



WHO COLLABORATING CENTRE FOR CHEMICAL REFERENCE SUBSTANCES

Report on the work in 1994

by M. Westermark

Newly established International Chemical Reference Substances, proposed by the WHO Collaborating Centre for Chemical Reference Substances on the basis of adequate testing and characterization, are included in the Centre's annual report. The report is circulated, *inter alia*, to members of the WHO Expert Advisory Panel on the International Pharmacopoeia and Pharmaceutical Preparations, who are requested to consider the proposals carefully together with the attached analytical documentation, and to notify the Centre of any reservations or adverse comments within three months of the date of mailing. In these cases the Centre will proceed with any consultations or additional analyses necessary for the validation.

If no adverse comments are received within the three-month period, the proposed new International Chemical Reference Substances may be considered *provisionally* adopted. They will be considered for *final* adoption during the subsequent meeting of the Expert Committee.

Kindly address your comments to Mrs M. Westermark, WHO Collaborating Centre for Chemical Reference Substances, Apoteksbolaget AB, Centrallaboratoriet, Prismavägen 2, S-10514 Stockholm, Sweden.

CONTENTS

| | page |
|--|------|
| Distribution of reference substances in 1994 | 2 |
| Establishment of reference substances in 1994 | 2 |
| Work on new reference substances completed in 1994 | 2 |
| Stability testing | 2 |
| Work in progress and future work | 3 |
| Administrative and financial matters | 3 |
| Acknowledgements | 3 |
| Appendix 1. Distribution of chemical reference substances in 1994 | 4 |
| Appendix 2. Distribution of ICRS to different WHO regions in 1994 | 6 |
| Appendix 3. Distribution of reference spectra to different WHO regions in 1994 | 7 |
| Appendix 4. International Chemical Reference Substances established in 1994 | 8 |
| Appendix 5. List of available International Chemical Reference Substances | 9 |
| Appendix 6. Stability testing - analytical report | 14 |
| Appendix 7. International Chemical Reference Substances - Project list 1995 | 22 |
| Appendix 8. International Chemical Reference Spectra - Project list 1995 | 23 |
| Appendix 9. Benzil, Control No. 294170 | 24 |
| Appendix 10. Calcium folinate, Control No. 194188 | 27 |
| Appendix 11. Erythromycin B, Control No. 194186 | 31 |
| Appendix 12. Erythromycin C, Control No. 194187 | 35 |
| Appendix 13. Gentamicin sulfate, Control No. 194183 | 39 |
| Appendix 14. Hydrocortisone sodium succinate, Control No. 194184 | 44 |
| Appendix 15. Levonorgestrel, Control No. 194182 | 49 |
| Appendix 16. Loperamide hydrochloride, Control No. 194185 | 53 |
| Appendix 17. Methotrexate, Control No. 194193 | 59 |
| Appendix 18. Nifurtimox, Control No. 194189 | 63 |
| Appendix 19. Praziquantel, Control No. 194191 | 67 |
| Appendix 20. Prednisolone sodium phosphate, Control No. 194190 | 71 |
| Appendix 21. Testosterone enantate, Control No. 194192 | 76 |

This document is not issued to the general public, and all rights are reserved by the World Health Organization (WHO). The document may not be reviewed, abstracted, quoted, reproduced or translated, in part or in whole, without the prior written permission of WHO. No part of this document may be stored in a retrieval system or transmitted in any form or by any means - electronic, mechanical or other - without the prior written permission of WHO.

Ce document n'est pas destiné à être distribué au grand public et tous les droits y afférents sont réservés par l'Organisation mondiale de la Santé (OMS). Il ne peut être commenté, résumé, cité, reproduit ou traduit, partiellement ou en totalité, sans une autorisation préalable écrite de l'OMS. Aucune partie ne doit être chargée dans un système de recherche documentaire ou diffusée sous quelque forme ou par quelque moyen que ce soit - électronique, mécanique, ou autre - sans une autorisation préalable écrite de l'OMS.

The views expressed in documents by named authors are solely the responsibility of those authors.

Les opinions exprimées dans les documents par des auteurs cités nommément n'engagent que lesdits auteurs.

Distribution of reference substances in 1994

During 1994 the total number of International Chemical Reference Substances distributed from the Centre was 751. Compared to the figures for 1993 this corresponds to a decrease of about eighteen per cent. The most frequently requested substances were in order of demand Folic acid, Propicillin potassium, Acetanilide m.p., Phenacetin m.p., Azobenzene m.p., Ethinylestradiol, Benzanilide m.p., and Sulfanilamide m.p. Detailed figures for the distribution of the individual substances are given in Appendix 1.

The substances were distributed to twenty-seven different countries during 1994. Details of the distribution are given in Appendix 2. Considering the distribution to different regions it is observed that about 5% of the substances went to the African Region, less than 1% to the Americas, 2 % to the Eastern Mediterranean, 80 % to Europe, 6% to South East Asia and 6% to the Western Pacific Region.

A comment to this is that there is a great variation in this pattern from year to year. The high figure for the European Region is however remarkable.

Distribution of reference spectra in 1994

The distribution of spectra to different WHO regions are given in Appendix 3. A total number of 206 spectra were distributed in 1994. 73% was sent to the Eastern Mediterranean Region and 27 % to South -East Asia.

Establishment of reference substances in 1994

In accordance with the procedure recommended by the WHO Expert Committee on Specifications for Pharmaceutical Preparations in its Thirty-second report (Technical Report Series No 823) ,seven International Chemical Reference Substances were established in 1994. The substances are listed in Appendix 4 to this report.

A complete list of all International Chemical Reference Substances available from the Centre in January 1995, with information about package sizes and control numbers for the current batches is given in Appendix 5 to this report. The list also includes thirteen substances mentioned below which are expected to be formally adopted by the Expert Committee in 1996.

Work on new reference substances completed in 1994

Work is being continued on new reference substances required to support specifications in the third edition of the International Pharmacopoeia. During 1994 twelve new reference substances for volume 3 and 4 were examined. They are Calcium folinate, Erythromycin B, Erythromycin C, Gentamicin sulfate, Hydrocortisone sodium succinate, Levonorgestrel, Loperamide hydrochloride, Methotrexate, Nifurtimox, Praziquantel, Prednisolone sodium phosphate and Testosterone enantate. The analytical reports are given in Appendices 10-21. These substances are considered suitable for adoption as International Chemical Reference Substances. The stock of Benzil Melting Point Reference Substance No 192170 was depleted and has been replaced by Benzil Melting Point Reference Substance, No 294170 during 1994. The analytical report is given in Appendix 9.

Stability testing

The regular stability monitoring of existing International Chemical Reference Substances was continued. This year fifteen substances were re-examined. The results are given in Appendix 6. Details about the analytical methods used can be obtained from the Centre.

Work in progress and future work

Work is continuously performed on the substances required to support the monographs in Volume 3 and 4 of the International Pharmacopoeia. For the moment work on fourteen of the twenty-eight substances, given in Appendix 7, is in progress at the Centre.

Administrative and financial matters

The total cost for running the Centre in 1994 was estimated at 462.727 US\$. The income from sales of reference substances was about 30.760 US\$ and the contribution received from the WHO headquarters was 16.000 US\$ which leaves a deficit of 415.967 US\$, covered by the support from the National Corporation of Swedish Pharmacies.

The fee remains 40 US\$ per package and a freight and handling charge of 10 US\$ is added to each order.

Acknowledgements

The Centre is grateful to the laboratories that have contributed to the work during 1994. This year we want to address our thanks to the European Pharmacopoeia Laboratory in Strasbourg, France and the Institute of Science and Forensic Medicine in Singapore.

The Centre is also very grateful to the pharmaceutical companies who have provided candidate materials and participated in the analytical testing. This year we want to give a special thanks to Abbott, North Chicago, USA; American Cyanamide Company, Pearl River, USA; Bayer AG, Wuppertal, Germany; Bayer Argentina S.A., Buenos Aires, Argentina; Diosynth BV, Oss, Netherlands; Janssen Pharmaceutica N.V., Beerse, Belgium; Merck Schuchardt, Hohenbrunn, Germany; Piriell SPA, Milano, Italy; Schering AG, Berlin, Germany and Upjohn, Kalamazoo, USA.

DISTRIBUTION OF CHEMICAL REFERENCE SUBSTANCES IN 1994

| | | | |
|--|---------|--|----------|
| Aceclidine salicylate | 2 items | Dapsone | -- items |
| p-Acetamidobenzalazine | 2 " | Desoxycortone acetate | -- " |
| Acetazolamide | 1 " | Dexamethasone | 1 " |
| Allopurinol | 4 " | Dexamethasone acetate | -- " |
| 2-Amino-5-nitrothiazole | 2 " | Dexamethasone phosphoric acid | -- " |
| 3-Aminopyrazole-4-carbox- amide hemisulfate | 2 " | Dexamethasone sodium phosphate | 4 " |
| Amitriptyline hydrochloride | 4 " | Diazepam | 6 " |
| Amodiaquine hydrochloride | -- " | Diazoxide | -- " |
| Amphotericin B | -- " | Dicloxacillin sodium | 2 " |
| Ampicillin (anhydrous) | 16 " | Dicolinium iodide | -- " |
| Ampicillin sodium | 5 " | Dicoumarol | -- " |
| Ampicillin trihydrate | 3 " | Diethylcarbamazine dihydrogen citrate | 1 " |
| Anhydrotetracycline hydro- chloride | 14 " | Digitoxin | 1 " |
| Atropine sulfate | 4 " | Digoxin | 12 " |
| Azathioprine | -- " | NN'-Di-(2,3-xylyl)anthra- nilamide | -- " |
| Bacitracin zinc | -- " | Dopamine hydrochloride | -- " |
| Beclometasone dipropionate | -- " | Emetine hydrochloride | -- " |
| Bendazol hydrochloride | 2 " | 4-Epianhydrotetracycline hydrochloride | 15 " |
| Benzobarbital | 2 " | 4-Epitetracycline hydrochloride | 5 " |
| Benzylamine sulfate | 2 " | Ergocalciferol | 1 " |
| Benzylpenicillin potassium | 2 " | Ergometrine hydrogen maleate | 1 " |
| Benzylpenicillin sodium | 20 " | Ergotamine tartrate | -- " |
| Bephenium hydroxynaphthoate | -- " | Erythromycin | 6 " |
| Betamethasone | 5 " | Estradiol benzoate | 1 " |
| Betamethasone valerate | 1 " | Estrone | 1 " |
| Betanidine sulfate | -- " | Etacrynic acid | -- " |
| Bupivacaine hydrochloride | -- " | Ethambutol hydrochloride | -- " |
| Caffeine | 1 " | Ethinylestradiol | 22 " |
| Carbamazepine | 1 " | Ethisterone | 1 " |
| Carbenicillin monosodium | -- " | Ethosuximide | -- " |
| Chloramphenicol | 4 " | Etocarlide | -- " |
| Chloramphenicol palmitate | -- " | Flucytosine | -- " |
| Chloramphenicol palmitate (Polymorph A) | -- " | Fluorouracil | 2 " |
| 5-Chloro-2-methylamino- benzophenone | 2 " | Fluphenazine decanoate dihydrochloride | -- " |
| 2-(4-Chloro-3-sulfamoyl- benzoyl)benzoic acid | 3 " | Fluphenazine enantate dihydrochloride | -- " |
| Chlorphenamine hydrogen maleate | 4 " | Fluphenazine hydrochloride | -- " |
| Chlorpromazine hydro- chloride | 2 " | Folic acid | 48 " |
| Chlortalidone | 1 " | 3-Formylrifamycin | -- " |
| Chlortetracycline hydrochloride | 2 " | Furosemide | 3 " |
| Cimetidine | 3 " | Griseofulvin | 2 " |
| Clomifene citrate | -- " | Haloperidol | 1 " |
| Clomifene citrate Z-isomer see Zuclofemifene | -- " | Hydrochlorothiazide | -- " |
| Cloxacillin sodium | 6 " | Hydrocortisone | 8 " |
| Colecalciferol | 4 " | Hydrocortisone acetate | 7 " |
| Cortisone acetate | 4 " | (-)-3(4-Hydroxy-3-methoxyphenyl) -2-hydrazino-2-methylalanine | -- " |

| | | | |
|--|----------|-----------------------------------|---------|
| (-)-3-(4-Hydroxy-3-methoxy-phenyl)-2-methylalanine | -- items | Phenoxymethylpenicillin potassium | 4 items |
| Ibuprofen | 6 " | Phenytoin | 8 " |
| Imipramine hydrochloride | 1 " | Prednisolone | 3 " |
| Indometacin | 11 " | Prednisolone acetate | 2 " |
| o-Iodohippuric acid | -- " | Prednisone | 11 " |
| Isoniazid | 1 " | Prednisone acetate | -- " |
| Lanatoside C | -- " | Probenecid | 4 " |
| Levodopa | 1 " | Procaine hydrochloride | 1 " |
| Levothyroxine sodium | -- " | Procarbazine hydrochloride | -- " |
| Lidocaine | -- " | Progesterone | 2 " |
| Lidocaine hydrochloride | 1 " | Propicillin potassium | 30 " |
| Liothyronine sodium | -- " | Propranolol hydrochloride | 2 " |
| Mefenamic acid | -- " | Propylthiouracil | -- " |
| Melting Point Reference Substances | | Pyrantel embonate | 1 " |
| Azobenzene | 24 " | Pyridostigmine bromide | -- " |
| Vanillin | 17 " | Reserpine | 1 " |
| Benzil | 20 " | Retinol acetate | |
| Acetanilide | 25 " | (solution à 25000 IU) | 18 " |
| Phenacetin | 25 " | Riboflavin | 14 " |
| Benzanilide | 22 " | Rifampicin | 1 " |
| Sulfanilamide | 22 " | Rifampicin quinone | 1 " |
| Sulfapyridine | 21 " | Sodium cromoglicate | 2 " |
| Dicyandiamide | 17 " | Spectinomycin hydrochloride | -- " |
| Saccharin | 19 " | Sulfamethoxazole | 3 " |
| Caffeine | 14 " | Sulfamethoxypyridazine | -- " |
| Phenolphthalein | 20 " | Sulfanilamide | -- " |
| Metazide | -- " | Sulfasalazine | -- " |
| Methaqualone | -- " | Testosterone propionate | 2 " |
| Methyldopa | 7 " | Tetracycline hydrochloride | 4 " |
| Methyltestosterone | -- " | Thioacetazone | 4 " |
| Meticillin sodium | -- " | 4,4'-Thiodianiline | -- " |
| Metronidazole | 7 " | Thyroxine sodium | |
| Nafcillin sodium | -- " | see Levothyroxine sodium | |
| Neamine hydrochloride | -- " | Tolbutamide | -- " |
| Neomycin B sulfate | 1 " | Tolnaftate | 2 " |
| Neostigmine metilsulfate | -- " | Trimethadione | -- " |
| Nicotinamide | 5 " | Trimethoprim | 2 " |
| Nicotinic acid | 7 " | Trimethylguanidine sulfate | -- " |
| Niridazole | -- " | Tubocurarine chloride | 1 " |
| Niridazole-chlorethyl-carboxamide | -- " | Vitamin A acetate (solution) | |
| Norethisterone | -- " | see Retinol acetate | |
| Norethisterone acetate | 2 " | Vincristine sulfate | -- " |
| Nystatin | 15 " | Warfarin | 1 " |
| Ouabain | -- " | Zuclomifene | 6 " |
| Oxacillin sodium | 16 " | | |
| Oxytetracycline dihydrate | 7 " | | |
| Oxytetracycline hydrochloride | 4 " | | |
| Papaverine hydrochloride | -- " | | |
| Phenethicillin potassium | -- " | | |
| Phenoxymethylpenicillin | 1 " | | |
| Phenoxymethylpenicillin calcium | -- " | | |

DISTRIBUTION OF INTERNATIONAL CHEMICAL REFERENCE SUBSTANCES
TO DIFFERENT WHO REGIONS IN 1994

| <i>WHO Regions</i> | <i>Number of ICRS distributed in 1994</i> |
|--|---|
| African Region (AFRO) | |
| Republic of Benin | 23 |
| Kenya | 3 |
| Lesotho | 5 |
| Malawi | 4 |
| Region of the Americas (AMRO) | |
| United States of America | 2 |
| Eastern Mediterranean Region (EMRO) | |
| Egypt | 5 |
| Iran | 8 |
| Syrian Arab Republic | 1 |
| European Region (EURO) | |
| Belgium | 24 |
| Denmark | 4 |
| Finland | 1 |
| France | 29 |
| Germany | 283 |
| Hungary | 9 |
| Italy | 15 |
| Norway | 12 |
| Slovakia | 2 |
| Spain | 1 |
| Sweden | 133 |
| Switzerland | 26 |
| United Kingdom | 68 |
| South-East Asia Region (SEARO) | |
| Bangladesh | 38 |
| India | 9 |
| Western Pacific Region (WPRO) | |
| Australia | 6 |
| Malaysia | 23 |
| New Zealand | 13 |
| The Philippines | 4 |

DISTRIBUTION OF INTERNATIONAL INFRARED REFERENCE SPECTRA
TO DIFFERENT WHO REGIONS IN 1994

| <i>WHO Regions</i> | <i>Number of IIRS distributed in 1994</i> |
|-------------------------------------|---|
| African Region (AFRO) | - |
| Region of the Americas (AMRO) | - |
| Eastern Mediterranean Region (EMRO) | |
| Cyprus | 150 |
| European Region (EURO) | - |
| South-East Asia Region (SEARO) | |
| Indonesia | 56 |
| Western Pacific Region (WPRO) | - |

INTERNATIONAL CHEMICAL REFERENCE SUBSTANCES ESTABLISHED IN 1994

| Reference Substance | Control Number | Analytical Report | Remarks |
|---|----------------|---------------------------------|-----------------------|
| 4-Epitetracycline hydrochloride | 293098 | WHO/PHARM/94.566 Appendix 9 | Replaces No 180098 |
| (-)-3-(4-Hydroxy-3- methoxyphenyl)-2- hydrazino-2-methyl- alanine (3-O-Methylcarbidopa) | 193180 | WHO/PHARM/94.566 Appendix 10 | |
| Liothyronine sodium | 193179 | WHO/PHARM/94.566 Appendix 11 | |
| Neamine hydrochloride | 193177 | WHO/PHARM/94.566 Appendix 12 | |
| Neomycin B sulfate | 193178 | WHO/PHARM/94.566 Appendix 13 | |
| Spectinomycin hydrochloride | 193176 | WHO/PHARM/94.566 Appendix 14 | |
| Vincristine sulfate | 193181 | WHO/PHARM/94.566 Appendix 15 | |

LIST OF AVAILABLE INTERNATIONAL CHEMICAL REFERENCE SUBSTANCES

1995

General information

International Chemical Reference Substances are established on the advice of the WHO Expert Committee on Specifications for Pharmaceutical Preparations. They are supplied primarily for use in physical and chemical tests and assays described in the specifications for quality control of drugs published in *The International Pharmacopoeia* or proposed in draft monographs.

Directions for use and the analytical data required for the tests specified in *The International Pharmacopoeia* are given in the certificates enclosed with the substances when distributed. More detailed analytical reports on the substances may be obtained on request from the WHO Collaborating Centre for Chemical Reference Substances.

International Chemical Reference Substances may also be used in tests and assays not described in *The International Pharmacopoeia*. However, the responsibility for assessing the suitability of the substances then rests with the user or with the pharmacopoeia commission or other authority that has prescribed the use of these substances.

It is generally recommended that the substances be stored protected from light and moisture and preferably at a temperature of about +5 °C. When special storage conditions are required this is stated on the label or in the accompanying leaflet.

The stability of the International Chemical Reference Substances stored at the Collaborating Centre is monitored by regular re-examination, and any deteriorated materials are replaced by new batches as necessary. Lists giving control numbers for the current batches are issued in the annual reports from the Centre and may be obtained on request.

Ordering Information

Orders for International Chemical Reference Substances should be sent to:

WHO Collaborating Centre for Chemical Reference Substances
APOTEKSBOLAGET AB
Centrallaboratoriet
S-105 14 STOCKHOLM
SWEDEN

(Telex: 115 53 APOBOL S)

(Fax: + 46 8 740 60 40)

International Chemical Reference Substances are supplied only in the standard packages indicated in the following list.

| <u>Reference substance</u> | <u>Package size</u> | <u>Control Number</u> |
|---|---------------------|-----------------------|
| Aceclidine salicylate | 100 mg | 172048 |
| p-Acetamidobenzalazine | 100 mg | 290042 |
| Acetazolamide | 100 mg | 186128 |
| Allopurinol | 100 mg | 287049 |
| 2-Amino-5-nitrothiazole | 25 mg | 186131 |
| 3-Aminopyrazole-4-carboxamide hemisulfate | 100 mg | 172050 |
| Amitriptyline hydrochloride | 100 mg | 181101 |
| Amodiaquine hydrochloride | 200 mg | 192160 |
| Amphotericin B | 400 mg | 191153 |
| Ampicillin (anhydrous) | 200 mg | 390001 |
| Ampicillin sodium | 200 mg | 388002 |
| Ampicillin trihydrate | 200 mg | 274003 |
| Anhydrotetracycline hydrochloride | 25 mg | 180096 |
| Atropine sulfate | 100 mg | 183111 |
| Azathioprine | 100 mg | 172060 |
| Bacitracin zinc | 200 mg | 192174 |
| Beclometasone dipropionate | 200 mg | 192175 |
| Bendazol hydrochloride | 100 mg | 173066 |
| Benzobarbital | 100 mg | 172051 |
| Benzylamine sulfate | 100 mg | 172052 |
| Benzylpenicillin potassium | 200 mg | 180099 |
| Benzylpenicillin sodium | 200 mg | 280047 |
| Bephenium hydroxynaphthoate | 100 mg | 183112 |
| Betamethasone | 100 mg | 183113 |
| Betamethasone valerate | 100 mg | 190145 |
| Betanidine sulfate | 100 mg | 172053 |
| Bupivacaine hydrochloride | 100 mg | 289054 |
| Caffeine | 100 mg | 181102 |
| Calcium folinate (Leucovorin calcium) | 100 mg | 194188 |
| Carbamazepine | 100 mg | 189143 |
| Carbenicillin monosodium | 200 mg | 383043 |
| Chloramphenicol | 200 mg | 486004 |
| Chloramphenicol palmitate | 1 g | 286072 |
| Chloramphenicol palmitate (Polymorph A) | 200 mg | 175073 |
| 5-Chloro-2-methylaminobenzophenone | 100 mg | 172061 |
| 2-(4-Chloro-3-sulfamoylbenzoyl)benzoic acid | 50 mg | 181106 |
| Chlorphenamine hydrogen maleate | 100 mg | 182109 |
| Chlorpromazine hydrochloride | 100 mg | 178080 |
| Chlortalidone | 100 mg | 183114 |
| Chlortetracycline hydrochloride | 200 mg | 187138 |
| Cimetidine | 100mg | 190150 |
| Clomifene citrate | 100 mg | 187136 |
| Clomifene citrate Z-isomer see Zuclomifene | | |
| Cloxacillin sodium | 200 mg | 274005 |
| Colecalciferol (Vitamin D ₃) | 500mg | 190146 |
| Cortisone acetate | 100 mg | 167006 |
| Dapsone | 100 mg | 183115 |
| Desoxycortone acetate | 100 mg | 167007 |
| Dexamethasone | 100 mg | 388008 |
| Dexamethasone acetate | 100 mg | 288009 |
| Dexamethasone phosphoric acid | 100 mg | 192161 |

| | <u>Package size</u> | <u>Control Number</u> |
|---|-------------------------|---------------------------|
| Dexamethasone sodium phosphate | 100 mg | 192158 |
| Diazepam | 100 mg | 172062 |
| Diazoxide | 100 mg | 181103 |
| Dicloxacillin sodium | 200 mg | 174071 |
| Dicolinium iodide | 100 mg | 172055 |
| Dicoumarol | 100 mg | 178077 |
| Diethylcarbamazine dihydrogen citrate | 100 mg | 181100 |
| Digitoxin | 100 mg | 277010 |
| Digoxin | 100 mg | 587011 |
| NN'-Di-(2,3-xylyl)anthranilamide | 50 mg | 173067 |
| Dopamine hydrochloride | 100 mg | 192159 |
| Emetine hydrochloride | 100 mg | 187134 |
| 4-Epianhydrotetracycline hydrochloride | 25 mg | 288097 |
| 4-Epitetracycline hydrochloride | 25 mg | 293098 |
| Ergocalciferol (Vitamin D ₂) | 500mg | 190147 |
| Ergometrine hydrogen maleate | 50 mg | 277012 |
| Ergotamine tartrate | 50 mg | 385013 |
| Erythromycin | 250 mg | 191154 |
| Erythromycin B | 150 mg | 194186 |
| Erythromycin C | 25 mg | 194187 |
| Estradiol benzoate | 100 mg | 167014 |
| Estrone | 100 mg | 279015 |
| Etacrynic acid | 100 mg | 281056 |
| Ethambutol hydrochloride | 100 mg | 179081 |
| Ethinylestradiol | 100 mg | 291016 |
| Ethisterone | 100 mg | 167017 |
| Ethosuximide | 100 mg | 179088 |
| Etocarlide | 100 mg | 172057 |
| Flucytosine | 100 mg | 184121 |
| Fluorouracil | 100 mg | 184122 |
| Fluphenazine decanoate dihydrochloride | 100 mg | 182107 |
| Fluphenazine enantate dihydrochloride | 100 mg | 182108 |
| Fluphenazine hydrochloride | 100 mg | 176076 |
| Folic acid | 100 mg | 388019 |
| 3-Formylrifamycin | 200 mg | 190149 |
| Framycetin sulfate | 200 mg | 193178 |
| Furosemide | 100 mg | 171044 |
| Gentamicin sulfate | 100 mg | 194183 |
| Griseofulvin | 200 mg | 280040 |
| Haloperidol | 100 mg | 172063 |
| Hydrochlorothiazide | 100 mg | 179087 |
| Hydrocortisone | 100 mg | 283020 |
| Hydrocortisone acetate | 100 mg | 280021 |
| Hydrocortisone sodium succinate | 200 mg | 194184 |
| (-)-3-(4-Hydroxy-3-methoxyphenyl)-2-hydrazino- 2-methylalanine (3-O-Methylcarbidopa) | 25 mg | 193180 |
| (-)-3-(4-Hydroxy-3-methoxyphenyl)-2-methyl- alanine | 25 mg | 179085 |
| Ibuprofen | 100 mg | 183117 |
| Imipramine hydrochloride | 100 mg | 172064 |
| Indometacin | 100 mg | 178078 |
| o-Iodohippuric acid | 100 mg | 171045 |
| Isoniazid | 100 mg | 185124 |
| Lanatoside C | 100 mg | 281022 |

| | <u>Package size</u> | <u>Control Number</u> |
|---|-------------------------|---------------------------|
| Levodopa | 100 mg | 172065 |
| Levonorgestrel | 200 mg | 194182 |
| Levothyroxine sodium | 100 mg | 189144 |
| Lidocaine | 100 mg | 181104 |
| Lidocaine hydrochloride | 100 mg | 181105 |
| Liothyronine sodium | 50 mg | 193179 |
| Loperamide hydrochloride | 100 mg | 194185 |
| Mefenamic acid | 100 mg | 173068 |
| <i>Melting Point Reference Substances</i> | | |
| Azobenzene (69 °C) | 4 g | 192168 |
| Vanillin (83 °C) | 4 g | 192169 |
| Benzil (96 °C) | 4 g | 294170 |
| Acetanilide (116 °C) | 4 g | 192171 |
| Phenacetin (136 °C) | 4 g | 192172 |
| Benzanilide (165 °C) | 4 g | 192173 |
| Sulfanilamide (166 °C) | 4 g | 192162 |
| Sulfapyridine (193 °C) | 4 g | 192163 |
| Dicyandiamide (210 °C) | 4 g | 192164 |
| Saccharin (229 °C) | 4 g | 192165 |
| Caffeine (237 °C) | 4 g | 192166 |
| Phenolphthalein (263 °C) | 4 g | 192167 |
| Metazide | 100 mg | 172058 |
| Methaqualone | 100 mg | 173069 |
| Methotrexate | 100 mg | 194193 |
| Methyldopa | 100 mg | 179084 |
| Methyltestosterone | 100 mg | 167023 |
| Meticillin sodium | 200 mg | 274024 |
| Metronidazole | 100 mg | 183118 |
| Nafcillin sodium | 200 mg | 272025 |
| Neamine hydrochloride | 0.5 mg | 193177 |
| Neomycin B sulfate see Framycetin sulfate | | |
| Neostigmine metilsulfate | 100 mg | 187135 |
| Nicotinamide | 100 mg | 179090 |
| Nicotinic acid | 100 mg | 179091 |
| Nifurtimox | 100 mg | 194189 |
| Niridazole | 200 mg | 186129 |
| Niridazole-chlorethylcarboxamide | 25 mg | 186130 |
| Norethisterone | 100 mg | 186132 |
| Norethisterone acetate | 100 mg | 185123 |
| Nystatin | 200 mg | 191152 |
| Ouabain | 100 mg | 283026 |
| Oxacillin sodium | 200 mg | 382027 |
| Oxytetracycline dihydrate | 200 mg | 189142 |
| Oxytetracycline hydrochloride | 200 mg | 189141 |
| Papaverine hydrochloride | 100 mg | 185127 |
| Phenethicillin potassium | 200 mg | 167028 |
| Phenoxymethylpenicillin | 200 mg | 179082 |
| Phenoxymethylpenicillin calcium | 200 mg | 179083 |
| Phenoxymethylpenicillin potassium | 200 mg | 176075 |
| Phenytoin | 100 mg | 179089 |
| Praziquantel | 100 mg | 194191 |
| Prednisolone | 100 mg | 389029 |
| Prednisolone acetate | 100 mg | 289030 |
| Prednisolone sodium phosphate | 200 mg | 194190 |

| | <u>Package size</u> | <u>Control Number</u> |
|--|-------------------------|---------------------------|
| Prednisone | 100 mg | 167031 |
| Prednisone acetate | 100 mg | 169032 |
| Probenecid | 100 mg | 192156 |
| Procaine hydrochloride | 100 mg | 183119 |
| Procarbazine hydrochloride | 100 mg | 184120 |
| Progesterone | 100 mg | 167033 |
| Propicillin potassium | 200 mg | 274034 |
| Propranolol hydrochloride | 100 mg | 187139 |
| Propylthiouracil | 100 mg | 185126 |
| Pyrantel embonate | 500 mg | 192157 |
| Pyridostigmine bromide | 100 mg | 182110 |
| Reserpine | 100 mg | 186133 |
| Retinol acetate (solution) | 5 caps. (*) | 791038 |
| Riboflavin | 250 mg | 382035 |
| Rifampicin | 200 mg | 191151 |
| Rifampicin quinone | 200 mg | 190148 |
| Sodium cromoglicate | 100 mg | 188140 |
| Spectinomycin hydrochloride | 200 mg | 193176 |
| Sulfamethoxazole | 100 mg | 179092 |
| Sulfamethoxypyridazine | 100 mg | 178079 |
| Sulfanilamide | 100 mg | 179094 |
| Sulfasalazine | 100 mg | 191155 |
| Testosterone enantate | 200 mg | 194192 |
| Testosterone propionate | 100 mg | 167036 |
| Tetracycline hydrochloride | 200 mg | 180095 |
| Thioacetazone | 100 mg | 171046 |
| 4,4'-Thiodianiline | 50 mg | 183116 |
| Thyroxine sodium see Levothyroxine sodium | | |
| Tolbutamide | 100 mg | 179086 |
| Tolnaftate | 100 mg | 176074 |
| Trimethadione | 200 mg | 185125 |
| Trimethoprim | 100 mg | 179093 |
| Trimethylguanidine sulfate | 100 mg | 172059 |
| Tubocurarine chloride | 100 mg | 170037 |
| Vitamin A acetate (solution) see Retinol acetate | | |
| Vincristine sulfate | 9.7 mg/vial | 193181 |
| Warfarin | 100 mg | 168041 |
| Zuclomifene | 50 mg | 187137 |

(*) About 9 mg in 250 mg oil per capsule

STABILITY TESTING

The stability on storage of the International Chemical Reference Substances is monitored by regular re-examination of the substances held in stock at the Centre. The results obtained for the substances re-examined in 1994 are summarized below. For comparison results obtained at earlier occasions are included in the summaries. The substances have been stored in tightly closed containers at +5 °C and in a relative humidity of about 30%. The following abbreviations are used in the tables:

| | |
|------|--|
| DSC | Differential Scanning Calorimetry |
| DTA | Differential Thermal Analysis |
| HPLC | High Performance Liquid Chromatography |
| IR | Infrared Spectrophotometry |
| KF | Karl Fischer titration |
| LOD | Loss on drying |
| TLC | Thin-layer Chromatography |
| PSA | Phase solubility analysis |
| TGA | Thermogravimetric analysis |

The estimates of total impurities by HPLC and by TLC are expressed as area per cent (area %), if not otherwise stated; by DSC and by DTA as mole per cent (mol %), and by PSA as weight per cent (w/w %). LOD and TGA (loss of weight) are expressed as weight per cent (w/w %). Assay values are calculated with reference to the dried or the anhydrous substance unless otherwise stated.

More details about the analytical methods used can be obtained from the Centre.

Anhydrotetracycline hydrochloride, Control No 180096

Initial analytical report: WHO/PHARM/81.508, Appendix 5

| Examination year: | 1980 | 1985 | 1994 |
|--------------------------|---------------|--------------|------|
| IR | conforms | - | - |
| TLC, % | one sec. spot | one sec spot | - |
| HPLC, % | 1.4 | 1.5 | 1.3 |
| TGA, % | - | - | 2.2 |
| Water(KF) , % | - | 2.4 | - |
| LOD, % | 1.2 | - | - |
| Assay, potentiometric, % | 93.9 | - | - |

Caffeine, Control No 181102

Initial analytical report: WHO/PHARM/82.509, Appendix 7

| Examination year: | 1981 | 1994 |
|--------------------------|--------------|------|
| IR | conforms | - |
| TLC, % | no sec spots | - |
| HPLC, % | 0.1 | <0.1 |
| TGA, % | - | <0.1 |
| LOD, % | <0.1 | - |
| Assay, potentiometric, % | 99.5 | - |
| Melting point, °C | 236 | 236 |

Chlorphenamine hydrogen maleate, Control No 182109

Initial analytical report: WHO/PHARM/83.510, Appendix 5

| Examination year: | 1982 | 1994 |
|--------------------------|----------|------|
| IR | conforms | - |
| TLC, % | <0.1 | - |
| HPLC, % | <0.1 | 0.1 |
| TGA, % | - | <0.1 |
| Water (KF), % | < 0.1 | - |
| LOD, % | 0.3 | - |
| Assay, potentiometric, % | 100.2 | - |

Epianhydrotetracycline hydrochloride, Control No 288097

Initial analytical report: WHO/PHARM/89.544, Appendix 9

| Examination year: | 1988 | 1994 |
|--------------------------|--------------|------|
| IR | conforms | - |
| TLC, % | one sec spot | - |
| HPLC, % | 4.7 | 4.9 |
| TGA, % | 5.4 | 5.3 |
| Water (KF), % | 5.4 | - |
| Assay, potentiometric, % | 99.6 | - |

Fluphenazine decanoate dihydrochloride, Control No 182107

Initial analytical report: WHO/PHARM/83.510, Appendix 6

| Examination year: | 1982 | 1994 |
|--------------------------|----------|--------|
| IR | conforms | - |
| TLC, % | 1 | - |
| HPLC, % | 0.8 | 0.9 |
| TGA, % | - | < 0.1% |
| LOD, % | <0.1 | - |
| Assay, potentiometric, % | 99.7 | - |

Fluphenazine enantate dihydrochloride, Control No 182108

Initial analytical report: WHO/PHARM/83.510 Appendix 7

| Examination year: | 1982 | 1994 |
|--------------------------|----------|------|
| IR | conforms | - |
| TLC, % | 1 | - |
| HPLC, % | 1.2 | 1.0 |
| TGA, % | - | 2.8 |
| Water(KF), % | 2.4 | - |
| LOD, % | 0.7 | - |
| Assay, potentiometric, % | 99.3 | - |

Fluphenazine hydrochloride, Control No 176076

Initial analytical report: WHO/PHARM/77.491 Appendix 5

| Examination year: | 1976 | 1988 | 1994 |
|--------------------------|-------------|-------------|-------------|
| IR | conforms | conforms | - |
| TLC, % | 2 sec spots | 4 sec spots | 4 sec spots |
| HPLC, % | 0.5 | 0.4 | 0.2 |
| TGA, % | - | < 0.1 | 0.4 |
| LOD, % | <0.1 | - | - |
| Assay, potentiometric, % | 100.2 | - | - |

Hydrocortisone. Control No 283020

Initial analytical report: WHO/PHARM/84.513, Appendix 11

| Examination year: | 1983 | 1989 | 1994 |
|------------------------------|-------------------|------|-------------|
| IR | conforms | - | conforms |
| TLC, % | 0.3 (4 sec spots) | - | 3 sec spots |
| HPLC, % | 0.3 | 0.6 | 0.3 |
| TGA, % | - | <0.1 | <0.1 |
| LOD, % | <0.1 | - | - |
| Assay, spectrophotometric, % | 99.9 | 99.7 | 99.6 |

The re-examination of this substance has been partially performed by the Department of Scientific Services, Institute of Science and Forensic Medicine, Singapore.

Hydrocortisone acetate. Control No 280021

Initial analytical report: WHO/PHARM/81.508, Appendix 11

| Examination year: | 1980 | 1989 | 1994 |
|------------------------------|-------------------|------|-------|
| IR | conforms | - | - |
| TLC, % | 0.4 (2 sec spots) | - | - |
| HPLC, % | <0.5 | 0.4 | 0.5 |
| TGA, % | - | 0.1 | <0.1 |
| LOD, % | 0.1 | - | - |
| Assay, spectrophotometric, % | 99.6 | 99.9 | 100.4 |

Lanatoside C .Control No 281022

Initial analytical report: WHO/PHARM/82.509, Appendix 12

| Examination year: | 1981 | 1987 | 1994 |
|---------------------------|-------------|-------------|------|
| IR | conforms | conforms | - |
| TLC, % | 5 sec spots | 5 sec spots | - |
| HPLC, % | 0.8 | 1.0 | 1.0 |
| TGA, % | - | - | 7.5 |
| LOD, % | 7.2 | 7.2 | - |
| Assay, % (potentiometric) | 99.9 | 99.9 | - |

Nystatin. Control No 191152

Initial analytical report: WHO/PHARM/92.558, Appendix 10

| Examination year: | 1991 | 1992 | 1993 | 1994 |
|---|--------------------|----------|-------------|---------|
| IR | conforms | - | - | - |
| HPLC, %, impurities, 304 nm | 5.9 | - | 7.9* | 8.7 |
| HPLC, assay, % | 100.0 | - | 100.0 | 100.5 |
| TGA, % | 5.0 | - | 5.2 | 5.3 |
| Water (KF), % | 4.8 | - | - | - |
| Microbiological assay, IU/mg (as is) | 6382 ** | 5208 *** | 4962*** | 4868*** |
| TLC, % | 4.1(3 sec spots) - | - | 3 sec spots | - |
| UV, assay, 304 nm, % | 100.0 | - | 100.0 | - |

* Increased values, due to new liquid chromatographic system with better separation efficiency

** EPCRS with wrong content on label, used as standard.

*** WHO 2nd Biol. standard with declared content 4855 IU/mg used as standard.

Prednisolone, Control No 389029

Initial analytical report: WHO/PHARM/90.547, Appendix 11

| Examination year: | 1989 | 1994 |
|------------------------------|----------|-------|
| IR | conforms | - |
| TLC, % | 0.2 | 0.6 |
| HPLC, % | 0.3 | 0.6* |
| TGA, % | 0.2 | 0.4 |
| Water (KF), % | 0.3 | - |
| Assay, spectrophotometric, % | 100.1 | 100.3 |

*New liquid chromatographic system

Prednisolone acetate, Control No 289030

Initial analytical report: WHO/PHARM/90.547, Appendix 12

| Examination year: | 1989 | 1994 |
|------------------------------|----------|------|
| IR | conforms | - |
| TLC, % | 0.3 | 0.4 |
| HPLC, % | 0.3 | 0.4 |
| TGA, % | <0.1 | <0.1 |
| Water (KF), % | <0.1 | - |
| Assay, spectrophotometric, % | 99.9 | 99.8 |

The re-examination of this substance has been partially performed by the Department of Scientific Services, Institute of Science and Forensic Medicine, Singapore.

Prednisone, Control No 167031

Initial analytical report: WHO/PHARM/67.441 Appendix 3

| Examination year: | 1966 | 1975 | 1984 | 1994 |
|------------------------------|--------------|--------------|-------------|------------------|
| IR | conforms | - | conforms | - |
| TLC, % | no sec spots | no sec spots | 2 sec spots | 0.2(3 sec spots) |
| HPLC, % | - | - | 0.7 | 0.5 |
| TGA, % | - | - | - | <0.1 |
| LOD, % | <0.1 | 0.1 | <0.1 | - |
| Assay, spectrophotometric, % | - | - | 99.5 | 100.9 |

Prednisone acetate, Control No 169032

Initial analytical report: WHO/PHARM/70.455, Appendix 4

| Examination year: | 1969 | 1975 | 1984 | 1994 |
|-------------------|-------------|-------------|-------------|------------------|
| IR | conforms | - | - | - |
| TLC, % | 3 sec spots | 2 sec spots | 2 sec spots | 0.6(4 sec spots) |
| HPLC, % | - | - | 1.5 | 0.8 |
| TGA, % | - | - | - | 0.1 |
| LOD, % | 0.1 | 0.3 | - | - |
| PSA, % | <0.5 | - | - | - |

INTERNATIONAL CHEMICAL REFERENCE SUBSTANCES PROJECT LIST 1995

The following additional International Chemical Reference Substances and are required to support specifications in the third edition of the International Pharmacopoeia:

Volume 3*Reference substances*

| | |
|-------------------------------|--------------------------------------|
| Doxorubicin hydrochloride (*) | Noroxymorphone hydrochloride |
| Fludrocortisone acetate(*) | (impurity in Naloxone hydrochloride) |
| | Paromomycin sulfate (*) |
| | Sulfacetamide(*) |

Volume 4*Reference substances*

| | |
|------------------------------------|--------------------------------|
| Amidotrizoic acid (*) | Paracetamol (*) |
| 3-Amino-2,4,6-triiodobenzoic acid | Piperazine adipate |
| Betamethasone sodium phosphate (*) | Piperazine citrate |
| Chloroquine sulfate (*) | Prednisolone sodium phosphate |
| Cisplatin (*) | Prednisolone succinate (*) |
| Dactinomycin | Sodium amidotrizoate |
| Iohexol | Streptomycin sulfate |
| Kanamycin monosulfate | Tamoxifen citrate (*) |
| Mebendazole (*) | Tamoxifen citrate E-isomer (*) |
| Medroxyprogesterone acetate | Thiopental sodium |
| Neomycin sulfate | Toluene-2-sulfonamide (*) |
| | Vinblastine sulfate |

(*) Denotes that work on the substance is in progress at the Centre.

INTERNATIONAL CHEMICAL REFERENCE SPECTRA PROJECT LIST 1995

The following International Chemical Reference Spectra are required to support specifications in the third edition of the International Pharmacopoeia.

Volume 3
Reference spectra

Diloxanide furoate
Mebendazole(*)
Metoclopramide hydrochloride(*)
Naloxone hydrochloride(*)
Nitrofurantoin
Pyrazinamide(*)
Spironolactone(*)
Sulfacetamide

Volume 4
Reference spectra

Disodium edetate
Ephedrine sulfate
Iopanoic acid
Iotroxic acid
Ketamine hydrochloride
Norethisterone enantate
Pentamidine isetionate
Timolol maleate

(*) Work in progress.

BENZIL

WHO Melting Point Reference Substance

Melting temperature 96 °C

Control No 294170

Analytical Report

Intended use

The stock of the current batch of the WHO Melting Point Reference Substance for benzil, Control No 192170, is depleted and has to be replaced.

The WHO Melting Point Reference Substance for benzil is supplied primarily for calibration of different instruments and methods for determination of melting temperatures against the method of the International Pharmacopoeia 3rd Ed.Vol. 1.

Material

About 4500 g of the sample (manufacturers batch no 44215938) were received at the WHO Centre in January 1994. The material is being stored in tightly closed containers at room temperature, protected from light.

Analytical data

Description

A yellow powder.

Evidence of chemical structure

Infrared spectrum

An infrared spectrum is given in Figure 1 (No W 294170T). The spectrum is concordant with the spectrum of benzil in The Aldrich Library of Infrared Spectra Ed.III.

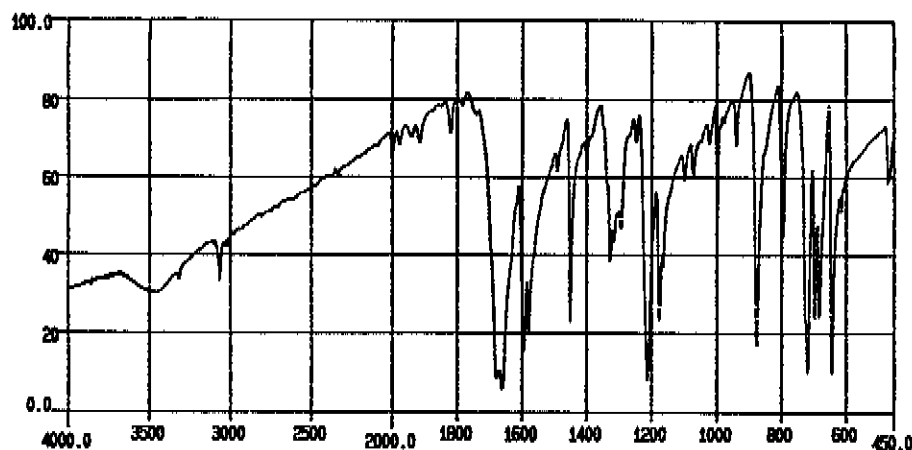


Figure 1. IR-spectrum of 1.5 mg of benzil Control No 294170 in 300 mg of KBr recorded against a KBr disc.
Instrument: Perkin-Elmer 1600 FTIR.

Purity

Melting point

A melting point determination was performed on a Mettler FP 81 according to the International Pharmacopoeia 3rd Ed. Vol. 1.

Melting point 95.9 °C (n=14, RSD=0.27%), at a heating rate of 1 °C/minute.

The same result was obtained for the previous batch of benzil ICRS 192170.

High performance liquid chromatography

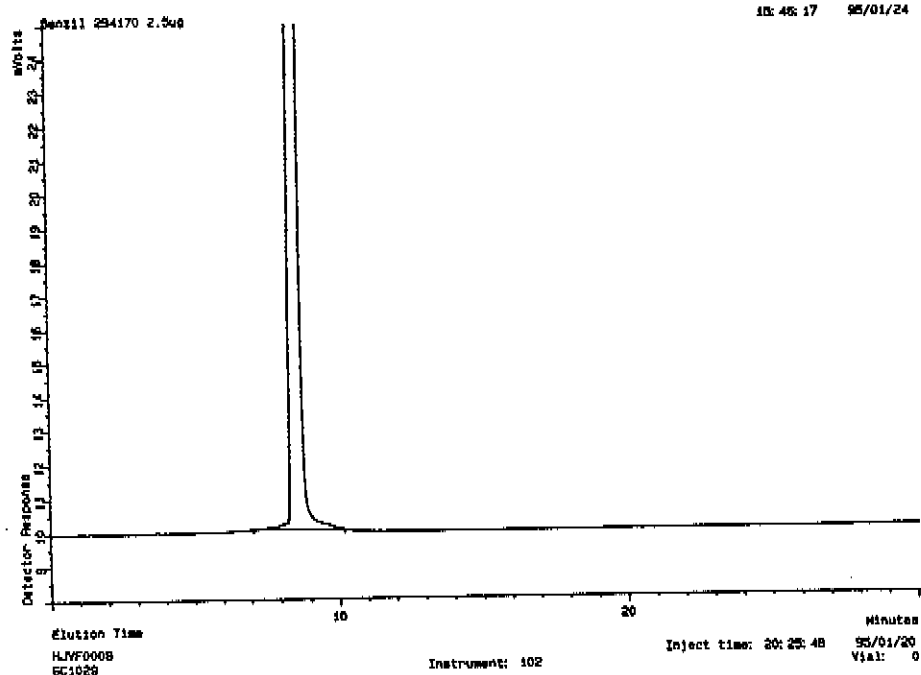


Figure 2. Chromatogram of benzil Control No 294170 monitored at 260 nm.

The following conditions were used:

Eluent: Acetonitrile: water (60:40)
Column: Brownlee Labs RP -18 OD-5A (250 x 4,6 mm, 5 µm)
Detector: Varian Polychrom 9065 operated at 260 and 210 nm
Pump: Varian 9012 operated at a flow rate of 1ml/min
Integrator: PeakPro (Beckman)
Sample: 0,13 mg dissolved in 1 ml of the eluent.
20 µl corresponding to 2,6 µg were injected.

No impurity peaks were detected by the above described liquid chromatographic system.

Minimum detectable quantity: 0.4 ng

Limit of quantification: 1.2 ng

Total solid impurities

Differential scanning calorimetry (DSC)

The amount of total solid impurities was estimated to about 0.24mol % (n=13, RSD=0.05%) determined by DSC-analysis. The determination was performed on 3 mg using a heating rate of 1 and 2 °C per minute.

Melting temperature(T_M): 94.1 °C

Onset: 93.5 °C

Instrument: Perkin Elmer DSC7 Differential Scanning Calorimeter

The same result was obtained for the previous batch ICRS 192170.

Data given by the manufacturer

Identification IR: Conforms

Assay: 99.9%

Melting point: 94 °C

Stability

No special stability studies were performed as it was considered that this substance, based on the experience of the stability of the previous lot, was stable and showed no signs of degradation when stored for 30 years at + 20 °C.

Conclusion

Benzil, Control No 294170, can be considered suitable as WHO Melting Point Reference Substance for the intended purpose with the melting point set to 96 °C.

APPENDIX 10

CALCIUM FOLINATE

(LEUCOVORIN CALCIUM)

Control No 194188

Analytical Report

Intended use

The monograph for Calcium folinate in the International Pharmacopoeia 3rd Ed. Vol. 3 requires a reference substance of calcium folinate to be used in the infrared spectrophotometric test for identity and in the liquid chromatographic assay.

Material

About 26 g of the sample were received at the WHO Centre in October 1992. The material is being stored in tightly closed containers at + 5 °C, protected from light.

CAUTION: As calcium folinate is a cytotoxic drug it should be handled with care. Avoid contact with the skin and inhalation of airborne particles.

Analytical data

Description

A light beige powder.

Evidence of chemical structure

Infrared spectrum

An infrared spectrum is given in Figure 1 (No W 194188T). The spectrum is concordant with the spectrum of the United States Pharmacopoeia reference standard (USPRS) lot I-1 for leucovorin calcium.

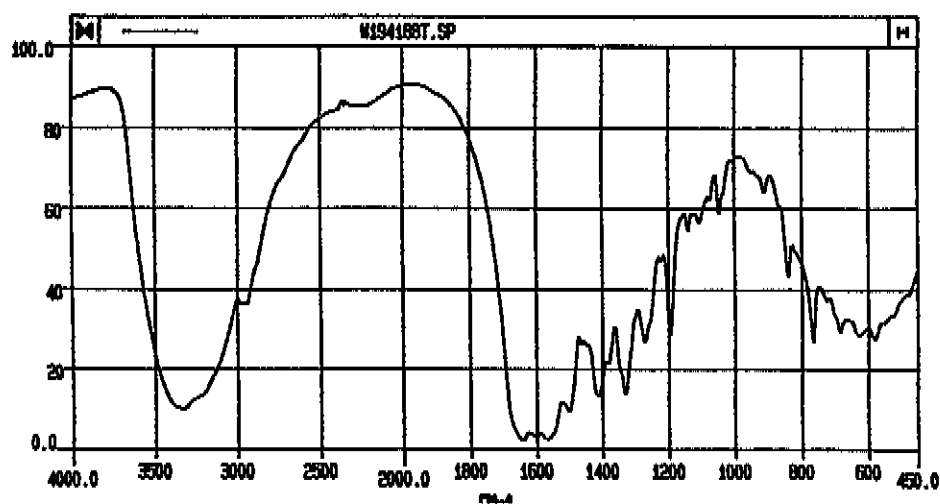


Figure 1. IR-spectrum of 1.3 mg of calcium folinate Control No 194188 in 300 mg KBr recorded against a KBr disc.
Instrument: Perkin-Elmer 1600 FTIR.

UV-spectrum

A UV-spectrum in 0.1 M phosphate buffer pH 7, according to Analytical Profiles Vol. 8, was recorded on a Varian Cary 5 spectrophotometer.

UV-maxima were observed at 220nm and at 287nm.

$A_{1cm}^{1\%} = 606$ at 287 nm (n=7, RSD =1.3 %)

$A_{1cm}^{1\%} = 673$ at 220 nm (n=7, RSD=1.3 %)

Calculations were performed with reference to the dried substance.

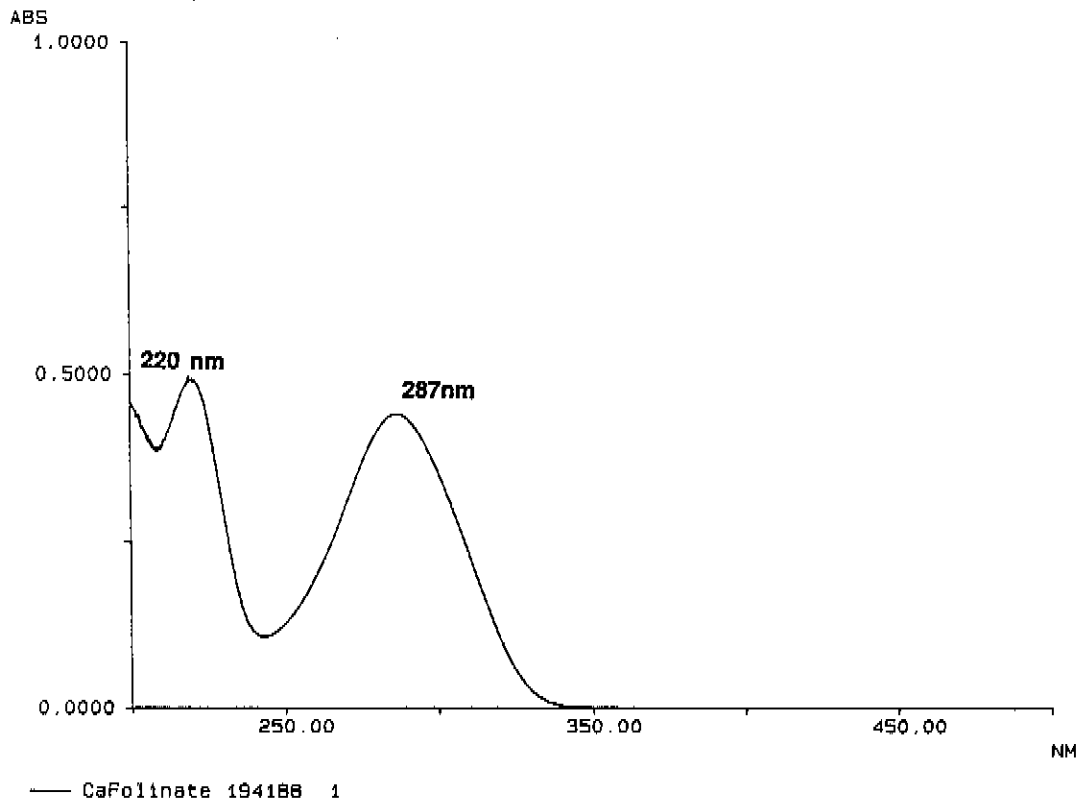


Figure 2. UV-spectrum of calcium folinate control No 194188. 10 µg / ml in 0.1M phosphate buffer pH = 7.

Specific optical rotation

$[\alpha]_D^{20} = + 17.8^\circ$ (n=2). The determination was performed in water at a concentration of 10 mg/ml. The result was calculated with reference to the dried substance.

AssayLiquid chromatographic assay

101.1 % (n=7, RSD= 0.4%) when determined at 287 nm against the USPRS for leucovorin calcium lot I-1 regarded as 100 %. The results are calculated on the dried substances. Statistical calculation shows a significant difference between the proposed ICRS and the USPRS at the 95% confidence level using unpaired t-test.

Spectrophotometric assay

99.2 % (n=7, RSD =1.3 %). The determination was performed in water at 287 nm and calculations were performed with reference to the dried substance. The USPRS lot I-1 for leucovorin calcium was used as standard and regarded as 100 %. The difference between the proposed ICRS and the USPRS is not statistically significant at the 95% confidence level using unpaired t-test.

Thermogravimetric analysis

When the substance was heated to 180 °C, a loss of 13.5% (w/w) was observed.(n=3, RSD = 3.6%).

Instrument: Perkin Elmer TGA 7 Thermogravimetric analyzer
Sample weight: 4.0 mg
Heating program: step 1: 5 °C/min, then holding 110 °C for 100 minutes
step 2: 5 °C/min, then holding 150 °C for 200 minutes
step 3: 5 °C/min, then holding 180 °C for 500 minutes

The corresponding results for the USPRS lot I-1 was 15.0% (w/w), (n=3, RSD=3.1%).

Water

13.0% w/w (n = 3, RSD = 2.0%) determined by Karl Fischer titration. The corresponding value for the USPRS lot I-1 for leucovorin calcium was 14.7% (n = 2, RSD = 1.0%).

Organic volatile compounds

< 0.1 % .The test included ethanol, methanol, dichloromethane, chloroform, benzene, trichloroethylene and dioxan. Only traces ,possibly originating from ethanol were found .

Instrument: Hewlett Packard 5890 A
Column: HP-5(30 m x0.53 mm)
Carrier gas: Helium(10 ml/min)
Detector: FID
Injector temperature:200 °C
Detector temperature:200°C
Temperature program:40°C for 9 min.,40°C/min to 240°C and holding 240°C for 6 minutes.

Purity

High performance liquid chromatography

The total amount of impurities estimated by peak area measurement was about 0.2 %.The liquid chromatographic system according to the International Pharmacopoeia 3rd Ed. Vol. 3 was used. A chromatogram is shown in Figure 3.

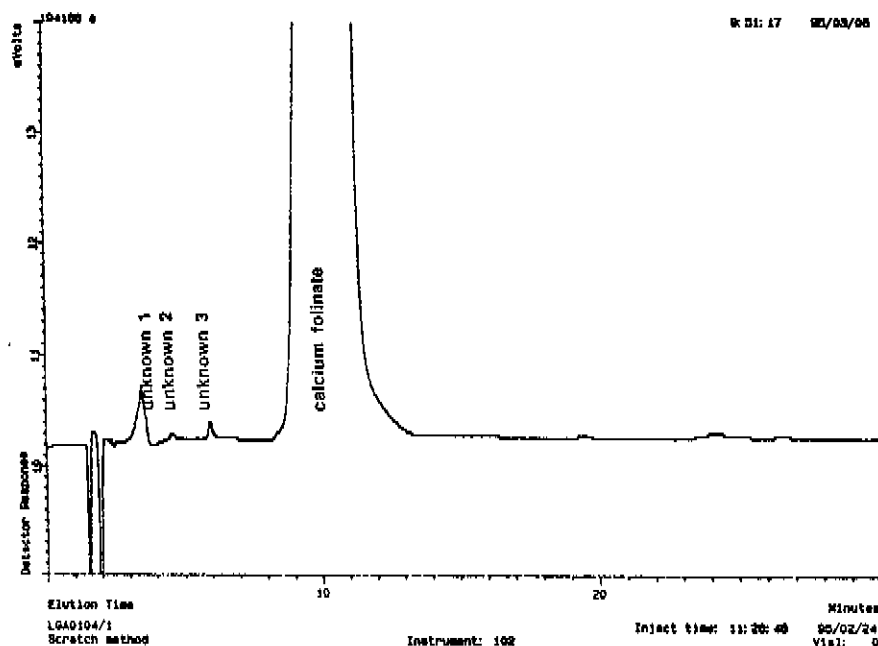


Figure 3. Chromatogram of calcium folinate Control No 194188 monitored at 220 nm.

At 287 nm one impurity was detected at about 3.5 min, estimated to less than 0.2% by peak area normalization. At 220 nm two additional impurities were detected in addition to the above mentioned, eluting after about 4.5 min and 6.0 min respectively. They were estimated by peak area normalization to less than 0.1 %.

The limit of detection for calcium folinate was about 20 ng at both wavelengths. The limit of quantitation was about 100ng.

Eluent: 835 ml of water were mixed with 15 ml of 25% tetrabutylammonium hydroxide. 125 ml of acetonitrile were added. The pH-value of the solution was adjusted to 7.5 with a 275 g/l solution of sodium dihydrogenphosphate. Water was finally added to a total volume of 1000 ml

Column: Kromasil C18, 4.6 x 250 mm.

Detector: Varian Polychrom 9065 operated at 220 and 287 nm.

Pump: Varian 9012 operated at a flow rate of 1.5 ml/min.

Integrator: PeakPro (Beckman)

Sample: 7.5 mg dissolved in 15 ml of a buffer prepared as the eluent, but omitting the acetonitrile. 20 µl corresponding to 10 µg were injected.

Diode-array detection

The chromatographic system described above was used to record UV-spectra for the detected peaks. The spectra for the detected impurity peaks were almost similar to that recorded for the calcium folinate peak. This means that UV-maxima were found at 220 and 275 nm.

Stability

Regular re-examinations of this ICRS when stored in dry state will be performed. During method development the stability in solution was investigated. The results are given below.

Sample

Solution in HPLC buffer pH=7.5, 48h,
dark, +8°C

Solution in HPLC buffer pH=7.5, 48h,
labwindow, +25°C

Status, monitored by HPLC
no degradation

slight degradation (0.1%)

Conclusion

Calcium folinate, Control No 194188, can be considered suitable as International Chemical Reference Substance for the intended purpose. When used in assays the content of calcium folinate is taken to be 99.8 % calculated with reference to the dried substance which corresponds to 86.3 % on the " as is basis".

APPENDIX 11

ERYTHROMYCIN B

Control No 194186

Analytical Report

Intended use

The International Chemical Reference Substance for Erythromycin B is intended to be used in chromatographic purity and identity tests of erythromycin. The monograph for Erythromycin is given in the International Pharmacopoeia 3rd Ed. Vol.3.

Material

About 6 g of the sample (manufacturers batch no Lot 771-91) were received at the WHO Centre in April 1994. This batch is the same as the European Pharmacopoeia Chemical Reference Substance (EPCRS) lot 1. The material is being stored in tightly closed containers at + 5 °C, protected from light.

Analytical data

Description

Fine white powder.

Evidence of chemical structure

Infrared spectrum

An infrared spectrum is given in Figure 1 (No W 194186).

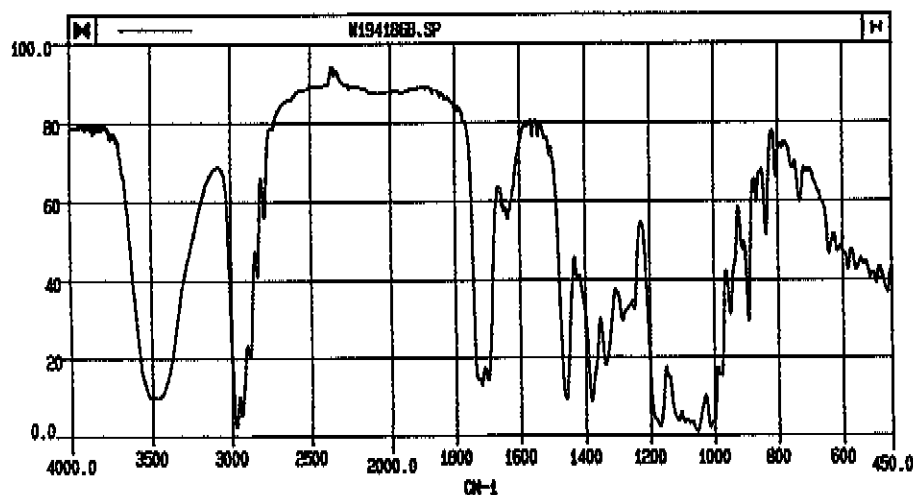


Figure 1. IR-spectrum of 2.6 mg of erythromycin B Control No 194186 in 300 mg KBr recorded against a KBr disc. Instrument: Perkin-Elmer 1600 FTIR.
Instrument: Perkin-Elmer 1600 FTIR.

Assay

Assay by titration: See manufacturer and collaborating laboratories.

Thermogravimetric analysis

When the substance was heated to 120 °C, a loss of 2.6% (n=4) was observed. No further loss was found with increasing temperature.

Instrument: Perkin Elmer TGA 7 Thermogravimetric analyzer.
Sample weight: 3 mg
Heating program: 5 °C/min from 20 - 120 °C and then holding 120 °C for 60 minutes to get loss on drying conditions and then further heating to 150 °C
Melting point: 198 -202 °C

Organic solvents

The content of organic solvents was tested by gas chromatography with the following conditions:

Instrument: Hewlett Packard 5890A
Column: HP-5 (30m x 0.53mm)
Carrier gas: Helium (10ml/min)
Detector: FID (range=1)
Injector temp.: 200 °C
Detector temp.: 200 °C
Temp. program: 40 °C for 9 min., 40 °C/min to 240 °C and holding 240 °C for 6 minutes

Two organic solvents were identified as acetonitrile and dichloromethane. Acetonitrile was estimated to 1.2% and dichloromethane to 0.4% by external standards.

Purity**Thin-layer chromatography**

The following thin-layer chromatographic system was used according to the system for erythromycin in the International Journal of Chromatography, 403 (1987) 343-349 and WHO/PHARM/92.558.

Thin-layer: Silica gel 60 F-254 (Merck)
Eluent: Diethyl ether: Methanol: Ammonia conc (90:9:2)
Sample: 100 µg of erythromycin B dissolved in methanol were applied.
Visualization: The thin-layer was treated with a solution of 0.15% xanthhydrol (w/v) in a mixture of conc. hydrochloric acid and acetic acid (90:7.5) and heating at 110-115 °C for 10 minutes.
Recommended from Chromatographic Society Bulletin 38 by Jan Hoogmartens et al.

Two faint secondary spots were detected visually at 365nm after spraying. The plate was also scanned at 530nm by densitometry. The impurities were estimated to be about 0.3%, with $R_f=0.1$ (0.1%) and $R_f=0.2$ (0.2%).

The detection limit of the system was about 0.08 µg (0.07%)

$R_f(\text{erythromycin B})=0.33$

$R_f(\text{erythromycin A})=0.25$

$R_f(\text{erythromycin C})=0.19$

TLC is not selective enough to separate erythromycin A from erythromycin B. However TLC can be used to complement the liquid chromatography.

High performance liquid chromatography

A liquid chromatographic system with UV detection performed with an alumina based CN-column, which gives a potentially greater separation efficiency compared to a C18-column, was used.

The total amount of impurities was estimated to be about 0.7% .

A chromatogram is shown in Figure 2.

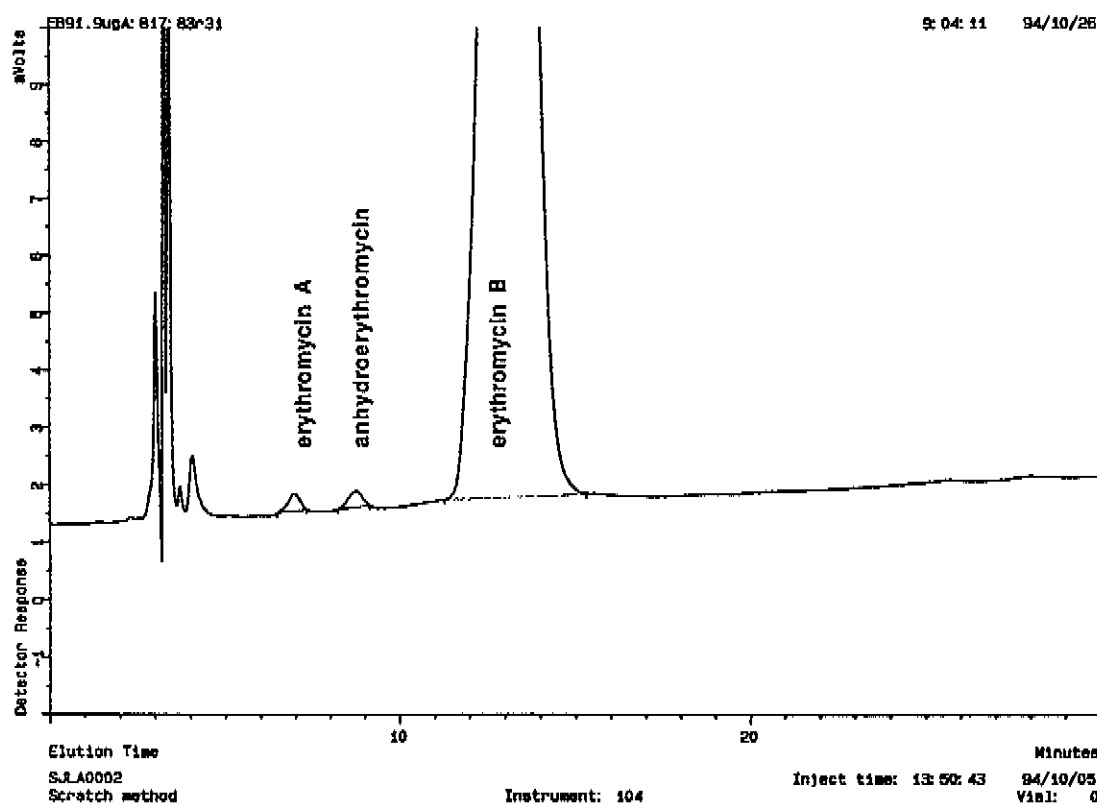


Figure 2. Chromatogram of erythromycin B Control No 194186 monitored at 215 nm.

The following conditions were used:

- Eluent: Acetonitrile/ Phosphate buffer pH 11 (17:83).
The buffer was prepared as follows: 2.7g KH_2PO_4 was dissolved in 1000 ml of water, pH adjusted to 11 with 10 M KOH.
- Column: Unisphere® - CN Alumina (4.6 x 250 mm) (Biotage)
- Detector: Waters Lambda-Max Model 481 LC Spectrophotometer operated at 215nm
- Pump: Waters 600 operated at a flow rate of 1ml/min
- Integrator: PeakPro (Beckman)
- Sample: 4.5mg/ml first dissolved in acetonitrile and thereafter buffer was added to obtain ACN/Buffer (30:70). 20 μl corresponding to 90 μg were injected.

NB: Erythromycin B is degraded in this eluent, why it is recommended to prepare fresh solutions.

As can be seen from figure 2 two possible impurities are observed. Erythromycin B is eluting at about 13 minutes. The two impurities were identified by external standards as erythromycin A eluting at about 7 minutes and anhydroerythromycin eluting at about 9 minutes. Erythromycin A was estimated to 0.29% and anhydroerythromycin was estimated to 0.35% by external standards. Erythromycin C was not found in the sample.

The detection limit for erythromycin B was 0.09 µg (0.1%).

The system peaks at about 3.5 minutes are rather high and probably due to the fact that the sample contains 1.2% acetonitrile and 0.4% dichloromethane (estimated by GC) and that the detection take place at 215 nm which is a universal wavelength.

Data given by the manufacturer

Identification (IR): The spectrum is consistent with the proposed structure
Moisture: 0.6%
Solvents: 0.4% Dichloromethane; 1.2% Acetonitrile
Impurities by HPLC: 1.1% "as is" (100% peak area percent)

The following test results were not directly used for the purity assignment but are included for information only.

Assay by HPLC: 98.9% "as is"
Assay by titration: 97.0% "as is"
Loss on Drying: 2.1%
Assigned Purity: 96.3% (based on correcting for HPLC-impurities, moisture and solvents)

Data given by collaborating laboratories

EPCRS lot 1 is from the same batch as ICRS 194186
Identity (TLC): complies
Related substances(HPLC): 0.2 % EP Lab, 0.7 % mean of collaborative study
Water content: 1.4 %
Assay (titration): 97.3 %
IR spectrum: complies
GC residual solvents :1.3%
Assigned value: 96.7 % of Erythromycin B

Stability

Stability studies have not been made, but regular re-examinations of the ICRS will be performed.

Conclusion

Erythromycin B, Control No 194186, can be considered suitable as International Chemical Reference Substance for the intended purpose. The value of 96.7% Erythromycin B can be set determined as 100 % -(water+solvents+impurities by HPLC).

APPENDIX 12

ERYTHROMYCIN C

Control No 194187

Analytical Report

Intended use

The International Chemical Reference Substance for Erythromycin C is intended to be used in chromatographic purity and identity tests of erythromycin. The monograph for Erythromycin is given in the International Pharmacopoeia 3rd Ed. Vol.3.

Material

About 5 g of the sample (manufacturers batch no lot 87-005-IQ) were received at the WHO Centre in April 1994. This batch is the same as the European Pharmacopoeia Chemical Reference Substance (EPCRS) lot 1. The material is being stored in tightly closed containers at + 5 °C, protected from light.

Analytical data

Description

White crystalline powder.

Evidence of chemical structure

Infrared spectrum

An infrared spectrum is given in Figure 1 (No W 194187). The spectrum is concordant with a spectrum from a previous lot of erythromycin C reference substance (lot 850-3).

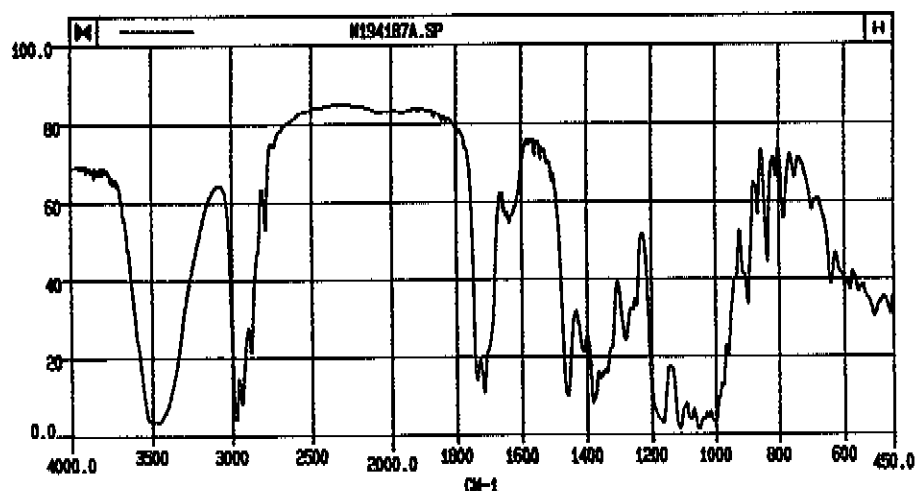


Figure 1. IR-spectrum of 2.6 mg of erythromycin C Control No 194187 in 300 mg KBr recorded against a KBr disc. Instrument: Perkin-Elmer 1600 FTIR.

Assay

Assay by titration: See manufacturer and collaborating laboratories.

Thermogravimetric analysis

When the substance was heated to 120 °C, a loss of 1.5%(n=4) was observed. No further loss was found with increasing temperature.

Instrument: Perkin Elmer TGA 7 Thermogravimetric analyzer.
Sample weight: 3.5 mg
Heating program: 5 °C/min from 20 °C and then holding 120 °C for 60 minutes to get loss on drying conditions and then further heating to 150 °C.
Melting point: 199 - 204 °C

Purity

Thin-layer chromatography

The following thin-layer system was used according to the system for Erythromycin in the International Journal of Chromatography, 403 (1987) 343-349 and WHO/PHARM/92.558.

Thin-layer: Silica gel 60 F-254 (Merck)
Eluent: Diethyl ether:Methanol:Ammonia conc (90:9:2)
Sample: 100 µg of erythromycin C dissolved in methanol were applied.
Visualization: The thin-layer was treated with a solution of 0.15% xanthhydrol (w/v) in a mixture of conc. hydrochloric acid and acetic acid (90:7.5) and heating at 110-115 °C for 10 minutes.
Recommended from Chromatographic Society Bulletin 38 by Jan Hoogmartens et al.

Three faint secondary spots were detected visually at 365nm after spraying. The plate was also scanned at 530 nm by densitometry. The impurities were estimated to be about 0.3%, with $R_f=0.06$ (0.1%); $R_f=0.1$ (0.1%) and $R_f=0.3$ (0.1%).

The detection limit of the system was about 0.05 µg (0.05%).

$R_f(\text{erythromycin C})=0.19$

$R_f(\text{erythromycin A})=0.25$

$R_f(\text{erythromycin B})=0.33$

TLC is not selective enough to separate erythromycin A from erythromycin B. However TLC can be used to complement the liquid chromatography.

High performance liquid chromatography

A liquid chromatographic system with UV detection performed with an alumina based CN-column, which gives a potentially greater separation efficiency compared to a C18-column, was used. The total amount of impurities were estimated to be about 0.5 %.

A chromatogram is shown in Figure 2.

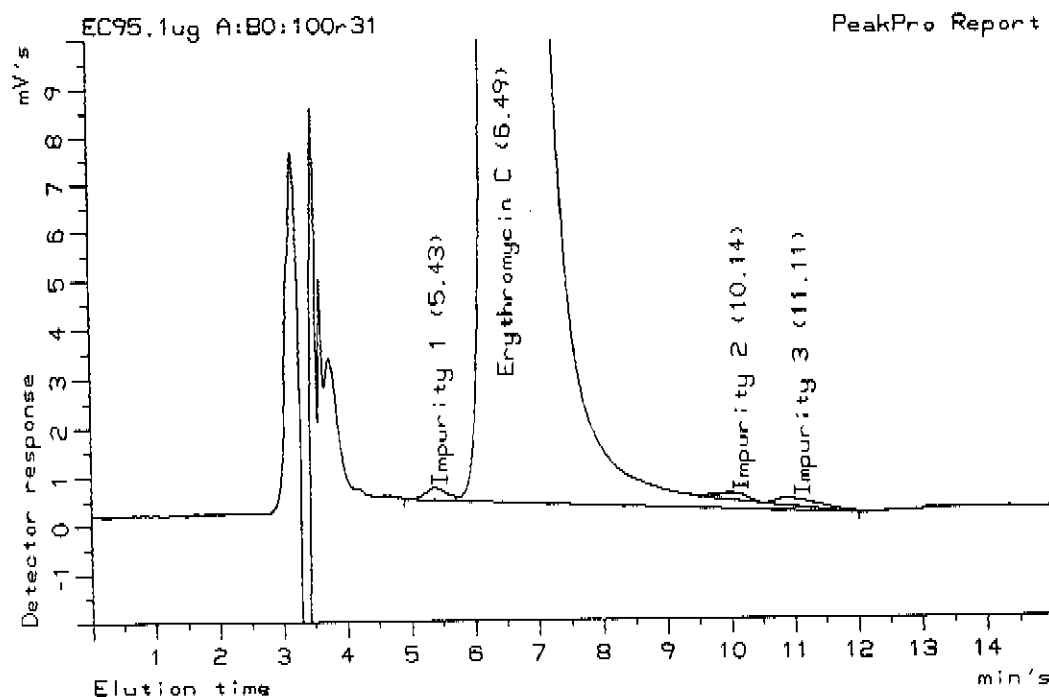


Figure 2. Chromatogram of erythromycin C Control No 194187 monitored at 215 nm.

The following conditions were used:

Eluent: Phosphate buffer pH 11.
The buffer was prepared as follows: 2.7g KH₂PO₄ was dissolved in 1000 ml of water, pH adjusted to 11 with 10 M KOH.

Column: Unisphere® - CN Alumina (4.6 x 250 mm) (Biotage)

Detector: Waters, Lambda-Max Model 481 LC Spectrophotometer operated at 215nm.

Pump: Waters 600 operated at a flow rate of 1ml/min.

Integrator: PeakPro (Beckman)

Sample: 4.5mg/ml first dissolved in acetonitrile and thereafter buffer was added to obtain ACN/Buffer (30:70). 20 µl corresponding to 90 µg were injected.

NB: Erythromycin C is degraded in this eluent, why it is recommended to prepare fresh solutions.

The detection limit for erythromycin C was 0.09 µg (0.1%).

As can be seen from figure 2 three possible impurities are observed.

Erythromycin C is eluting at about 6.5 minutes and the impurities at about 5.4, 10.1 and 11.1 minutes. The impurities were estimated to be about 0.5% by peak area normalization.

Erythromycin B was not found in the sample, when using acetonitrile/ phosphate buffer pH 11 (17:83) as the eluent.

The system peaks at about 3.5 minutes are rather high and probably due to the fact that the sample is not dissolved in the eluent and that the detection take place at 215 nm which is a universal wavelength.

Data given by the manufacturer

| | |
|---------------------------|--|
| Identification (IR): | The spectrum is consistent with the proposed structure and compares to the previous RS (lot 850-3) |
| NMR: | Spectra are consistent with the proposed structure |
| Loss on drying: | 0.4% |
| Moisture by Karl Fischer: | 1.7 % |
| Residue on ignition: | 0.03% |
| Residual solvents by GC: | 0.01% Acetonitrile; 0.23% dichloromethane (EtOH, EtOAc and Acetone : none) |
| Assay (HPLC): | 99.5% as is |
| Bioassay vs. Ery A USP: | 443 mcg/mg |
| TLC Impurities: | 0.5% |
| Assay by titration: | 98.5% as is |
| Assigned purity: | 97.5% |

Data given by collaborating laboratories

| | |
|---|---|
| EPCRS lot 1 is from the same batch as ICRS 194187 | |
| Identity(TLC): | Complies |
| Related substances(HPLC): | 0.86 % EP lab.0.7 % mean of collaborative study |
| Water content: | 1.7% |
| Assay by titration: | 97.7% EP lab. |
| IR spectrum : | Complies |
| GC ,residual solvents: | 0.003% |
| Assigned value: | 97.7 % of Erythromycin C |

Stability

Stability studies have not been made, but regular re-examinations of the ICRS will be performed.

Conclusion

Erythromycin C, Control No 194187, can be considered suitable as International Chemical Reference Substance for the intended purpose. The value of 98 % Erythromycin C can be set determined as 100 % -(water + solvents + impurities by HPLC).

APPENDIX 13

GENTAMICIN SULFATE

Control No 194183

Analytical Report

Intended use

The monograph for Gentamicin sulfate in the International Pharmacopoeia 3rd Ed. Vol. 3 requires a reference substance of gentamicin sulfate to be used in the thin-layer chromatographic test for identity.

Material

About 20 g of the sample (manufacturers batch no GENTA 422/R) was received at the WHO Centre in February 1993. The material is being stored in tightly closed containers at + 5 °C, protected from light. This ICRS is of the same origin as the European Pharmacopoeia Chemical Reference Substance (EPCRS) batch 2 and the 2nd International Biological Standard for gentamicin.

Analytical data

Description

A white powder.

Evidence of chemical structure

Infrared spectrum

An infrared spectrum is given in Figure 1 (No W 194183). The spectrum is concordant with the spectrum of the United States Pharmacopoeia Reference Standard (USPRS) lot J.

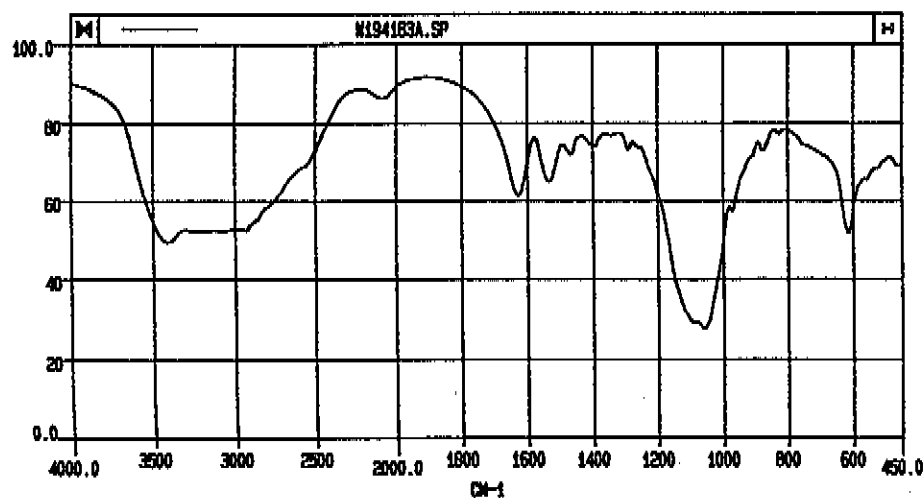


Figure 1. IR-spectrum of 1.6 mg of gentamicin sulfate Control No 194183 in 300 mg KBr recorded against a KBr disc.
Instrument: Perkin-Elmer 1600 FTIR.

Thin-layer chromatography

See purity tests.

High performance liquid chromatography with mass-spectrometric detection

Samples of gentamicin sulfate ICRS 194183 run with MS-detection show presence of C₁ (MW = 477.6), C_{1a} (MW = 449.5) and C₂/C_{2a} (MW = 463.6).

Eluent: 0.11 M TFA (pH 3.5 with ammoniumhydroxide) : methanol (95:5) 1 ml/minute
Column: BondClone C18 (4.6x300 mm, 10µm)
Detector: Finnigan MAT SSQ 7000, operating in ESI mode

UV-spectrum

There was no significant absorbance of a 0.6 mg/ml solution in water in the range 200 nm to 500 nm. $A_{1\text{cm}}^{1\%}$ is about 1 at 200 nm.

Specific optical rotation

$[\alpha]_D^{20^\circ} = +120.0^\circ$ calculated on the dried substance (limits + 107° to + 121°).

Sulfate

32.8% sulfate calculated on dried substance for the ICRS and 31.9% for the EPCRS, analyzed by ion chromatography.

Instrument: Dionex 2000i
Column: Dionex HPIC AS4A
Eluent: Na₂CO₃/NaHCO₃ 1.8/1.7 mM

AssayLiquid chromatographic assay

It is difficult to perform an assay for a substance consisting of at least four components. However this attempt was done by assuming that the sum of the four gentamicin components (C₁, C_{1a}, C_{2a} and C₂) is a measure of the gentamicin concentration (Journal of Chromatography 389 (1987) 306-311). By comparing the proposed ICRS with EPCRS batch 2 and USPRS lot J in this way it was found that the proposed ICRS and EPCRS were equal in area count and the USPRS obtained about 3 % higher value.

Microbiological assay

626 IU/mg "as is" with limits of error 96-104%. The EPCRS batch 2, 616 IU/mg "as is" was used as standard.

Thermogravimetric analysis

When the substance was heated to 110°C, a loss of 10.9% of weight was observed (n=7, RSD=2.0%). It is important to check the water content at the actual analysis occasion as the substance is hygroscopic.

Instrument: Perkin Elmer TGA 7 Thermogravimetric analyzer
Sample weight: 6 mg
Heating rate: 5 °C/min to 110 °C and thereafter holding 110 °C for 3 hours
Melting point: Decomposition above 220 °C

Water: 8.4 % (n=2) determined by Karl Fischer titration. As the substance is hygroscopic the content of water can vary depending on when the sample is analyzed. Values from 4.7 % (manufacturer) to 9.5 % (collaborating laboratory) have been reported.

Organic volatile compounds

About 0.04% methanol.

Instrument: Hewlett Packard 5890A
Column: HP-5 (30m x 0.53mm)
Carrier gas: Helium (10ml/min)
Detector: FID
Injector temperature: 200 °C
Detector temperature: 200 °C
Temperature program: 40 °C for 9 min., 40 °C/min to 240 °C and holding 240 °C for 6 minutes

Purity

Thin-layer chromatography

Three principal spots and two secondary spots were detected. The following thin-layer chromatographic system according to the International Pharmacopoeia 3rd Ed. Vol. 3 was used.

Thin-layer: Silica gel 60 TLC (Merck) and Silica gel 60 HPTLC (Merck)
Eluent: Chloroform : methanol : water (1:1:1), allow to separate and use the lower layer.
Sample: 20 and 100 µg of gentamicin sulfate dissolved in water were applied.
Visualization: Visualization in day-light and scanning with a Desaga CD60 densitometer after spraying with triketohydrindene 250mg/100ml in pyridine : acetone (1:1).

Three main spots were clearly detected in the proposed ICRS after spraying, corresponding to the three main spots in the USPRS lot J and in the EPCRS batch 2.

| | TLC | HPTLC |
|--|------|-------|
| R _f value (gentamicin sulfate spot 1) | 0.08 | 0.13 |
| R _f value (gentamicin sulfate spot 2) | 0.12 | 0.18 |
| R _f value (gentamicin sulfate spot 3) | 0.15 | 0.22 |

Two additional secondary spots were detected after spraying. One rather big spot stayed at the application area and additionally one very weak spot was detected at R_f = 0.05.

The same results were obtained for the EPCRS batch 2, but the USPRS lot J had a weaker spot at the application area and no extra spot at R_f = 0.05.

The spot at the application area corresponds to about 9.5 % of the total area for ICRS and EPCRS and about 5.4% for USPRS. The spot at R_f = 0.05 corresponds to about 0.5 % for ICRS and EPCRS.

The detection limit of the system was about 0.5 µg (0.5%) after spraying.

An attempt was made to use a silica gel 60 F-254 plate and scan the plate at 254 nm before spraying, however the result was less successful than with the sprayed plate.

High performance liquid chromatography

A liquid chromatographic system with pre-column derivatization according to the European Pharmacopoeia was used.

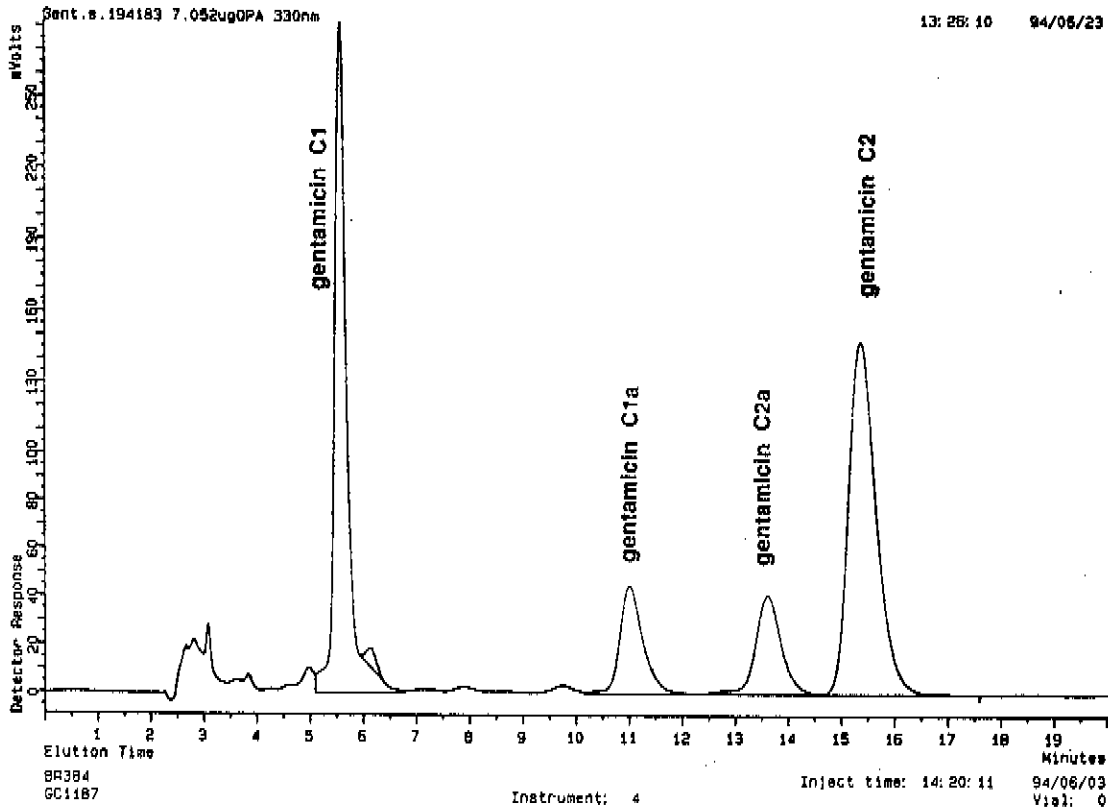


Figure 2. Chromatogram of gentamicin sulfate Control No 194183 monitored with precolumnderivatization.

The following conditions were used:

- Reagent: Dissolve 2.47 g of boric acid in 75 ml water, adjust pH to 10.4 using a 45 % potassium hydroxide and dilute to 100 ml. Dissolve 1.0 g of o-phtalaldehyde in 5 ml methanol, add 95 ml of the boric acid solution and 2 ml of thioglycolic acid and adjust pH to 10.4.
- Derivatization: Dissolve 10 mg of gentamicin sulfate in 10 ml water, add 5 ml methanol and 4 ml o-phtalaldehyde reagent. Mix and dilute to 25 ml with methanol. Heat in a water-bath at 60 °C for 15 minutes, cool to roomtemperature and use immediately.
- Eluent: Methanol : 5.5 g heptanesulfonate in 50 ml glacial acetic acid and 250 ml water (70:30).
- Column: Brownlee Labs RP18 OD -5A
- Sample: See derivatization.
- Detector: Waters Lambda Max model 480 operated at 330 nm.
- Integrator: PeakPro (Beckman)

Purity calculations of all peaks (normalized areas) in the above liquid chromatographic system give the following result:

| | ICRS | EPCRS | USPRS |
|----------------------------|---------|---------|---------|
| Gentamicin C ₁ | 34.5 | 34.5 | 37.6 |
| Gentamicin C _{1a} | 10.4 | 10.5 | 20.6 |
| Gentamicin C _{2a} | 10.7 | 10.7 | 10.3 |
| Gentamicin C ₂ | 40.2 | 39.9 | 29.1 |
| Sum of impurity peaks | 4.2 | 4.4 | 2.4 |
| | 100.0 % | 100.0 % | 100.0 % |

There are about twelve impurity peaks in the chromatograms. The total amount of impurity was estimated to about 4.2% for the proposed ICRS 194183. The corresponding figure for gentamicin sulfate EPCRS batch 2 was 4.4% and for the USPRS lot J 2.4%. However, as no impurity reference substance was available, this figure can only be used as a comparison of the quality between the three substances. It is probably not possible to perform a correct peak area normalization using precolumn derivatized samples.

Gentamicins

As can be seen from figure 2 there are four peaks corresponding to the four components gentamicin C₁, C_{1a}, C_{2a} respectively C₂. The peaks eluting before 4 minutes originates from the blank. The gentamicin C₁ peak is not properly separated from some smaller peaks with close retention times.

| Gentamicin sulfate | comp. C ₁ | comp. C _{1a} | comp. C _{2a} | comp. C ₂ | n | RSD (%) |
|--------------------|----------------------|-----------------------|-----------------------|----------------------|----|---------|
| ICRS 194183 | 35.6% | 11.1% | 10.8% | 41.1% | 16 | 1.6 |
| EPCRS Batch 2 | 36.0% | 11.4% | 10.9% | 40.8% | 8 | 1.6 |
| USPRS Lot J | 38.5% | 21.3% | 10.5% | 29.7% | 8 | 0.6 |

Data given by the manufacturer

| | |
|------------------------------------|--|
| Description: | Corresponding |
| Solubility: | Corresponding |
| Identification IR: | Corresponding |
| TLC: | Corresponding |
| Sulfates: | Corresponding |
| Assay: | 630 µg/mg (as gentamicin on dry basis) |
| pH: | 4.0 |
| Specific optical rotation: | + 116.3° (on dry basis) |
| Sulfate: | 32.3% (on dry basis) |
| Sulfated ash: | 0.2% |
| Water (K. Fischer): | 4.7% |
| Methanol: | 0.65% |
| Appearance of solution: | Corresponding |
| Abnormal toxicity: | Non toxic |
| Pyrogens: | Pyrogen-free |
| Loss on drying: | 4.4% |
| Content of Gentamicins (HPLC) | |
| C ₁ : | 36.3% |
| C _{1a} : | 11.1% |
| C _{2a} + C ₂ : | 52.6% |

Stability

Stability studies were not performed on this substance. It is already documented that gentamicin sulfate is hygroscopic and is degrading at humid atmosphere even in the absence of light. The decomposition being faster at higher temperatures (WHO/PHARM/86.529). Regular reexaminations of the ICRS will be performed.

Conclusion

Gentamicin sulfate, Control No 194183, can be considered suitable as International Chemical Reference Substance for the intended purpose.

HYDROCORTISONE SODIUM SUCCINATE

Control No 194184

Analytical Report

Intended use

The monograph for Hydrocortisone sodium succinate in the International Pharmacopoeia 3rd Ed. Vol. 3 requires a reference substance of hydrocortisone sodium succinate to be used in the infrared spectrophotometric test and in the thin-layer chromatographic test for identity as well as in the spectrophotometric assay.

Material

About 50 g of the sample (manufacturers batch no 395KR) were received at the WHO Centre in March 1994. The material is being stored in tightly closed containers at + 5 °C, protected from light.

Analytical data

Description

A white powder.

Evidence of chemical structure

Infrared spectrum

An infrared spectrum is given in Figure 1 (No W194184T). The spectrum is concordant with the spectrum of the British Pharmacopoeia Chemical Reference Substance (BPCRS) lot 1669.

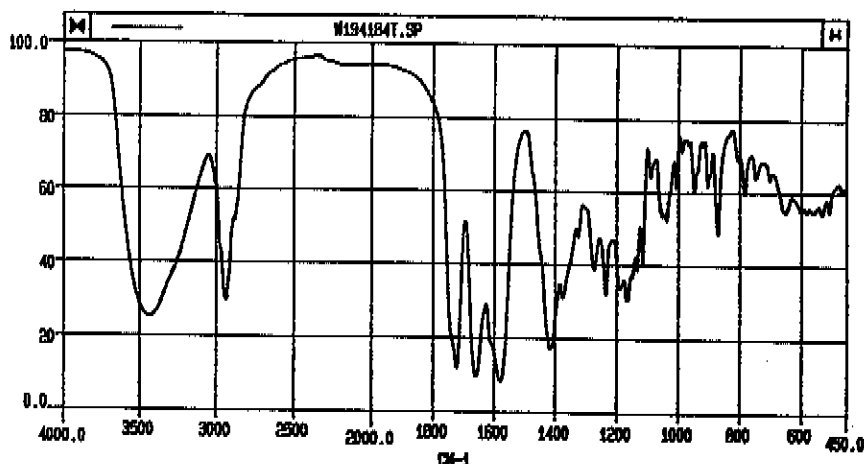


Figure 1. IR-spectrum of 1.5 mg of hydrocortisone sodium succinate Control No 194184 in 300 mg KBr recorded against a KBr disc.
Instrument: Perkin-Elmer 1600 FTIR.

Specific optical rotation

$[\alpha]_D^{20} = +147$ (n=10, RSD=0.6 %) calculated with reference to the dried substance. The determination was performed in ethanol at a concentration of 10 mg/ml.

UV-spectrum

A UV-spectrum in water was recorded on a Varian Cary 5 spectrophotometer. The spectrum is given in Figure 2. A maximum was observed at 248 nm.

$A_{1cm}^{1\%} = 335$ calculated with reference to the dried substance (n=7, RSD=1.5%).

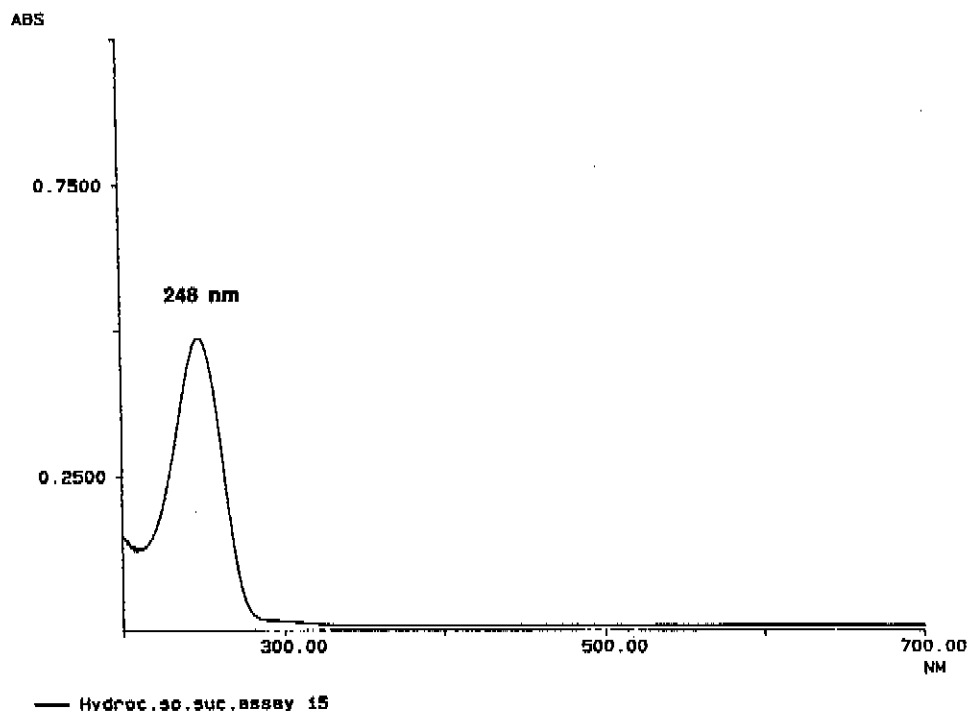


Figure 2. UV-spectrum of hydrocortisone sodium succinate Control No 194184, 11 µg/ml in water.

Sodium

The sodium content was determined to 4.7% (calculated on dried substance) by potentiometric titration according to the International Pharmacopoeia 3rd Ed. Vol. 3.

Assay

Liquid chromatographic assay

99.8 % (n=14, RSD=2.1 %) calculated with reference to the dried substance. The determination was performed against the BPCRS lot 1669 regarded as 100%. The difference between the proposed ICRS and the BPCRS is not statistically significant at the 95% confidence level using unpaired t-test. The determination was performed with the liquid chromatographic method described below under purity.

Spectrophotometric assay

101.2 % (n=7, RSD= 1.5 %) calculated with reference to the dried substance. The BPCRS lot 1669 was used as standard and regarded as 100 %. The difference is not statistically significant at the 95% confidence level using unpaired t-test. The determination was performed according to the International Pharmacopoeia 3rd Ed. Vol. 3.

Thermogravimetric analysis

When the substance was heated to 110°C, a loss of 3.6% (w/w) was observed.

N.B. The substance is very hygroscopic and therefore difficult to weigh accurately. To obtain secure values of the water content, for example when performing an assay, it is recommended to first let the substance be equilibrated with the actual relative humidity for 24 hours at the analysis occasion. After that the analysis and the water determination can be performed. See also stability. After this procedure at 70 % RH, a loss of 12.9 % (w/w) was observed.

Instrument: Perkin Elmer TGA 7 Thermogravimetric analyzer.
Sample weight: 4 mg
Heating rate: 5 °C/min, then holding 110 °C for 120 minutes.
Melting point: about 169-171 °C

The corresponding result for BPCRS lot 1669 was 4.5%.

Water

12.9 % determined by Karl Fischer titration after equilibration of the substance at 70 % RH. Compare thermogravimetric analysis.

Organic volatile compounds

About 0.1 % unknown.

Instrument: Hewlett Packard 5890A
Column: HP-5 (30m x 0.53mm)
Carrier gas: Helium (10ml/min)
Detector: FID
Injector temperature: 200°C
Detector temperature: 200°C
Temperature program: 40°C for 9 min., 40°C/min to 240°C and holding 240°C for 6 minutes

PurityThin-layer chromatography

The total amount of impurities was estimated to about 2.3 % by densitometry at 254 nm. The main spot tails and has $R_f=0.12$. Four extra spots were observed at $R_f=0.20$ (1.7%), $R_f=0.41$ (0.4%), $R_f=0.49$ (0.1%) and $R_f=0.55$ (0.1%). The following thin-layer chromatographic system according to the International Pharmacopoeia 3rd Ed. Vol. 3 was used.

Thin-layer: Silica gel 60 F-254 TLC (Merck) and silica gel 60 F-254 HPTLC (Merck)
Eluent: Dichloromethane:ether:methanol:water(77:15:8:1.2)
Sample: 20 and 100 µg of hydrocortisone sodium succinate dissolved in methanol were applied.
Detection: Scanning by Desaga CD60 densitometer at 254 nm (before spraying)
Visualization: Visualization in day-light after spraying with blue tetrazolium (2 mg/ml) and sodium hydroxide (120 mg/ml methanol) mixed 1:3

Only one secondary spot was detected after spraying with $R_f = 0.2$. The detection limit of the system was about 0.1 µg before spraying when examining at 254 nm and 0.1 µg after spraying.

Similar results were obtained for the BPCRS lot 1669.

High performance liquid chromatography

The total amount of impurities estimated by peak area measurement was 1.9 %. The following liquid chromatographic system according to Dix Smith, Journal of Chromatography, 164, 1979 page 129 was used:

- Eluent: Acetonitrile: 2.9 %(v/v) glacial acetic acid, pH=2.5 (30:70). pH=2.7 in the mixture.
- Column: Brownlee Labs RP -18 OD -5A (250x4.6 mm, 5µm)
- Detector: Waters Lambda-Max Model 481 operated at 250 nm and Varian 9065 Diode array
- Pump: Waters 600E and Varian 9012 operated at a flow rate of 1ml/min.
- Integrator: PeakPro (Beckman)
- Sample: 1 mg/ml dissolved in the eluent.

As can be seen from figure 3, two major impurities can be observed at 8.5 respectively 10 minutes, further nine small impurities can be detected. The peak eluting at 8.5 minutes corresponds to hydrocortisone, and is estimated to be 0.69% when compared to a hydrocortisone standard. All the other impurity peaks are of unknown identity. The impurity peak eluting at 10 minutes is estimated to 0.83% and the 9 smaller peaks together correspond to about 0.35% by peak area normalization.

The HPLC system is linear in the range 2ng-20µg and the limit of quantification was found to be 2 ng for hydrocortisone sodium succinate.

When the test solutions were stored at room temperature for 6 days, the peak eluting at 12.8 minutes is increasing from 0.06% to 0.18% and the hydrocortisone peak is increasing from 1.04% to 1.31%.

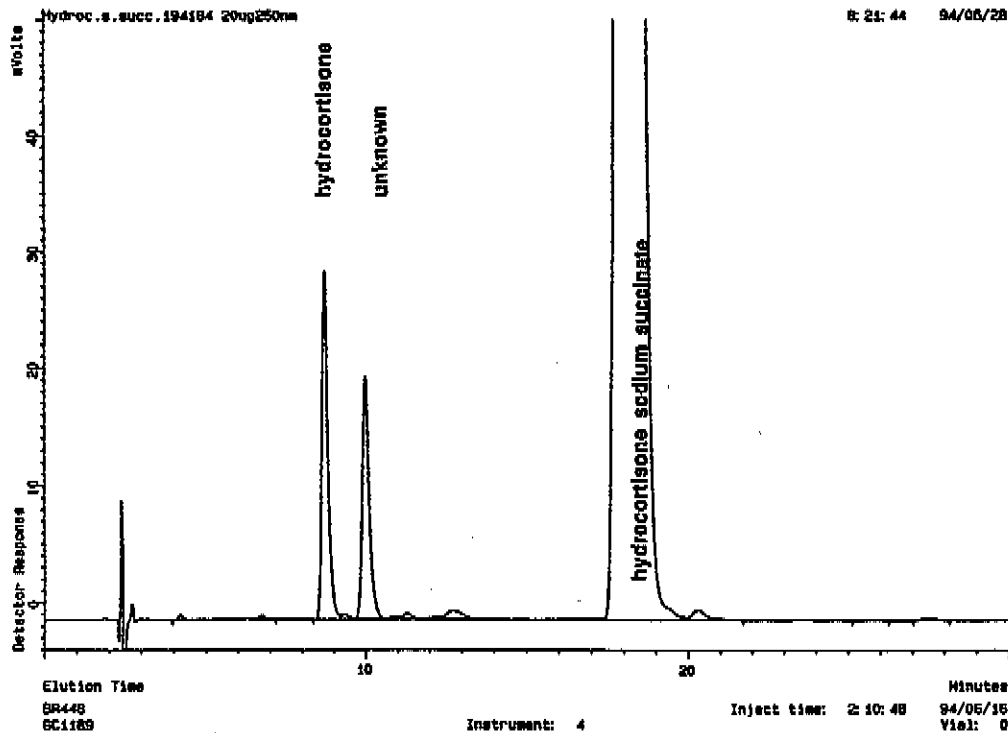


Figure 3. Chromatogram of hydrocortisone sodium succinate Control No 194184 monitored at 250 nm.

Diode-array detection

The spectra of the main peak and the six largest impurity peaks have very similar shape in the wavelength range 220-267 nm, with maxima at about 245 nm.

Data given by the manufacturer

| | |
|---------------------------|----------|
| Assay | 98.1 % |
| Description | conforms |
| Identity IR | conforms |
| Identification UV | conforms |
| Loss on drying | 0.9 % |
| Sodium content | 4.80 % |
| Specific optical rotation | 145° |

Stability

Hydrocortisone sodium succinate was exposed to air at different relative humidities at room temperature (about 20°C) for a period of 12 weeks as described in WHO/PHARM/82.509. Hydrocortisone sodium succinate is very hygroscopic, within one day, when stored at 98 % RH, the substance gains more than 50% in weight, which corresponds to a water content of about 36%. If the substance with this high content of water is exposed to lower relative humidity, it loses weight very rapidly. Hydrocortisone sodium succinate is extremely sensitive in both picking up and losing water. It is recommended to equilibrate the substance for 24 hours at the relative humidity that exists at the analysis occasion, before determining the water content and weighing the substance for the assay.

Results from the stability study is given below.

| | | | | | | |
|--------------------------------|--------|------|------|-------|-------|-------|
| Relative humidity of the air | silica | 11% | 22% | 55% | 76% | 98% |
| Water content of the substance | 3.2% | 5.4% | 6.8% | 15.1% | 19.6% | 36.0% |

The samples were investigated by HPLC after the 12 weeks storage at different humidities. For system see purity. The hydrocortisone peak and most of the other impurity peaks increase with increased RH value of storage. When analyzed the following results were obtained.

| | | | | | | |
|------------------------------|--------|-------|-------|-------|-------|-------|
| Relative humidity of the air | silica | 11% | 22% | 55% | 76% | 98% |
| HPLC purity | 97.5% | 97.2% | 96.9% | 93.3% | 92.4% | 89.7% |

A degradation was observed most pronounced at the highest relative humidities.

When stored at higher temperatures (50 °C and 70 °C) hydrocortisone sodium succinate is subject to decomposition as described in WHO/PHARM/86.529.

Regular reexaminations of the ICRS when stored at + 5 °C will be performed.

Conclusion

Hydrocortisone sodium succinate, Control No 194184, can be considered suitable as International Chemical Reference Substance for the intended purpose. The content of hydrocortisone sodium succinate when used in the spectrophotometric assay is taken to be 100 % calculated with reference to the dried substance. It is important to equilibrate the substance in the humidity at the analysis occasion and then determine the water content and perform the assay at the same time.

APPENDIX 15

LEVONORGESTREL

Control No 194182

Analytical Report

Intended use

The monograph for Levonorgestrel in the International Pharmacopoeia 3rd Ed. Vol. 3 requires a reference substance for levonorgestrel to be used in the infrared spectrophotometric and thin-layer chromatographic tests for identity and in the spectrophotometric assay.

Material

About 70 g of the sample (manufacturers batch no 33055945) were received at the WHO Centre in March 1994. The material is being stored in tightly closed containers at + 5 °C, protected from light.

Analytical data

Description

A white or almost white crystalline powder.

Evidence of chemical structure

Infrared spectrum

An infrared spectrum is given in Figure 1 (No W 194182). The spectrum is concordant with the spectrum of the French Pharmacopoeia Reference Substance (FPRS) lot 1 of levonorgestrel.

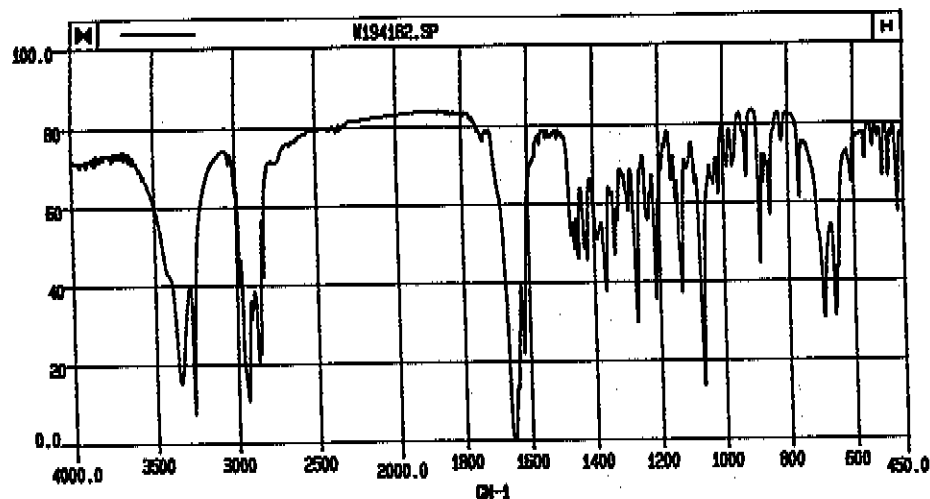


Figure 1. IR-spectrum of 1.30 mg of levonorgestrel Control No 194182 in 300 mg KBr recorded against a KBr disc.
Instrument: Perkin-Elmer, 1600 FTIR.

UV-spectrum

A UV-spectrum in methanol is given in Figure 2. λ max in methanol = 241nm.

$A_{1cm}^{1\%} = 553$ at 241 nm (n=6, RSD= 0.7%) .

The humidity content of the substance was negligible (<0.1%) .

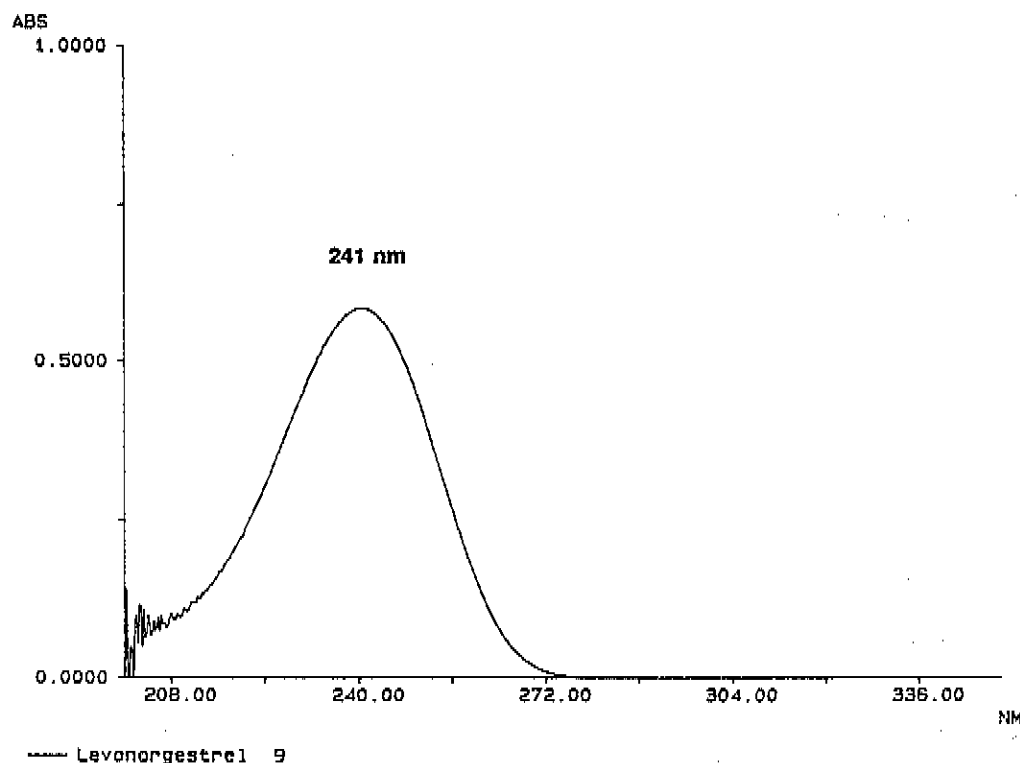


Figure 2. UV-pectrum of levonorgestrel Control No 194182 10 μ g /ml in methanol .

Assay

Spectrophotometric assay

100.2% (n=6, RSD= 0.7%) determined in methanol at 241nm. The FPRS lot 1 was used as reference and regarded as 100%. The humidity contents in the substances were negligible (< 0.1 %). The difference is not statistically significant at the 95 % confidence level using unpaired t-test.

Thermogravimetric analysis

When the substance was heated to 200 °C, a loss of < 0.1% of weight was observed.

Instrument: Perkin Elmer TGA 7 Thermogravimetric analyzer
Sample weight: 3 mg
Heating rate: 5 °C/min
Melting point: about 234-240 °C

The corresponding result for FPRS lot 1 was < 0.1% .

Purity

Thin-layer chromatography

The following thin-layer chromatographic system was used according to the International Pharmacopoeia 3rd Ed. Vol. 3.

Thin-layer: Silica gel 60 F-254 (Merck)
Eluent: Chloroform : acetone (80:20)
Sample: 100 µg of levonorgestrel dissolved in chloroform were applied.
Visualization: Evaluation was made by densitometry at 240 nm with a Desaga Densitometer CD 60.
After the evaluation the plate was treated with a mixture of 50 ml of methanol and 10 ml of sulfuric acid conc. and heated at 105 °C for 1 minute.
The spots were examined in UV-light at 365 nm.

Five faint secondary spots were detected visually and by scanning at 240 nm before spraying. Only one was strong enough to be determined. The detection limit of the system was about 0.02 µg (0.02 %). It was 17β-Hydroxy-18-methyl-4-estren-3-one and was estimated by an external standard to be 0.1 % in the proposed ICRS.
After treating the plate with sulfuric acid one additional faint spot was detected at 365nm.

Rf (levonorgestrel) = 0.45
Rf (17β-Hydroxy-18-methyl-4-estren-3-one) = 0.35

The same amount of spots was found in the FPRS lot 1 , with a content of 17β-Hydroxy-18-methyl-4-estren-3-one estimated to 0.2 %.

High performance liquid chromatography

A total amount of 0.3 % impurities was found (n=12 ,RSD=0.05 % on the main peak, RSD= 10 % calculated on 0.2 % impurity level).

A liquid chromatographic system with UV detection according to M. Gazdag, G. Szepesi and K. Mihálufi, Journal of Chromatography, 450 (1988) 145-155 was used.

A chromatogram is shown in Figure 3.

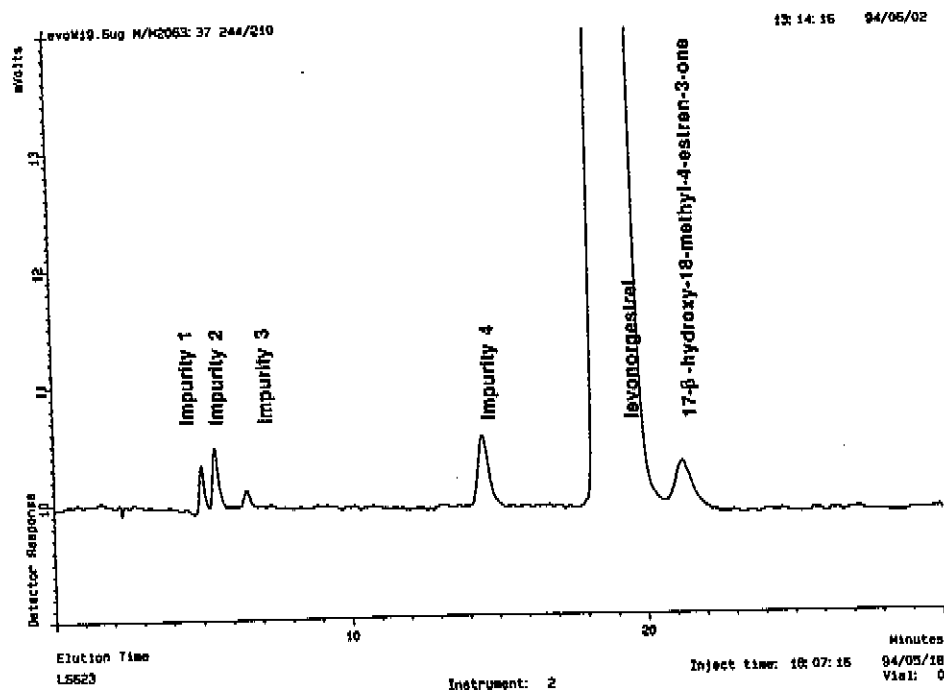


Figure 3. Chromatogram of levonorgestrel Control No 194182 monitored at 244 nm .

The following conditions were used:

Eluent: Methanol : Water (63:37)
Column: Brownlee Labs RP -18 OD -5A
Detector: Varian 9065 Polychrome Diode Array Detector operated at 244 nm.
Pump: Varian 9012 operated at a flow rate of 1ml/min.
Integrator: PeakPro (Beckman)
Sample: 1mg/ml , first dissolved in methanol and then water was added to obtain the same composition as the eluent. 20 µl corresponding to 20 µg were injected.

The detection limit for levonorgestrel was 0.002 µg (0.01 %).
As can be seen from figure 3 five impurities are observed. They were estimated to be about 0.3 % in the proposed ICRS by normalization against the main peak. 17β-Hydroxy-18-methyl-4-estren-3-one was identified by an external standard and was eluting at about 21.5 minutes. It was estimated to be about 0.1 % in the proposed ICRS 194182 .

Diode-array detection

Spectra were recorded for the four impurities. They had UV maxima at about 234 to 244nm. Levonorgestrel had UV maximum at 243 nm and was eluting at about 19 minutes.

Data given by the manufacturer

| | |
|-----------------------|-----------------------|
| IR | passes test |
| Melting range | 237.5-238 °C |
| Specific opt. rot. | - 32.3 ° |
| Color, solution | passes test |
| Related subst . | ≤ 0.5 % (single spot) |
| Sum of second . spots | ≤ 2 % |
| Dichloromethane | 0.07 % |
| Sulfated ash | ≤ 0.01 % |
| Loss on drying | 0.02 % |
| Assay | 99.1 % |

Stability

Stability studies were not performed as this substance was not suspected to degrade easily. Regular re-examinations of the ICRS will be performed.

Conclusion

Levonorgestrel, Control No 194182, can be considered suitable as International Chemical Reference Substance for the intended purpose. When used in the spectrophotometric assay the content is taken to be 100 %.

APPENDIX 16

LOPERAMIDE HYDROCHLORIDE

Control No 194185

Analytical Report

Intended use

The monograph for Loperamide hydrochloride in the International Pharmacopoeia 3rd Ed. Vol 3 requires a reference substance of loperamide hydrochloride to be used in the infrared spectrophotometric and in the ultraviolet spectrophotometric test for identity.

Material

About 26 g of the sample (manufacturers batch no ZR0018553 BEA 141) were received at the WHO Centre in October 1992. The material is being stored in tightly closed containers at + 5 °C, protected from light.

This reference substance is of the same batch as the European Pharmacopoeia Chemical Reference Substance (EPCRS) lot 1.

Analytical data

Description

A white powder.

Evidence of chemical structure

Infrared spectrum

An infrared spectrum is given in Figure 1 (No W 194185). The spectrum is concordant with the spectrum of the United States Pharmacopoeia Reference Standard (USPRS) lot G.

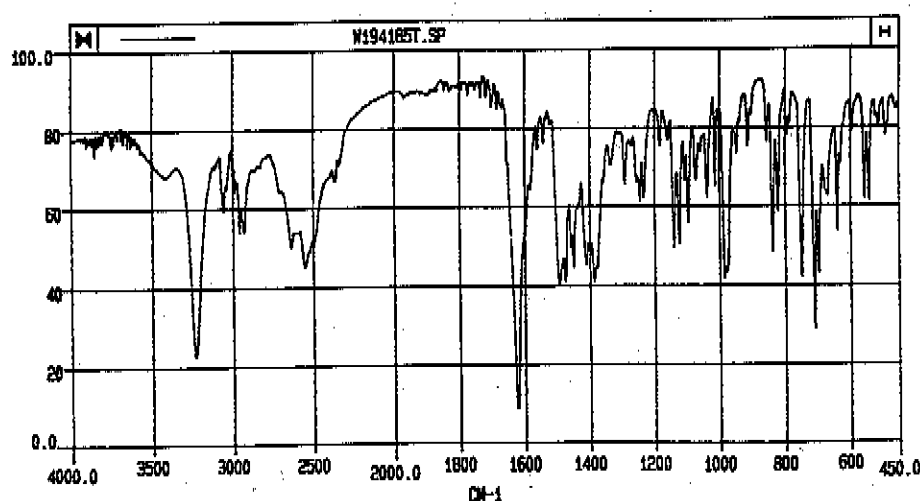


Figure 1. IR-spectrum of 1.2 mg of loperamide hydrochloride Control No 194185 in 300 mg KBr recorded against a KBr disc.
Instrument: Perkin-Elmer 1600 FTIR.

UV-spectrum

A UV-spectrum in 0.1 M hydrochloric acid : 2-propanol (10:90)according to the International Pharmacopeia was recorded on a Varian Cary 5 spectrophotometer (concentration 0.4mg/ml).Three UV-maxima were observed at 253 nm, 259 nm and 265 nm as well as a shoulder at 273 nm.

$A_{1cm}^{1\%} = 10$ at 253 nm (n=5 ,RSD=2.6 %)

$A_{1cm}^{1\%} = 12$ at 259 nm (n=5,RSD=2.0 %)

$A_{1cm}^{1\%} = 11$ at 265 nm (n=5,RSD=2.2 %)

The results are calculated with reference to the dried substance.

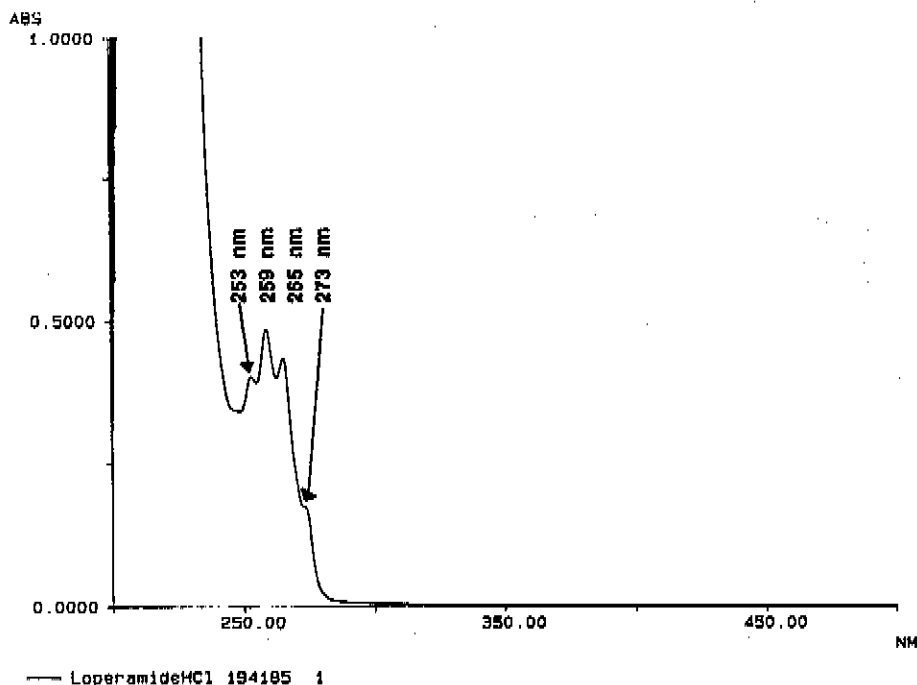


Figure 2. *UV-spectrum of loperamide hydrochloride Control No 194185 in hydrochloric acid / 2-propanol 0.4 mg/ml.*

Chlorides

Positive identity by ion-chromatography (6.0 % chlorides).

Assay

Liquid chromatographic assay

101.4 % (n=13 ,RSD= 1.2 %) when determined against the USPRS lot G taken to be 100 % according to the method described under purity as system 1.The determinations were performed on the dried substances.Statistical calculations show no significant difference between the two substances at the 95% confidence level using unpaired t-test .

Spectrophotometric assay

101.4 % (n=7 ,RSD=2.0%) when determined against the USPRS lot G taken to be 100 % according to the method described under UV-spectrum at 259 nm.The determinations were performed on the dried substances.

Statistical calculations show no significant difference between the two substances when compared at the 95% confidence level using unpaired t-test.

Thermogravimetric analysis

When the substance was heated to 120°C, a loss of < 0.1% (w/w) was observed.

Instrument: Perkin Elmer TGA 7 Thermogravimetric analyzer.
Sample weight: 3 mg
Heating rate: 5 °C/min, then holding 120°C for 120 minutes.
Melting point: about 224 °C

The corresponding result for USPRS lot G was <0.1 %.

Purity

Thin-layer chromatography

No secondary spots were detected. The thin-layer chromatographic system described in the International Pharmacopoeia Vol. 3 was used.

Thin-layer: Silica gel 60 F-254 (Merck)
Eluent: Chloroform : Methanol : Formic acid (85 : 10 : 5)
Sample: 100 and 200 µg of loperamide hydrochloride dissolved in chloroform were applied.
Visualization: Scanning at 220 nm with a Desaga CD60 densitometer was performed and visualization in day-light after treating with iodine vapours.

R_f for loperamide hydrochloride is 0.2. This R_f -value corresponds to that of the USPRS lot G of loperamide hydrochloride. Detection limit is 0.2 µg (0.1 %) both visually after developing in iodine vapour and when scanning at 220 nm.

High performance liquid chromatography

A total amount of < 0.1 % impurities was found.

System 1:

A liquid chromatographic system from the European Pharmacopoeia with gradient elution and detection at 218 nm was performed.

A chromatogram is shown in Figure 3.

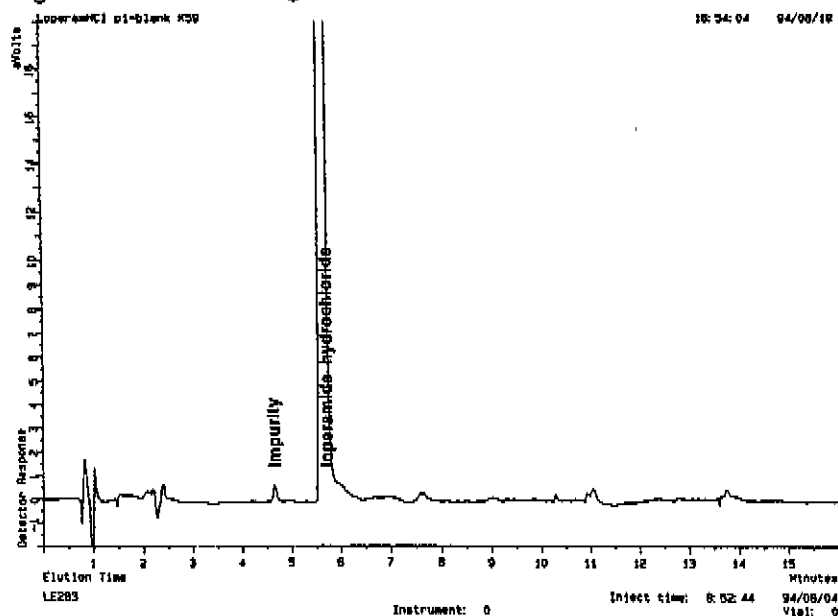


Figure 3. Chromatogram of loperamide hydrochloride Control No 194185, a blank gradient chromatogram is subtracted from the loperamide hydrochloride chromatogram.

The following conditions were used:

Eluent: Acetonitrile: 0.05 M tetrabutylammonium hydrogen sulfate in a linear gradient as follows.

| Time (minutes) | Proportions |
|----------------|-------------|
| 0 | 10 : 90 |
| 10 | 70 : 30 |
| 11 | 10 : 90 |
| 16 | 10 : 90 |

Column: Kromasil C18 (150 x 4.6 mm) 5 μ m.

Detector: Hewlett Packard series 1050 diode array detector operated at 218 nm.

Pump: Hewlett Packard series 1050 operated at a flow rate of 2ml/min.

Integrator: PeakPro (Beckman)

Sample: 1.0 mg/ml dissolved in methanol. 20 μ l were injected.

As can be seen from figure 3 one possible impurity is observed at about 4.5 minutes. Although the gradient is subtracted from the loperamide chromatogram disturbances from the gradient appears as extra peaks. This is the reason why the chromatogram was also run isocratically, see system 2 below. The limit of quantification for this system was estimated to about 0.01 μ g (0.05 %). The limit of detection was just slightly below that, because of the interference from the gradient.

System 2:

An isocratic liquid chromatographic system with detection at 218 nm was run. A chromatogram is shown in Figure 4.

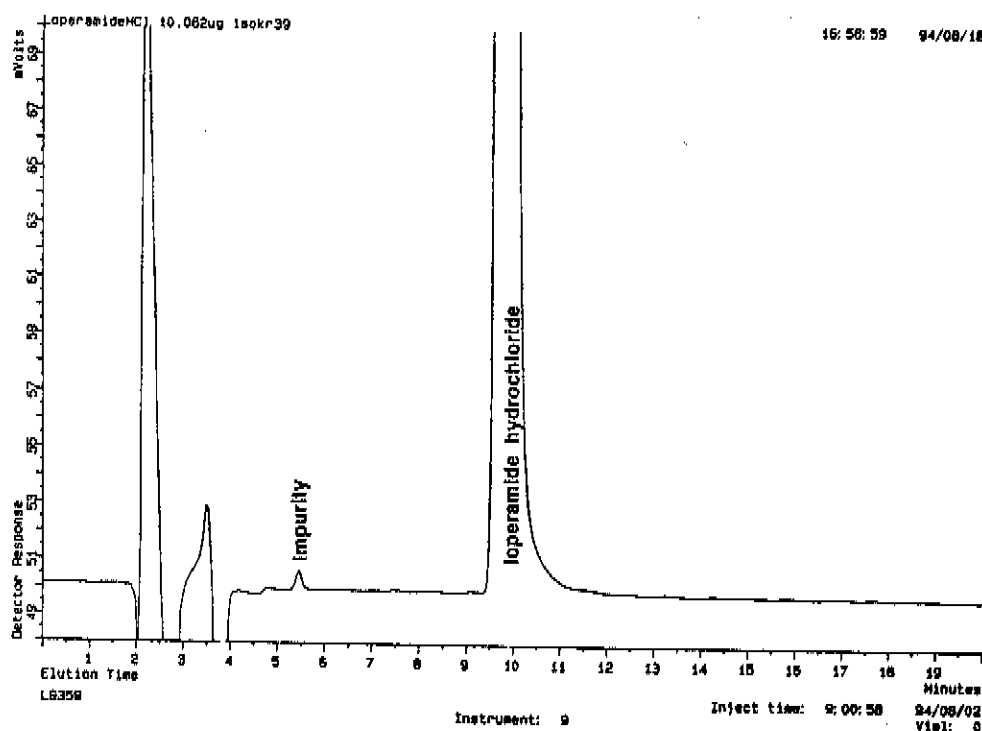


Figure 4. Chromatogram of loperamide hydrochloride Control No 194185.

The following conditions were used:

Eluent: Acetonitrile: 0.05 M Tetrabutylammonium hydrogen sulfate (30 : 70).
Column: Brownlee Labs RP-18 OD-5A
Detector: Hewlett Packard series 1050 diode array detector operated at 218 nm.
Pump: Hewlett Packard series 1050 operated at a flow rate of 1ml/min.
Integrator: PeakPro (Beckman)
Sample: 1.0 mg/ml dissolved in methanol. 20 µl were injected.

As can be seen from figure 4 one impurity is observed at about 5.5 minutes. It was estimated to be about 0.05% in the proposed ICRS and about 0.04% in the USPRS (lot G). However, as no impurity reference substance was available, this figure can only be used as a comparison of the quality between the two substances. The amount of the impurity is close to detection limit and RSD for the determination is about 10%. The chromatogram show some disturbances in the beginning of the chromatogram before 4.5 minutes due to that methanol was used as solvent for the sample. The limit of quantification for this system was estimated to be 0.01 µg (0.05 %) and the limit of detection to be about 0.005 µg.

Diode-array detection

When the UV-spectra of the main peak and the impurity are compared they show the same maxima at about 210 nm and 263 nm.

Total solid impurities

Differential scanning calorimetry (DSC)

DSC is not suitable for purity determination as loperamide hydrochloride melts with decomposition. DSC curves as well as infrared spectra for the possible polymorphic forms are published in Florey, Analytical Profiles, Vol 19. Two polymorphic forms are reported, 224.4 °C(T_M) for polymorph I and 218.4 °C(T_M) for polymorph II. Our results indicate that the ICRS consists predominantly of polymorph I.

Melting temperature: 224.3 °C (T_M)
Onset: 223.0 °C
Instrument: Perkin Elmer DSC7 Differential Scanning Calorimeter.
Sample weight: 2mg
Heating rate: 2 °C/minute

Data given by the manufacturer

Appearance: complies
Melting range: 223.6 - 225.4 °C
TLC-identity: complies
UV-spectrum: maxima at 253.0, 259.0, 266.0 and 274.0 nm.
IR: complies
TLC-purity: complies (individual spot \leq 0.5%; total \leq 1%)
Colour and clarity of solution: complies
% transmittance: 96%
Loss on drying: \leq 0.1% (w/w)
Sulfated ash: \leq 0.01% (w/w)
Heavy metals: \leq 20 ppm
Chlorine (total): 13.73% (w/w)
Arsenic: \leq 2 ppm
Base titration (assay): 100.3% (w/w)

Data given by the collaborating laboratories

EP lab
EPCRS lot 1 is from the same batch as ICRS 194185
IR spectrum : complies with K.Florey
TLC: complies
Reaction of chlorides: complies
HPLC purity: 0.1 %
Loss on drying,water: 0.1%
Assay,titration: 100.1%
Sulfated ash: 0.01 %

Stability

Stability studies were not performed as this substance is not suspected to degrade easily.Regular reexaminations of the ICRS will be performed.

Conclusion

Loperamide hydrochloride, Control No 194185 can be considered suitable as International Chemical Reference Substance for the intended purpose.

APPENDIX 17

METHOTREXATE

Control No 194193

Analytical Report

Intended use

The monograph for Methotrexate in the International Pharmacopoeia 3rd Ed. Vol. 3 requires a reference substance of methotrexate to be used in the infrared spectrophotometric test for identity and in the liquid chromatographic assay.

Material

About 15 g of the sample (manufacturers batch no OMTT174A) were received at the WHO Centre in April 1994. The material is being stored in tightly closed containers at + 5 °C, protected from light.

CAUTION: As methotrexate is a cytotoxic drug it should be handled with care. Avoid contact with the skin and inhalation of airborne particles.

Analytical data

Description

An orange-yellow powder.

Evidence of chemical structure

Infrared spectrum

An infrared spectrum is given in Figure 1 (No W 194193B). The spectrum is concordant with the spectra of the United States Pharmacopoeia Reference Standard (USPRS) lot H and of the European Pharmacopoeia Chemical Reference Substance (EPCRS) lot 2 for methotrexate.

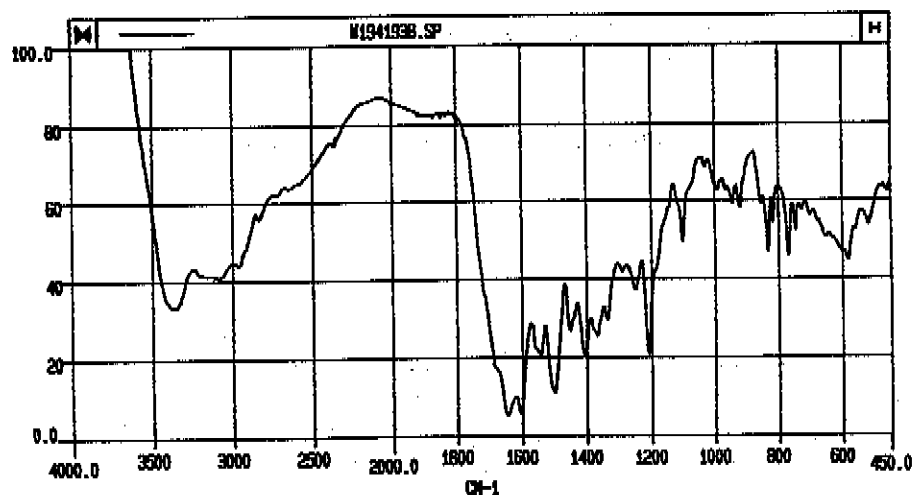


Figure 1. IR-spectrum of 0.9 mg of methotrexate Control No 194193 in 300 mg KBr recorded against a KBr disc.

Instrument: Perkin-Elmer 1600 FTIR.

UV-spectrum

A UV-spectrum in 0.1M sodium hydroxide according the International Pharmacopoeia was recorded on a Varian Cary 5 spectrophotometer .

UV-maxima were observed at 371nm, 303 nm, 258 nm and 221nm.

$A_{1cm}^{1\%} = 179$ at 371 nm (n=6 ,RSD=0.6 %)

$A_{1cm}^{1\%} = 554$ at 303 nm (n=6,RSD=0.6 %)

$A_{1cm}^{1\%} = 550$ at 258 nm (n=6,RSD=0.6 %)

The results are calculated with reference to the dried substance.

The ratio between 303nm/371 nm is 3.1 (limit 2.8-3.3 according to the International Pharmacopoeia)

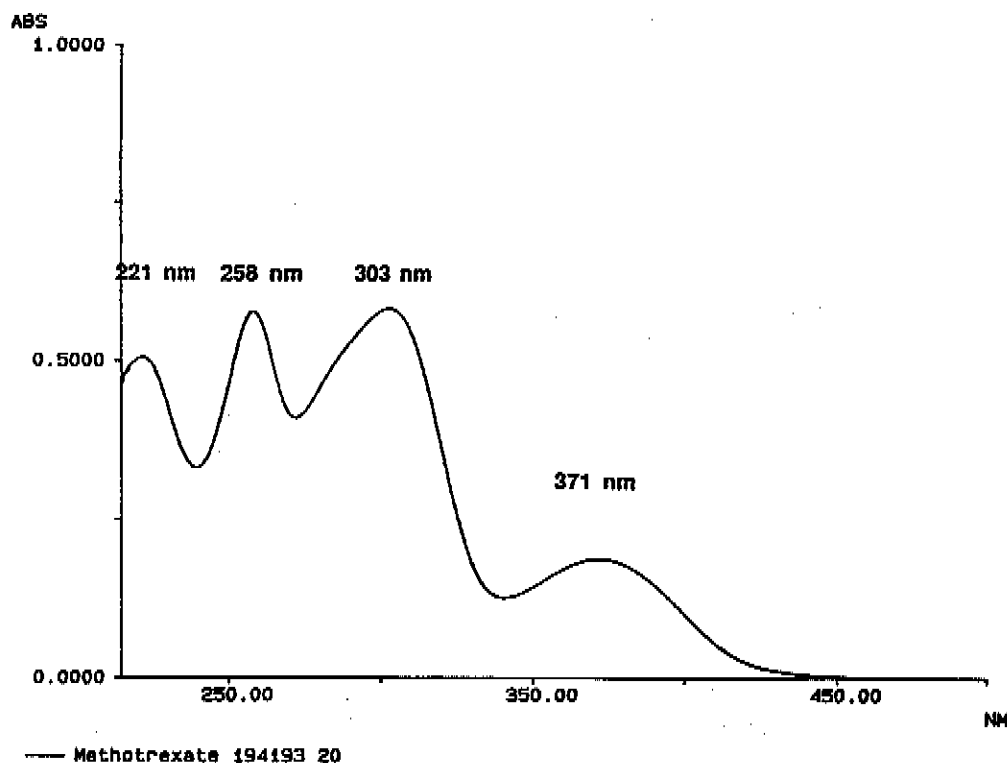


Figure 2. UV-spectrum of methotrexate Control No 194193 in 0.1 M NaOH 10 µg/ml.

Specific optical rotation

$[\alpha]_D^{20} = + 22.3^\circ$ calculated on anhydrous basis. (limits +19-24°Ph. Int.)

Assay

Liquid chromatographic assay

99.5% (n=9, RSD = 0.6%) when determined against the USPRS lot H taken to be 99.8% according to the liquid chromatographic method described under purity.

The assay was performed at 302 nm and 254nm. The determinations were performed on the dried substances. Statistical calculation shows no significant difference at the 95% confidence level using unpaired t-test between the proposed ICRS and the USP reference substance.

Spectrophotometric assay

100.4%(n=6 ,RSD=0.6 %) when determined against the USPRS lot H taken to be 100 %.The determination was performed at 303 nm using the method described under UV-spectrum above and calculations were performed on the anhydrous basis. The difference is not

statistically significant at the 95% confidence level using unpaired t-test.

Thermogravimetric analysis

When the substance was heated to 110 °C, a loss of 10.1% (w/w) was observed (n=7, RSD=4.8%).

Instrument: Perkin Elmer TGA 7 Thermogravimetric analyzer
Sample weight: 3.5 mg
Heating rate: 5 °C/min, then holding 110 °C for 60 minutes
Melting point: about 185-204 °C with decomposition.

The corresponding result for the USPRS lot H was 9.9% (w/w), (n=3, RSD=5.4%).

Purity

High performance liquid chromatography

A total amount of 0.5 % impurities was found.

A liquid chromatographic system with UV- detection at 302nm and at 254nm was performed. A chromatogram is shown in Figure 3.

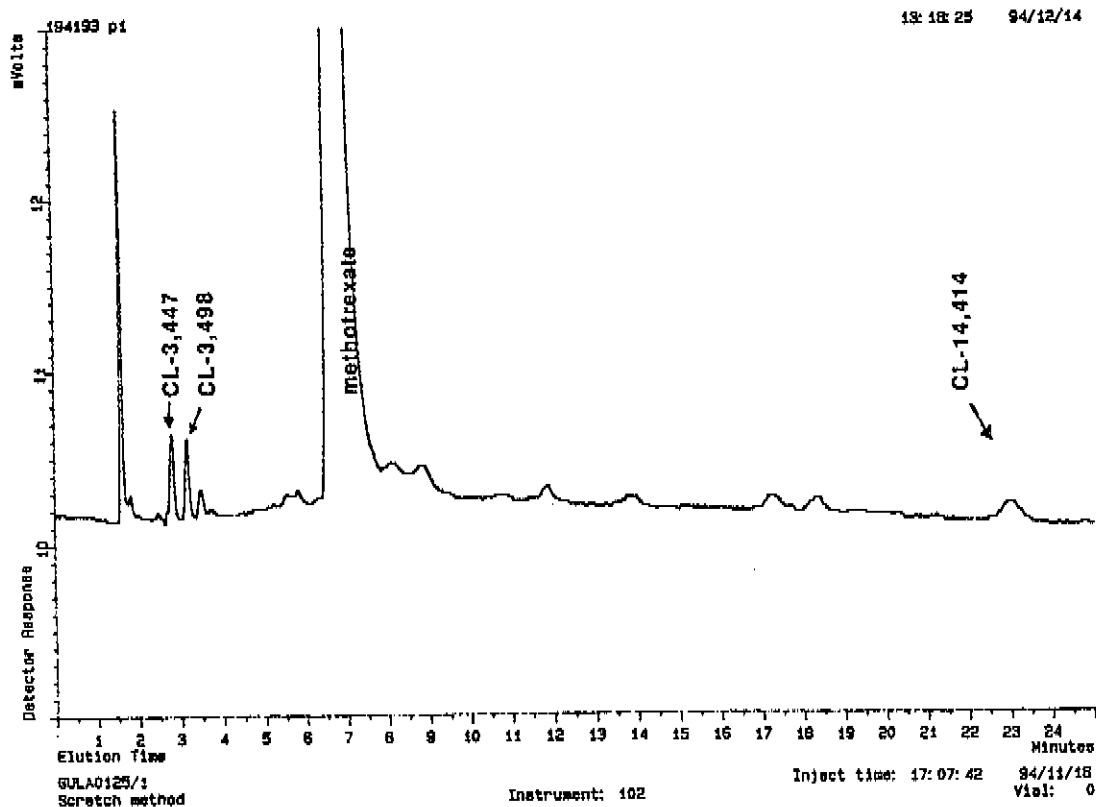


Figure 3. Chromatogram of methotrexate Control No 194193 monitored at 254nm.

The following conditions were used:

Eluent: Acetonitrile: buffer (10:90)
Buffer: 22.4g di-sodiumhydrogenphosphate dihydrate + 7.8g citric acid monohydrate in 1000 ml water ; pH = 6.0.
Column: Kromasil C18, 4.6 x 250 mm.
Detector: Varian Polychrom 9065 operated at 254 and 302 nm.

Pump: Varian 9012 operated at a flow rate of 1.2 ml/min.
Integrator: PeakPro (Beckman)
Sample: 5mg dissolved in 10 ml of the eluent.

As can be seen from Figure 3 several impurities were found. Three of the impurities were identified by means of impurity references of the related compounds CL 3,447 , CL 3,498 and CL 14,414 obtained from the manufacturer. The content of these impurities in the ICRS was calculated with use of these references as external standards. The other impurities were estimated by peak area normalization.

| | | Purity | CL 3,447 | CL 3,498 | CL 14,414 | others |
|------------|--------|--------|----------|----------|-----------|--------|
| Prop. ICRS | 302 nm | 99.7% | 0.07% | 0.13% | <0.02% | <0.1% |
| | 254 nm | 99.5% | 0.07% | 0.14% | 0.02% | 0.25% |

The corresponding purity for the USPRS lot H used in the assays was 99.8 %.

The limit of quantification (for methotrexate) was 10 ng at 302 nm and 7.5 ng at 254 nm.
The limit of detection was 5 ng at 302 nm and 3.5 ng at 254 nm.

Diode-array detection

The chromatographic system described above was used to record UV-spectra for the observed impurities. The spectra for the peaks identified as CL 3,447 and 3,498 was concordant with those recorded for the impurity standards. All of the impurities as well as the main peak had similar spectra with maxima at about 258 nm and 304 nm. A notable difference in specific absorbance between the different impurity standards was noted, with an especially high value for CL 14,414.

Data given by the manufacturer

Potency: 99.9% anhydrous.

Stability

The substance is reported to be gradually affected by light. Regular re-examinations of the ICRS when stored in the dark at +5 °C will be performed.

A small examination of the stability during analysis time was performed. The following results were obtained.

| <u>Sample</u> | <u>Status, monitored by HPLC</u> |
|--|----------------------------------|
| Solution in HPLC eluent 20h, dark, +8°C | no degradation |
| Solid 24h, labwindow, +25°C | no degradation |
| Solution in HPLC eluent, 24h, labwindow, +25°C | slight degradation (0.2%) |

Conclusion

Methotrexate, Control No 194193, can be considered suitable as International Chemical Reference Substance for the intended purpose. The content of methotrexate is taken to be 89.4 % determined on the "as is " basis calculated as 100-(water+solvents+impurities by HPLC). This content corresponds to 99.5 % when calculated on the dried substance.

APPENDIX 18

NIFURTIMOX

Control No 194189

Analytical Report

Intended use

The monograph for Nifurtimox in the International Pharmacopoeia 3rd Ed. Vol. 3 requires a reference substance of nifurtimox to be used in the infrared spectrophotometric and thin-layer chromatographic tests for identity.

Material

About 50 g of the sample (manufacturers batch no 910583B) were received at the WHO Centre in June 1994. The material is being stored in tightly closed containers at + 5 °C, protected from light.

Analytical data

Description

An orange-yellow powder.

Evidence of chemical structure

Infrared spectrum

An infrared spectrum is given in Figure 1 (No W 194189). The spectrum is concordant with the spectrum of the French Pharmacopoeia Reference Substance (FPRS) lot 126083B of nifurtimox.

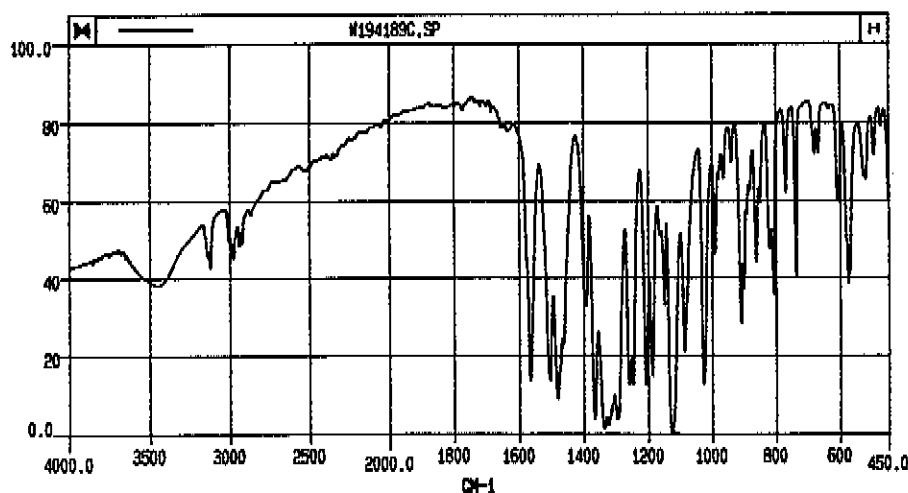


Figure 1. IR-spectrum of 0.9 mg of nifurtimox Control No 194189 in 300 mg of KBr recorded against a KBr disc.

Instrument: Perkin-Elmer 1600 FTIR.

UV-spectrum

A UV-spectrum in methanol was recorded on a Varian Cary 5 spectrophotometer. The spectrum is given in Figure 2.

UV-maxima were observed at 391 nm and 274 nm.

$A_{1\text{cm}}^{1\%} = 645$ at 391 nm (n=6, RSD= 0.6 %)

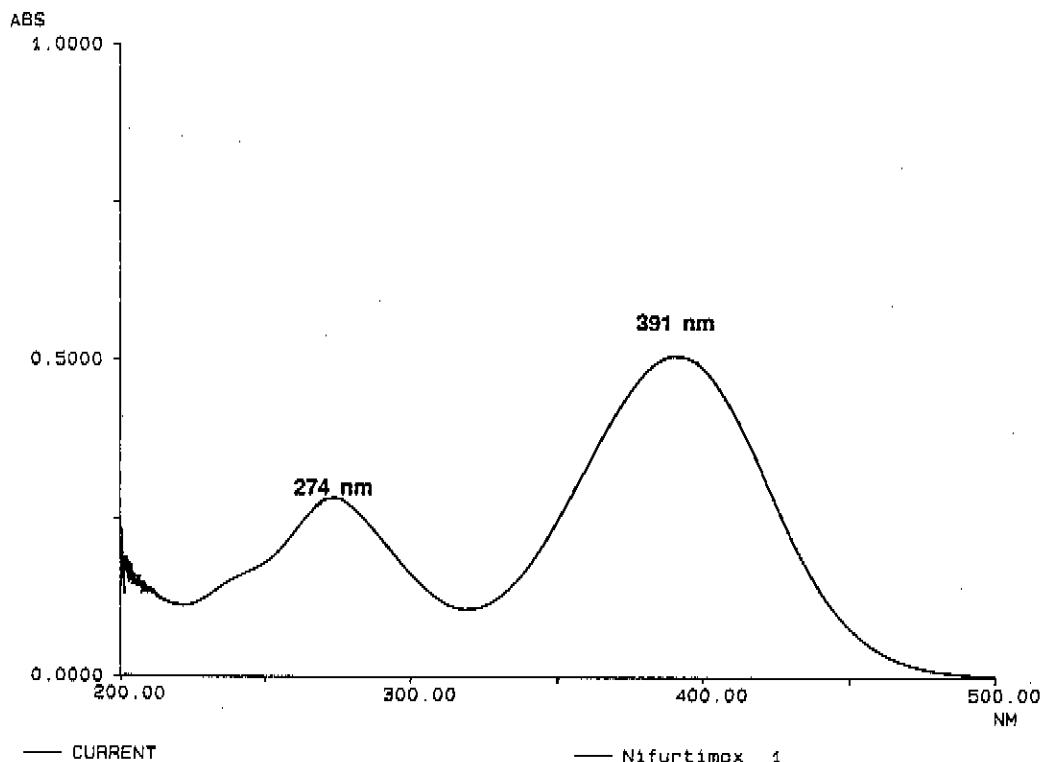


Figure 2. UV-spectrum of nifurtimox Control No 194189 , 7 $\mu\text{g}/\text{ml}$ in methanol.

Assay

Liquid chromatographic assay

100.9 % (n=6, RSD=0.4%) when determined against the FPRS lot 126083B regarded as 100 %. The difference between the proposed ICRS and the FPRS is statistically significant at the 95% confidence level using unpaired t-test. The determination was performed with the liquid chromatographic method described under purity.

Spectrophotometric assay

99.5 % when determined in methanol at 391 nm. The FPRS lot 126083B was used as standard and was regarded as 100 %. The result is not statistically significant at the 95% confidence level using unpaired t-test (n=6, RSD=0.6 %).

Thermogravimetric analysis

When the substance was heated to 110 $^{\circ}\text{C}$, a loss of weight of < 0.1% (w/w) was observed.

Instrument: Perkin Elmer TGA 7 Thermogravimetric analyzer
Sample weight: 4 mg
Heating rate: 5 $^{\circ}\text{C}/\text{min}$, then holding 110 $^{\circ}\text{C}$ for 90 minutes
Melting point: About 178-182 $^{\circ}\text{C}$

The corresponding result for FPRS lot 126083B was a loss of weight of <0.1% (w/w).

Purity

Thin-layer chromatography

No secondary spots were detected in nifurtimox Control No 194189.

The following thin-layer chromatographic system according to the International Pharmacopoeia 3rd Ed. Vol. 3 was used.

Thin-layer: Silica gel 60 F-254 (Merck)
Eluent: Ethylacetate : hexane (50 : 50), unsaturated chamber
Sample: 100 µg of nifurtimox dissolved in acetone were applied
Visualization: Visualization in day-light, UV-light of 254nm and by scanning at 270 nm and 395 nm with a Desaga CD60 densitometer.

R_f (nifurtimox) = 0.31.

The same results were obtained for the FPRS lot 126083B except two minor impurities at $R_f = 0.57$ and $R_f = 0.67$, only detectable when scanning at 270 nm. Detection limit for nifurtimox was 0.06 µg (0.06 %) when scanning at 395 nm and 0.1µg (0.1 %) when scanning at 270 nm.

High performance liquid chromatography

A liquid chromatographic system with UV-spectrophotometric detection at 391nm was performed.

A chromatogram is shown in Figure 3.

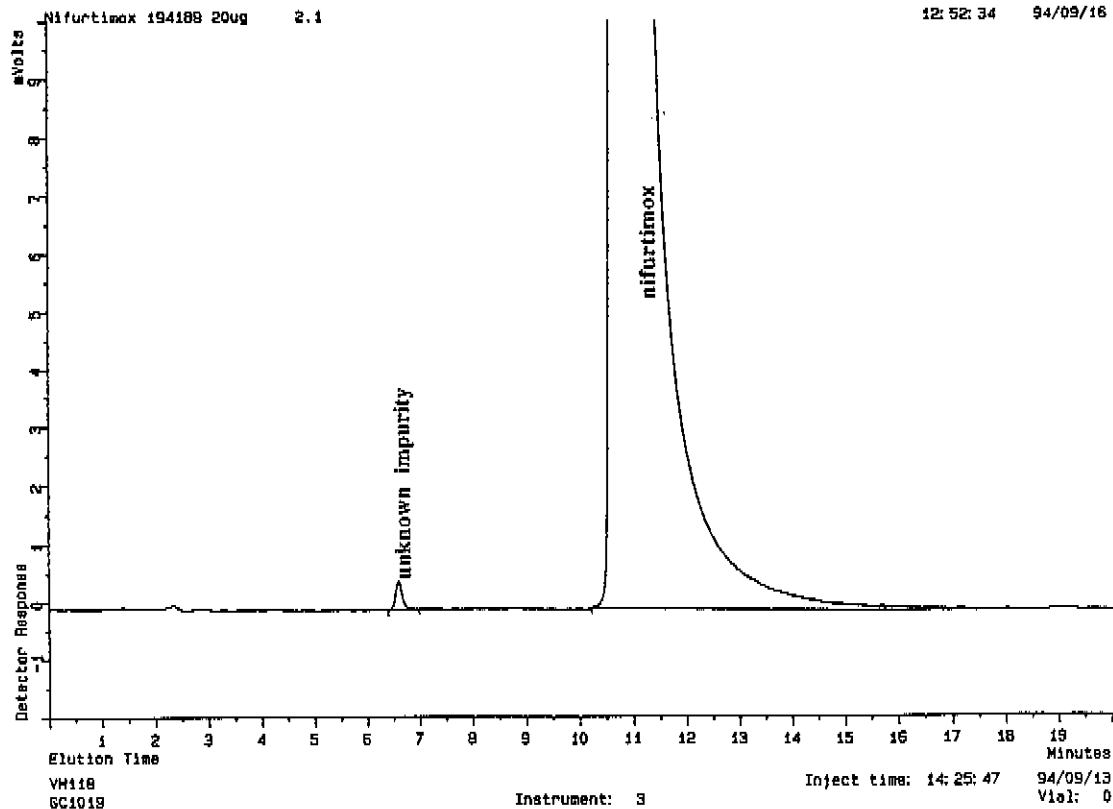


Figure 3. Chromatogram of nifurtimox Control No 194189 .

The following conditions were used:

Eluent: Acetonitrile: 0.01 M sodiumheptanesulphonate pH 1.8 with phosphoric acid (35:65)
Column: Brownlee labs RP-18 Sphere-5 OD-5A (250 x 4.6 mm, 5µm)
Detector: Varian UV 100 operated at 391 nm
Pump: Varian Vista 5500 operated at a flow rate of 1ml/min
Integrator: PeakPro (Beckman)
Sample: 1 mg dissolved in 0.05 ml dimethylformamide and eluent to 1 ml.
20 µl corresponding to 20 µg were injected.

As can be seen from figure 3 one impurity was eluting at about 6.5 minutes. It was estimated to about 0.03% (n=6) by peak area normalization in the proposed ICRS and 0.05% (n=6) in the FPRS lot 126083B. However, as no impurity reference substance was available, this figure can only be used as a comparison of the quality between the two substances. Minimum detectable quantity for nifurtimox was 3 ng and the limit of quantification 10ng.

Total solid impurities

Differential scanning calorimetry (DSC)

The amount of total solid impurities was estimated to 0.2 mol % (n=7, RSD=0.05%). The determination was performed on 3 mg using a heating rate of 1°C and 2 °C per minute.

Melting temperature (T_M): 179.1 °C
Onset: 178.7 °C
Instrument: Perkin Elmer DSC7 Differential Scanning Calorimeter

The corresponding result for the FPRS lot 126083B was 0.5 mol %.

Data given by the manufacturer

Appearance: Yellow powder
Identity (IR): Complies
Assay (98.0 - 102 %): 100.6%

Stability

Stability studies were not performed as this substance was not suspected to degrade easily. Regular re-examinations of the ICRS will be performed.

Conclusion

Nifurtimox, Control No 194189, can be considered suitable as International Chemical Reference Substance for the intended purpose.

APPENDIX 19

PRAZIQUANTEL

Control No 194191

Analytical Report

Intended use

The monograph for Praziquantel in the International Pharmacopoeia 3rd Ed. Vol. 3 requires a reference substance of praziquantel to be used in the infrared spectrophotometric and the thin-layer chromatographic tests for identity, in the thin-layer test for related substances and in the infrared spectrophotometric assay.

Material

About 50 g of the sample (manufacturers batch no 728252D) were received at the WHO Centre in May 1994. The material is being stored in tightly closed containers at + 5 °C, protected from light.

Analytical data

Description

A white powder.

Evidence of chemical structure

Infrared spectrum

The spectrum of praziquantel Control No 194191 is concordant with the spectra of the United States Pharmacopoeia Reference Standard (USPRS) lot F-1 and the European Pharmacopoeia Chemical Reference Substance (EPCRS) lot 1.

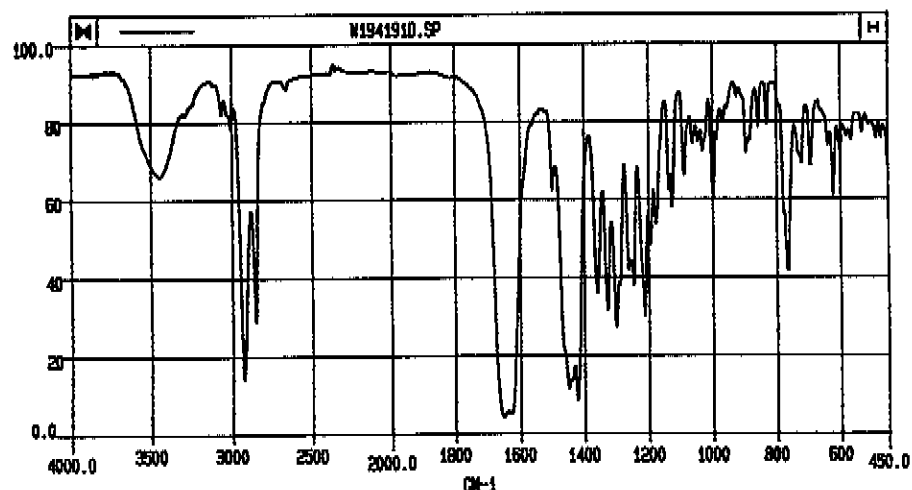


Figure 1. IR-spectrum of 1.3 mg of praziquantel Control No 194191 in 300 mg of KBr recorded against a KBr disc.

Instrument: Perkin-Elmer 1600 FTIR.

UV-spectrum

A UV-spectrum in ethanol was recorded on a Varian Cary 5 spectrophotometer. The spectrum is given in Figure 2.

λ -max in ethanol were observed at 264 and 271 nm.

$A_{1cm}^{1\%} = 11.3$ at 264 nm (n=6, RSD=0.5 %)

$A_{1cm}^{1\%} = 9.5$ at 271 nm (n=6, RSD=0.5%)

The calculations were performed on the dried substance.

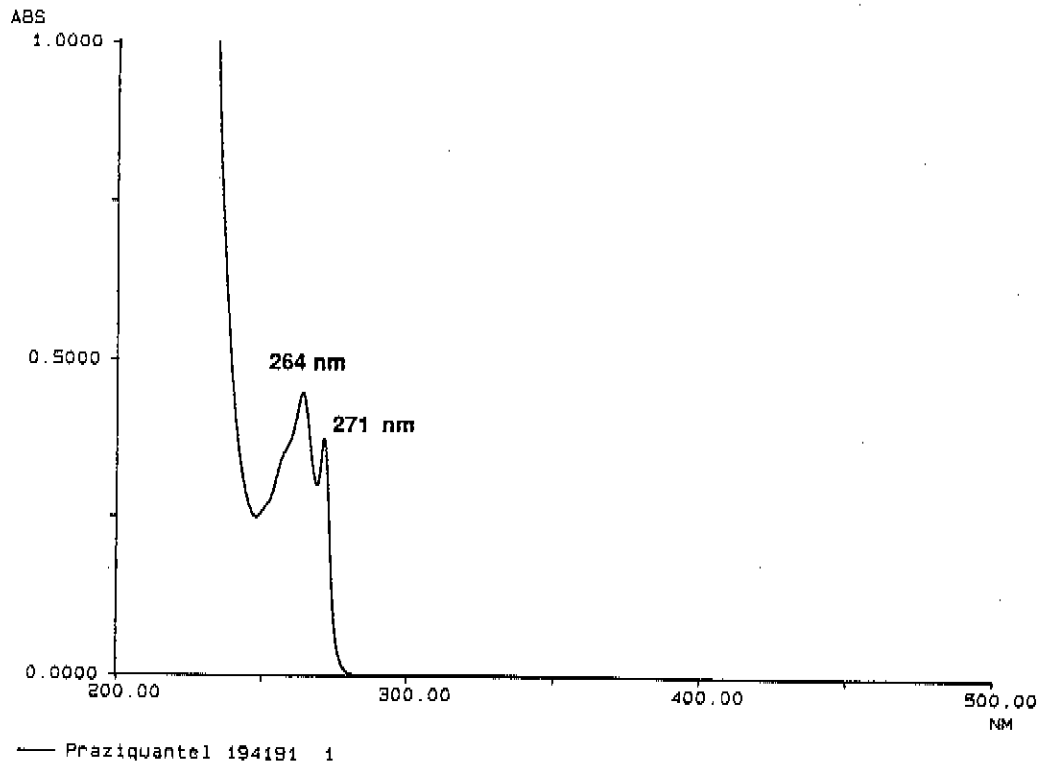


Figure 2. UV-spectrum of praziquantel Control No 194191, 395 µg/ml in ethanol.

Assay

Liquid chromatographic assay

100.3 % (n=6, RSD=0.8 %) when determined against the USPRS lot F-1 regarded as 100 %. The calculations were performed on the dried substance. The difference between the proposed ICRS and the USPRS is not statistically significant at the 95% confidence level using unpaired t-test. The determination was performed with the liquid chromatographic method described under purity.

Spectrophotometric assay

100.2 % (n=6, RSD=0.5 %) when determined against the USPRS lot F-1 regarded as 100 %. The calculations were performed on the dried substance. The difference is not statistically significant at the 95% confidence level using unpaired t-test.

Thermogravimetric analysis

When the substance was heated to 110 °C, a loss of weight of < 0.1 % (w/w) was observed.

Instrument: Perkin Elmer TGA 7 Thermogravimetric analyzer
Sample weight: 3 mg
Heating rate: 5 °C/min, then holding 110 °C for 120 minutes
Melting point: About 136-140 °C according to Merck Index

The corresponding results for the USPRS lot F-1 and the EPCRS lot 1 were < 0.1%, respectively.

Purity

Thin-layer chromatography

No secondary spots were observed when the plate was evaluated visually. When the plate was scanned at 235 nm one impurity with $R_f=0.23$ and traces of another two with $R_f=0.10$ and 0.29 were found. The impurity with $R_f=0.23$ corresponds to 1,2,3,6,7,11 β -hexahydro-4H-pyrazino[2,1a]isoquinolin-4-one and was estimated to 0.2% against the corresponding external standard. Corresponding results were observed for the EPCRS and the USPRS, respectively. R_f (praziquantel) = 0.40. The detection limit of the system was about 0.3 μ g for praziquantel when scanning at 235 nm. Praziquantel impurity EPCRS, ((RS)-2-benzoyl-1,2,3,6,7,11 β -hexahydro-4H-pyrazino[2,1a]isoquinolin-4-one) with $R_f=0.33$ was not detected in the samples.

The following thin-layer chromatographic system according to the International Pharmacopoeia 3rd Ed. Vol. 3 was used.

Thin-layer: Silica gel 60 (Merck)
Eluent: Toluene:methanol (85:15)
Sample: 100 μ g of praziquantel in ethanol were applied
Visualization: Scanning by densitometry at 235nm with a Desaga CD60 densitometer and visualization with iodine vapours

High performance liquid chromatography

The total amount of impurities present was estimated by peak area normalization to 0.1%(n=6). A chromatogram is shown in Figure 3.

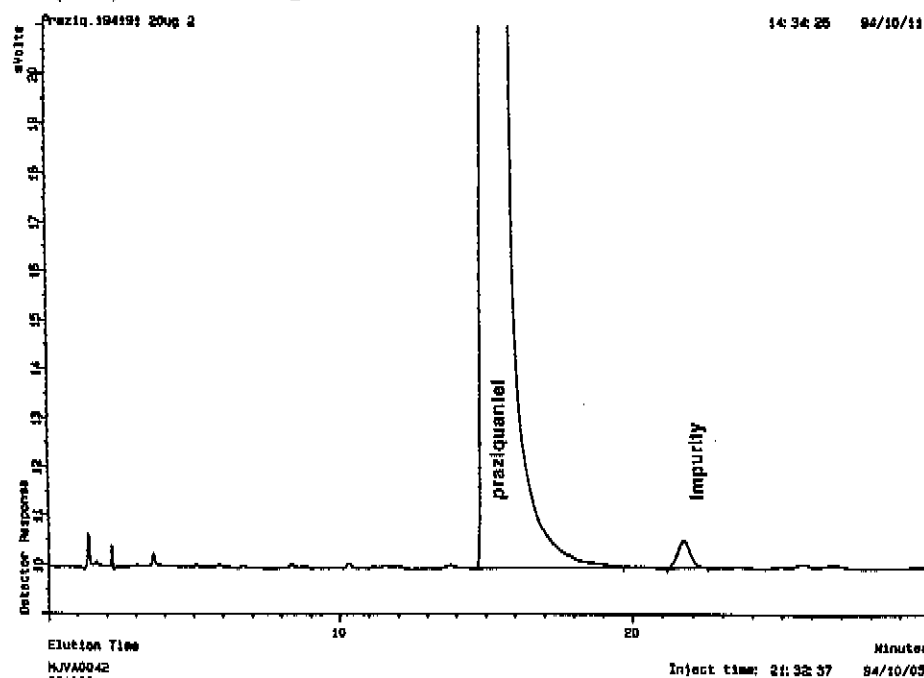


Figure 3. Chromatogram of praziquantel Control No 194191 monitored at 210 nm.

The following conditions were used:

Eluent: Acetonitrile: Water (40:60)
Column: Brownlee Labs RP -18 Spheri-5 OD -5A (250 x 4.6 nm, 5µm)
Detector: Varian 9065 Polychrom operated at 210 nm. For choice of wavelength see Diode-array detection.
Pump: Varian 9012 operated at a flow rate of 1ml/min
Integrator: PeakPro (Beckman)
Sample: 1 mg/ml dissolved in the eluent. 20 µl corresponding to 20 µg were injected.

The impurity peak eluting at 22 minutes does not correspond to praziquantel impurity EPCRS, ((RS)-2-benzoyl-1,2,3,6,7,11β-hexahydro-4H-pyrazino[2,1a]isoquinolin-4-one) which elutes at 8-9 minutes.

Similar results were obtained for USPRS lot F-1 and EPCRS lot 1.

Diode -array detection

The chromatographic system above was evaluated with a Varian 9065 Polychrom detector. UV-spectra were recorded for praziquantel and the impurity eluting at 22 minutes. Both praziquantel and the impurity had their strongest UV-maxima at wavelengths below 220 nm. 210 nm was chosen as detection wavelength. In other reference materials tested additional impurities with UV-maxima at 250 nm were found. Therefore it is also convenient to monitor at this wavelength at the stability testing or when replacing a batch of this ICRS.

Data given by the manufacturer

| | | |
|---------------------|-----------------|---------------|
| Identity IR: | Corresponds | |
| Melting point: | 139.2 - 140.4°C | (136-142°C) |
| Loss on drying: | <0.01% | (≤0.5%) |
| Sulfated ash: | <0.1% | (≤0.1%) |
| Heavy metals: | <0.002% | (≤0.002%) |
| Phosphate: | <0.05% | (≤0.05%) |
| Related substances: | | |
| a) benzoylcompound | 0.01% | (≤0.2%) |
| b) dehydrocompound | <0.01% | (≤0.2%) |
| c) formylcompound | <0.01% | (≤0.2%) |
| Assay (I.T.) | 99.8% | (98.5-101.0%) |

Stability

Stability studies were not performed as this substance was not suspected to degrade easily. Regular re-examinations of the ICRS will be performed.

Conclusion

Praziquantel, Control No 194191, can be considered suitable as International Chemical Reference Substance for the intended purpose. When used in chemical assays the content of praziquantel is taken to be 100 %.

APPENDIX 20

PREDNISOLONE SODIUM PHOSPHATE

Control No 194190

Analytical Report

Intended use

The monograph for Dexamethasone sodium phosphate in the International Pharmacopoeia 3rd Ed. Vol. 3 requires a reference substance of prednisolone sodium phosphate to be used in the thin-layer chromatographic test for identity. The monograph for Prednisolone sodium phosphate injection in the International Pharmacopoeia 3rd Ed. Vol. 4 requires a reference substance of prednisolone sodium phosphate to be used in the thin-layer chromatographic test for identity and in the spectrophotometric assay.

Material

About 50 g of the sample (manufacturers batch no 00004467) were received at the WHO Centre in June 1994. The material is being stored in tightly closed containers at + 5 °C, protected from light.

Analytical data

Description

A white powder.

Evidence of chemical structure

Infrared spectrum

An infrared spectrum is given in Figure 1 (No W 194190). The spectrum is concordant with the spectra of the European Pharmacopoeia Chemical Reference Substance (EPCRS) batch 1 and of the British Pharmacopoeia Chemical Reference Substance (BPCRS) lot 1688.

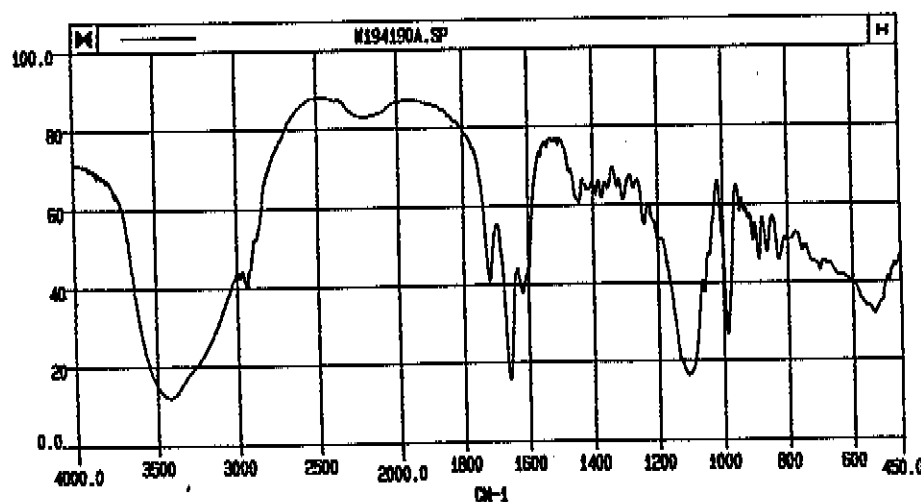


Figure 1. IR-spectrum of 1.2 mg of Prednisolone sodium phosphate Control No 194190 in 300 mg KBr recorded against a KBr disc.
Instrument: Perkin-Elmer 1600 FTIR.

UV-spectrum

A UV-spectrum in water at a concentration of 0.02 mg/ml was recorded on a Varian Cary 5 spectrophotometer. The spectrum is given in Figure 2.

λ -max in water is 247 nm.

$A_{1cm}^{1\%} = 308$ (n=8, RSD=1.3 %) The result is calculated with reference to the dried substance.

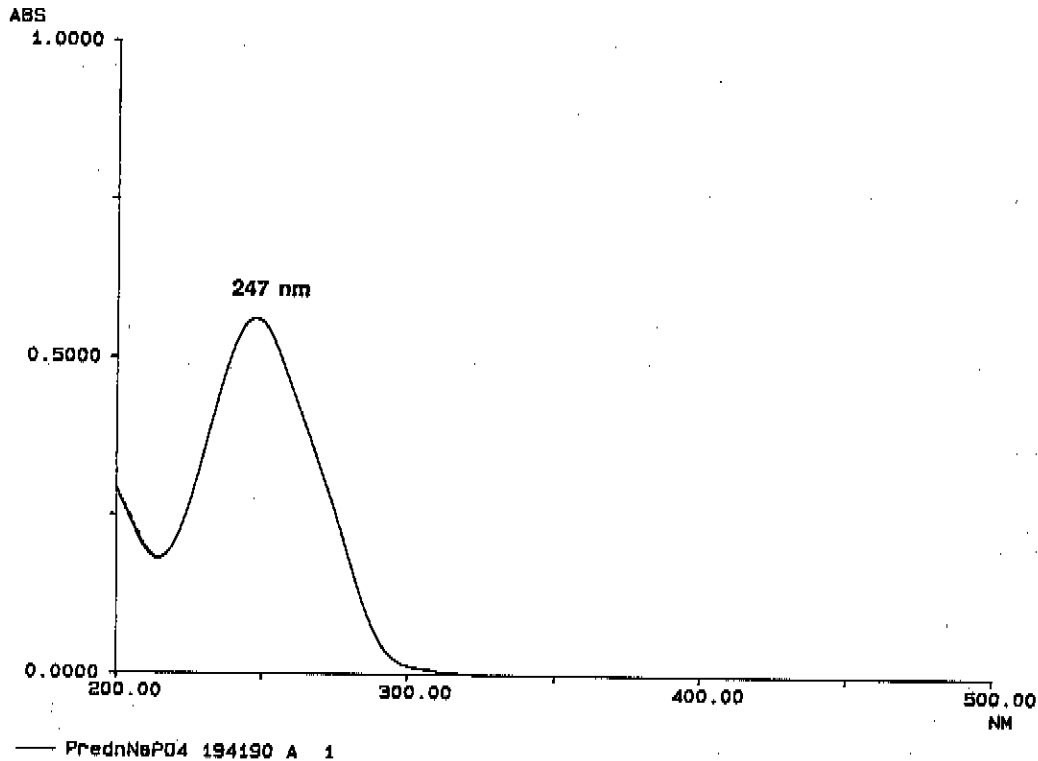


Figure 2. UV-spectrum of prednisolone sodium phosphate Control No 194190 ,20 μ g/ml in water.

Specific optical rotation

$[\alpha]_D^{20} = + 94.7^\circ$. Determined in water at a concentration of 10 mg/ml. The calculation is performed on the anhydrous substance.

Assay

Liquid chromatographic assay

Several assays were performed with the method described below under purity. Due to the hygroscopicity of the substance and difficulties with the reproducibility of the chromatographic system, varying results were obtained. If taking the BPCRS, lot 1688 to 100% the proposed ICRS is about 97% (n=4, RSD=2%) calculated on a dry basis. If taking the EPCRS, batch 1 to 100% the proposed ICRS is about 103% (n=4, RSD=2%). As these reference substances are impure the results are only approximate.

Spectrophotometric assay

99 % (n=8, RSD=1.3 %) when calculated against the theoretical value for prednisolone (419 according to Ph.Int. Vol.4).

Taking the BPCRS lot 1688 to 100% (n=8, RSD=1.3%), the proposed ICRS is 97.3% (n=8, RSD=1.3%), calculated on the dry basis. Taking the EPCRS batch 1 to 100% (n=6, RSD=0.4%), the proposed ICRS is 99.9%, calculated on the dry basis. However as these reference substances are impure these figures are only approximate.

Statistical calculations using unpaired t-test show no significant difference at the 95% confidence level between the proposed ICRS and the EPCRS. Between the proposed ICRS and the BPCRS there is a significant difference at the 95% confidence level.

Thermogravimetric analysis

When the substance was heated to 110°C, a loss of 6.1% (w/w) was observed (n=6, RSD=1.2 %).

Instrument: Perkin Elmer TGA 7 Thermogravimetric analyzer.
Sample weight: 4 mg
Heating rate: 5 °C/min, then holding 110°C for 900 minutes.
Melting point: about 230 °C with decomposition.

Because of degradation the value never stabilized. The given value is calculated as the loss of weight until the point where the derivative becomes constant.

The corresponding value for the EPCRS batch 1 was 9.8% and for the BPCRS lot 1688 8.9%.

Water

5.8 % (n=2) determined with Karl Fischer titration.

Organic volatile compounds

No organic volatiles were detected (<0.01 %). The test included dichloromethane, chloroform, benzene, trichlorethylene and dioxan.

Instrument: Hewlett Packard 5890A
Column: HP-5 (30m x 0.53mm)
Carrier gas: Helium (10ml/min.)
Detector: FID
Injector temperature: 200°C
Detector temperature: 200°C
Temperature program: 40°C for 9 min., 40°C/min to 240°C and holding 240°C for 6 minutes.

Purity

Thin-layer chromatography

Four secondary spots were detected. Their total amount was estimated to be about 0.8 %. The following thin-layer chromatographic system according to the International Pharmacopoeia 3rd Ed. Vol. 4 was used.

Thin-layer: Silica gel 60 F-254 (Merck) and HPTLC.
Eluent: 1-butanol : acetic anhydride : water (60 : 20 : 20)
Sample: 100 and 200 µg of prednisolone sodium phosphate dissolved in methanol were applied.
Visualization: Scanning at 247 nm with a Desaga CD60 densitometer was performed as well as visualization in day-light and 365 nm after spraying with 20% ethanolic sulfuric acid.

R_f (prednisolone sodium phosphate)=0.5. This R_f -value corresponds to that of the BPCRS lot 1688 of prednisolone sodium phosphate. The detection limit is 0.1 µg (0.05%) both when scanning at 247 nm and visually at 254 nm before spraying. After spraying the detection limit is 0.2 µg in daylight and 0.5 µg in 365 nm.

High performance liquid chromatography

A total amount of about 1 % impurities was found. From this figure 0.1 % was identified to be prednisolone. A chromatogram is shown in Figure 3. As can be seen from figure 3 several impurities were found. Two of the impurities were identified by means of impurity reference substances. The content of each identified impurity was calculated with use of these external standards. The other impurities were estimated by peak area normalization.

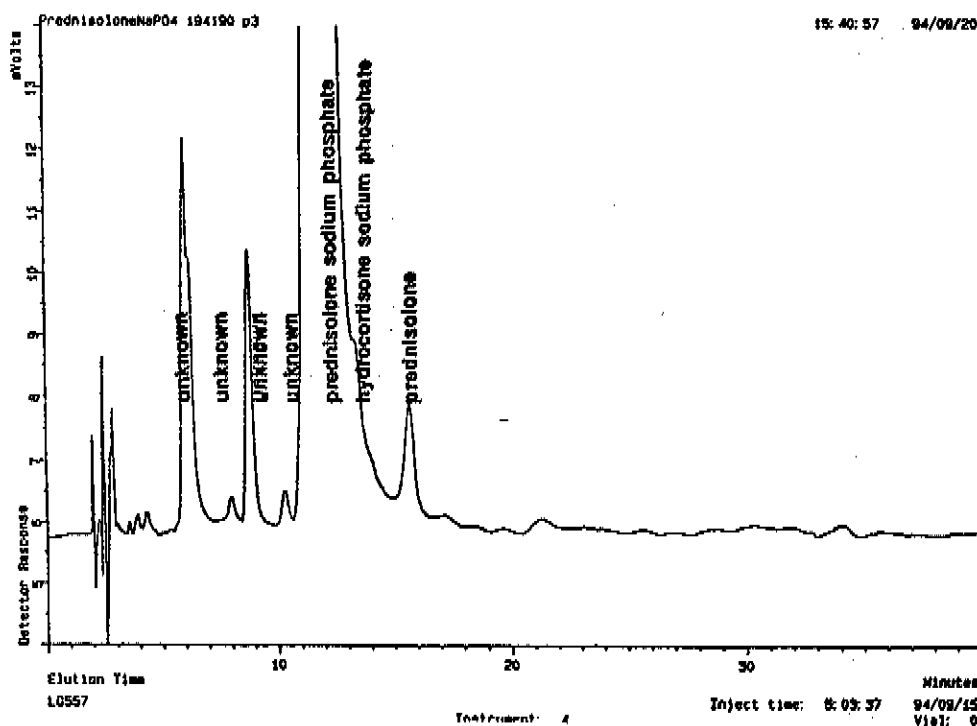


Figure 3. Chromatogram of prednisolone sodium phosphate ,Control No 194190 at 247 nm.

The following conditions were used:

- Eluent: Acetonitrile:Buffer 25:75
- Buffer: 1.36 g potassium dihydrogen phosphate + 0.6 g hexylamine in 185 ml water
- Column: CT-Sil C18, 4.6 x 250 mm.
- Detector: Waters Lambda-Max Mod 481 operated at 247 nm.
- Pump: Waters 600E operated at a flow rate of 1 ml/min.
- Integrator: PeakPro (Beckman)
- Sample: 1.0 mg/ml dissolved in acetonitrile:water 25:75

Below follows a comparison with the two other reference substances that were used in the assays and which were found to be of too low quality for assay purposes.

| | <u>Prop ICRS 194190</u> | <u>EPCRS batch 1</u> | <u>BPCRS lot 1688</u> |
|---------------------------------|-------------------------|----------------------|-----------------------|
| Prednisolone | 0.1% | 0.3% | <0.1% |
| Hydrocortisone sodium phosphate | traces | 0.2% | 0.2% |
| Others | 0.9% | 3.9% | 1.8% |

Data given by the manufacturer

| | |
|-------------------------------|--|
| Identification IR: | conforms |
| Water: | 5.3% |
| Assay (BP): | 98.5% (on the anhydrous substance) |
| (USP): | 96.9% (on the anhydrous substance) |
| Test for sodium: | positive |
| Test for phosphate: | positive |
| Specific rotation: | $[\alpha]_D^{25^\circ}$ in phosphate buffer pH 7.0 c=1cm: +96.6° (anhydrous substance) |
| | $[\alpha]_D^{20^\circ}$ in water c=1cm: +96.2° (anhydrous substance) |
| pH in water: | 1% solution:8.3; 5% solution: 8.2 |
| Phosphate: | 0.15% |
| Free prednisolone and others: | conform requirements |
| Related substances: | conform requirements |
| Selenium: | < 0.0001% |

Stability

Samples of prednisolone sodium phosphate No 194190 were exposed to air of different relative humidity (RH) during a period of about 2 months. The gain of weight occurred already after 24 hours and remained thereafter constant.

| RH | 0%(silica gel) | 11% | 22% | 55% | 76% | 98% |
|---|---------------------|-------|--------|----------------------|-------|-------------|
| Thermogravimetric analysis,loss of weight | 6.8 % | 8.9 % | 11.3 % | 22.4 % | 27.2% | 44.8% |
| Appearance | -----unchanged----- | | | ----stearin-like---- | | semi-liquid |

No signs of chemical degradation were found in any of the samples when investigated by the liquid chromatographic method described under purity.

Regular re-examinations of the ICRS will be performed.

Conclusion

Prednisolone sodium phosphate, Control No 194190 can be considered suitable as International Chemical Reference Substance for the intended purpose. The content of prednisolone sodium phosphate when used in the spectrophotometric assay is taken to be about 93 % when calculated on the "as is" basis as (100 % -impurities by HPLC- volatiles by TG) ,this corresponds to 99 % when calculated with reference to the anhydrous substance.As the substance is hygroscopic careful control of the content of water is necessary .

In the method described in Ph.Int. Vol. 4 for prednisolone sodium phosphate injection, the reference substance is calibrated against the theoretical absorptivity value for prednisolone at each analysis occasion.

TESTOSTERONE ENANTATE

Control No 194192

Analytical Report

Intended use

The monograph for Testosterone enantate in the International Pharmacopoeia 3rd Ed. Vol. 3 requires a reference substance for testosterone enantate to be used in the infrared spectrophotometric and thin-layer chromatographic tests for identity and in the spectrophotometric assay.

Material

About 70 g of the sample (manufacturers batch no 42052091) were received at the WHO Centre in March 1994. The material is being stored in tightly closed containers at + 5 °C, protected from light.

Analytical data

Description

White to cream-white crystalline powder.

Evidence of chemical structure

Infrared spectrum

An infrared spectrum is given in Figure 1 (No W 194192). The spectrum is concordant with the spectra of the United States Pharmacopoeia Reference Standard (USPRS) lot J and the British Pharmacopoeia Chemical Reference Substance (BPCRS) batch 1196.

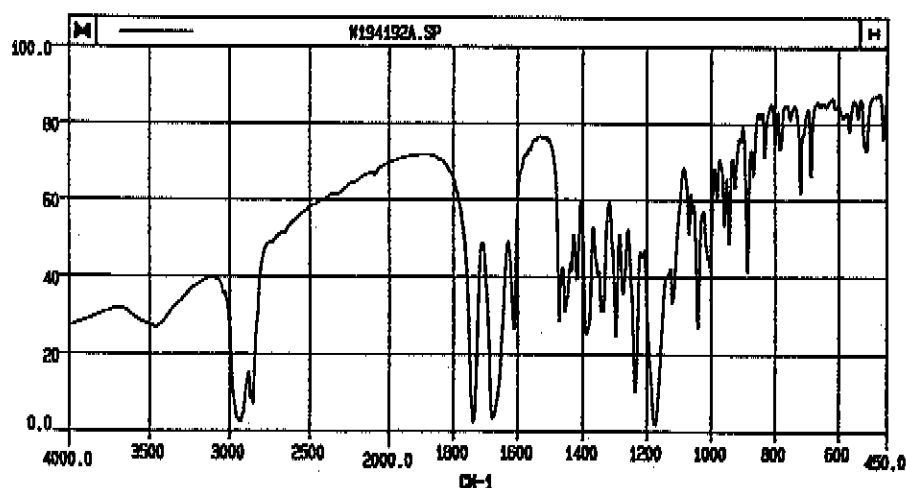


Figure 1. IR-spectrum of 2.5 mg of testosterone enantate Control No 194192 in 300 mg of KBr recorded against a KBr disc.
Instrument: Perkin-Elmer 1600 FTIR.

UV-spectrum

A UV-spectrum in ethanol was recorded on a Varian Cary 5 spectrophotometer. The spectrum is given in Figure 2. λ -max in ethanol was observed at 240 nm.

$A_{1\text{cm}}^{1\%} = 423$ (n=6, RSD=0.3 %)

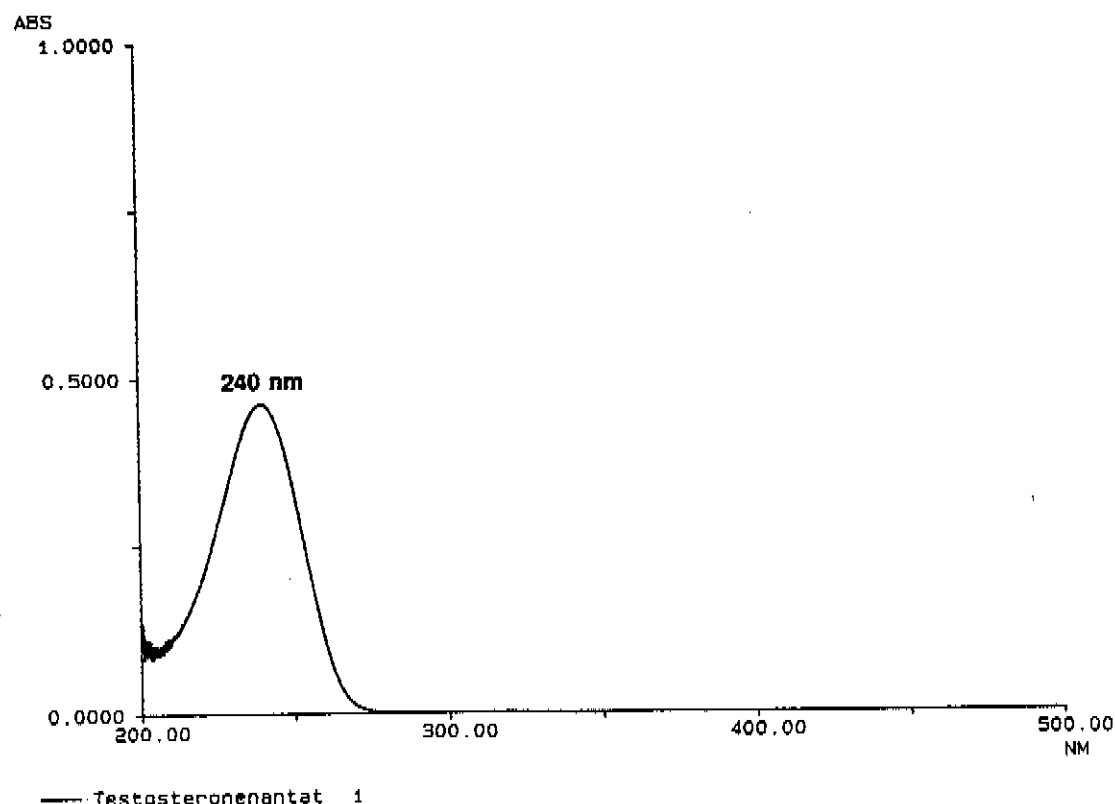


Figure 2. UV-spectrum of testosterone enantate Control No 194192, 13 $\mu\text{g/ml}$ in ethanol.

Specific optical rotation

$[\alpha]_D^{20^\circ} = +77.3^\circ$. Determined in dioxan at a concentration of 10 mg/ml.

Thin-layer chromatography identity test

The system described in the International Pharmacopoeia 3rd Ed. Vol. 3 page 313 is not suitable because testosterone enantate is retained at the starting point. A more suitable system is described under purity.

Assay

Liquid chromatographic assay

99.5 % (n=12, RSD=0.6%) when determined at 241nm with the HPLC system described under purity. The USPRS lot J was used as reference and regarded as 100 %. The difference is statistically significant at the 95% confidence level when using unpaired t-test.

Spectrophotometric assay

100.2%(n=6, RSD=0.3 %) when determined at 240 nm in ethanol against the USPRS lot J, regarded as 100 %. The difference is not statistically significant at the 95% confidence level using unpaired t-test.

Loss on drying

< 0.1%(n=3) when dried to constant weight in vacuum over phosphorus pentoxide at ambient temperature.

Purity

Free heptanoic acid

The test was performed according to the International Pharmacopoeia 3rd Ed. Vol.3 page 313. 0.3 ml of 0.01 M sodium hydroxide was required to neutralize the free acid (limit 0.6 ml).

Thin-layer chromatography

One secondary spot was detected when evaluated by densitometry at 241 nm. Its total amount was estimated to be about 0.1 %. After spraying the same spot was found. The detection limit of this system was about 0.1µg (0.1 %) for testosterone enantate. R_f (testosterone enantate) = 0.64.

The following thin-layer chromatographic system according to the International Pharmacopoeia 3rd Ed. Vol. 3 was used.

Thin-layer: Silica gel 60 (Merck)
Eluent: Dichloroethane:methanol:water (92:8:0.5)
Sample: 100 µg of testosterone enantate dissolved in ethanol were applied.
Visualization: Examination by densitometry at 241 nm with a Desaga CD60 densitometer.
After drying in air and heating to 110 °C, the hot plate was sprayed with sulfuric acid/ethanol TS followed by heating to 110 °C for 10 minutes and examination in UV-light of 365 nm.

In USPRS lot J no extra spots were found. In the BPCRS batch 1196 seven secondary spots were detected.

High performance liquid chromatography

The total amount of impurities estimated by peak area measurement was about 0.2 %. A chromatogram is shown in Figure 3. Three impurities were found. None of them was identical to testosterone capronate, which elutes at 13 minutes.

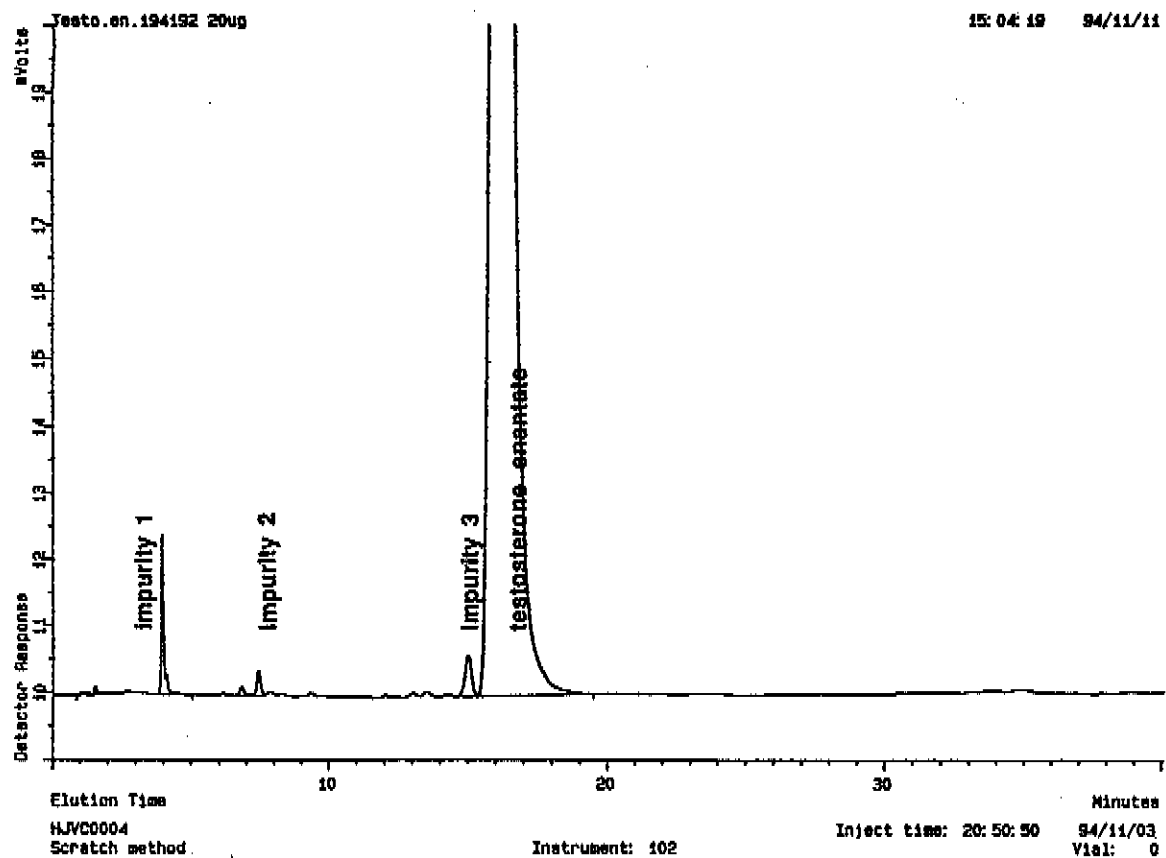


Figure 3. Chromatogram of testosterone enantate Control No 194192 monitored at 241 nm.

The following conditions were used:

Eluent: Acetonitrile: water (73: 27)
Column: Hichrom Spherisorb S5C8 (250 x 4.6 mm)
Detector: Varian 9065 Polychrom at 241 nm
Pump: Varian 9012 operated at a flow rate of 1ml/min.
Integrator: PeakPro (Beckman)
Sample: 1 mg/ml dissolved in the eluent. 20 μ l corresponding to 20 μ g were injected.
No degradation was observed when the sample was stored in the dark at + 8 °C for 24 hours in the eluent.

Minimum detectable quantity for testosterone enantate was 2 ng and the limit of quantification 5ng (0.03 %).

In USPRS lot J 0.2 % impurities were found, but it did not show the same impurity pattern as the ICRS. In the BPCRS batch 1196 about twenty different impurities were detected. The BPCRS was also found to be more unstable in solution than the ICRS.

Diode-array detection

The impurities and the main peak were evaluated with the Varian 9065 Polychrom detector. They had all similar spectra with UV maxima at about 240 nm. This wavelength was chosen giving the best sensitivity compared to e.g. 210nm.

Data given by the manufacturer

| | |
|----------------------------|---|
| Appearance: | white to cream-white crystalline powder |
| Identity (IR): | passes test |
| Melting range: | 36-37.5 °C |
| Specific optical rotation: | + 79.3° |
| Related substances: | passes test (no foreign steroid \geq 1%) passes test (the sum of foreign steroids \leq 2%) |
| Enanthic acid | \leq 0.01 % |
| Water | 0.01 % |
| Sulfated ash | \leq 0.01 % |
| Assay | 99.2% |

Stability

Stability studies were not performed as this substance was not suspected to degrade easily. However as the substance melts at about 37 °C it must be protected from higher temperatures. Regular re-examinations of the ICRS will be performed.

Conclusion

Testosterone enantate, Control No 194192, can be considered suitable as International Chemical Reference Substance for the intended purpose. The content of testosterone enantate when used in the spectrophotometric assay is taken to be 100 % calculated with reference to the dried substance.
