylalanyl-L-arginine 4-nitroanilide acetate or D-prolyl-L-phenylalanyl-L-arginine-4-nitroanilide dihydrochloride), dissolved in buffer B. Record the rate of change in absorbance per minute for 2 to 10 minutes at the wavelength specific for the substrate used. Prepare a blank for each mixture of sample or standard using buffer B instead of prekallikrein substrate.

Correct ΔA per minute by subtracting the value obtained for the corresponding blank. Plot a calibration curve using the values thus obtained for the reference preparation and the respective concentrations. Use the curve to determine the PKA activity of the preparation being examined.

REAGENTS

Buffer A

Tris(hydroxymethyl)methylamine	6.055	g
Sodium chloride	1.17	g
Hexadimethrine bromide	0.050	g
Sodium azide	0.100	g

Dissolve the ingredients in *water*, adjust to pH 8.0 with 2 M *hydrochloric acid* and dilute to 1000 ml with *water.*

Buffer B

Tris(hydroxymethyl)methylamine	6.055	g
Sodium chloride	8.77	g

Dissolve the ingredients in *water*, adjust to pH 8.0 with 2 M *hydrochloric acid* and dilute to 1000 ml with *water.*

14.2.3 Determination of Hemoglobin Concentration by Hemiglobincyanide Method

There are three methods for estimation of hemoglobin concentration by using a spectrometer or photoelectric colourimeter, i.e. alkaline-hematin, oxyhemoglobin (HbO_2) and hemiglobincyanide or cyanomethemoglobin (HiCN) methods. A major advantage of the hemiglobincyanide (HiCN) method is the availability of a stable and reliable reference solution.

The hemiglobincyanide (HiCN) method is provided for determination of hemoglobin concentration in human blood. The basis of the method is dilution of blood in the solution containing potassium cyanide and potassium hexacyanoferrate(III). Hemoglobin (Hb) and its derivatives, methemoglobin (Hi) and carboxyhemoglobin (HbCO), but not sulphemoglobin (SHb) are oxidized to methemoglobin in the presence of alkaline potassium hexacyanoferrate(III). Methemoglobin then reacts with potassium cyanide to form hemiglobincyanide (HiCN) which has maximum absorption at 540 nm. The colour intensity measured at 540 nm is proportional to the total hemoglobin concentration.

CYANIDE-HEXACYANOFERRATE(III) SOLUTION

Potassium hexacyanoferrate(III)	200	mg
Potassium cyanide[1]	50	mg
Potassium dihydrogenphosphate	140	mg
Non-ionic detergent[2]	0.5	ml
Water to	1000	ml

The solution should be clear and pale yellow in colour. When measured against *water* as a blank in a spectrophotometer at a wavelenght of 540 nm, the optical density must read zero. The pH of the solution should lie between 7.0 and 7.4 and must be checked regularly, at least once a month. If stored between 4° and 25° in a brown borosilicate glass bottle, the solution will be kept for several months. It must not be allowed to freeze, as this can result in its decomposition and must be discarded if it becomes turbid, or if the pH is found to be outside the 7.0 to 7.4 range or if it has an absorbance other than zero at 540 nm against a water blank.

Instead of the solution described above, Drabkin's cyanide-hexacyanoferrate(III) solution (Drabkin's reagent) or similar products, some of which are available in powder or tablet form, are also used in clinical hemoglobinometry. It should, however, be borne in mind that with these solutions complete conversion of hemoglobin derivatives to HiCN takes about 20 minutes and that the resulting solution is often slightly turbid, giving results that are too high.

[1] Highly poisonous. Great care must be taken when handling this reagent and to ensure safe storage.

[2] A suitable non-ionic detergent, i.e. nonylphenylpolyethylene glycol or equivalent, may be used.

HEMIGLOBINCYANIDE REFERENCE SOLUTION

Solutions of HiCN are available commercially as reference solutions that conform to the international specifications. They contain 550 to 850 mg of hemoglobin per litre and the exact concentration is indicated on the label. The solution is dispensed in sealed appropriate containers. In use, the reference solution is regarded as a dilution of whole blood, and the original Hb that it represents is obtained by mutiplying the figure stated on the label by the dilution to be applied to the blood sample.

Blood sample The blood sample may be taken from a freely bleeding capillary puncture (finger, heel in infants) or may be collected by venesection. The capillary puncture must be deep enough to allow the blood to flow freely. A venous specimen may be collected into any solid anticoagulant (EDTA, mixture of ammonium and potassium oxalate or heparin). The blood should be well mixed before sampling, either by gentle inversion at least 20 times, or, preferably, on a mechanical mixing device for 2 to 3 minutes.

Procedure Dilute 20.0 µl of the blood sample in 5.0 ml of *Cyanide-hexacyanoferrate(III) solution*. Mix well and allow to stand for at least 3 minutes to ensure the completion of the reaction. Measure the absorbances of *Hemiglobincyanide reference solution* and blood sample at the maximum at about 540 nm (Appendix 2.2), against *Cyanide-hexacyanoferrate(III) solution* as a blank.

Determine total hemoglobin concentration (g/dl) of blood sample by the following formula:

$$\text{Hb (g/dl)} = (A_u / A_s)(C_s)(\text{Dilution factor} / 100)$$

in which C_s is the concentration, g/dl, of Hb in *Hemiglobincyanide reference solution*, and A_s and A_u are the absorbances of *Hemiglobincyanide reference solution* and blood sample, respectively.

To prepare a calibration curve, set up series of five tubes. Pipette into these tubes the following amounts of Hemiglobincyanide Reference Solution.

Tube No.	Hemiglobincyanide Reference Solution (ml)	Cyanide-hexacyanoferrate(III) Solution (ml)
1	6.0	0.0
2	4.5	1.5
3	3.0	3.0
4	1.5	4.5
5	0.0	6.0

The hemoglobin concentration in the five tubes will be, respectively: labelled value (full strength); ¾ times labelled value; ½ times labelled value; ¼ times labelled value; and zero strength (blank). Measure the absorbances of five tubes at the maximum at about 540 nm (Appendix 2.2), against *Cyanide-hexacyanoferrate(III) solution* as a blank. Construct a calibration curve by plotting the absorbance reading on the vertical axis and hemoglobin concentration on the horizontal axis of graph paper. The points should fall on a straight line passing through zero. Determine total hemoglobin concentration (g/dl) of blood sample by reading from the calibration curve.

14.5 IMMUNOCHEMICAL METHODS

Immunochemical methods are based on the selective, reversible and non-covalent binding of antigens by antibodies. These methods are employed to detect or quantify either antigens or antibodies. The formation of an antigen-antibody complex may be detected and the amount of complex formed may be measured by a variety of techniques. The provisions of this general method apply to immunochemical methods using labelled or unlabelled reagents, as appropriate.

The results of immunochemical methods depend on the experimental conditions and the nature and quality of the reagents used. It is essential to standardize the components of an immunoassay and to use, wherever available, international reference preparations for immunoassays.

The reagents necessary for many immunochemical methods are available as commercial assay kits, that is a set including reagents (particularly the antigen or the antibody) and materials intended for the *in vitro* estimation of a specified substance as well as instructions for their proper use. The kits are used in accordance with the manufacturers' instructions; it is important to ascertain that the kits are suitable for the analysis of the substance to be examined, with particular reference to selectivity and sensitivity.

METHODS IN WHICH A LABELLED ANTIGEN OR A LABELLED ANTIBODY IS USED

Methods using labelled substances may employ suitable labels such as enzymes, fluorophores, luminophores and radioisotopes. Where the label is a radioisotope, the method is described as a "radioimmunoassay". The recommendations for the measurement of radio activity given under radiopharmaceutical preparations are applicable to immunoassays involving radioisotopes. All work with radioactive materials must be carried out in conformity with national legislation and internationally accepted codes of practice for protection against radiation hazards.

Radioimmunoassay methods (RIA) The principle of the assay method is that radioactive labelled antigen competes with the non-labelled antigen of a sample under test for the antibody with which the labelled and non-labelled antigens are mixed. The more of the antigen in the test sample the less chance the labelled antigen has of combining with a limited number of antibody molecules that are available in the antibody serum. The amount of labelled antigen bound to antibody is detemined by the amount of unlabelled antigen that competes in test sample. Thus, by measuring the quantity of labelled antigen combined with antibody (using isotope counting equipment) a measure of the antigen in the test sample can be obtained.

Enzyme-linked immunosorbent assay methods (ELISA) Where the label is enzyme, the method is described as Enzyme-linked immunosorbent assay. The method involves coupling to enzymes which give a coloured soluble reaction products after enzyme substrate, a colourless substrate, is added. The enzymes such as *horseradish peroxidase* or *alkaline phosphatase* are linked to either antibody or antigen molecules. The presence of the enzyme-linked molecule is detected by means of the enzyme substrate and can be measured by a spectrophotometer. Either the labelled antigen or the antibody can be attached to an insoluble support, such as plastic beads or plastic agglutination plates.

METHODS IN WHICH AN UNLABELLED ANTIGEN OR ANTIBODY IS USED

Immunoprecipitation methods Immunoprecipitation methods include flocculation and precipitation reactions. When a solution of an antigen is mixed with its corresponding antibody under suitable conditions, the reactants form flocculating or precipitating aggregates. The ratio of the reactants that gives the shortest flocculation time or the most marked precipitation is called the optimal ratio, and is usually produced by equivalent amounts of antigen and antibody. Immunoprecipitation can be assessed visually or by light-scattering techniques (nephelometric or turbidimetric assay). An increase insensitivity can be obtained by using antigen- or antibody-coated particles (such as latex) as reactants.

In flocculation methods, stepwise dilutions of one of the reactants are usually used whereas, in immunodiffusion (ID) methods, the dilution is obtained by diffusion in a gel medium: concentration gradients of one or both of the reactants are obtained, thus creating zones in the gel medium where the ratio of the reactants favours precipitation. While flocculation methods are performed in tubes, immunodiffusion methods may be performed using different supports such as tubes, plates, slides, cells or chambers.

Where the immunoprecipitating system consists of one antigen combining with its corresponding antibody, the system is referred to as simple; when it involves related but not serologically identical reactants, the system is complex and where several serologically unrelated reactants are involved, the system is multiple.

In simple diffusion methods, a concentration gradient is established for only one of the reactants diffusing from an external source into the gel medium containing the corresponding reactant at a comparatively low concentration.

Single radial immunodiffusion (SRID) is a simple quantitative immunodiffusion technique. When the equilibrium between the external and the internal reactants has been established, the circular precipitation area, originating from the site of the external reactant, is directly proportional to the amount of the antigen applied and inversely proportional to the concentration of the antibody in the gel.

In *double diffusion methods*, concentration gradients are established in a neutral (inert) gel by allowing both reactants to diffuse into the gel from separate sites.

Comparative double diffusion methods are used for qualitatively comparing various antigens versus a suitable antibody or *vice versa*. The comparison is based on the presence or absence of interaction between the precipitation patterns. Reactions of identity, non-identity or partial identity of antigens or antibodies can be distinguished.

Agglutination methods In this reaction the antigen is part of the surface of some particulate material such as a red cell, bacterium or perhaps an inorganic particle (e.g. polystyrene latex) which has been coated with antigen. Antibody added to a suspension of such particles combines with the surface antigens and links them together to form clearly visible aggregates or agglutinates. This clearly visible agglutinates is the end-point of the test. The reciprocal of the antiserum dilution made at the end-point is known as the titre of the antiserum and is a measure of the number of antibody unit per volume of serum.

Immunoelectrophoretic methods Immunoelectrophoresis (IE) is a qualitative technique combining two methods: gel electrophoresis followed by immunodiffusion.

Crossed immunoelectrophoresis is a modification of the IE method. It is suitable for both qualitative and quantitative analysis. The first part of the procedure is an ordinary gel electrophoresis, after which a longitudinal gel strip, containing the separated fractions to be determined, is cut out and transferred to another plate. The electrophoresis in the second direction is carried out at an angle of 90° to the previous electrophoretic run in a gel containing a comparatively low concentration of antibodies corresponding to the antigens. For a given antibody concentration and gel thickness, the relationship between the area of the respective precipitation peaks and the amount of the corresponding antigen is linear.

Electroimmunoassay, often referred to as rocket immunoelectrophoresis, is a rapid quantitative method for determining antigens with a charge differing from that of the antibodies or *vice versa*. The electrophoresis of the antigen to be determined is carried out in a gel containing a comparatively lower concentration of the corresponding antibody. The test material and dilutions of a standard antigen used for calibration are introduced into different wells in the gel. During electrophoresis, migrating peak-shaped precipitation zones originating from the wells are developed. The front of the precipitate becomes stationary when the antigen is no longer in excess. For a given antibody concentration, the relationship between the distance travelled by the precipitate and the amount of antigen applied is linear.

Counter-immunoelectrophoresis is a rapid quantitative method allowing concentration gradients of external antigen and external antibody to be established in an

electric field depending on the different charges. Dilutions of a standard for calibration and dilutions of the test material are introduced into a row of wells in a gel and a fixed amount of the corresponding reactant is introduced into an opposite row of wells. The titre of the test material may be determined as the highest dilution showing a precipitation line.

A number of modifications of crossed immunoelectrophoresis and electroimmunoassay methods exist.

Other techniques combine separation of antigens by molecular size and serological properties.

Visualization and characterization of immunoprecipitation line These may be performed by selective or non-selective stains, by fluorescence, by enzyme or isotope labelling, or by other relevant techniques. Selective staining methods are usually performed for characterization of non-protein substances in the precipitates.

In translucent gels such as agar or agarose, the precipitation line becomes clearly visible in the gel, provided that the concentration of each of the reactants is appropriate.

14.6 NUCLEIC ACID AMPLIFICATION TECHNIQUES

INTRODUCTION

Nucleic acid amplification techniques are based on two different approaches:

1. amplification of a target nucleic acid sequence using, for example, polymerase chain reaction (PCR), ligase chain reaction (LCR), or isothermal ribonucleic acid (RNA) amplification:

2. amplification of a hybridization signal using, for example, for deoxyribonucleic acid (DNA), the branched DNA (bDNA) method. In this case signal amplification is achieved without subjecting the nucleic acid to repetitive cycles of amplification.

In this general appendix, the PCR method is described as the reference technique. Alternative methods may be used, if they comply with the quality requirements described below.

SCOPE

This section establishes the requirements for sample preparation, *in vitro* amplification of DNA sequences and detection of the specific PCR product. With the aid of PCR, defined DNA sequences can be detected. RNA sequences can also be detected following reverse transcription of the RNA to complementary DNA (cDNA) and subsequent amplification.

PRINCIPLE OF THE METHOD

PCR is a procedure that allows specific *in vitro* amplification of segments of DNA or of RNA after reverse transcription into cDNA.

Following denaturation of double-stranded DNA into single-stranded DNA, two synthetic oligonucleotide primers of opposite polarity, anneal to their respective complementary sequences in the DNA to be amplified. The short double-stranded regions, which form as a result of specific base pairing between the primers and the complementary DNA sequence, border the DNA segment to be amplified and serve as starting positions for *in vitro* DNA synthesis by means of a heat-stable DNA polymerase.

Amplification of the DNA occurs in cycles consisting of:

– heat denaturation of the nucleic acid (target sequence) into two single strands;

– specific annealing of the primers to the target sequence under suitable reaction conditions;

– extension of the primers, which are bound to both single strands, by DNA polymerase at a suitable temperature (DNA synthesis).

Repeated cycles of heat denaturation, primer annealing and DNA synthesis results in an exponential amplification of the DNA segment limited by the primers.

The specific PCR product known as an amplicon can be detected after or during amplification by a variety of methods of appropriate specificity and sensitivity. However, only post-PCR detection will be described herein.

TEST MATERIAL

Because of the high sensitivity of PCR, the samples must be optimally protected against external contamination with target sequences. Sampling, storage and transport of the test material are performed under conditions that minimize degradation of the target sequence. In the case of RNA target sequences, special precautions are necessary since RNA is highly sensitive to degradation by ribonucleases. Care must be taken since some added reagents, such as anticoagulants or preservatives, may interfere with the test procedure.

TEST METHOD

Prevention of contamination

The risk of contamination requires a strict segregation of the areas depending on the material handled and the technology used. Points to consider include movement of personnel, gowning, material flow and air supply, and decontamination procedures.

The system should be subdivided into compartments such as:

– master-mix area (area where exclusively template-free material is handled, e.g., primers, buffers, etc.);

– pre-PCR (area where reagents, samples and controls are handled);

– PCR amplification (amplified material is handled in a closed system);

– post-PCR detection (the only area where the amplified material is handled in an open system).

Sample preparation

When preparing samples, the target sequence to be amplified needs to be efficiently extracted or liberated from the test material in a reproducible manner and in such a way that amplification under the selected reac-

tion conditions is possible. A variety of physico-chemical extraction procedures and/or enrichment procedures may be employed.

Additives present in test material may interfere with PCR. The procedures described under *Internal control* must be used as a control for the presence of inhibitors originating from the test material.

In the case of RNA-templates, care must be taken to avoid ribonuclease activity.

Amplification

PCR amplification of the target sequence is conducted under optimized cycling conditions (temperature profile for denaturation of double-stranded DNA, annealing and extension of primers; incubation times at selected temperatures; ramp rates). These depend on various parameters such as:

– the length and base composition of primer and target sequences;

– the type of DNA polymerase, buffer composition and reaction volume used for the amplification;

– the type of thermocycler used and the thermal conductivity rate between the apparatus, reaction tube and reaction fluid.

Detection

The amplicon generated by PCR may be identified by size, sequence, chemical modification or a combination of these parameters. Characterization by size may be achieved by gel electrophoresis (using agarose or polyacrylamide slab gels or capillary electrophoresis) or column chromatography (for example, HPLC). Characterization by sequence composition may be achieved by the specific hybridization of probes having a sequence complementary to the target sequence or by cleavage of the amplified material reflecting target-specific restriction-enzyme sites. Characterization by chemical modification may be achieved, for example, by incorporation of a fluorophore into the amplicons and subsequent detection of fluorescence following excitation.

Detection of amplicons may also be achieved by using probes labelled to permit a subsequent radio isotopic or immuno-enzyme-coupled detection.

EVALUATION AND INTERPRETATION OF RESULTS

A valid result is obtained within a test only if the positive control(s) is unambiguously positive and the negative control(s) is unambiguously negative. Due to the very high sensitivity of the PCR method and the inherent risk of contamination, it is necessary to confirm positive results by repeating the complete test procedure in duplicate, where possible, on a new aliquot of the sample. The sample is considered positive if at least one of the repeat tests gives a positive result.

QUALITY ASSURANCE

Validation of the PCR assay system

The validation programme must include validation of instrumentation and the PCR method employed.

Appropriate official working reference preparations or in-house reference preparations calibrated against International Standards for the target sequences for which the test system will be used are indispensable for validation of a PCR test.

During validation the positive cut-off point must be determined. The positive cut-off point is defined as the minimum number of target sequences per volume of the sample which can be detected in 95 per cent of test runs. The positive cut-off point depends on interrelated factors such as the volume of the sample extracted and the efficacy of the extraction methodology, the transcription of the target RNA into cDNA, the amplification process and the detection.

To define the detection limit of the assay system, reference must be made to the positive cut-off point for each target sequence and the test performance above and below the positive cut-off point.

Quality control of reagents

All reagents crucial for the methodology used have to be controlled prior to use in routine applications. Their acceptance or withdrawal is based on predefined quality criteria.

Primers are a crucial component of the PCR assay and as such their design, their purity and the validation of their use in a PCR assay require careful attention. Each new batch of primers is tested for specificity, amplification efficiency and absence of inhibitory impurities before acceptance. Primers may be modified (for example, by conjugation with a fluorophore or antigen) in order to permit a specific method of detection of the amplicon, provided such modifications do not inhibit accurate and efficient amplification of the target sequence.

Run controls

EXTERNAL CONTROLS

In order to minimize the risk of contamination and to ensure adequate sensitivity, the following external controls are included in each PCR test:

– positive control: this contains a defined number of target-sequence copies, the number being determined individually for each assay system and indicated as a multiple of the positive cut-off value of the test system;

– negative control: a sample of the same matrix already proven to be free of the target sequences;

INTERNAL CONTROLS

Internal controls are defined as nucleic acid sequences containing the primer binding sites. Internal controls must be amplified with similar efficacy as the target sequence to be tested, but the amplicons must be clearly discernible. Internal controls must be of the same type of nucleic acid (DNA or RNA) as the material to be tested. The internal control is preferably added to the test material before isolating the nucleic acid and therefore acts as an overall control (extraction, reverse transcription, amplification, detection).

External quality assessment

Participation in external quality assessment programmes is an important PCR quality assurance procedure for each laboratory and each operator.

APPENDIX 15
ASSAY OF BIOLOGICAL PRODUCTS

15.1 HUMAN BLOOD AND BLOOD PRODUCTS

15.1.1 Determination of ABO Group of Donors

Examine the blood for both antigens on the red cells and antibodies in the serum or plasma using either manual or automated techniques.

For manual tests, collect a few ml of fresh blood without anticoagulant in a narrow test-tube, allow to clot, and remove the serum. Stir the clot or a small part of it with *saline TS*, centrifuge the resulting suspension, and resuspend the cells in *saline TS* to make a 2 to 3 per cent v/v suspension of packed cells.

For automated tests, collect a few ml of blood and prevent from clotting by the addition of 1 volume of a 10 per cent w/v solution of *dipotassium edetate* for each 32 volumes of blood collected. Separate the plasma and cellular components and prepare a suspension of red cells in *saline TS* suitable for the type of instrument to be used.

TESTS FOR ANTIGENS

Mix separate portions of the red cell suspension with Anti-A Blood-grouping Reagent, Anti-B Blood-grouping Reagent and Anti-A,B (group O) Blood-grouping Reagent, the specificities of which have been demonstrated by testing with red cells of known groups A, B and O, and determine the antigens present by examination of the resulting pattern of agglutination.

Manual tests Mix 1 volume of the appropriate ABO Blood-grouping Reagent with 1 volume of red cell suspension in a test-tube. Allow the tubes to stand at room temperature for 1 to 2 hours and then tap them gently to disperse the deposit of cells. Examine the contents of the tubes macroscopically for agglutination.

Automated tests Perform the tests with appropriate automatic equipment[1] according to the manufacturer's instructions. Mix the red cell suspension with the appropriate ABO Blood-grouping Reagent. Allow agglutination to take place and determine its degree either by visual inspection after the agglutinates have been deposited upon filter paper or by the use of a suitable photometric detector fitted with an automatic means of recording.

TESTS FOR ANTIBODIES

Mix separate portions of the serum or plasma with suspensions of human red cells of group A (sub-groups A_1 and A_2), group B and group O, and determine the antibodies present by examination of the resulting pattern of agglutination by either manual or automated tests as described above under the Tests for Antigens.

When the above tests are used to determine the blood group of an intended recipient rather than that of a donor, it should be noted that antibody development may be incomplete in infants under 1 year old; in such circumstances the blood group of the child is based solely on the antigens present on the red cells.

ABO BLOOD-GROUPING REAGENTS

(**Note** ABO Blood-grouping Reagents are normally selected on the basis of their suitability for use in manual tests. When such reagents are to be used in automated tests, their suitability must be reassessed by performing automated tests with a large number of red cell suspensions of different ABO group including examples of the A_1, A_2, A_2B, and Ax sub-groups.)

ABO Blood-grouping Reagents are derived from the sera or defibrinated plasma of selected persons of the appropriate ABO blood group who may have been deliberately immunized with either red cells or group-specific substance of the appropriate blood group or groups. Alternatively they are derived from the sera of lower animals or from cultures of mammalian lymphocytes. For preparations of human origin, use only material that has been tested with negative results for the presence of hepatitis B surface antigen, HIV antibodies, and other blood borne infectious agents by suitably sensitive methods. However, the reagent cannot be assumed to be free from infectious agents. Care must be taken in the use and disposal of each container and its contents.

ABO Blood-grouping Reagents may be issued as liquids or they may be prepared by constitution from the dried reagents. Liquid reagents are clear or faintly opalescent, yellowish or colourless fluids without turbidity. They may contain a suitable antimicrobial preservative. Dried reagents are pale yellow powders or friable solids.

ABO Blood-grouping Reagents are of three types, Anti-A Blood-grouping Reagent, Anti-B Blood-grouping Reagent and Anti-A,B (Group O) Blood-grouping Reagent.

Anti-A Blood-grouping Reagent agglutinates human red cells containing A antigens, including sub-groups A_1 and A_2 but rarely agglutinates those red cells classified as Ax; that is, it agglutinates red cells of blood groups A and AB that include the sub-groups A_1, A_2, A_1B, and A_2B but does not agglutinate red cells of the sub-groups Ax or AxB. The reagent does not agglutinate human red cells that do not contain A antigens, i.e. red cells of blood groups O and B. The reagent is shown not to agglutinate, under the conditions specified for use, any of a comprehensive panel of group O or group B red cells chosen to bear a wide range of red cell antigens, or to contain antibodies to the serum protein

[1]Instruments based on continuous flow system or a discrete sample system may be used.

factors Gm or Km. It does not agglutinate group O or group B red cells coated with IgG.

Anti-B Blood-grouping Reagent agglutinates human red cells containing B antigens, i.e. red cells of blood groups B and AB including sub-groups A_1B and A_2B. The reagent does not agglutinate human red cells that do not contain B antigens, i.e. red cells of blood groups O and A including sub-groups A_1 and A_2. The reagent is shown not to agglutinate, under the conditions specified for use, any of a comprehensive panel of group O or A red cells chosen to bear a wide range of red cell antigens, or to contain antibodies to the serum protein factors Gm or Km. It does not agglutinate group O or group A red cells coated with IgG.

Anti-A,B (Group O) Blood-grouping Reagent agglutinates human red cells containing A or B antigens; that is, it agglutinates red cells of blood groups A, B and AB including the sub-groups A_1, A_2, Ax, A_1B, A_2B, and AxB. The reagent does not agglutinate human red cells that do not contain A or B antigens, i.e. red cells of blood group O. The reagent is shown not to agglutinate, under the conditions specified for use, any of a comprehensive panel of group O cells chosen to bear a wide range of red cell antigens, or to contain antibodies to the serum protein factors Gm or Km. It does not agglutinate group O red cells coated with IgG.

ABO Blood-grouping Reagents, reconstituted where necessary as stated on the label, comply with the following requirements.

Avidity On a microscopic slide, mix a suitable volume of the reagent with an equal volume of a 5 to 10 per cent v/v suspension of human red cells of each of the groups or sub-groups given below; use a separate portion of the reagent for each type of red cell. The time taken for agglutination to appear first to the unaided eye is not more than twice that taken when the procedure is carried out using the appropriate National or International Standard of Blood-typing Serum in place of the reagent being examined.

ANTI-A BLOOD-GROUPING REAGENT Use human red cells of sub-groups A_1 and A_2B and preferably also use cells of sub-groups A_1 and A_2B; the appropriate National or International Standard is that for Anti-A blood-typing serum.

ANTI-B BLOOD-GROUPING REAGENT Use human red cells of sub-group B; the appropriate National or International Standard is that for Anti-B blood-typing serum.

ANTI-A, B (GROUP O) BLOOD-GROUPING REAGENT Use human red cells of sub-groups A_1 and A_2 and of group B; the appropriate National or International Standard is that for Anti-A, B blood-typing serum.

In addition carry out the procedure described above at a temperature of 20° to 25° using red cells of subgroup Ax; agglutination first appears to the unaided eye in not more than 2 minutes.

Potency The potency of an ABO Blood-grouping Reagent is determined by comparing its "saline agglutinin" antibody activity with that of the appropriate National or International Standard for Blood-typing serum.

The determination is carried out by simultaneously titrating the reagent being examined and the Standard against suspensions of human red cells of the groups or sub-groups given below.

ANTI-A BLOOD-GROUPING REAGENT Not less than 64 IU of anti-A antibody per ml; that is, for each type of red cell against which it is titrated, the titre of the reagent being examined is not less than one quarter of that of the Standard, irrespective of the actual titres obtained.

Use human red cells of sub-groups A_1 and A_2B and preferably also use cells of sub-groups A_2 and A_1B.

ANTI-B BLOOD-GROUPING REAGENT Not less than 64 IU of anti-B antibody per ml; that is, the titre of the reagent being examined is not less than one quarter of that of the Standard, irrespective of the actual titres obtained.

Use human red cells of group B.

ANTI-A, B (GROUP O) BLOOD-GROUPING REAGENT Not less than 64 IU of anti-A antibody and of anti-B antibody per ml; that is, the titre of reagent being examined is not less than one quarter of that of the Standard, irrespective of the actual titres obtained.

Use human red cells of sub-groups A_1 and A_2 and of group B.

In addition undiluted Anti-A,B Blood-grouping Reagent gives easily detectable agglutination with at least one example of red cells of sub-group Ax.

Sterility Comply with the "Sterility Test" (Appendix 10.1).

Packaging and storage ABO Blood-grouping Reagents shall be kept in sterile containers, sealed so as to exclude micro-organisms.

Liquid ABO Blood-grouping Reagents which do not contain an antimicrobial preservative shall be kept frozen, preferably at a temperature below –30°.

Liquid ABO Blood-grouping Reagents containing an antimicrobial preservative shall be kept at a temperature of 2° to 8°; they shall not be frozen unless the antimicrobial preservative has been shown to be innocuous to the reagent in the frozen state.

Dried ABO Blood-grouping Reagents shall be kept at a temperature not exceeding 20°.

Labelling The label states (1) the name "Anti-A Blood-grouping Reagent", "Anti-B Blood-grouping Reagent" or "Anti-A,B (Group O) Blood-grouping Reagent", as appropriate; (2) the number of IU of the relevant antibody or antibodies per ml; (3) a number or other indication by which the history of the preparation may be traced; (4) the date after which the preparation may not be expected to retain its activity; (5) the conditions under which it shall be stored; (6) for a reagent containing an antimicrobial preservative the name and

concentration of the preservative and that the reagent must not be frozen unless the preservative has been shown to be innocuous to the reagent in the frozen state; (7) for dried reagents the nature and volume of the solvent to be used for reconstitution.

A leaflet included in the package should give the above information together with the appropriate method for using the reagent.

15.1.2 Determination of Rh Group of Donors

Examine the blood for antigens on the red cells using either manual or automated techniques. (Since antibodies of the Rh system are found only in the blood of persons who have been immunized, accidentally or deliberately, with human red cells bearing Rh antigens, it is necessary to depend solely on the reaction of the red cells to determine the Rh group of a donor.)

For manual and automated tests prepare suitable suspensions of human red cells as described under the "Determination of ABO Group of Donors" (Appendix 15.1.1) or, alternatively, for manual tests, prepare a suitable suspension from blood that has been prevented from clotting by the addition of a suitable anticoagulant.

(**Note** If the indirect antiglobulin test is performed, it is necessary to prepare a 10 per cent v/v suspension of red cells.)

TESTS FOR D ANTIGENS

Mix the red cell suspension either with IgM, Anti-D Blood-grouping Reagent or with IgG Anti-D Blood-grouping Reagent, the specificities of which have been demonstrated by testing with D-positive red cells (preferably of the genotype R_1r) and D-negative red cells, and determine whether the anti-D antigen is present by examination for agglutination. If the IgG reagents is used, it is necessary to enhance the agglutination either by the addition of a suitable proteolytic enzyme or by the addition of *bovine serum albumin* or by testing the sensitized red cells with an antiglobulin reagent.

Manual tests Use one of the following methods. The phenotype D^u is rarely detected using IgM Anti-D Blood-grouping Reagent, IgG Anti-D Blood-grouping Reagent should therefore be used, preferably in accordance with method (i) or (iii) below.

Using IgM Anti-D Blood-grouping Reagent: mix 1 volume of the reagent with 1 volume of red cell suspension in a test-tube. Incubate the tubes at 37° for 1 to 2 hours and then tap them gently to disperse the deposit of cells. Examine the contents of the tubes macroscopically for agglutination.

Using IgG Anti-D Blood-grouping Reagent:

(i) Mix 1 volume of the reagent with 1 volume of red cell suspension in a test-tube and add an appropriate volume of a 0.5 per cent v/v suspension of a suitable preparation of activated papain at pH 5.4. Incubate the tubes at 37° for 30 minutes and then tap them gently to disperse the deposit of cells. Examine the contents of the tubes macroscopically for agglutination.

(ii) Mix 1 volume of the reagent with 1 volume of red cell suspension in a test-tube. Incubate the tubes at 37° for 1 hour and then add 1 volume of a 30 per cent v/v solution of *bovine serum albumin* in such a manner that it displaces the supernatant liquid in contact with the sedimented red cells and taking care not to disturb the red cell deposit. Incubate the tubes at 37° for a further 30 minutes and then tap them gently to disperse the deposit of cells. Examine the contents of the tubes macroscopically for agglutination.

(iii) Perform an indirect antiglobulin test treating a 10 per cent v/v suspension of red cells in *saline TS* as follows. Add 4 volumes of the reagent to 2 volumes of the red cell suspension. Incubate the tubes at 37° for 45 minutes. Prepare a positive control by using known D-positive red cells (preferably of the genotype R_1r) and a negative control by using known D-negative cells. After incubation, wash the test and control cells four times in *saline TS*, removing the supernatant liquid as completely as possible each time. Resuspend the cells in *saline TS* to give an approximately 10 per cent v/v suspension of packed cells. Mix 1 drop of this suspension with an equal volume of an appropriate dilution of a suitable antihuman IgG reagent on a tile. Gently rock the tile and examine for positive reactions, shown by agglutination that develops over a period of 5 minutes. (After that time non-specific agglutination is liable to occur.) Alternatively wash the test and control cells with *saline TS* either manually or by means of an automated mechanical device, and then add the appropriate volume of suitable dilution of anti-human IgG reagent. After further centrifugation examine the red cell deposit for agglutination.

Automated tests Perform tests as described in the "Determination of ABO Group of Donors" but using the appropriate Rh Blood-grouping Reagent in place of the ABO Blood-grouping Reagent.

TEST FOR C AND E ANTIGENS

The blood of donors that has been shown by the tests described above to be D-negative should additionally be tested for the presence of C and E antigens. Only donors whose blood is negative for all three antigens may be described as Rh negative (rr).

Tests for C and E antigens are carried out as described in the test for D antigens above but using the appropriate Anti-C or Anti-E Rh Blood-grouping Reagent.

In automated systems samples of blood are usually tested simultaneously for all three antigens.

Rh BLOOD-GROUPING REAGENTS

(**Note** Rh Blood-grouping Reagents are normally selected on the basis of their suitability for use in manual tests. When such reagents are to be used in automated tests their suitability must be assessed by performing automated tests with a large number of red cell suspensions embracing a wide variety of Rh phenotypes. IgM Anti-D Blood-grouping Reagent is simple to use but not necessarily the most reliable. IgG Anti-D

Blood-grouping Reagent is much more readily available and is therefore more commonly used.)

Rh Blood-grouping Reagents are derived from the sera or defibrinated plasma of one or more persons immunized by the appropriate antigen of the Rh system or from cultures of mammalian lymphocytes. Only material that has been tested with negative results for the presence of hepatitis B surface antigen, HIV antibodies, and other blood borne infectious agents by suitably sensitive methods is used. However, the reagent cannot be assumed to be free from infectious agents. Care must be taken in the use and disposal of each container and its contents.

Rh Blood-grouping Reagents may be issued as liquids or they may be prepared by reconstitution from the dried reagents. Liquid reagents are clear or faintly opalescent, yellowish or colourless fluids without turbidity. They may contain a suitable antimicrobial preservative. Dried reagents are pale yellow powders or friable solids.

Rh Blood-grouping Reagents are of four types: IgM Anti-D Blood-grouping Reagent, IgG Anti-D Blood-grouping Reagent, Anti-C Blood-grouping Reagent, and Anti-E Blood-grouping Reagent.

IgM Anti-D Blood-grouping Reagent agglutinates D-positive cells suspended in *saline TS*. The reagent should be shown not to agglutinate, under the conditions specified for use, any of a comprehensive panel of cells which do not contain the D antigen and which are chosen to bear a wide range of red cell antigens.

IgG Anti-D Blood-grouping Reagent agglutinates D-positive human red cells in the presence of a 20 to 30 per cent w/v solution of *bovine serum albumin*. The reagent suspended also coats D-positive human red cells in *saline TS* so that these cells may be subsequently agglutinated by an anti-IgG antiglobulin reagent. By chemical modification of IgG, IgG Anti-D Blood grouping Reagent may agglutinate D-positive human red cells suspended in *saline TS*. The reagent should be shown not to agglutinate or to coat, under the conditions specified for use, any of a comprehensive panel of cells that do not contain the D antigen and which are chosen to bear a wide range of red cell antigens.

Anti-C Blood-grouping Reagent may consist predominantly of IgM or of IgG anti-C antibodies and tests appropriate to the immunological class of the antibody present should be carried out. (See tests described under IgM Anti-D Blood-grouping Reagent and IgG Anti-D Blood-grouping Reagent, respectively.)

For detection of the C antigen in D-negative donors, anti-C, D (that is, anti-G) may be used. The anti-C activity should be capable of reacting with a C^w antigen when in the combination, C^w/c. Anti-G which reacts with either C or D or both C and D antigens will not, if present, interfere when used with DE-negative samples. An alternative anti-C reagent may be used, derived from C-negative, D, E-positive individuals. The anti-C must be capable of detecting a C^w antigen when in the combination C^w/c, and capable of detecting C in *cis* position with both e and E (Ce, CE). These reagents often lack the ability to react with CE (CDE, CdE) complexes. Anti-C Blood-grouping Reagent must be shown not to agglutinate, under the conditions specified for use, any of a panel of cells which do not bear the C or C^w antigens (or D antigen in the case of anti-G) chosen to bear a wide range of red cell antigens including A, B and E.

Anti-E Blood-grouping Reagent may consist predominantly of IgM or of IgG anti-E antibodies and tests appropriate to the immunological class of the antibody present should be carried out. (See tests described under IgM Anti-D Blood-grouping Reagent and IgG Anti-D Blood-grouping Reagent, respectively.)

Many examples of anti-E reagent contain a proportion of anti-cE, an Rh antibody that agglutinates only those human red cells in which the c and E antigens are expressed by c and E genes contained within the same Rh gene complex; that is in *cis* position, and that does not agglutinate cells that contain an E antigen that is the expression of a gene complex, such as CDE and CdE that does not contain e. Anti-E Blood-grouping Reagent should therefore be shown to agglutinate human red cells that contain the E antigen that is not the expression of a gene complex that also contains e. The reagent should be shown not to agglutinate, under the conditions specified for use, any of a comprehensive panel of cells chosen to bear a wide range of red cell antigens. Alternatively an anti-D, E reagent may be used.

Rh Blood-grouping Reagents, reconstituted where necessary as stated on the label, comply with the following requirements.

Potency

IgM ANTI-D BLOOD-GROUPING REAGENT Contains anti-D as a "saline agglutinin" in such quantities that it gives a positive reaction at a dilution of 1 in 32 against red cells known to contain the D antigen.

IgG ANTI-D BLOOD-GROUPING REAGENT The potency of IgG Anti-D Blood-grouping Reagent is determined by comparing its "albumin agglutinin" antibody activity with that of the appropriate National or International Standard of Blood-typing serum.

The determination is carried out by simultaneously titrating the reagent being examined and the reference preparation against suspensions of human red cells containing the D antigen. It contains not less than 32 IU of anti-D antibody per ml; that is, the titre of the reagent being examined is not less than one half of that of the appropriate reconstituted Standard irrespective of the titre obtained.

ANTI-C BLOOD-GROUPING REAGENT Contains anti-C antibody in such quantities that it gives a positive reaction at a dilution of 1 to 8 with red cells known to contain the C antigen.

ANTI-E BLOOD-GROUPING REAGENT Contains anti-E antibody in such quantities that it gives a positive reaction at a dilution of 1 in 8 with red cells known to contain the E antigen.

Sterility Comply with the "Sterility Test" (Appendix 10.1).

Packaging and storage Rh Blood-grouping Reagents shall be kept in sterile containers sealed so as to exclude micro-organisms.

Liquid Rh Blood-grouping Reagents that contain no antimicrobial preservative shall be kept frozen, preferably at a temperature below –30°.

Liquid Rh Blood-grouping Reagents containing an antimicrobial preservative shall be kept at a temperature of 2° to 8°: they shall not be frozen unless the antimicrobial preservative has been shown to be innocuous to the reagent in the frozen state.

Dried Rh Blood-grouping Reagents shall be kept at a temperature not exceeding 20°.

Labelling The label states (1) the name "IgM Anti-D Blood-grouping Reagent", "IgG Anti-D Blood grouping Reagent", "Anti-C Blood-grouping Reagent", or "Anti-E Blood-grouping Reagent", as appropriate; (2) the agglutination titre; (3) a number or other indication by which the history of the preparation may be traced; (4) for a reagent containing an antimicrobial preservative the name and concentration of the preservative and that the reagent must not be frozen unless the preservative has been shown to be innocuous to the reagent in the frozen state; (5) for dried reagents the nature and volume of the solvent to be used for reconstitution.

A leaflet included in the package should give the above information together with the appropriate method for using the reagent.

(**Note** There are two other nomenclatures which may be used for the Rh blood group system. C is sometimes termed rh' or Rh2, D termed Rh_0 or Rh1, E termed rh'' or Rh3, Ce termed rh_i or Rh7 and cE termed Rh27.)

15.1.3 Biological Assay of Human Coagulation Factor VIII

Human coagulation factor VIII is assayed by its biological activity as a cofactor in the activation of factor X by activated factor IX (factor IXa) in the presence of calcium ions and phospholipids. The potency of a factor VIII preparation is estimated by comparing the quantity necessary to achieve a certain rate of factor Xa formation in a test mixture containing the substances that take part in the activation of factor X, and the quantity of the International Standard, or of a reference preparation calibrated in International Units, required to produce the same rate of factor Xa formation.

The chromogenic assay method consists of two consecutive steps: the factor VIII-dependent activation of factor X in a coagulation-factor reagent composed of purified components, and the enzymatic cleavage of a chromogenic factor Xa substrate to yield a chromophore that can be quantified spectrophotometrically. Under appropriate assay conditions, there is a linear relation between the rate of factor Xa formation and the factor VIII concentration. The assay is summarized in Fig. 1.

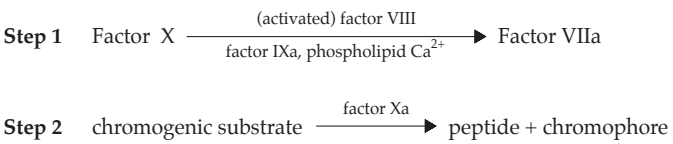

Fig. 1 Schematic Representation of the Assay of Human Coagulation Factor VIII

Both steps employ reagents that may be obtained commercially from a variety of sources. Although the composition of individual reagents may be subject to some variation, their essential features are described in the following specification. Deviations from this description may be permissible provided that it has been shown, using the International Standard for Human Blood Coagulation Factor VIII Concentrate as the standard, that the results obtained do not differ significantly.

It is important to demonstrate by validation the suitability of the kit used, notably by checking the time course of factor Xa generation in order to determine the time taken to reach 50 per cent of the maximal factor Xa generation.

REAGENTS

The coagulation factor reagent comprises purified proteins derived from human or bovine sources. These include factor X, factor IXa, and a factor VIII activator, usually thrombin. These proteins are partly purified, preferably to at least 50 per cent, and do not contain impurities that interfere with the activation of factor VIII or factor X. Thrombin may be present in its precursor form prothrombin, provided that its activation in the reagent is sufficiently rapid to give almost instantaneous activation of factor VIII in the assay. Phospholipid may be obtained from natural sources or be synthetically prepared, and must, to a substantial extent, consist of the species phosphatidylserine. The components of the complete reagent are usually divided into at least two separate reagents, each lacking the ability to generate factor Xa on its own. One of the reagents contains calcium ions. After reconstitution, the reagents may be combined provided that no substantial amounts of factor Xa are generated in the absence of factor VIII. In the final incubation mixture, factor VIII must be the only rate-limiting component.

The second step comprises the quantification of the formed factor Xa, employing a chromogenic substrate that is specific for factor Xa. Generally this consists of a derivatized short peptide of between 3 and 5 amino acids, joined to a chromophore group. On cleavage of this group from the peptide substrate, its chromophoric properties shift to a wavelength allowing its spectrophotometric quantification. The substrate must also contain appropriate inhibitors to stop further factor Xa generation, e.g. chelating agents, and to suppress thrombin activity.

ASSAY

Reconstitute the entire contents of one ampoule of the reference preparation and the preparation to be examined; use immediately. Add sufficient prediluent to the reconstituted preparations to produce solutions containing 0.5 to 2.0 IU per ml.

The prediluent consists of hemophilia A plasma, or of an artificially prepared reagent that contains sufficient von Willebrand factor and that give results that do not differ significantly from those obtained employing hemophilia plasma. The prediluted materials must be stable beyond the time required for the assay.

Prepare further dilutions of reference and test preparations using non-chelating, appropriately buffered solution, for example, *tris(hydroxymethyl)methylamine* or *imidazole*, containing 1 per cent of *bovine serum albumin* or *human albumin*. Prepare at least two dilution series of at least three further dilutions for each material. Prepare the dilutions such that the final factor VIII concentration in the reaction mixture is preferably below 0.01 IU per ml, during the step of factor Xa generation.

Prepare a control solution that includes all components except factor VIII.

Prepare all dilutions in plastic tubes and use immediately.

Step 1 Mix prewarmed dilutions of the factor VIII reference preparation and of the preparation to be examined with an appropriate volume of the prewarmed coagulation factor reagent or a combination of its separate constituents, and incubate the mixture in plastic tubes or microplate wells at 37°. Allow the activation of factor X to proceed for a suitable time, terminating the reaction (step 2) when the factor Xa concentration has reached approximately 50 per cent of the maximal (plateau) level. Appropriate activation times are usually between 2 and 5 minutes.

Step 2 Terminate the activation by addition of a prewarmed reagent containing a chromogenic substrate. Quantify the rate of substrate cleavage, which must be linear with the concentration of factor Xa formed, by measuring the absorbance change at an appropriate wavelength using a spectrophotometer, either monitoring the absorbance continuously, thus allowing the initial rate of substrate cleavage to be calculated, or terminating the hydrolysis reaction after a suitable interval by lowering the pH by addition of a suitable reagent, such as a 50 per cent v/v solution of *acetic acid*, or a 1 M citrate buffer solution pH 3. Adjust the hydrolysis time to achieve a linear development of chromophore over time. Appropriate hydrolysis times are usually between 3 and 15 minutes, but deviations are permissible if better linearity of the dose-response relationship is thus obtained.

Calculate the potency of the test preparation as described in the "Statistical Analysis of Results of Biological Assays and Tests" (Appendix 9).

15.1.4 Biological Assay of Human Coagulation Factor IX

The principle of the assay is to measure the ability of a factor IX preparation to reduce the prolonged coagulation time of factor IX-deficient plasma. The reaction is accelerated by addition of a reagent containing phospholipid and a contact activator, e.g. kaolin, silica or ellagic acid. The potency is assessed by comparing the dose-response curve of the preparation to be examined to that of a reference preparation, calibrated in International Units.

Reconstitute separately the preparation being examined and the reference preparation as stated on the label and use immediately. Where applicable, determine the amount of heparin present, (Appendix 14.2.1), and neutralize the heparin, for example by addition of *protamine sulfate* (10 µg of protamine sulfate neutralizes 1 IU of heparin). Predilute the preparation being examined and the reference preparation in factor IX-deficient plasma (for example *plasma substrate* 2) to produce solution containing 0.5 to 2.0 IU per ml. Prepare at least three dilutions for each material, preferably in duplicate, using a suitable buffer solution (for example *imidazole buffer solution pH* 7.3) containing 1 per cent w/v solution of *bovine serum albumin* or *human albumin*. Use this dilutions immediately.

Use an apparatus suitable for measurement of coagulation times or carry out the assay with incubation tubes maintained in a water-bath at 37°. Place in each tube 0.1 ml of factor IX-deficient plasma (for example *plasma substrate* 2) and 0.1 ml of one of the dilutions of the reference preparation or of the preparation to be examined. Add to each tube 0.1 ml of a suitable Activated Partial Thromboplastin Time (APTT) reagent containing phospholipid and contact activator and incubate the mixture for a recommended time at 37°. To each tube, add 0.1 ml of a 0.37 per cent w/v solution of *calcium chloride* previously heated to 37°. Using a timer, measure the coagulation time, i.e. the interval between the moment of the addition of the calcium chloride and the first indication of the formation of fibrin. The volumes given above may be adapted to the APTT reagent and apparatus used. Calculate the potency of the test preparation as described in the "Statistical Analysis of Results of Biological Assays and Tests (Appendix 9).

15.1.5 Biological Assay of Human Coagulation Factor X

Human coagulation factor X is assayed following specific activation to form factor Xa. Factor Xa is estimated by comparing its activity in cleaving a specific chromogenic peptide substrate with the same activity of the International Standard or of a reference preparation calibrated in International Units.

The chromogenic assay method consists of two steps: snake venom-dependent activation of factor X, followed by enzymatic cleavage of a chromogenic factor Xa substrate to form a chromophore that can be quanti-

fied spectrophotometrically. Under appropriate assay conditions, there is a linear relation between factor Xa activity and the cleavage of the chromogenic substrate.

REAGENTS

Russell's viper venom specific factor X activator (RVV) A protein derived from the venom of Russell's viper (*Vipera russelli*) which specifically activates factor X. Reconstitute according to the manufacturer's instructions. Store the reconstituted preparation at 4° and use within 1 month.

Factor Xa chromogenic substrate Specific chromogenic substrate for factor Xa such as: *N*-α benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-4-nitroanilide dihydrochloride, *N*-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-4-nitroanilide hydrochloride, methanesulfonyl-D-leucyl-glycyl-L-arginine-4-nitroanilide, methoxycarbonyl-D-cyclohexylalanyl-glycyl-L-arginine-4-nitroanilide acetate. Reconstitute according to the manufacturer's instructions.

Dilution buffer Solution containing 0.37 per cent w/v of *tris(hydroxymethyl)methylamine*, 1.8 per cent w/v of *sodium chloride*, 0.21 per cent w/v of *imidazole*, 0.002 per cent w/v of *hexadimethrine bromide* and 0.1 per cent w/v of *bovine serum albumin* or *human albumin*. Adjust to pH 8.4 if necessary, using *hydrochloric acid*.

METHOD

Test solution Dilute the preparation being examined with dilution buffer to obtain a solution containing 0.18 IU of factor X per ml. Prepare at least three further dilutions in dilution buffer.

Reference solution Dilute the reference preparation to be examined with dilution buffer to obtain a solution containing 0.18 IU of factor X per ml. Prepare at least three further dilutions in dilution buffer.

Warm all solutions to 37° in a water-bath shortly before the test.

The following working conditions apply to microtitre plates. If the assay is carried out in tubes, the volumes are adjusted while maintaining the proportions in the mixture.

Using a microtitre plate maintained at 37°, add 12.5 µl of each dilution of the test solution or the reference solution to each of a series of wells. To each well add 25 µl of RVV and incubate for exactly 90 seconds. To each well add 150 µl of factor X chromogenic substrate, diluted 1 in 6 in dilution buffer.

Read the rate of change of absorbance at 405 nm (Appendix 2.2) continuously over a period of 3 minutes and obtain the mean rate of change of absorbance (ΔA/minute). If continuous monitoring is not possible, read the absorbance at 405 nm at suitable consecutive intervals, for instance 40 seconds, plot the absorbances against time on a linear graph and calculate ΔA/minute as the slope of the line. From the ΔA/minute values of each individual dilution of standard and test preparations, calculate the potency of the preparation being examined and check the validity of the assay by the "Statistical Analysis of Results of Biological Assay and Tests" (Appendix 9).

15.1.6 Biological Assay of Anti-Rh_0 (D) Immunoglobulin

Method A

The potency of anti-Rh_0 (D) immunoglobulin is determined by comparing the quantity necessary to produce agglutination of Rh_0 (D)-positive red blood cells with the quantity of a reference preparation, calibrated in International Units, required to produce the same effect.

Use pooled Rh_0 (D)-positive red blood cells, collected not more than 7 days earlier and suitably stored, obtained from not less than four group O R_1R_1 donors. To a suitable volume of the cells, previously washed three times with *saline TS*, add an equal volume of *bromelains TS*, allow to stand at 37° for 10 minutes, centrifuge, remove the supernatant liquid and wash three times with *saline TS*. Suspend 20 volumes of the red blood cells in a mixture of 15 volumes of inert serum, 20 volumes of a 30 per cent w/v solution of *bovine serum albumin* and 45 volumes of *saline TS*. Stand the resulting suspension in iced water, stirring continuously.

Using a calibrated automated dilutor, prepare suitable dilutions of the preparation being examined and of the reference preparation using as diluent a solution containing 0.5 per cent w/v of *bovine serum albumin* in *saline TS*.

Use a suitable apparatus for automatic continuous analysis. The following protocol is usually suitable: maintain the temperature in the manifold, except for the incubation coils, at 15.0°. Pump into the manifold of the apparatus the red blood cell suspension at a rate of 0.1 ml per minute and a 0.3 per cent w/v solution of *methylcellulose 450* at a rate of 0.05 ml per minute. Introduce the dilutions of the preparation being examined and the reference preparation at a rate of 0.1 ml per minute for 2 minutes, followed by the diluent solution at a rate of 0.1 ml per minute for 4 minutes before the next dilution is introduced.

Introduce air at a rate of 0.6 ml per minute. Incubate at 37° for 18 minutes and then disperse the rouleaux[1] by introducing at a rate of 1.6 ml per minute *saline TS* containing a suitable wetting agent (for example, *polysorbate 20* at a final concentration of 0.02 per cent w/v) to prevent disruption of the bubble pattern. Allow the agglutinates to settle and decant twice, first at 0.4 ml per minute and then at 0.6 ml per minute. Lyse the unagglutinated red blood cells with a solution containing 0.5 per cent w/v of *octoxinol 10*, 0.02 per cent w/v of *potassium hexacyanoferrate(III)*, 0.1 per cent w/v of *sodium hydrogencarbonate* and 0.005 per cent w/v of *potassium cyanide* at a rate of 2.5 ml per minute. A ten-minute delay coil is introduced to allow for conversion

[1]An abnormal group of red blood cells, adhering together like a roll of coins.

of the hemoglobin. Continuously record the absorbance (Appendix 2.2), of the hemolysate at a wavelength between 540 and 550 nm. Determine the range of antibody concentrations over which there is a linear relationship between concentration and the resultant change in absorbance (ΔA). From the results, prepare a standard curve and use the linear portion of the curve to determine the activity of the preparation being examined.

Calculate the potency of the preparation to be examined using the "Statistical Analysis of Results of Biological Assay and Test" (Appendix 9).

Method B

The potency of anti-Rh_0 (D) immunoglobulin is determined by competitive enzyme-linked immunoassay on erythrocyte-coated microtitre plates. The method is based on the competitive binding between a polyclonal anti-Rh_0 (D) immunoglobulin preparation and a biotinylated monoclonal anti-Rh_0 (D) antibody directed against a D-antigen specific epitope. The activity of the preparation to be examined is compared with a reference preparation calibrated in International Units.

MATERIALS

Reagents not specified are of analytical grade.

Phosphate-buffered saline (PBS) Dissolve 8.0 g of *sodium chloride*, 0.76 g of *anhydrous disodium hydrogenphosphate*, 0.2 g of *potassium chloride*, 0.2 g of *potassium dihydrogenphosphate* and 0.2 g of *sodium azide* in *water* and dilute to 1000 ml with the same solvent.

Tris-buffered saline (TBS) Dissolve 8.0 g of *sodium chloride* and 0.6 g of *tris(hydroxymethyl)methylamine* in *water*. Adjust to pH 7.2 (Appendix 4.11) with 1 M *hydrochloric acid* and dilute to 1000 ml with the same solvent.

Papain solution Prepare a solution by stirring 1 g of *papain* at 37° for 30 minutes in 10 ml of 0.067 M *phosphate buffer solution pH 5.4*, centrifuge at 10,000 × *g* for 5 minutes and filter through a membrane with a pore size of 0.22 µm. To activate, combine 1 ml of the filtrate with 1 ml of a 4.844 per cent w/v solution of L-*cysteine* and 1 ml of a 0.372 per cent w/v solution of *sodium edetate* and dilute to 10 ml with 0.067 M *phosphate buffer solution pH 5.4*. Freeze in aliquots at –20° or below.

Red blood cells Use pooled Rh_0 (D)-positive red blood cells obtained from not less than three group O R_2R_2 donors. Wash the cells four times with PBS. Centrifuge the cells at 1800 × *g* for 5 minutes, mix a suitable volume of prewarmed packed cells with a suitable volume of prewarmed papain solution (2 volumes to 1 volume has been found suitable) and incubate at 37° for 10 minutes. Wash the cells four times with PBS. Store at 4° in an appropriate stabilizer for up to 1 week.

Biotinylated Brad-5 Use according to instructions.

Alkaline phosphatase-conjugated avidin streptavidin reagent Preferably modified to combine high specific activity with low non-specific binding. Use according to instructions.

Substrate solution Use para-nitrophenyl phosphate according to instructions.

Cell fixation buffer Dissolve 18.02 g of *dextroxe*, 4.09 g of *sodium chloride*, 1.24 g of *boric acid*, 10.29 g of *sodium citrate* and 0.74 g of *sodium edetate* in *water*. Adjust to pH 7.2 to 7.3 (Appendix 4.11) using 1 M *sodium hydroxide* or 1 M *hydrochloric acid*, dilute to 1000 ml with *water*. Use directly from storage at 4°.

Glutaraldehyde solution Immediately before use, add 90 µl of a 25 per cent w/v solution of *glutaraldehyde* to 24 ml of cold PBS.

Microtitre plates Plates to be coated with red blood cells are flat-bottomed polystyrene plates with surface properties optimized for enzyme immunoassay and high protein-binding capacity. Plates used to prepare immunoglobulin dilutions are U or V-bottomed polystyrene or polyvinyl chloride plates.

METHOD

Prepare a 0.1 per cent v/v suspension of papain-treated red blood cells in cold cell fixation buffer. Pipette 50 µl into each well of the flat-bottomed microtitre plate.

Centrifuge the plate at 350 × *g* for 3 minutes, preferably at 4°. Without removing the supernatant, gently add 100 µl of *glutaraldehyde solution* to each well and leave for 10 minutes. Drain the wells by quickly inverting the plate and wash three times with 250 to 300 µl of PBS. This may be done manually or using a suitable automated plate washer. Either carry out the assay as described below, or store the plate at 4° after draining off the PBS and adding 100 µl of cell fixation buffer per well and sealing with plastic film. Plates can be stored at 4° for up to 1 month.

Test solutions For freeze-dried preparations, reconstitute as stated on the label. Prepare four independent replicates of five serial twofold dilutions starting with 30 IU per ml in PBS containing 1 per cent w/v of *bovine serum albumin*. If necessary, adjust the starting dilution to obtain responses falling in the linear portion of the dose-response curve.

Reference solutions Reconstitute the reference preparation according to instructions. Prepare four independent replicates of five serial twofold dilutions starting with 30 IU per ml in PBS containing 1 per cent w/v of *bovine serum albumin*.

Using U or V-bottomed microtitre plates, add 35 µl of each of the dilutions of the test solution or reference solution to each of a series of wells. To each well add 35 µl of *biotinylated Brad-5* at 250 ng per ml.

Empty the wells of the red cell-coated plate by inverting and draining on a paper towel. Add 250 µl of PBS containing 2 per cent w/v of *bovine serum albumin* and leave at room temperature for 30 minutes.

Empty the wells of the red cell-coated plate by inverting and draining on a paper towel and transfer 50 µl from each of the dilutions of the test solution or reference solution containing biotinylated Brad-5 into the wells. Use 50 µl of PBS containing 1 per cent w/v of *bovine serum albumin* as negative control. Seal the plate with plastic film and incubate at room temperature for 1 hour.

Remove liquid from the wells of the red cell-coated plate and wash three times with 250 to 300 µl of TBS.

Dilute the *alkaline phosphatase-conjugated avidin/streptavidin reagent* in TBS containing 1 per cent w/v solution of *bovine serum albumin* and add 50 µl to each well. Incubate for 30 minutes at room temperature. Remove liquid from the wells of the red cell-coated plate and wash three times with 250 to 300 µl of TBS.

Add 100 µl of substrate solution to each of the wells and incubate at room temperature for 10 minutes in the dark. To stop the reaction, add 50 µl of 3 M *sodium hydroxide* to each of the wells.

Measure the absorbances at 405 nm and substract the negative control reading. Use the absorbance values in the linear range of the titration curve to estimate the potency of the preparation to be examined by the "Statistical Analysis of Results of Biological Assays and Tests" (Appendix 9).

Method C

The potency of anti-Rh_0 (D) immunoglobulin is determined by flow cytometry in a microtitre plate format. The method is based on the specific binding between anti-Rh_0 (D) immunoglobulin and Rh_0 (D)-positive red blood cells. The activity of the preparation to be examined is compared with a reference preparation calibrated in International Units.

MATERIALS

Reagents not specified are of analytical grade.

PBS Dissolve 8.0 g of *sodium chloride*, 0.76 g of *disodium hydrogenphosphate*, 0.2 g of *potassium chloride* and 0.2 g of *potassium dihydrogenphosphate* in *water* and dilute to 1000 ml with the same solvent.

PBS-BSA solution PBS containing 1 per cent w/v of *bovine serum albumin*.

Red blood cells Use Rh_0 (D)-positive red blood cells obtained from a group OR_1R_1 donor within 2 weeks of collection. Store if necessary in an appropriate stabilizer at 4°. Wash the cells at least twice with *PBS-BSA solution* and prepare a suspension containing 1×10^4 cells per microlitre but not more than 5×10^4 cell per microlitre in *PBS-BSA solution*.

Use Rh_0 (D)-negative red blood cells obtained from a group Orr donor and prepared similarly.

Secondary antibody Use a suitable fluorescent dye conjugated anti-IgG antibody-fragment specific for human IgG or parts of it. Store and use according to the manufacturer's instructions.

Microtitre plates Use flat-bottomed plates without surface treatment for enzyme immunoassays.

METHOD

Test solutions For freeze-dried preparations, reconstitute as stated on the label. Prepare at least three independent replicates of at least three serial 1.5 or twofold dilutions starting with a concentration in the range of 1.2 to 0.15 IU per ml using *PBS-BSA solution* as diluent. If necessary, adjust the starting dilution to obtain responses falling in the liner portion of the dose-response curve.

Reference solutions Reconstitute the reference preparation according to instructions. Prepare at least three independent replicates of at least three serial 1.5 or twofold dilutions starting with a concentration in the range of 1.2 to 0.15 IU per ml using *PBS-BSA solution* as diluent. If necessary, adjust the starting dilutions to obtain responses falling in the linear portion of the dose-response curve.

Distribute 50 µl of the Rh_0 (D)-positive red blood cells into each well of a microtitre plate. Add 50 µl of each of the dilutions of the test solutions or reference solution to each of a series of wells. Use 50 µl of *PBS-BSA solution* as negative control. Distribute 50 µl of the Rh_0 (D)-negative red blood cells into four wells of the same microtitre plate and add 50 µl of the lowest dilution of the test preparation. To monitor spurious reactions distribute 50 µl of the Rh_0 (D)-positive red blood cells into four wells of the same microtitre plate and add 50 µl of *PBS-BSA solution*. Seal with plastic film and incubate at 37° for 40 minutes.

Centrifuge the plates at 50 × *g* for 3 minutes, discard the supernatant and wash the cells with 200 to 250 µl of *PBS-BSA solution*. Repeat this at least once.

Centrifuge the plates at 50 × *g* for 3 minutes, discard the supernatant and add 50 µl of the secondary antibody diluted with *PBS-BSA solution* to a suitable protein concentration. Seal with plastic film and incubate, protected from light, at room temperature for 20 minutes.

Centrifuge the plates at 50 × *g* for 3 minutes, discard the supernatant and wash the cells with 200 to 250 µl of *PBS-BSA solution*. Repeat this at least once.

Centrifuge the plates at 50 × *g* for 3 minutes, resuspend the cells into 200 to 250 µl of PBS. Transfer the cell suspension into a tube suitable for the flow cytometry equipment available and further dilute by adding PBS to allow a suitable flow rate.

Proceed immediately with measurement of the median fluorescence intensity in a flow cytometer. Record at least 10,000 events without gating but excluding debris.

Use the median fluorescence intensity in the linear range of the dose response curve to estimate the potency of the preparation to be examined by the "Statistical Analysis of Results of Biological Assays and Test" (Appendix 9).

15.1.7 Biological Assay of Human Coagulation Factor II

Human coagulation factor II is assayed following specific activation to form factor IIa. Factor IIa is estimated by comparing its activity in cleaving a specific chromogenic peptide substrate with the same activity of the International Standard or of a reference preparation calibrated in International Units.

The chromogenic assay method consists of two steps: snake venom-dependent activation of factor II, followed by enzymatic cleavage of a chromogenic factor IIa substrate to form a chromophore that can be quantified spectrophotometrically. Under appropriate assay conditions, there is a linear relation between factor IIa activity and the cleavage of the chromogenic substrate.

REAGENTS

Viper venom specific factor II activator (ECARIN) A protein derived from the venom of the saw-scaled viper (*Echis carinatus*) which specifically activates factor II. Reconstitute according to the manufacturer's instructions. Store the reconstituted preparation at 4° and use within 1 month.

Factor II a chromogenic substrate Specific chromogenic substrate for factor IIa such as: *H*-D phenylalanyl-L-pipecolyl-L-arginine-4-nitroanilide dihydrochloride, 4-toluenesulfonyl-glycyl-prolyl-L-arginine-4-nitroanilide, *H*-D-cyclohexylglycyl-α-aminobutyryl-L-arginine-4-nitroanilide, D-cyclohexylglycyl-L-alanyl-L-arginine-4-nitroanilide diacetate. Reconstitute according to the manufacturer's instructions.

Dilution buffer Solution containing 0.606 per cent w/v of *tris(hydroxymethyl)aminomethane*, 1.753 per cent w/v of *sodium chloride*, 0.279 per cent w/v of *(ethylenedinitrilo)tetra-acetic acid* and 0.1 per cent w/v of *bovine serum albumin* or *human albumin*. Adjust to pH 8.4 if necessary, using *hydrochloric acid*.

METHOD

Test solution Dilute the preparation being examined with dilution buffer to obtain a solution containing 0.015 IU of factor II per ml. Prepare at least three further dilutions in dilution buffer.

Reference solution Dilute the reference preparation to be examined with dilution buffer to obtain a solution containing 0.015 IU of factor II per ml. Prepare at least three further dilutions in dilution buffer.

Warm all solutions to 37° in a water-bath shortly before the test.

The following working conditions apply to microtitre plates. If the assay is carried out in tubes, the volumes are adjusted while maintaining the proportions in the mixture.

Using a microtitre plate maintained at 37°, add 25 µl of each dilution of the test solution or the reference solution to each of a series of wells. To each well add 125 µl of dilution buffer, then 25 µl of ecarin and incubate for exactly 2 minutes. To each well add 25 µl of factor IIa chromogenic substrate.

Read the rate of change of absorbance at 405 nm (Appendix 2.2) continuously over a period of 3 minutes and obtain the mean rate of change of absorbance (ΔA/minute). If continuous monitoring is not possible, read the absorbance at 405 nm at suitable consecutive intervals, for instance 40 seconds, plot the absorbances against time on a linear graph and calculate ΔA/minute as the slope of the line. From the ΔA/minute values of each individual dilution of standard and test preparations, calculate the potency of the preparation being examined and check the validity of the assay by the "Statistical Analysis of Results of Biological Assay and Tests" (Appendix 9).

15.1.8 Biological Assay of Human Coagulation Factor VII

Human coagulation factor VII is assayed by its biological activity as a factor VIIa-tissue factor complex in the activation of factor X in the presence of calcium ions and phospholipids. The potency of a factor VII preparation is estimated by comparing the quantity necessary to achieve a certain rate of factor Xa formation in a test mixture containing the substances that take part in the activation of factor X, and the quantity of the International Standard, or of a reference preparation calibrated in International Units, required to produce the same rate of factor Xa formation.

The chromogenic assay method consists of two consecutive steps: the factor VII-dependent activation of factor X reagent mixture containing tissue factor, phospholipids and calcium ion, followed by enzymatic cleavage of a chromogenic factor Xa substrate into a chromophore that can be quantified spectrophotometrically. Under appropriate assay conditions, there is a linear relation between the rate of factor Xa formation and the factor VII concentration. The assay is summarized in Fig. 1.

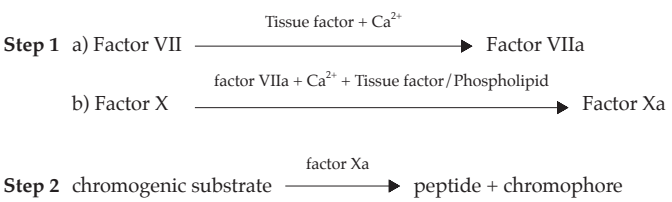

Fig. 1 Schematic Representation of the Assay of Human Coagulation Factor VII

Both steps employ reagents that may be obtained commercially from a variety of sources. Although the composition of individual reagents may be subject to some variation, their essential features are described in the following specification.

REAGENTS

The coagulation factor reagent comprises purified proteins derived from human or bovine sources. These include factor X and thromboplastin tissue factor/phospholipids as factor VII activator. These proteins are partly purified and do not contain impurities that interfere with the activation of factor VII or factor X. Factor X is present in amounts giving a final concentration during the first step of the assay of 10 to 350 nmol per litre, preferably 14 to 70 nmol per litre. Thromboplastin from natural sources (bovine or rabbit brain) or synthetic preparations may be used as the tissue factor/phospholipids component. Thromboplastin suitable for use in prothrombin time determination is diluted 1:5 to 1:50 in buffer such that the final concentration of Ca^{2+} is 15 to 25 nmol per litre. The final factor Xa generation is performed in a solution containing human or bovine albumin at a concentration such that adsorption losses do not occur and which is appropriately buffered at pH 7.3 to 8.0. In the final incubation mixture, factor VII must be the only rate-limiting component and each reagent component must lack the ability to generate factor Xa on its own.

The second step comprises the quantification of the formed factor Xa employing a chromogenic substrate that is specific for factor Xa. Generally this consists of a short peptide of between three and five amino acids, bound to a chromophore group. On cleavage of this group from the peptide substrate, its absorption maximum shifts to a wavelength allowing its spectrophotometric quantification. The substrate is usually dissolved in *water* and used at a final concentration of 0.2 to 2 nmol per litre. The substrate may also contain appropriate inhibitors to stop further factor Xa generation (addition of edetate).

ASSAY

Reconstitute the entire contents of one ampoule of the reference preparation and the preparation to be examined by adding the appropriate quantity of *water*; use within 1 hour. Add sufficient prediluent to the reconstituted preparations to produce solutions containing 0.5 to 2.0 IU of factor VII per ml.

(**Note** Prepare all dilutions in plastic tubes and use within 1 hour.)

Prepare further dilutions of reference and test preparations using an isotonic non-chelating buffer containing 1 per cent of *bovine serum albumin* or *human albumin*, buffered preferably between pH 7.3 and 8.0. Prepare at least three separate, independent dilutions for each material, preferably in duplicates. Prepare the dilutions such that the final factor VII concentration is below 0.005 IU per ml.

Prepare a control solution that includes all components except factor VII.

Step 1 Mix dilutions of the factor VII reference preparation and the preparation to be examined with an appropriate volume of the prewarmed coagulation factor reagent or a combination of its separate constituents, and incubate the mixture in plastic tubes or microplate wells at 37°. The concentrations of the various components during the factor Xa generation must be as specified above under the description of the reagents.

Allow the activation of factor X to proceed for a suitable time, usually terminating the reaction before the factor Xa concentration has reached its maximal level in order to obtain a satisfactory linear dose-response relationship. The activation time is also chosen to achieve linear production of factor Xa in time. Appropriate activation times are usually between 2 and 5 minutes, but deviations are permissible if acceptable linearity of the dose-response relationship is thus obtained.

Step 2 Terminate the activation by the addition of a prewarmed reagent containing a chromogenic substrate. Quantify the rate of substrate cleavage, which must be linear with the concentration of factor Xa formed, by measuring the absorbance change at an appropriate wavelength using a spectrophotometer, either monitoring the absorbance continuously, thus allowing the initial rate of substrate cleavage to be calculated, or terminating the hydrolysis reaction after a suitable interval by lowering the pH by addition of a suitable reagent, such as 50 per cent v/v solution of *acetic acid* or a 1 M citrate buffer solution pH 3. Adjust the hydrolysis time to achieve a linear development of chromophore with time. Appropriate hydrolysis times are usually between 3 and 15 minutes, but deviations are permissible if better linearity of the dose-response relationship is thus obtained.

Check the validity of the assay and calculate the potency of the test preparation as described in the "Statistical Analysis of Results of Biological Assays and Tests" (Appendix 9).

15.1.9 Biological Assay of Human von Willebrand Factor

The biological functions of human von Willebrand factor are numerous. At present, its ristocetin cofactor activity and its collagen binding activity can be utilized for assays. The potency of human von Willebrand factor is determined by comparing, in given conditions, its activity with the same activity of a reference preparation calibrated against the International Standard, in International Units where applicable.

RISTOCETIN COFACTOR ASSAY

The ristocetin cofactor activity of von Willebrand factor is determined by measuring agglutination of a suspension of platelets in the presence of ristocetin A.

The assay can be carried out for quantitative determinations by using automated instruments, or for semi-quantitative determinations by visually assessing the end-point of agglutination in a dilution series. Quantitative assays are preferred.

Reagents

SUSPENSION OF PLATELETS Use standardized and, for example, formaldehyde- or paraformaldehyde-fixed preparations of freshly isolated and washed human platelets. The suspension may also be freeze-dried. An appropriate amount of ristocetin A is added if necessary. Some platelet reagents may already contain ristocetin A.

REFERENCE PREPARATION The reference preparation for von Willebrand factor is the WHO International Standard for von Willebrand factor concentrate.

Method

SEMI-QUANTITATIVE ASSAY Prepare suitable dilutions of the preparation to be examined and of the reference preparation, using as diluent a solution containing 1 to 5 per cent w/v of *human albumin* in *saline TS*. Add to each dilution an appropriate amount of the suspension of platelets and, if necessary, of ristocetin A. Mix on a glass slide by moving it gently in circles for 1 minute. Allow to stand for a further 1 minute and read the result against a dark background with side lighting. The last dilution which clearly shows visible agglutination indicates the ristocetin cofactor titre of the sample. Use diluent as a negative control.

QUANTITATIVE ASSAY Reconstitute the entire contents of one ampoule of the reference preparation and the preparation to be examined by adding the appropriate quantity of the recommended diluent (for example *water*); use immediately. Add sufficient prediluent to the reconstituted preparations to produce solutions containing 0.5 to 2.0 IU per ml. The prediluent consists of an isotonic non-chelating buffer, for example, 1 to 5 per cent of *bovine serum albumin* or *human albumin*, and *tris(hydroxymethyl)methylamine* or *imidazole*, appropriately buffered.

The test is performed in accordance with the manufacturer's instructions with at least two dilution series with as many dilutions as are needed to obtain a total of at least three different concentrations in the linear of the assay.

Check the validity of the assay and calculate the potency of the test preparation as described in the "Statistical Analysis of Results of Biological Assay and Tests" (Appendix 9).

COLLAGEN-BINDING ASSAY

Collagen-binding is determined by an enzyme-linked immunosorbent assay on collagen-coated microtitre plates. The method is based on the specific binding of von Willebrand factor to collagen fibrils and the subsequent binding of polyclonal anti-von Willebrand factor antibody conjugated to an enzyme, which on addition of a chromogenic substrate yields a product that can be quantitated spectrophotometrically. Under appropriate conditions, there is a linear relationship between von Willebrand factor collagen-binding and absorbance.

Reagents

COLLAGEN Use native equine or human fibrils of collagen type I or III. For ease of handling, collagen solutions may be used.

COLLAGEN DILUENT Dissolve 50 g of *dextrose* in *water*, adjust to pH 2.7 to 2.9 with 1 M *hydrochloric acid* and dilute to 1000 ml with *water*.

PHOSPHATE-BUFFER SALINE (PBS) Dissolve 8.0 g of *sodium chloride*, 1.05 g of *disodium hydrogenphosphate dihydrate*, 0.2 g of *sodium dihydrogenphosphate* and 0.2 g of *potassium chloride* in *water*. Adjust to pH 7.2 using 1 M *sodium hydroxide* or 1 M *hydrochloric acid* and dilute to 1000 ml with *water*.

WASHING BUFFER PBS containing 0.1 per cent w/v solution of *polysorbate 20*.

BLOCKING REAGENT PBS containing 0.1 per cent w/v of *polysorbate 20* and 1 per cent w/v of *bovine serum albumin*.

DILUTION BUFFER PBS containing 0.1 per cent w/v of *polysorbate 20* and 5 per cent w/v of *bovine serum albumin*.

CONJUGATE Rabbit anti-human von Willebrand factor serum horseradish peroxidase conjugate. Use according to the manufacturer's instructions.

SUBSTRATE SOLUTION Immediately before use, dissolve a tablet of o-phenylenediamine dihydrochloride and a tablet of urea hydrogen peroxide in 20 ml of *water* or use a suitable volume of *hydrogen peroxide TS* (e.g., 1 drop of *hydrogen peroxide (10 volumes) TS*). Protect from light.

MICROTITRE PLATES Flat-bottomed polystyrene plates with surface properties optimized for enzyme immunoassay and high protein-binding capacity.

Method

TEST SOLUTIONS Reconstitute the preparation to be examined as stated on the label. Dilute with dilution buffer to produce a solution containing approximately 1 IU of von Willebrand factor. Prepare two series of at least three further dilutions using dilution buffer.

REFERENCE SOLUTIONS Reconstitute the reference preparation as directed. Dilute with dilution buffer to produce a solution containing approximately 1 IU of von Willebrand factor. Prepare two series of at least three further dilutions using dilution buffer.

Allow the solution of collagen to warm to room temperature. Dilute with collagen diluent to obtain a solution containing 30 to 75 µg per ml of collagen, and mix gently to produce a uniform suspension of collagen fibrils. Pipette 100 µl into each well of the microtitre plate. Cover the plate with plastic film and incubate at

37° overnight. Empty the wells of the collagen-coated plate by inverting and draining on a paper towel. Add 250 µl of washing buffer. Empty the wells of the plate by inverting and draining on paper towel. Repeat this operation three times. Add 250 µl of blocking reagent to each well, cover the plate with plastic film and incubate at 37° for 1 hour. Empty the wells of the plate by inverting and draining on a paper towel. Add 250 µl of washing buffer. Empty the wells of the plate by inverting and draining on a paper towel. Repeat this operation three times.

Add 100 µl each of the test solutions or reference solutions to the wells of the plate. Add 100 µl of dilution buffer to a series of wells to serve as negative control. Cover the plate with plastic film and incubate at 37° for 2 hours. Empty the wells of the plate by inverting and draining on a paper towel. Add 250 µl of washing buffer. Empty the wells of the plate by inverting and draining on a paper towel. Repeat this operation three times.

Prepare a suitable dilution of the conjugate (for example, a dilution factor of 1 to 4000) with PBS containing 0.5 per cent of *bovine serum albumin* and add 100 µl to each well. Cover the plate with plastic film and incubate at 37° for 2 hours. Empty the wells of the plate by inverting and draining on a paper towel. Add 250 µl of washing buffer. Empty the wells of the plate by inverting and draining on a paper towel. Repeat this operation three times.

Add 100 µl of substrate solution to each of the wells and incubate at room temperature for 20 minutes in the dark. Add 100 µl of 1 M *hydrochloric acid* to each of the wells.

Measure the absorbance at the maximum at 492 nm (Appendix 2.2). Use absorbance values to estimate the potency of the preparation to be examined using the "Statistical Analysis of Results of Biological Assay and Tests" (Appendix 9).

The assay is invalid if the absorbances measured for the negative controls are greater than 0.05.

15.1.10 Test for Anti-D Antibodies in Human Immunoglobulin for Intravenous Administration

REAGENTS

Phosphate-buffered saline (PBS) Dissolve 8.0 g of *sodium chloride*, 0.76 g of *anhydrous disodium hydrogenphosphate*, 0.2 g of *potassium chloride*, and 0.2 g of *potassium dihydrogenphosphate* in *water* and dilute to 1000 ml with the same solvent. If the solution has to be kept for several days, 0.2 g of *sodium azide* may be added in order to avoid microbial contamination.

Papain solution Use serological-grade papain from a commercial source, the activity of which has been validated.

Red blood cells Use pooled D-positive red blood cells from not less than three donors, preferably of group OR_2R_2. D-positive red blood cells may also be obtained from OR_1R_1 or OR_1R_2 donors. Mixing phenotypes has not been tested and is therefore not recommended.

Use pooled D-negative red blood cells, preferably from three donors of group Orr. When only one donor of group Orr is available, D-negative red blood cells from only one donor may be used.

Wash the cells four times with PBS or until the supernatant is clear. Centrifuge the cells at 1800 × *g* for 5 minutes to pack. Treat the packed cells with papain solution according to the manufacturer's instructions.

Store red blood cells for not more than one week in a preservative solution. A preparation of the following composition is appropriate:

Trisodium citrate	8	g
Dextrose	20	g
Citric acid	0.5	g
Sodium chloride	4.2	g
Inosine	0.938	g
Adenosine triphosphate (ATP)	0.4	g
Chloramphenicol	0.34	g
Neomycin sulfate	0.1	g
Water to	1000	ml

Microtitre plates Use V-bottomed rigid microtitre plates.

Reference substances Immunoglobulin (anti-D antibodies test) RS and Immunoglobulin (anti-D antibodies test negative control) RS are suitable for use as the reference preparation and negative control, respectively.

METHOD

The test described in this Appendix is performed at room temperature on the reference solutions, the negative control solutions and the test solutions at the same time and under identical conditions.

Reference solutions and negative control solutions Reconstitute the reference preparation and the negative control according to instructions. The immunoglobulin G (lgG) concentration is 5 per cent w/v solution in each of the reconstituted preparations. Make a twofold dilution of each reconstituted preparation with PBS containing 0.2 per cent w/v solution of *bovine serum albumin* to give solutions containing 2.5 per cent w/v solution of IgG. Prepare seven further serial twofold dilutions of each preparation using PBS containing 0.2 per cent w/v solution of *bovine serum albumin* to extend the dilution range to 1/256 (0.0195 per cent w/v solution of IgG). Add 20 µl of each dilution to the microtitre plate.

Test solutions Dilute the preparation to be examined with PBS containing 0.2 per cent w/v solution of *bovine serum albumin* to give a starting IgG concentration of 2.5 per cent w/v solution. For a 5 per cent w/v solution of products, this is a twofold dilution; adjust the dilution factor accordingly for samples that are not 5 per cent w/v solution to give a starting concentration of 2.5 per cent w/v solution for testing. This 2.5 per cent w/v solution is assigned a nominal twofold dilu-

tion factor for comparison with the reference preparations, even if this does not reflect the true dilution factor used to achieve 2.5 per cent w/v solution. Prepare seven further serial twofold dilutions of each preparation using PBS containing 0.2 per cent w/v solution of *bovine serum albumin* to extend the nominal dilution range to 1/256 (0.0195 per cent w/v solution of IgG) for comparison with the reference preparations over the same IgG concentration range. Make two independent sets of dilutions. Add 20 µl of each dilution to the microtitre plate.

Prepare 3 per cent v/v suspensions of papain-treated D-positive (preferably OR_2R_2, but OR_1R_1 or OR_1R_2 may also be used) and D-negative (Orr) red blood cells in PBS containing 0.2 per cent w/v solution of *bovine serum albumin*. Add 20 µl of D-positive cells to one dilution series of each of the preparation to be examined, the reference preparation and the negative control, and 20 µl of D-negative cells to the other dilution series of each of the preparation to be examined, the reference preparation and the negative control. Mix by shaking the plate on a shaker for 10 seconds.

Centrifuge the plate at 80 × *g* for 1 minute to pack the cells. Place the plate at an angle of approximately 70°. Read after at least 3 minutes and once the cells have streamed in the wells containing the negative control and the wells where the D-negative cells have been added. A cell button at the bottom of the well indicates a positive result. A stream of cells represents a negative result.

Record the end-point titre as the reciprocal of the highest dilution that gives rise to a positive result.

The negative control must have a titre not more than two, otherwise an investigation of the test reagents and conditions has to be performed.

The titre of the preparation to be examined is not more than the titre of the reference preparation when all preparations are titrated from a 2.5 per cent w/v solution.

15.1.11 Test for Anticomplementary Activity of Immunoglobulin

For the measurement of anticomplementary activity (ACA) of immunoglobulin, a defined amount of test material (10 mg of immunoglobulin) is incubated with a defined amount of guinea-pig complement (20 CH_{50}) and the remaining complement is titrated; the anticomplementary activity is expressed as the percentage consumption of complement relative to the complement control considered as 100 per cent.

The hemolytic unit of complement activity (CH_{50}) is the amount of complement that, in the given reaction conditions, will produce the lysis of 2.5×10^8 out of a total of 5×10^8 optimally sensitized red blood cells.

REAGENTS

Magnesium and calcium stock solution Dissolve 1.103 g of *calcium chloride* and 5.083 g of *magnesium chloride* in *water* and dilute to 25 ml with the same solvent.

Barbital buffer stock solution Dissolve 207.5 g of *sodium chloride* and 25.48 g of *barbital sodium* in 4000 ml of *water* and adjust to pH 7.3 using 1 M *hydrochloric acid*. Add 12.5 ml of *magnesium and calcium stock solution* and dilute to 5000 ml with *water*. Filter through a membrane filter (pore size 0.22 µm). Store at 4° in glass containers.

Gelatin solution Dissolve 12.5 g of *gelatin* in about 800 ml of *water* and heat to boiling in a water-bath. Cool to 20° and dilute to 10 litres with *water*. Filter through a membrane filter (pore size 0.22 µm). Store at 4°. Use clear solutions only.

Citrate solution Dissolve 8.0 g of *sodium citrate*, 4.2 g of *sodium chloride* and 20.5 g of *dextrose* in 750 ml of *water*. Adjust to pH 6.1 using a 10 per cent w/v solution of *citric acid* and dilute to 1000 ml with *water*.

Gelatin barbital buffer solution Add 4 volumes of *gelatin solution* to 1 volume of *barbital buffer stock solution* and mix. Adjust to pH 7.3, if necessary, using 1 M *sodium hydroxide* or 1 M *hydrochloric acid*. Maintain at 4°. Prepare fresh solutions daily.

Stabilized sheep blood Collect one volume of sheep blood into one volume of *citrate solution* and mix. Store at 4° for not less than 7 days and not more than 28 days. (Stabilized sheep blood or sheep red blood cells are available from a number of commercial sources.)

Hemolysin Antiserum against sheep red blood cells prepared in rabbits. (Such antisera are available from a number of commercial sources.)

Guinea-pig complement Prepare a pool of serum from the blood of not less than 10 guinea-pigs. Separate the serum from the clotted blood by centrifugation at about 4°. Store the serum in small amounts below –70°.

METHOD

Preparation of a standardized 5 per cent v/v suspension of sheep red blood cell Separate sheep red blood cells by centrifuging an appropriate volume of stabilized sheep blood and wash the cells at least three times with *gelatin barbital buffer solution* and prepare as a 5 per cent v/v suspension in the same solution. Measure the cell density of the suspension as follows. Add 0.2 ml to 2.8 ml of *water* and centrifuge the lysed solution for 5 minutes at 1000 × *g*; the cell density is suitable if the absorbance (Appendix 2.2) of the supernatant liquid at 541 nm is 0.62±0.01. Correct the cell density by adding *gelatin barbital buffer solution* according to the expression:

$$V_f = \frac{V_i \times A}{0.62},$$

where V_f = final adjusted volume,
V_i = the initial volume, and
A = absorbance of the original suspension at 541 nm.

The adjusted suspension contains about 1×10^9 cells per ml.

HEMOLYSIN TITRATION

Prepare hemolysin dilutions as shown in Table 1.

Table 1

Required Dilution of Hemolysin	Prepared Hemolysin Dilutions		
	Gelatin Barbital Buffer Solution		Hemolysin
	Volume (ml)	Dilution (1/...)	Volume (ml)
7.5	0.65	undiluted	0.1
10	0.90	undiluted	0.1
75	1.80	7.5	0.2
100	1.80	10	0.2
150	1.00	75	1.0
200	1.00	100	1.0
300	1.00	150	1.0
400	1.00	200	1.0
600	1.00	300	1.0
800	1.00	400	1.0
1200	1.00	600	1.0
1600	1.00	800	1.0
2400	1.00	1200	1.0
3200*	1.00	1600	1.0
4800*	1.00	2400	1.0

*discard 1.0 ml of the mixture.

Add 1.0 ml of a 5 per cent v/v suspension of *sheep red blood cell* to each tube of the hemolysin dilution series, starting at the 1:75 dilution, and mix. Incubate at 37° for 30 minutes.

Transfer 0.2 ml of each of these incubated mixtures to new tubes and add 1.10 ml of *gelatin barbital buffer solution* and 0.2 ml of diluted *guinea-pig complement* (for example, 1:150). Perform this in duplicate.

As the unhemolyzed cell control, prepare three tubes with 1.4 ml of *gelatin barbital buffer solution* and 0.1 ml of a 5 per cent v/v suspension of *sheep red blood cell*.

As the fully hemolyzed control, prepare three tubes with 1.4 ml of *water* and 0.1 ml of a 5 per cent v/v suspension of *sheep red blood cell*.

Incubate all tubes at 37° for 60 minutes and centrifuge at 1000 × *g* for 5 minutes. Measure the absorbance (Appendix 2.2) of the supernatants at 541 nm and calculate the percentage degree of hemolysis in each tube using the formula:

$$\frac{A_a - A_1}{A_b - A_1} \times 100 ,$$

where A_a = absorbance of tubes with hemolysin dilution,

A_b = mean absorbance of the three tubes with full hemolysis, and

A_1 = mean absorbance of the three tubes with no hemolysis.

Plot the percentage degree of hemolysis (*Y*) as the ordinate against the corresponding reciprocal value of the hemolysin dilution as the abscissa on linear graph paper. Determine the optimal dilution of the hemolysin from the graph by inspection. Select a dilution such that further increase in the amount of hemolysin does not cause appreciable change in the degree of hemolysis. This dilution is defined as one minimal hemolytic unit (1 MHU) in 1.0 ml. The optimal hemolytic hemolysin dilution for preparation of sensitized sheep red blood cells contains 2 MHU per ml.

The hemolysin titration is not valid unless the maximum degree of hemolysis is 50 to 70 per cent. If the maximum degree of hemolysis is not in this range, repeat the titration with more or less diluted complement solution.

PREPARATION OF OPTIMIZED SENSITIZED SHEEP RED BLOOD CELLS (HEMOLYTIC SYSTEM)

Prepare an appropriate volume of diluted hemolysin containing 2 MHU per ml and an equal volume of the standardized 5 per cent v/v suspension of sheep red blood cell. Add the hemolysin dilution to the standardized cell suspension and mix. Incubate at 37° for 15 minutes, store at 2° to 8° and use within 6 hours.

TITRATION OF COMPLEMENT

Prepare an appropriate dilution of complement (for example, 1:250) with *gelatin barbital buffer solution* and perform the titration in duplicate as shown in Table 2.

Table 2

Tube Number	Volume of Diluted Complement (for Example 1/250) (ml)	Volume of Gelatin Barbital Buffer Solution (ml)
1	0.1	1.2
2	0.2	1.1
3	0.3	1.0
4	0.4	0.9
5	0.5	0.8
6	0.6	0.7
7	0.7	0.6
8	0.8	0.5
9	0.9	0.4
10	1.0	0.3
11	1.1	0.2
12	1.2	0.1
3 tubes as cell control at 0 per cent hemolysis	–	1.3
3 tubes at 100 per cent hemolysis	–	1.3 ml of water

Add 0.2 ml of sensitized sheep red blood cells to each tube, mix well and incubate at 37° for 60 minutes. Cool the tubes in an ice bath and centrifuge at 1000 × *g* for 5 minutes. Measure the absorbance of the supernatant liquid at 541 nm and calculate the degree of hemolysis (Y) using the formula:

$$\frac{A_c - A_1}{A_b - A_1},$$

where A_c = absorbance of tubes 1 to 12,
A_b = mean absorbance of tubes with 100 per cent hemolysis, and
A_1 = mean absorbance of cell controls with 0 per cent hemolysis.

Plot $Y/(1–Y)$ as the abscissa against the amount of diluted complement in ml as the ordinate on log–log graph paper. Fit the best line to the points and determine the ordinate for the 50 per cent hemolytic complement dose where $Y/(1–Y) = 1.0$. Calculate the activity in hemolytic units (CH_{50} per ml) from the formula:

$$\frac{C_d}{C_a \times 5},$$

where C_d = reciprocal value of the complement dilution,
C_a = volume of complement in ml resulting in 50 per cent hemolysis, and
the scaling factor to take account of the number of red blood cells is 5.

The test is not valid unless the plot is a straight line between 15 per cent and 85 per cent hemolysis and the slope is 0.15 to 0.40, and preferably 0.18 to 0.30.

TEST FOR ANTICOMPLEMENTARY ACTIVITY

Prepare a complement dilution having 100 CH_{50} per ml by diluting titrated *guinea-pig complement* with *gelatin barbital buffer solution*. If necessary, adjust the immunoglobulin being examined to pH 7. Prepare incubation mixtures as shown in Table 3 for an immunoglobulin containing 50 mg per ml.

Table 3

	Immunoglobulin Being Examined	Complement Control (in duplicate)
Immunoglobulin (50 mg/ml)	0.2 ml	–
Gelatin barbital buffer	0.6 ml	0.8 ml
Complement	0.2 ml	0.2 ml

Carry out the test in parallel on the immunoglobulin being examined and prepare ACA negative and positive controls using Human Immunogobulin RS, as indicated in the leaflet accompanying the reference preparation. Higher of lower volumes of sample and of *gelatin barbital buffer solution* are added if the immunoglobulin concentration varies from 50 mg per ml; for example, 0.47 ml of *gelatin barbital buffer solution* is added to 0.33 ml of immunoglobulin containing 30 mg per ml to give 0.8 ml. Close the tubes and incubate at 37° for 60 minutes. Add 0.2 ml of each incubation mixture to 9.8 ml of *gelatin barbital buffer solution* to dilute the complement. Perform complement titrations as described above on each tube to determine the remaining complement activity (see Table 2). Calculate the anticomplementary activity of the preparation being examined relative to the complement control considered as 100 per cent from the formula:

$$\frac{a - b}{a} \times 100,$$

where a = mean complement activity (CH_{50} per ml) of complement control, and
b = complement activity (CH_{50} per ml) of tested sample.

The test is not valid unless the anticomplementary activities found for ACA negative control and ACA positive control are within the limits stated in the leaflet accompanying the reference preparation and the complement activity of the complement control (a) is in the range 80 to 120 CH_{50} per ml.

15.2 ANTISERA

15.2.7 Biological Assay of Cobra Antivenin

The potency of Cobra Antivenin is determined by estimating the dose necessary to protect mice against the lethal effect of a fixed dose of the cobra venom.

SUGGESTED METHOD

Preparation of cobra test venoms

Caution Because of the allergic properties of cobra venoms, suitable precautions should be taken to avoid inhalation of venom dust.

Use venoms that have the normal physico-chemical, toxicological and immunological characteristics of venoms from the particular species of cobra (*Naja kaouthia*). They are preferably freeze-dried and stored in the dark at 2° to 8°.

Selection of test venom Select a venom for use as a test venom by determining the following quantity.

LD_{50} The smallest quantity of venom that, when injected into mice, causes the death within 48 hours of one half of the mice injected.

Determination of the dose of test venom Prepare graded dilutions of the reconstituted venom in *saline TS* or other isotonic diluent in such a manner that the middle dilution contains in 0.25 ml the dose expected to be the LD_{50}. Dilute with an equal volume of the same diluent. Using at least four mice, each weighing 18 to 20 g, for each dilution inject 0.5 ml intravenously into each mouse. Observe the mice for 48 hours and record the number of deaths. Calculate the LD_{50} by standard statistical methods.

Determination of the potency of the antivenin Dilute the reconstituted test venom so that 0.25 ml contains the test dose of 5 LD_{50}. Prepare 1.5- to 2.5-fold serial dilutions of the antiserum being examined in

saline TS or other isotonic diluent. Use a sufficient number and range of dilutions to enable a mortality curve between 20 and 80 per cent mortality to be established and to permit an estimation of the statistical variation.

Prepare mixtures such that 5 ml of each mixture contains 2.5 ml of one of the dilutions of the antiserum being examined and 2.5 ml of the dilution of the test venom. Allow the mixtures to stand in a water-bath at 37° for 30 minutes. Using not less than six mice, each weighing 18 to 20 g, for each mixture inject 0.5 ml intravenously into each mouse. Observe the mice for 48 hours and record the number of deaths. Calculate the ED_{50} by standard statistical methods. At the same time verify the number of LD_{50} in the test dose of venom, using the method described above. The potency is expressed in terms of the number of LD_{50} or the quantity of test venom in mg that is neutralized by 1 ml or some other defined volumes of the antivenin.

15.3 VACCINES

15.3.1 Biological Assay of Adsorbed Diphtheria Vaccine

The potency of Adsorbed Diphtheria Vaccine is determined by comparing the dose of the vaccine required to protect guinea-pigs from the effects of either an erythrogenic dose of diphtheria toxin administered intradermally or a lethal dose of diphtheria toxin administered subcutaneously with the dose of a reference preparation, calibrated in International Units, needed to give the same protection.

The International Unit is the activity contained in a stated amount of the International Standard which consists of a quantity of diphtheria toxoid adsorbed on aluminium hydroxide.

The design of the assay described below follows a parallel-line model with three dilutions for the test and reference preparations. Once the analyst has sufficient experience with this method for a given vaccine, it is possible to apply a simplified model using a single dilution for both test and reference preparations. Such a model enables the analyst to determine whether the potency of the test preparation is significantly higher than the minimum required but does not give information on linearity, parallelism and the dose-response curve.

METHOD OF INTRADERMAL CHALLENGE

Selection and distribution of the test animals Use in the test, healthy, white guinea-pigs from the same stock and of a size suitable for the prescribed number of challenge sites, the difference in body weight between the heaviest and the lightest animal being not more than 100 g. Distribute the guinea-pigs in not less than six equal groups; use groups containing a number of animals sufficient to obtain results that fulfil the requirements for a valid assay prescribed below. If the challenge toxin to be used has not been shown to be stable or has not been adequately standardized, include five guinea-pigs as unvaccinated controls. Use guinea-pigs of the same sex or with males and females equally distributed between the groups.

Selection of the challenge toxin Select a preparation of diphtheria toxin containing 67 to 133 lr/100 in one Lf and 25,000 to 50,000 minimal reacting doses for guinea-pig skin in one Lf. If the challenge toxin preparation has been shown to be stable, it is not necessary to verify the activity for every assay.

Preparation of the challenge toxin solution Immediately before use, dilute the challenge toxin with a suitable diluent to obtain a challenge toxin solution containing about 0.0512 Lf in 0.2 ml. Prepare from this a further series of five fourfold dilutions containing about 0.0128, 0.0032, 0.0008, 0.0002, and 0.00005 Lf in 0.2 ml.

Determination of potency of the vaccine Using *saline TS*, prepare dilutions of the vaccine to be examined and of the reference preparation, such that for each, the dilutions from a series differing by not more than 2.5-fold steps and in which the intermediate dilutions, when injected subcutaneously at a dose of 1.0 ml per guinea-pig, will result in an intradermal score of approximately 3 when the animals are challenged. Allocate the dilutions one to each of the groups of guinea-pigs and inject subcutaneously 1.0 ml of each dilution into each guinea-pig in the group to which that dilution is allocated. After 28 days, shave both flanks of each guinea-pig and inject 0.2 ml of each of the six toxin dilutions intradermally into six separate sites on each of the vaccinated guinea-pigs in such a way as to minimize interference between adjacent sites.

Determination of the activity of the challenge toxin If necessary, inject the unvaccinated control animals with dilutions containing 80, 40, 20, 10, and 5 millionths of an Lf of the challenge toxin.

Reading and interpretation of results Examine all injection sites 48 hours after injection of the challenge toxin and record the incidence of specific diphtheria erythema. Record also the number of sites free from such reactions as the intradermal challenge score. Tabulate together the intradermal challenge scores for all the animals receiving the same dilution of vaccine and use those data with a suitable transformation, such as $(score)^2$ or arcsin $((score/6)^2)$, to obtain an estimate of the relative potency for each of the test preparations by parallel-line quantitative analysis.

Requirements for a valid assay The test is not valid unless:

– for both the vaccine to be examined and the reference preparation, the mean score obtained at the lowest dose level is less than three and the mean score at the highest dose level is more than three;

– if applicable, the toxin dilution that contains 40 millionths of an Lf gives a positive erythema in at least 80 per cent of the control guinea-pigs and the

dilution containing 20 millionths of an Lf gives a positive erythema in less than 80 per cent of the guinea-pigs (if these criteria are not met, a different toxin has to be selected);

– the confidence limits ($P = 0.95$) are not less than 50 per cent and not more than 200 per cent of the estimated potency;

– the statistical analysis shows no deviation from linearity and parallelism.

The test may be repeated but when more than one test is performed, the results of all valid tests must be combined in the estimate of potency.

METHOD OF LETHAL CHALLENGE

Selection and distribution of the test animals Use in the test healthy guinea-pigs from the same stock, each weighing 250 to 350 g. Distribute the guinea-pigs in not less than six equal groups; use groups containing a number of animals sufficient to obtain results that fulfil the requirements for a valid assay prescribed below. If the challenge toxin to be used has not been shown to be stable or has not been adequately standardized, include four further groups of five guinea-pigs as unvaccinated controls. Use guinea-pigs of the same sex or with males and females equally distributed between the groups.

Selection of the challenge toxin Select a preparation of diphtheria toxin containing not less than 100 LD_{50} per millilitre. If the challenge toxin preparation has been shown to be stable, it is not necessary to verify the lethal dose for every assay.

Preparation of the challenge toxin solution Immediately before use, dilute the challenge toxin with a suitable diluent to obtain a challenge toxin solution containing approximately 100 LD_{50} per millilitre. If necessary, dilute portions of the challenge toxin solution 1 in 32, 1 in 100 and 1 in 320 with the same diluent.

Determination of potency of the vaccine Using *saline TS*, prepare dilutions of the vaccine to be examined and of the reference preparation, such that for each, the dilutions form a series differing by not more than 2.5-fold steps and in which the intermediate dilutions, when injected subcutaneously at a dose of 1.0 ml per guinea-pig, protect approximately 50 per cent of the animals from the lethal effects of the subcutaneous injection of the quantity of diphtheria toxin prescribed for this test. Allocate the dilutions one to each of the groups of guinea-pigs and inject subcutaneously 1.0 ml of each dilution into each guinea-pig in the group to which that dilution is allocated. After 28 days, inject subcutaneously into each animal 1.0 ml of the challenge toxin solution (100 LD_{50}).

Determination of the activity of the challenge toxin If necessary, allocate the challenge toxin solution and the three dilutions made from it, one to each of the four groups of five guinea-pigs and inject subcutaneously 1.0 ml of each solution into each guinea-pig in the group to which that solution is allocated.

Reading and interpretation of results Count the number of surviving guinea-pigs 4 days after injection of the challenge toxin. Calculate the potency of the vaccine to be examined relative to the potency of the reference preparation on the basis of the proportion of animals surviving in each of the groups of vaccinated guinea-pigs, using the "Statistical Analysis of Results of Biological Assay and Tests" (Appendix 9).

Requirements for a valid assay The test is not valid unless:

– for the vaccine to be examined and the reference preparation the 50 per cent protective dose lies between the largest and smallest doses of the preparations given to the guinea-pigs;

– if applicable, the number of animals that die in the four groups of five injected with the challenge toxin solution and its dilutions indicates that the challenge dose was approximately 100 LD_{50};

– the confidence limits ($P = 0.95$) are not less than 50 per cent and not more than 200 per cent of the estimated potency;

– the statistical analysis shows no deviation from linearity and parallelism.

The test may be repeated but when more than one test is performed the results of all valid tests must be combined in the estimate of potency.

15.3.2 Biological Assay of Adsorbed Pertussis Vaccine

The potency of Adsorbed Pertussis Vaccine is determined by comparing the dose necessary to protect mice against the effects of a lethal dose of *Bordetella pertussis*, administered intracerebrally, with the quantity of a reference preparation, calibrated in International Units, needed to give the same protection.

The International Unit is the activity contained in a stated amount of the International Standard which consists of a quantity of dried pertussis vaccine.

Selection and distribution of the test animals Use in the test, healthy mice less than 5 weeks old of a suitable strain from the same stock, the difference in weight between the heaviest and the lightest being not more than 5 g. Distribute the mice in six groups of not less than 16 and four groups of 10. The mice must all be of the same sex or the males and females should be distributed equally between the groups.

Selection of the challenge strain and preparation of the challenge suspension Select a suitable strain of *B. pertussis* capable of causing the death of mice within 14 days of intracerebral injection. If more than 20 per cent of the mice die within 48 hours of the injection the strain is not suitable. Make one subculture from the strain and suspend the harvested *B. pertussis* in a solution containing 1 per cent w/v of *casein hydrolysate* and 0.6 per cent w/v of *sodium chloride* and having a pH of 7.0 to 7.2 or in another suitable solution. Determine the opacity of the suspension. Prepare a series of dilutions in the same solution and allocate each dilution to a group of 10 mice. Inject intracerebrally into each mouse

a dose (0.02 ml or 0.03 ml) of the dilution allocated to its group. After 14 days, count the number of mice surviving in each group. From the results, calculate the expected opacity of a suspension containing 100 LD_{50} in each challenge dose. For the test of the vaccine to be examined make a fresh subculture from the same strain of *B. pertussis* and prepare a suspension of the harvested organisms with an opacity corresponding to about 100 LD_{50} in each challenge dose. Prepare three dilutions of the challenge suspension.

Determination of potency of the vaccine Prepare three serial dilutions of the vaccine to be examined and three similar dilutions of the reference preparation such that in each the intermediate dilution may be expected to protect about 50 per cent of the mice from the lethal effects of the challenge dose of *B. pertussis*. Suggested doses are 1/8, 1/40 and 1/200 of the human dose of the vaccine to be examined and 0.5 IU, 0.1 IU and 0.02 IU of the reference preparation, each dose being contained in a volume not exceeding 0.5 ml. Allocate six dilutions one to each of the groups of not less than 16 mice and inject intraperitoneally into each mouse one dose of the dilution allocated to its group. After 14 to 17 days inject intracerebrally into each animal in the groups of not less than 16, one dose of the challenge suspension. Allocate the challenge suspension and the three dilutions made from it one to each of the groups of 10 mice and inject intracerebrally one dose of each suspension into each mouse in the group to which that suspension is allocated. Exclude from consideration any mice that die within 48 hours of challenge. Count the number of mice surviving in each of the groups after 14 days. Calculate the potency of the vaccine to be examined relative to the potency of the reference preparation on the basis of the numbers of animals surviving in each of the groups of not less than 16.

Requirements for a valid assay The test is not valid unless:

– for both the vaccine to be examined and the reference preparation, the 50 per cent protective dose lies between the largest and the smallest doses given to the mice;

– the number of animals which die in the four groups of 10 injected with the challenge suspension and its dilutions indicates that the challenge dose is approximately 100 LD_{50};

– the statistical analysis shows no deviation from linearity or parallelism.

The test may be repeated but when more than one test is performed, the results of all valid tests must be combined.

15.3.3 Biological Assay of Adsorbed Tetanus Vaccine

The potency of Adsorbed Tetanus Vaccine is determined by administration of the vaccine to animals (guinea-pigs or mice) followed either by challenge with tetanus toxin (method A or B) or by determination of the titre of antibodies against tetanus toxoid in the serum of the guinea-pigs (method C). In both cases, the potency of the vaccine is calculated by comparison with a reference vaccine, calibrated in International Units.

Adsorbed Tetanus Vaccine Reference Preparation is calibrated in International Units with reference to the National or International Standard.

SUGGESTED METHOD

The method chosen for assay of tetanus vaccine (adsorbed) depends on the intended purpose.

Method A or B may be used for routine assay of batches of vaccine but in the interests of animal welfare, method C is used wherever possible.

Method C may be used after verification of the suitability of the method for the product. For this purpose, a suitable number of batches (usually 3) are assayed by method C and method A or B. Where different vaccines (monovalent or combinations) are prepared from tetanus toxoid of the same origin, suitability demonstrated for the combination with the highest number of components can be assumed to be valid for combinations with less components and for monovalent vaccine.

For combinations with a whole-cell pertussis component, a separate demonstration of equivalence must be made for the highest combination.

The design of the assays described below uses multiple dilutions for the test and reference preparations. Based on the potency data obtained in multidilution assays, it may be possible to decrease the number of animals needed to obtain a statistically significant result by applying a simplified model using a single dilution of both test and reference preparations. Such a model enables the analyst to determine whether the potency of the test preparation is significantly higher than the minimum required but does not give information on the dose-response curves and their linearity, parallelism and significant slope. The simplified model may lead to a considerable reduction in the number of animals required and its use must be considered in accordance with the provisions of the National Authority.

Where a single-dilution assay is used, production and test consistency over time are monitored via suitable indicators and by carrying out a full multiple-dilution assay periodically, for example every 2 years. For serological assays, suitable indicators to monitor test consistency are:

– mean and standard deviation of relative antitoxin titres or scores of the serum samples obtained after administration of a fixed dose of the vaccine reference preparation,

– antitoxin titres or scores of run controls (positive and negative serum samples),

– ratio of antitoxin titres or scores for the positive serum control and the serum samples corresponding to the reference vaccine.

Method A. Challenge Test in Guinea-pigs

Selection and distribution of the test animals Use

in the test healthy guinea-pigs from the same stock, each weighing 250 to 350 g. Distribute the guinea-pigs in not less than six equal groups; use groups containing a number of animals sufficient to obtain results that fulfil the requirements for a valid assay prescribed below. If the activity of the challenge toxin has to be determined, include three further groups of five guinea-pigs as unvaccinated controls. Use guinea-pigs of the same sex or with the males and females equally distributed between the groups.

Selection of the challenge toxin Select a preparation of tetanus toxin containing not less than 50 times the 50 per cent paralytic dose per millilitre. If the challenge toxin preparation has been shown to be stable, it is not necessary to verify the paralytic dose for every assay.

Preparation of the challenge toxin solution Immediately before use, dilute the challenge toxin with a suitable diluent (for example, peptone buffered saline solution pH 7.4) to obtain a stable challenge toxin solution containing approximately 50 times the 50 per cent paralytic dose per millilitre. If necessary, use portions of the challenge toxin solution diluted 1 to 16, 1 to 50 and 1 to 160 with the same diluent to determine the activity of the toxin.

Dilution of the test and reference preparations Using *saline TS*, prepare dilutions of the vaccine to be examined and of the reference preparation, such that for each, the dilutions form a series differing by not more than 2.5-fold steps and in which the intermediate dilutions, when injected subcutaneously at a dose of 1.0 ml per guinea-pig, protect approximately 50 per cent of the animals from the paralytic effects of the subcutaneous injection of the quantity of tetanus toxin prescribed for this test.

Immunization and challenge Allocate the dilutions, one to each of the groups of guinea-pigs and inject subcutaneously, 1.0 ml of each dilution into each guinea-pig in the group to which that dilution is allocated. After 28 days, inject subcutaneously into each animal 1.0 ml of the challenge toxin solution (containing 50 times the 50 per cent paralytic dose).

Determination of the activity of the challenge toxin If necessary, allocate the three dilutions made from the challenge toxin solution, one to each of the three groups of five guinea-pigs, and inject subcutaneously 1.0 ml of each solution into each guinea-pig in the group to which that solution is allocated. The activity and stability of the challenge toxin are determined by carrying out a suitable number of determinations of the 50 per cent paralytic dose. It is then not necessary to repeat the determination for each assay.

Reading and interpretation of results Examine the guinea-pigs twice daily. Remove and humanely kill all animals showing definite signs of tetanus paralysis. Count the number of guinea-pigs without paralysis 5 days after injection of the challenge toxin. Calculate the potency of the vaccine to be examined relative to the potency of the reference preparation on the basis of the proportion of challenged animals without paralysis in each of the groups of vaccinated guinea-pigs, using the "Statistical Analysis of Results of Biological Assay and Tests" (Appendix 9).

Requirements for a valid assay The test is not valid unless:

– for both the vaccine to be examined and the reference preparation the 50 per cent protective dose lies between the largest and smallest doses of the preparations given to the guinea-pigs,

– if applicable, the number of paralyzed animals in the three groups of five injected with the dilutions of the challenge toxin solution indicates that the challenge was approximately 50 times the 50 per cent paralytic dose,

– the confidence limits ($P = 0.95$) are not less than 50 per cent and not more than 200 per cent of the estimated potency,

– the statistical analysis shows significant slope and no deviation from linearity and parallelism of the dose-response lines (Appendix 9).

The test may be repeated but when more than one test is performed, the results of all valid tests must be combined in the estimate of potency.

Method B. Challenge Test in Mice

Selection and distribution of the test animals Use in the test healthy mice from the same stock, about 5 weeks old and from a strain shown to be suitable. Distribute the mice in not less than six equal groups; use groups containing a number of animals sufficient to obtain results that fulfil the requirements for a valid assay prescribed below. If the challenge toxin to be used has not been shown to be stable or has not been adequately standardized, include three groups of not less than five mice to serve as unvaccinated controls. Use mice of the same sex or with males and females equally distributed between the groups.

Selection of the challenge toxin Select a preparation of tetanus toxin containing not less than 100 times the 50 per cent paralytic dose per millilitre. If the challenge toxin preparation has been shown to be stable, it is not necessary to verify the paralytic dose for every assay.

Preparation of the challenge toxin solution Immediately before use, dilute the challenge toxin with a suitable diluent (for example, peptone buffered saline solution pH 7.4) to obtain a stable challenge toxin solution containing approximately 50 times the 50 per cent paralytic dose in 0.5 ml. If necessary, use portions of the challenge toxin solution diluted 1 to 16, 1 to 50 and 1 to 160 with the same diluent to determine the activity of the toxin.

Dilution of the test and reference preparations Using *saline TS*, prepare dilutions of the vaccine to be examined and of the reference preparation, such that for each, the dilutions form a series differing by not more

than 2.5-fold steps and in which the intermediate dilutions, when injected subcutaneously at a dose of 0.5 ml per mouse, protect approximately 50 per cent of the animals from the paralytic effects of the subcutaneous injection of the quantity of tetanus toxin prescribed for this test.

Immunization and challenge Allocate the dilutions, one to each of the groups of mice and inject subcutaneously 0.5 ml of each dilution into each mouse in the group to which that dilution is allocated. After 28 days, inject subcutaneously into each animal 0.5 ml of the challenge toxin solution (containing 50 times the 50 per cent paralytic dose).

Determination of the activity of the challenge toxin If necessary, allocate the three dilutions made from the challenge toxin solution, one to each of the three groups of not less than five mice and inject subcutaneously 0.5 ml of each solution into each mouse in the group to which that solution is allocated.

Reading and interpretation of results Examine the mice twice daily. Remove and humanely kill all animals showing definite signs of tetanus paralysis. Count the number of mice without paralysis 4 days after injection of the challenge toxin. Calculate the potency of the vaccine to be examined relative to the potency of the reference preparation on the basis of the proportion of challenged animals without paralysis in each group of vaccinated mice, using the "Statistical Analysis of Results of Biological Assay and Tests" (Appendix 9)

Requirements for a valid assay The test is not valid unless:

– for both the vaccine to be examined and the reference preparation the 50 per cent protective dose lies between the largest and smallest doses of the preparations given to the mice,

– if applicable, the number of paralyzed animals in the three groups of not less than five injected with the dilutions of the challenge toxin solution indicates that the challenge dose was approximately 50 times the 50 per cent paralytic dose,

– the confidence limits ($P = 0.95$) are not less than 50 per cent and not more than 200 per cent of the estimated potency,

– the statistical analysis shows a significant slope and no deviation from linearity and parallelism of the dose-response lines (Appendix 9).

The test may be repeated but when more than one test is performed the results of all valid tests must be combined in the estimate of potency.

Method C. Determination of Antibodies in Guinea-pigs

Selection and distribution of the test animals Use in the test healthy guinea-pigs from the same stock, each weighing 250 to 350 g. Use guinea-pigs of the same sex or with males and females equally distributed between groups. Distribute the guinea-pigs in not less than six equal groups; use groups containing a number of animals sufficient to obtain results that fulfil the requirements for a valid assay. Use a further group of non-vaccinated guinea-pigs of the same origin to provide a negative serum control. If test consistency has been demonstrated, a reference negative serum control may be used.

Dilution of the test and reference preparation Using *saline TS*, as diluent, prepare serial dilutions of the vaccine to be examined and the reference preparation; series differing by 2.5- to fivefold steps have been found suitable. Use not less than three dilutions within the range for example 0.5 to 16 IU/ml for each series. Use dilutions for immunization preferably within 1 hour of preparation. Allocate one dilution to each group of guinea-pigs.

Immunization Inject subcutaneously in the nape of each guinea-pig 1.0 ml of the dilution allocated to its group.

Blood sampling Thirty-five to 42 days after immunization, take a blood sample from each vaccinated and control guinea-pig using a suitable method.

Preparation of serum samples Avoid frequent freezing and thawing of serum samples. To avoid microbial contamination, it is preferable to carry out manipulations in a laminar-flow cabinet.

Determination of antibody titre Determine the relative antibody titre of each serum sample by a suitable immunochemical method (Appendix 14.5). Enzyme-linked immunosorbent assay (ELISA); either indirect ELISA or competitive ELISA (Toxin-Binding Inhibition; ToBI) has been found suitable.

Calculation of potency Calculate the potency of the vaccine to be examined in International Units relative to the reference preparation, using the "Statistical Analysis of Results of Biological Assay and Tests" (Appendix 9). (**Note** International Units of potency refer to the reference vaccine and not to the International Units of antitoxin of the reference guinea-pig serum.)

Requirements for a valid assay The test is not valid unless:

– the confidence limits ($P = 0.95$) are not less than 50 per cent and not more than 200 per cent of the estimated potency,

– the statistical analysis shows significant slope and no deviation from linearity and parallelism of the dose-response lines (Appendix 9).

The test may be repeated but when more than one test is performed, the results of all valid tests must be combined in the estimate of potency.

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Periodic Table of the Elements

1																	18
1 **H** hydrogen 1.00794(7)	2		Key:									13	14	15	16	17	2 **He** helium 4.002602(2)
3 **Li** lithium 6.941(2)	4 **Be** beryllium 9.012182(3)		Atomic number **Symbol** Name Standard atomic weight									5 **B** boron 10.811(7)	6 **C** carbon 12.0107(8)	7 **N** nitrogen 14.0067(2)	8 **O** oxygen 15.9994(3)	9 **F** fluorine 18.9984032(5)	10 **Ne** neon 20.1797(6)
11 **Na** sodium 22.98976928(2)	12 **Mg** magnesium 24.3050(6)	3	4	5	6	7	8	9	10	11	12	13 **Al** aluminium 26.9815386(8)	14 **Si** silicon 28.0855(3)	15 **P** phosphorus 30.973762(2)	16 **S** sulfur 32.065(5)	17 **Cl** chlorine 35.453(2)	18 **Ar** argon 39.948(1)
19 **K** potassium 39.0983(1)	20 **Ca** calcium 40.078(4)	21 **Sc** scandium 44.955912(6)	22 **Ti** titanium 47.867(1)	23 **V** vanadium 50.9415(1)	24 **Cr** chromium 51.9961(6)	25 **Mn** manganese 54.938045(5)	26 **Fe** iron 55.845(2)	27 **Co** cobalt 58.933195(5)	28 **Ni** nickel 58.6934(2)	29 **Cu** copper 63.546(3)	30 **Zn** zinc 65.409(4)	31 **Ga** gallium 69.723(1)	32 **Ge** germanium 72.64(1)	33 **As** arsenic 74.92160(2)	34 **Se** selenium 78.96(3)	35 **Br** bromine 79.904(1)	36 **Kr** krypton 83.798(2)
37 **Rb** rubidium 85.4678(3)	38 **Sr** strontium 87.62(1)	39 **Y** yttrium 88.90585(2)	40 **Zr** zirconium 91.224(2)	41 **Nb** niobium 92.90638(2)	42 **Mo** molybdenum 95.94(2)	43 **Tc** technetium [97.9072]	44 **Ru** ruthenium 101.07(2)	45 **Rh** rhodium 102.90550(2)	46 **Pd** palladium 106.42(1)	47 **Ag** silver 107.8682(2)	48 **Cd** cadmium 112.411(8)	49 **In** indium 114.818(3)	50 **Sn** tin 118.710(7)	51 **Sb** antimony 121.760(1)	52 **Te** tellurium 127.60(3)	53 **I** iodine 126.90447(3)	54 **Xe** xenon 131.293(6)
55 **Cs** caesium 132.9054519(2)	56 **Ba** barium 137.327(7)	57-71 lanthanoids	72 **Hf** hafnium 178.49(2)	73 **Ta** tantalum 180.94788(2)	74 **W** tungsten 183.84(1)	75 **Re** rhenium 186.207(1)	76 **Os** osmium 190.23(3)	77 **Ir** iridium 192.217(3)	78 **Pt** platinum 195.084(9)	79 **Au** gold 196.966569(4)	80 **Hg** mercury 200.59(2)	81 **Tl** thallium 204.3833(2)	82 **Pb** lead 207.2(1)	83 **Bi** bismuth 208.98040(1)	84 **Po** polonium [208.9824]	85 **At** astatine [209.9871]	86 **Rn** radon [222.0176]
87 **Fr** francium [223]	88 **Ra** radium [226]	89-103 actinoids	104 **Rf** rutherfordium [261]	105 **Db** dubnium [262]	106 **Sg** seaborgium [266]	107 **Bh** bohrium [264]	108 **Hs** hassium [277]	109 **Mt** meitnerium [268]	110 **Ds** darmstadtium [271]	111 **Rg** roentgenium [272]							

57 **La** lanthanum 138.90547(7)	58 **Ce** cerium 140.116(1)	59 **Pr** Praseodymium 140.90765(2)	60 **Nd** neodymium 144.242(3)	61 **Pm** promethium [145]	62 **Sm** samarium 150.36(2)	63 **Eu** europium 151.964(1)	64 **Gd** gadolinium 157.25(3)	65 **Tb** terbium 158.92535(2)	66 **Dy** dysprosium 162.500(1)	67 **Ho** holmium 164.93032(2)	68 **Er** erbium 167.259(3)	69 **Tm** thulium 168.93421(2)	70 **Yb** ytterbium 173.04(3)	71 **Lu** lutetium 174.967(1)
89 **Ac** actinium [227]	90 **Th** thorium 232.03806(2)	91 **Pa** protactinium 231.03588(2)	92 **U** uranium 238.02891(3)	93 **Np** neptunium [237]	94 **Pu** plutonium [244]	95 **Am** americium [243]	96 **Cm** curium [247]	97 **Bk** berkelium [247]	98 **Cf** californium [251]	99 **Es** einsteinium [252]	100 **Fm** fermium [257]	101 **Md** mendelevium [258]	102 **No** nobelium [259]	103 **Lr** lawrencium [262]

Notes

1. "Aluminum" and "cesium" are commonly used alternative spellings for "aluminium" and "caesium". For elements that have no stable or long-lived nuclides, the mass number of the nuclide with the longest confirmed half-life is listed between square brackets.
2. IUPAC 2005 standard atomic weights are listed with uncertainties in the last figure in parentheses (M. E. Wieser, "Atomic weights of the elements 2005 (IUPAC Technical Report)," *Pure Appl. Chem.*, *78*(11), 2006, pp. 2054-2056.).

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