



WHO COLLABORATING CENTRE FOR CHEMICAL REFERENCE SUBSTANCES

Report on the work in 1993

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.

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### Distribution of reference substances in 1993

During 1993 the total number of International Chemical Reference Substances distributed from the Centre was 920. Compared to the figures for 1992 this corresponds to a decrease of about 18 per cent. The most frequently requested substances were in order of demand Tetracycline hydrochloride, Rifampicin quinone, 3-Formylrifamycin, Erythromycin, Melting point reference substances and Oxytetracycline hydrochloride. Detailed figures for the distribution of the individual substances are given in Appendix 1.

The substances were distributed to 34 different countries during 1993. Details of the distribution are given in Appendix 2. Considering the distribution to different regions it is observed that no substances went to the African Region, 8.5% to the Americas, 32% to the Eastern Mediterranean, 53.5% to Europe, 3% to South East Asia and 3% to the Western Pacific Region.

A comment to this is that there is a great variation in this pattern from year to year. Last year eg. 22% went to the African Region and only 5% to the Eastern Mediterranean Region.

### Distribution of reference spectra in 1993

During 1993 International Infrared Reference Spectra (IIRS) were available for the first time. The distribution of spectra to different WHO regions are given in Appendix 3. A total number of 765 spectra were distributed. 40% was sent to the African Region, 8.6% to the Americas, 18% to the Eastern Mediterranean, 15.8% to Europe, 6.6% to South East Asia and 11% to the Western Pacific Region. The requests for spectra were intense in the beginning when the information about them was new, by the end of 1993 the requests have been rare.

### Establishment of reference substances in 1993

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), 8 International Chemical Reference Substances were established in 1993. 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 1994, with information about package sizes and control numbers for the current batches is given in Appendix 5 to this report. The list also includes 7 substances mentioned below which are expected to be formally adopted by the Expert Committee in 1994.

### Work on new reference substances completed in 1993

Work is being continued on new reference substances required to support specifications in the third edition of the International Pharmacopoeia. During 1993 six new reference substances for volume 3 were examined. They are (-)-3-(4-Hydroxy-3-methoxyphenyl)-2-hydrazino-2-methylalanine, Liothyronine sodium, Neamine hydrochloride, Neomycin B sulfate, Spectinomycin hydrochloride and Vincristine sulfate. The analytical reports are given in Appendices 10-15. These substances are considered suitable for adoption as International Chemical Reference Substances. The stock of 4-Epitetracycline ammonium salt No 180098 was depleted and has been replaced by 4-Epitetracycline hydrochloride, No 293098 during 1993. 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. Due to suspected stability problems one extended special stability investigation of nystatin in dry state was performed. The results together with comparisons with the WHO 2nd Biological standard and the Ph Eur CRS lot 1 are given in Appendix 7.

#### Work in progress and future work

Work is continuously performed on the substances required to support the monographs in Volume 3 of the International Pharmacopoeia. For the moment work on eight of the 15 substances, given in Appendix 8, is in progress at the Centre.

During 1993 four scholarship holders visited the Centre for longer periods namely Dr Ian Matondo, Mr Henry Chavanika, Mrs Charity Kanjere, all three from the Zimbabwe Regional Drug Control Laboratory, as well as Mrs Wong-Neo Geok Eng from the Department of Scientific Services, Institute of Science and Forensic Medicine, Singapore. They studied administrative aspects of the handling of reference substances as well as different analytical techniques used in the testing of International Chemical Reference Substances.

The Centre has also participated in two scientific meetings during 1993. The first was a meeting on Quality Assurance in Laboratories, held in Jönköping, Sweden. The other was an International Symposium on Purity Determination of Drugs in Stockholm, Sweden, where a lecture with the title "Purity determination of reference substances" was given.

#### Administrative and financial matters

The total cost for running the Centre in 1993 was estimated at 467.848 US\$. The income from sales of reference substances was about 38.330 US\$ and the contribution received from the WHO headquarters was 18.000 US\$ which leaves a deficit of 411.518 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 1993. This year we want to address our thanks to the European Pharmacopoeia Laboratory in Strasbourg, France and the Therapeutic Goods Administration Laboratories, Canberra, Australia.

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 Bracco Industries Chimica SPA, Milano, Italy; Merck and Co Inc., Rahway, NJ, USA; Roussel UCLAF, Romainville, France and Upjohn PTY Limited, Rydalmere, USA.

## APPENDIX 1

## DISTRIBUTION OF CHEMICAL REFERENCE SUBSTANCES IN 1993

Aceclidine salicylate	-- items	Clomifene citrate Z-isomer	
p-Acetamidobenzalazine	1 "	see Zuclomifene	
Acetazolamide	2 "	Cloxacillin sodium	5 items
Allopurinol	3 "	Colecalciferol	6 "
2-Amino-5-nitrothiazole	1 "	Cortisone acetate	4 "
3-Aminopyrazole-4-carbox- amide hemisulfate	1 "	Dapsone	4 "
Amitriptyline hydrochloride	4 "	Desoxycortone acetate	2 "
Amodiaquine hydrochloride	-- "	Dexamethasone	5 "
Amphotericin B	4 "	Dexamethasone acetate	6 "
Ampicillin (anhydrous)	5 "	Dexamethasone phosphoric acid	-- "
Ampicillin sodium	7 "	Dexamethasone sodium phosphate	2 "
Ampicillin trihydrate	12 "	Diazepam	5 "
Anhydrotetracycline hydro- chloride	18 "	Diazoxide	1 "
Atropine sulfate	16 "	Dicloxacin sodium	4 "
Azathioprine	1 "	Dicolinium iodide	-- "
Bacitracin zinc	2 "	Dicoumarol	1 "
Beclometasone dipropionate	-- "	Diethylcarbamazine	
Bendazol hydrochloride	-- "	dihydrogen citrate	1 "
Benzobarbital	-- "	Digitoxin	3 "
Benzylamine sulfate	-- "	Digoxin	12 "
Benzylpenicillin potassium	12 "	NN' -Di-(2,3-xylyl)anthra- nilamide	1 "
Benzylpenicillin sodium	18 "	Dopamine hydrochloride	-- "
Bephenium hydroxynaphthoate	-- "	Emetine hydrochloride	2 "
Betamethasone	2 "	4-Epianhydrotetracycline	
Betamethasone valerate	2 "	hydrochloride	16 "
Betanidine sulfate	1 "	4-Epitetracycline ammonium	
Bupivacaine hydrochloride	4 "	salt	12 "
Caffeine	2 "	Ergocalciferol	8 "
Carbamazepine	7 "	Ergometrine hydrogen maleate	4 "
Carbenicillin monosodium	2 "	Ergotamine tartrate	2 "
Chloramphenicol	9 "	Erythromycin	27 "
Chloramphenicol palmitate	7 "	Estradiol benzoate	1 "
Chloramphenicol palmitate (Polymorph A)	2 "	Estrone	1 "
5-Chloro-2-methylamino- benzophenone	4 "	Etacrylic acid	-- "
2-(4-Chloro-3-sulfamoyl- benzoyl)benzoic acid	1 "	Ethambutol hydrochloride	3 "
Chlorphenamine hydrogen maleate	-- "	Ethinylestradiol	2 "
Chlorpromazine hydro- chloride	4 "	Ethisterone	2 "
Chlortalidone	-- "	Ethosuximide	3 "
Chlortetracycline hydrochloride	17 "	Etocarlide	-- "
Cimetidine	4 "	Flucytosine	2 "
Clomifene citrate	3 "	Fluorouracil	3 "
		Fluphenazine decanoate	
		dihydrochloride	10 "
		Fluphenazine enantate	
		dihydrochloride	1 "
		Fluphenazine hydrochloride	2 "
		Folic acid	14 "
		3-Formylrifamycin	28 "

Furosemide	11 items	Oxytetracycline dihydrate	5 items
Griseofulvin	2 "	Oxytetracycline hydrochloride	20 "
Haloperidol	9 "	Papaverine hydrochloride	5 "
Hydrochlorothiazide	3 "	Phenethicillin potassium	2 "
Hydrocortisone	7 "	Phenoxyethylpenicillin	5 "
Hydrocortisone acetate	4 "	Phenoxyethylpenicillin calcium	-- "
(-)-3-(4-Hydroxy-3-methoxy- phenyl)-2-methylalanine	1 "	Phenoxyethylpenicillin potassium	3 "
Ibuprofen	4 "	Phenytoin	9 "
Imipramine hydrochloride	5 "	Prednisolone	6 "
Indometacin	4 "	Prednisolone acetate	4 "
o-Iodohippuric acid	1 "	Prednisone	4 "
Isoniazid	6 "	Prednisone acetate	4 "
Lanatoside C	3 "	Probenecid	-- "
Levodopa	2 "	Procaine hydrochloride	4 "
Levothyroxine sodium	3 "	Procarbazine hydrochloride	1 "
Lidocaine	9 "	Progesterone	3 "
Lidocaine hydrochloride	6 "	Propicillin potassium	1 "
Mefenamic acid	3 "	Propranolol hydrochloride	6 "
Melting Point Reference Substances		Propylthiouracil	3 "
(set of 13 substances)	20 "	Pyrantel embonate	-- "
<i>Individual Melting Point</i>		Pyridostigmine bromide	2 "
<i>Reference Substances</i>		Reserpine	4 "
Azobenzene	15 "	Retinol acetate	
Vanillin	10 "	(solution à 25000 IU)	8 "
Benzil	6 "	Riboflavin	7 "
Acetanilide	-- "	Rifampicin	8 "
Phenacetin	6 "	Rifampicin quinone	31 "
Benzanilide	14 "	Sodium cromoglicate	3 "
Sulfanilamide	8 "	Sulfamethoxazole	10 "
Sulfopyridine	6 "	Sulfamethoxypyridazine	-- "
Dicyandiamide	6 "	Sulfanilamide	5 "
Saccharin	7 "	Sulfasalazine	1 "
Caffeine	9 "	Testosterone propionate	3 "
Phenolphthalein	14 "	Tetracycline hydrochloride	32 "
Metazide	-- "	Thioacetazone	1 "
Methaqualone	-- "	4,4'-Thiodianiline	-- "
Methyldopa	7 "	Thyroxine sodium	
Methyltestosterone	2 "	see Levothyroxine sodium	
Meticillin sodium	3 "	Tolbutamide	2 "
Metronidazole	15 "	Tolnaftate	-- "
Nafcillin sodium	2 "	Trimethadione	1 "
Neostigmine metilsulfate	2 "	Trimethoprim	14 "
Nicotinamide	9 "	Trimethylguanidine sulfate	-- "
Nicotinic acid	5 "	Tubocurarine chloride	2 "
Niridazole	2 "	Vitamin A acetate (solution)	
Niridazole-chlorethyl- carboxamide	-- "	see Retinol acetate	
Norethisterone	3 "	Warfarin	4 "
Norethisterone acetate	4 "	Zuclomifene	6 "
Nystatin	3 "		
Ouabain	1 "		
Oxacillin sodium	9 "		

APPENDIX 2

DISTRIBUTION OF INTERNATIONAL CHEMICAL REFERENCE SUBSTANCES  
TO DIFFERENT WHO REGIONS IN 1993

<i>WHO Regions</i>	<i>Number of ICRS distributed in 1993</i>
<b>African Region (AFRO)</b>	—
<b>Region of the Americas (AMRO)</b>	
Argentina	10
Brasil	9
Cuba	55
Mexico	4
<b>Eastern Mediterranean Region (EMRO)</b>	
Cyprus	2
Egypt	83
Iran	5
Jordan	7
Morocco	98
Oman	101
<b>European Region (EURO)</b>	
Belgium	26
Bulgaria	15
Croatia	6
Denmark	10
Finland	9
France	4
Germany	99
Greece	3
Hungary	4
Italy	17
Netherlands	6
Norway	1
Russian Federation	86
Slovakia	12
Spain	2
Sweden	117
Switzerland	22
United Kingdom	53
<b>South-East Asia Region (SEARO)</b>	
India	5
Indonesia	10
Mongolia	13
<b>Western Pacific Region (WPRO)</b>	
Australia	5
The Philippines	12
Singapore	9

APPENDIX 3DISTRIBUTION OF INTERNATIONAL INFRARED REFERENCE SPECTRA  
TO DIFFERENT WHO REGIONS IN 1993

<i>WHO Regions</i>	<i>Number of IIRS distributed in 1993</i>
<b>African Region (AFRO)</b>	
Congo	56
Lesotho	Binder
Nigeria	250
<b>Region of the Americas (AMRO)</b>	
Costa Rica	16
Jamaica	50
<b>Eastern Mediterranean Region (EMRO)</b>	
Egypt	20
Iran	50
Jordan	31
Oman	37
<b>European Region (EURO)</b>	
Malta	71
Switzerland (WHO)	50
<b>South-East Asia Region (SEARO)</b>	
Indonesia	50
<b>Western Pacific Region (WPRO)</b>	
Malaysia	34
The Phillipines	50

APPENDIX 4

## INTERNATIONAL CHEMICAL REFERENCE SUBSTANCES ESTABLISHED IN 1993

Reference Substance	Control Number	Analytical Report	Remarks
Amodiaquine hydrochloride	192160	WHO/PHARM/93.564 Appendix 7	
Bacitracin zinc	192174	WHO/PHARM/93.564 Appendix 8	
Beclometasone dipropionate	192175	WHO/PHARM/93.564 Appendix 9	
Dexamethasone phosphoric acid	192161	WHO/PHARM/93.564 Appendix 10	
Dexamethasone sodium phosphate	192158	WHO/PHARM/93.564 Appendix 11	
Dopamine hydrochloride	192159	WHO/PHARM/93.564 Appendix 12	
Probenecid	192156	WHO/PHARM/93.564 Appendix 13	
Pyrantel embonate (Pyrantel pamoate)	192157	WHO/PHARM/93.564 Appendix 14	

APPENDIX 5

LIST OF AVAILABLE INTERNATIONAL CHEMICAL REFERENCE SUBSTANCES

1994

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>Package size</u>	<u>Control Number</u>
Lidocaine	100 mg	181104
Lidocaine hydrochloride	100 mg	181105
Liothyronine sodium	50 mg	193179
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	192170
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
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	200 mg	193178
Neostigmine metilsulfate	100 mg	187135
Nicotinamide	100 mg	179090
Nicotinic acid	100 mg	179091
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
Phenoxyethylpenicillin	200 mg	179082
Phenoxyethylpenicillin calcium	200 mg	179083
Phenoxyethylpenicillin potassium	200 mg	176075
Phenytoin	100 mg	179089
Prednisolone	100 mg	389029
Prednisolone acetate	100 mg	289030
Prednisone	100 mg	167031
Prednisone acetate	100 mg	169032
Probenecid	100 mg	192156
Procaine hydrochloride	100 mg	183119

	<u>Package size</u>	<u>Control Number</u>
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 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

APPENDIX 6

## 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 1993 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 (mole %), 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.

Allopurinol, Control No 287049

Initial analytical report: WHO/PHARM/88.537, Appendix 6

Examination year:	1987	1993
IR	conforms	—
TLC, %	no sec. spots	<0.1 one spot
HPLC, %	0.02	<0.1 (new system)
TGA, %	<0.1	~0.1

3-Aminopyrazole-4-carboxamide hemisulfate, Control No 172050

Initial analytical report: WHO/PHARM/72.471, Appendix 10

Examination year:	1972	1975	1981	1985	1993
IR	conforms	—	—	conforms	—
TLC, %	no sec spots	—	—	2 faint sec spots (250 µg)	<0.1 1 faint sec. spot
HPLC, %	—	—	—	—	0.05
TGA, %	—	—	—	—	5.0
Water (KF) %	13.7	11.2	10.8	10.4* } 5.2**	—
Assay (potentiometric)	99.8	99.6	99.5	100.3	—

\* old dispensed vials

\*\* bulk, dispensed 1985

Amitriptyline hydrochloride, Control No 181101

Initial analytical report: WHO/PHARM/82.509, Appendix 6

Examination year:	1982	1985	1993
IR	conforms	conforms	—
TLC, %	<0.1	<0.1	—
HPLC, %	<0.1	<0.1	<0.1
TGA, %	—	—	0.2
KF, %	—	0.1	—
LOD, %	0.1	0.1	—
Assay, potentiometric, %	100.3	100.3	—
Assay, HPLC, %	—	—	100.0
DTA, %	0.3	0.4	—
PSA, %	0.3	—	—

Betamethasone valerate, Control No 190145

Initial analytical report: WHO/PHARM/91.555, Appendix 8

Examination year:	1990	1993
IR	conforms	-
TLC, %	0.2	-
HPLC, %	0.3	0.2
TGA, %	<0.1	0.2
Assay, titration, %	99.8	-
Assay, % (spectrophotometric)	100.3	-

Chlortetracycline hydrochloride, Control No 187138

Initial analytical report: WHO/PHARM/88.537, Appendix 7

Examination year:	1987	1993
IR	conforms	-
TLC, %	<0.1	-
HPLC, %	0.5 (tetracycline)	0.7 (tetracycline) 0.8 (unknown)
TGA, %	0.15	0.23
Assay, microbiologically	1001 IU/mg	1043 IU/mg*
Assay, % (HPLC)	-	100.0*

\* WHO 2nd Biological Standard, 1000 IU/mg used as reference substance.

Ergometrine hydrogen maleate, Control No 277012

Initial analytical report: WHO/PHARM/78.494, Appendix 5

Examination year:	1977	1982	1989	1993
IR	conforms	—	—	—
TLC, %	1 (3 sec spots)	(2-3 sec spots)	0.8 (4 sec spots)	—
HPLC, %	0.8	0.9	—	0.5
TGA, %	—	—	0.3	0.5
LOD, %	0.8	—	0.3	—
Assay, % (potentiometric)	100.0	99.9	—	—

Ergotamine tartrate, Control No 385013

Initial analytical report: WHO/PHARM/86.527, Appendix 6

Examination year:	1985	1993
IR	conforms	—
TLC, %	two sec spots	—
HPLC, %	0.2 (210 nm)	1.1* (235 nm)
TGA, %	—	4.2
LOD, %	2.4	3.2
Assay, titration, %	99.2	—
GC, methanol, %	0.9	—

\* Higher figure due to a new system with better separation efficiency, not degradation.

Estrone, Control No. 279015

Initial analytical report: WHO/PHARM/79.499, Appendix 6

Examination year:	1979	1984	1993
IR	conforms	—	—
TLC, %	0.2	—	—
HPLC, %	≈0.15 (280 nm)	0.1 (280 nm)	0.1 (278 nm) 0.2 (220 nm)
TGA, %	—	—	<0.1
LOD, %	<0.1	—	—

Griseofulvin, Control No 280040

Initial analytical report: WHO/PHARM/81.508, Appendix 10

Examination year:	1980	1988	1993
IR	conforms	conforms	—
TLC, %	one spot	0.5-1	0.4
HPLC, %	0.6	0.6 (236 nm) 0.9 (291 nm)	0.8 (236 nm*) 1.1 (291 nm)
TGA, %	—	<0.1	<0.1
LOD, %	0.1	—	—
Assay, % (potentiometric)	100.0	99.5	—

\* Increased values, due to better separation efficiency on the column, not degradation.

Ibuprofen, Control No 183117

Initial analytical report: WHO/PHARM/84.513, Appendix 12

Examination year:	1983	1993
IR	conforms	—
TLC, %	0.7	—
HPLC, %	0.7 (264 nm)	0.6 (220 nm) 0.5 (263 nm)
TGA, %	—	<0.1
LOD, %	<0.1	—
Assay, % (HPLC)	—	99.6
Assay, % (titration)	99.5	—

Imipramine hydrochloride, Control No 172064

Initial analytical report: WHO/PHARM/73.475, Appendix 7

Examination year:	1972	1977	1983	1988	1993
IR	conforms	conforms	—	conforms	—
TLC, %	4 sec. spots	3 sec. spots	3 sec. spots	3 sec. spots	4 sec. spots 0.1
HPLC, %, 237 nm	—	—	—	—	0.1
HPLC, assay, %	—	—	—	—	100.5*
TGA, %	—	—	—	—	0.06
LOD, %	<0.1	0.05	<0.1	0.08	—
Assay, % (titration)	99.9	99.1	99.1	99.2	—
DSC, %	0.2	—	—	0.2	—

\* EPCRS batch 1, with a content of 99.7% was used as reference.

Metronidazole. Control No 183118

Initial analytical report: WHO/PHARM/84.513, Appendix 13

Examination year:	1983	1993
IR	conforms	-
TLC, %	0.1	-
HPLC, %	<0.2	0.1
TGA, %	-	<0.1
LOD, %	<0.1	-
Assay (titrimetric), %	100.1	-

Nystatin. Control No 191152

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

Examination year:	1991	1992	1993
HPLC, %, impurities, 304 nm	5.0	-	7.9*
HPLC, assay, %	100.0	-	100.0
TGA, %	5.0	-	5.2
Microbiological assay	6382 IU/mg**	5208 IU/mg***	4968 IU/mg***
TLC, %	4.1	-	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. No degradation of the ICRS was observed, see separate investigation in Appendix 7 of this report.

Propranolol hydrochloride, Control No 187139

Initial analytical report: WHO/PHARM/88.537, Appendix 13

Examination year:	1987	1993
IR	conforms	-
TLC, %	< 0.1	-
TGA, %	-	<0.1
HPLC, %	0.2	0.2
LOD, %	<0.1	-
DTA, %	0.2	-
Assay, % (spectrophotometric)	100.5	-
Assay, % (titrimetric)	100.0	-
Assay, % (HPLC)	100.6	-

Tetracycline hydrochloride, Control No 180095

Initial analytical report: WHO/PHARM/81.508, Appendix 12

Examination year:	1980	1985	1989	1993
HPLC, weight %				
of 4-epitetracycline	1.3	0.7	0.7	≈1.0
anhydrotetracycline	0.1	0.1	<0.05	-
4-epianhydrotetracycline	<0.5	0.05	<0.05	-
unidentified, area %	~0.05	0.1	-	-
Loss on drying, %	0.25	-	-	-
Water (KF), %	0.4	0.3	-	-
TLC	one sec spot	-	-	-
Assay, % (potentiometric)	99.6	-	-	-
TGA, %	-	-	0.3	0.3

APPENDIX 7**Stability study of nystatin in dry state**

Monika Westemark, WHO Collaborating Centre for Chemical Reference Substances.

Background:

When the International Chemical Reference Substance for Nystatin, ICRS 191152, was established in 1991, it was assayed microbiologically against the EPCRS with a declared content of 4980 IU/mg (November 1991). About one year later (August 1992), the microbiological assay was repeated but now with the WHO 2nd Biological standard with a declared content of 4855 IU/mg as reference standard. The result for the International Chemical Reference Substance showed a decrease from 6106 IU/mg to 5208 IU/mg in one year. As we did not suspect the ICRS to be very unstable, we performed this stability study (July - August 1993) on stressed samples of the ICRS, to see under what circumstances degradation occurs. The samples were tested with different techniques as HPLC, TLC, TG, UV and microbiological assay, to see the extent of degradation revealed.

According to the definition in Ph. Int and Ph. Eur, nystatin is a mixture of substances produced by the growth of certain strains of streptomyces noursei. It contains mainly tetraenes, the principal component being nystatin A<sub>1</sub>. The potency is not less than 4400 IU per milligram, calculated with reference to the dried substance.

Conclusion:

The ICRS 191152 for nystatin is a stable substance under normal storage conditions. To obtain significant degradation it has to be stressed. Nystatin is most sensitive to heat and light and to some extent to humidity. The EPCRS probably did not have the potency that was stated on the label 1991, which caused a misleading high value on the ICRS. See also concluding Table at the end of this report.

Samples tested:

The following samples were included in this study.

1. The International Chemical Reference Substance for Nystatin, ICRS 191152.
2. WHO 2nd Biological Standard, 1982 for Nystatin with a declared content of 4855 IU/mg.
3. The European Pharmacopoeia CRS lot 1 for Nystatin with a declared content of 4980 IU/mg.
4. Humidity-stressed ICRS 191152, 20 °C, RH 98%, 14 days, open containers in the dark.
5. Heat-stressed ICRS 191152, 105 °C, 20 hours, open containers in the dark.
6. Light-stressed ICRS 191152, 20 °C, 18 days, open container in daylight, lab-window.

Containers:

The 2nd Biological standard is dispensed in closed ampoules of uncoloured glass.

The EPCRS is dispensed in capped vials of uncoloured glass.

The ICRS is dispensed in capped vials of coloured glass.

Results from different analytical techniques:

The different nystatin samples were investigated with the following analytical methods.

- A. Liquid chromatography, assay + purity + diode-array
- B. Microbiological assay
- C. Thin-layer chromatography
- D. UV spectrophotometry
- E. Thermogravimetric analysis

Results and description of the methods are given below. If not otherwise stated all calculations are performed on the dried substances.

A. Liquid chromatography*System 1: Assay (+ purity)*

The following system was used.

Column: Kromasil C-18, 150 mm x 3 mm

Eluent: 30% acetonitrile in 0.067 M phosphate buffer, pH 6.5. The buffer was prepared by dissolving 7.428 g  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  and 12.466 g  $\text{KH}_2\text{PO}_4$  in 2000 ml of water.

Detector: Shimadzu SPD-6AV or Varian UV 100, operated at 304 nm.

Pump: LDC Constametric III or Varian Vista 5500, operated at a flow rate of 1 ml/min.

Integrator: PeakPro (Beckman)

Sample: 0.01 mg/ml dissolved in methanol for assay, (better stability and solubility) or in the eluent for purity determinations.

The assay was also repeated a second time on another Kromasil column. The results are given in Table 1.

Table 1

Substance	Assay, expressed as % of ICRS  (n=2) Column 1	Assay, expressed as % of ICRS  (n=2)		% impurities, expressed as area percent  *
		Column 2		
		Analysis 1	Analysis 2	
ICRS 191152	100.0%	100.0%	100.0%	6.9
WHO 2nd Biol.	85.6%	85.8%	88.1%	11.3
EPCRS, Batch 1	79.5%	79.0%	79.3%	12.0
ICRS 191152, 98% RH 14 days, 20 °C	95.9%	-	-	6.1
ICRS 191152, 105 °C 20 hours	40.8%	-	-	10.7
ICRS 191152, day-light 18 days, 20 °C	88.3%	-	-	9.3

(- = samples finished, see system 2 purity)

(\* system 2, Table 2, is more optimized for purity determination)

A chromatogram showing the result from the assay is given in Figure 1.

When 2 series of six sequential injections were made of a solution of 20 µg/ml of ICRS 191152 in methanol RSD was found to be about 0.5%.

10:35:16 93/09/10

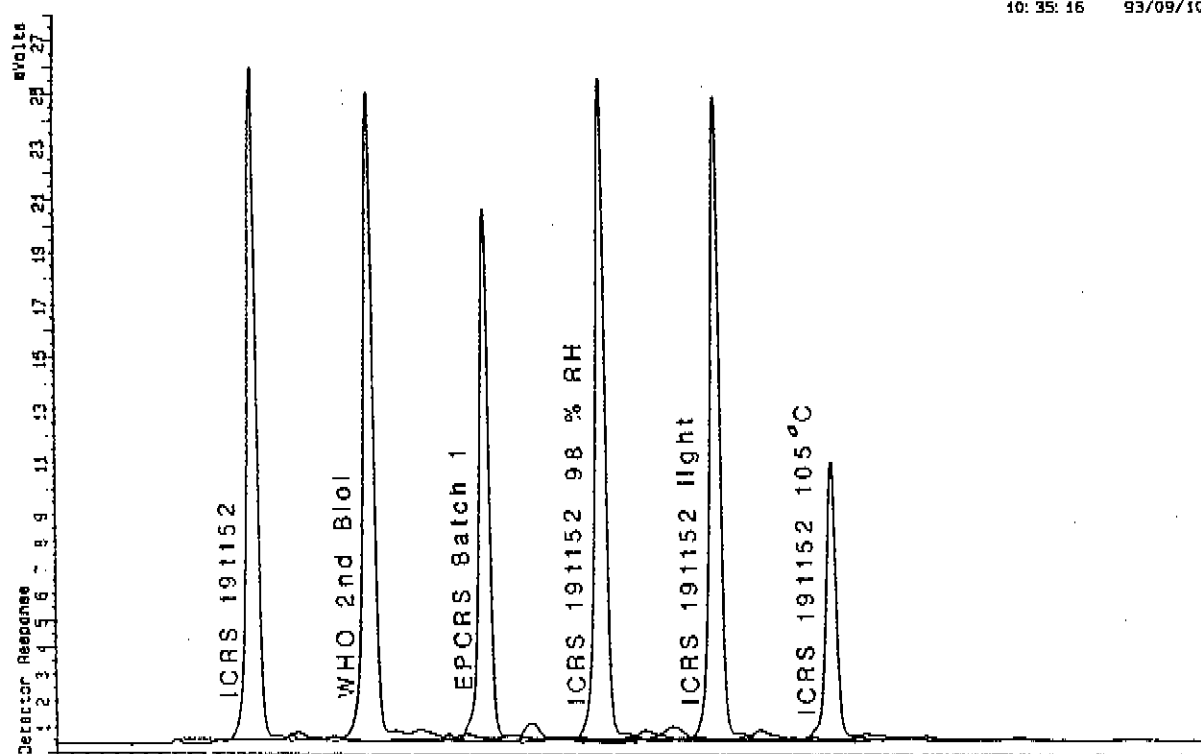


Figure 1. *Liquid chromatograms from assay of different nystatin samples.*

The ICRS 191152 has the highest content of nystatin A<sub>1</sub>. Degradation occurs when the substance is stressed, most by heat and light, but to some extent also in high humidity.

**System 2: Purity**  
Column: Vydac 300Å, 250 mm x 4.6 mm  
Eluent: Acetonitrile:Methanol:Buffer as system 1 (28:6:66 or 29:6:65)  
Detector: Waters Lambda Max 481  
Varian 9065 Polychrom operated at 302 nm.  
Pump: Waters 600 E or Varian 9012, operated at a flow rate of 1 ml/min.  
Integrator: PeakPro (Beckman)  
Sample: 0.5 mg/ml dissolved in mobile phase.

The analysis was performed at two occasions.

The results are given in Table 2.

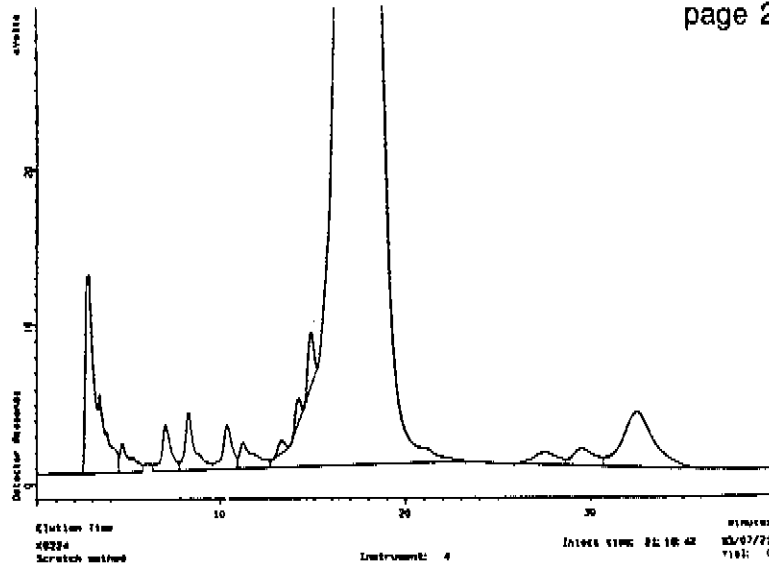
Table 2

Substance	% impurities, expressed as area %	
	Analysis 1	Analysis 2
ICRS 191152	8.1% (n=4)	7.6% (n=2)
WHO 2nd Biol.	12.9% (n=2)	11.3% (n=2)
EPCRS, Batch 1	10.0% (n=2)	12.2% (n=2)
ICRS 191152, 98% RH 14 days, 20 °C	9.0% (n=2)	6.8% (n=2)
ICRS 191152, 105 °C 20 hours	15.1% (n=2)	14.6% (n=2)
ICRS 191152, day-light 18 days, 20 °C	9.8% (n=2)	10.3% (n=2)

Chromatograms from the purity testing are given for ICRS 191152, WHO 2nd Biological Standard and the EPCRS in Figure 2 and for the stressed samples of ICRS 191152 in Figure 3.

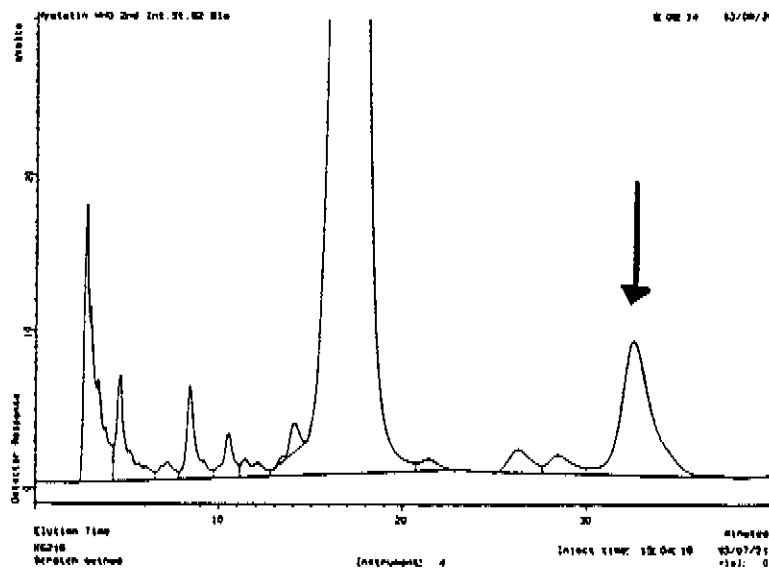
ICRS 191152

8.1%



WHO 2nd  
Biol Standard

12.9%



EPCRS  
Batch 1

9.0%

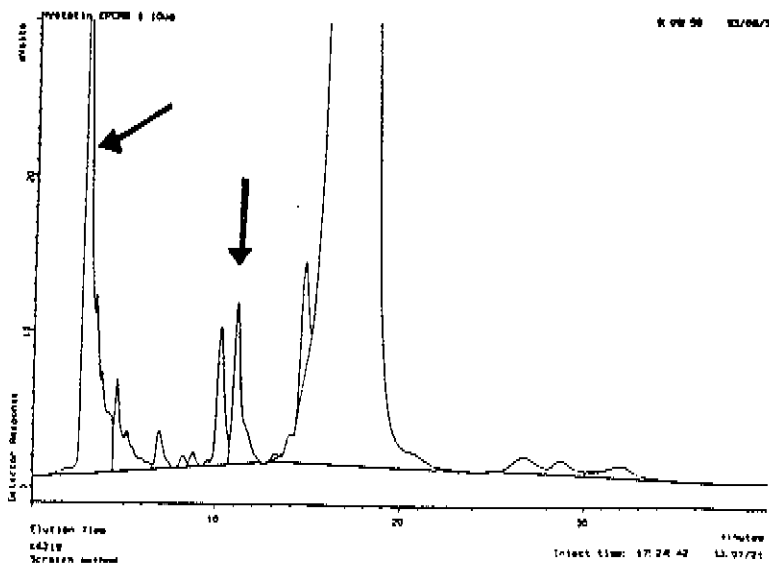
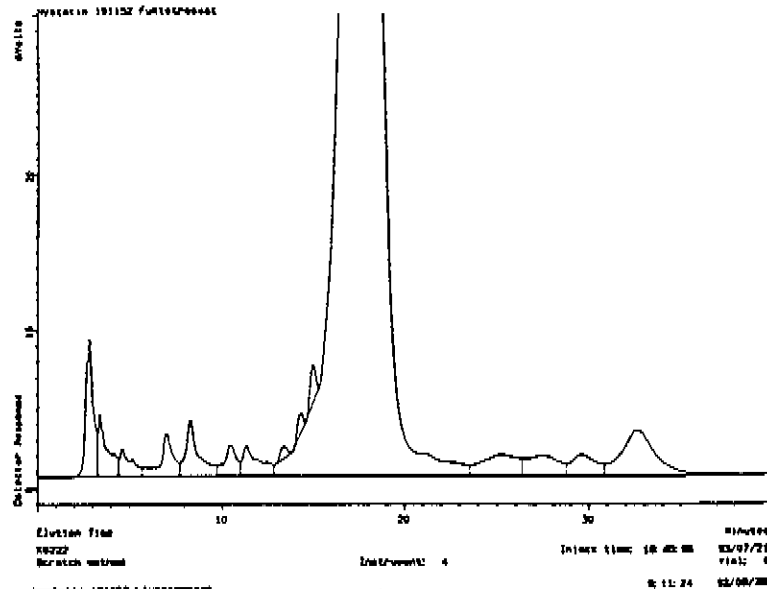


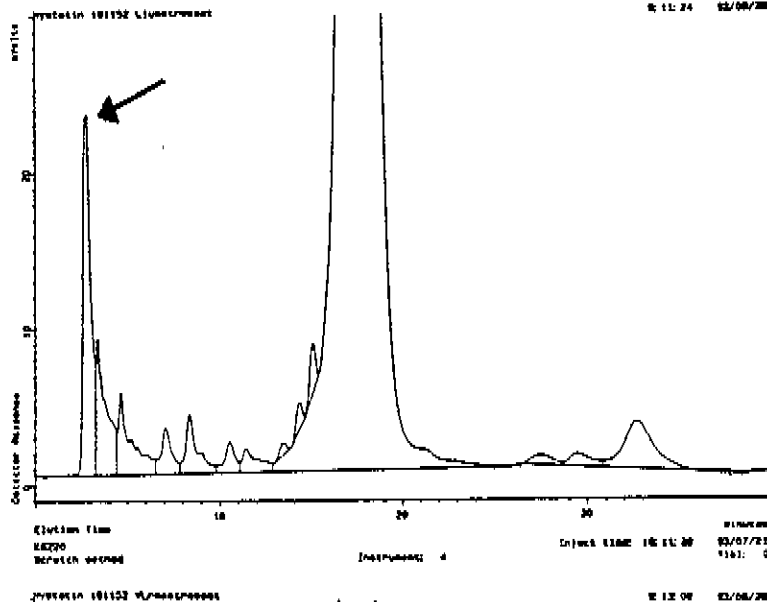
Figure 2. Chromatograms from purity testing of different reference substances of nystatin.

The impurity patterns differs somewhat. The WHO 2nd Biological Standard has a large peak at about 33 minutes, estimated to 4.6%. This peak is also present in the ICRS 191152 (about 2%), but not in the EPCRS. This peak was found to decrease in the stressed samples of ICRS 191152, especially the heat-stressed cf Figure 3. EPCRS has a somewhat different impurity pattern, e.g. the peak at about 11 minutes which also was found to be formed in the heat-stressed sample (cf Figure 3). EPCRS also has early eluting impurities, before 4 minutes, peaks that also increased in the heat-stressed sample of ICRS 191152 (cf Figure 3).

ICRS 191152  
98% RH  
14 days 20 °C  
9.0%



ICRS 191152  
daylight  
18 days 20 °C  
9.8%



ICRS 191152  
105 °C 20 hours  
15.1%

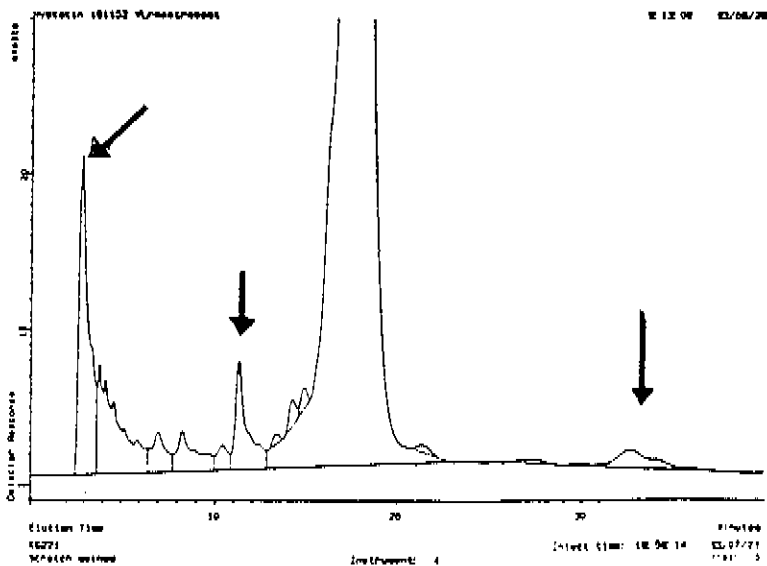


Figure 3. Chromatograms from purity testing of stressed samples of ICRS 191152.

It is clearly shown that heat-stress gives an increase in degradation products (early eluting peaks) and also a significant decrease in content of the nystatin A<sub>1</sub> peak (see assay). This is also observed for the light-stressed sample.

When these purity patterns on the Vydac column (system 2) were compared to results from the Kromasil column (system 1), we found a different selectivity and retention order of the peaks. The system on the Vydac column was not suitable for assay. We did not obtain reproducible results, probably due to the high loading on this column (adsorption?). We also saw a tendency of deterioration of the column; this is probably the reason why the impurity values shows slight differences between analysis 1 and 2.

#### Diode-array detection

All extra peaks that were found during purity testing with the Vydac column (system 2) were scanned with a Varian 9065 Polychrom detector. UV-maxima for nystatin A<sub>1</sub> and for the main part of extra peaks were found at 229, 292, 306 and 321 nm. The only exception from this is the first peak in all chromatograms, it elutes at 2.8-3 min and shows a different spectrum with maxima at 234 and 273 nm.

#### B. Microbiological assay

The samples studied were also tested by the microbiological assay according to Ph. Eur 2nd Ed. The WHO 2nd Biological Standard with a declared content of 4855 IU/mg was used as reference standard.

The results are given in Table 3.

Table 3

Substance	IU/mg	% of ICRS "as is"
ICRS 191152	4968	100.0
WHO 2nd Biol.	declared 4855	97.7
EPCRS, Batch 1	4221 (declared 4980)	85.0
ICRS 191152, 98% RH 14 days, 20 °C	4605	92.7
ICRS 191152, 105 °C 20 hours	1930	38.8
ICRS 191152, day-light 18 days, 20 °C	4074	82.0

The results are in acceptable agreement with the liquid chromatographic assay (cf Table 1). Except for the WHO 2nd Biological Standard, which gives a high microbiological value, but not chemically. It could possibly have an explanation in the large impurity at 33 minutes (see Figure 2), which is absent in the EPCRS.

The ICRS 191152 stored under normal conditions, +5 °C in closed vials, was also followed microbiologically for a period of about 1.5 years.

The results are given in Table 4.

Table 4

Date of analysis	ICRS 191152 IU/mg	Reference standard used
November 1991	6106	EPCRS declared content 4980 IU/mg
August 1992	5208 (n=6)	WHO 2nd Biol. Stand. declared content 4855 IU/mg
March 1993	5166 (n=2)	WHO 2nd Biol. Stand. declared content 4855 IU/mg
June 1993	4968 (n=2)	WHO 2nd Biol. Stand. declared content 4855 IU/mg

The decrease in content between November 1991 and August 1992 is a result of the change of reference standard. The EPCRS was found to have a lower content than was stated on the label (see Table 3). The raw data from the rest of the table were treated statistically with unpaired t-test, and no statistically significant degradation was found at the 95% confidence level.

C. Thin-layer chromatography

The samples were also examined by thin-layer chromatography.

The following thin-layer chromatographic system was used:

- System: According to Journal of Chromatography, 216 (1981) 367-373.
- Thin-layer: Silica gel 60, F-254 (Merck).
- Eluent: n-Amyl alcohol:Glacial acetic acid:Water (2:1:1)
- Sample: 100 µg of nystatin were applied; 5 mg/ml solutions were made in methanol.
- Visualization: UV-light at 254 nm, evaluation by densitometry at 304 nm.

Three secondary spots with  $R_f=0.46$ , 0.57 and 0.65 were detected at 304 nm in ICRS 191152 as well as in the WHO 2nd Biol. Stand. and the EPCRS.

$R_f$  (nystatin A<sub>1</sub>) = 0.43

Most degradation was observed for the heat-stressed sample. On scanning at 304 nm the ICRS 191152 was found to be the purest substance.

The results are given in figure 4.

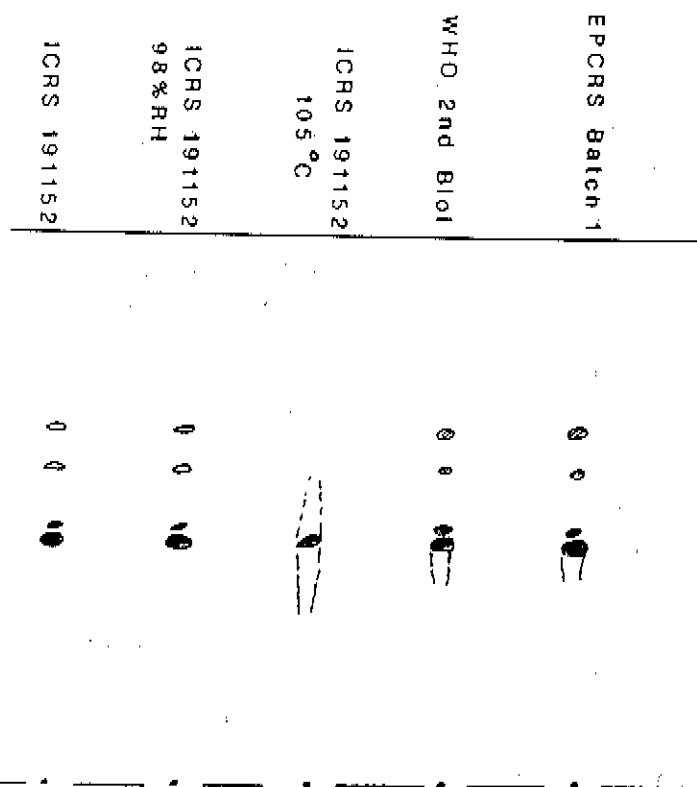


Figure 4 *Thin-layer chromatograms of nystatin.*

D. UV-spectrophotometry

The samples were also analyzed by a simple UV-spectrophotometric assay. The samples were dissolved in methanol and the absorbance measured at 304 nm.

The results are given in Table 5.

Table 5

Substance	Assay, expressed as % of ICRS
ICRS 191152	100.0
WHO 2nd Biol.	97.1
EPCRS, Batch 1	86.4
ICRS 191152, 98% RH 14 days, 20 °C	94.9
ICRS 191152, 105 °C 24 hours	63.0
ICRS 191152, day-light 18 days, 20 °C	-

(- sample not available)

Even this non-selective method is to some extent stability indicating.

E. Thermogravimetric analysis

The substances investigated were heated to 140 °C. The results of loss in weight calculated to 120 °C is given in table 6.

Instrument: Perkin Elmer TGA 7 Thermogravimetric analyzer  
Sample weight: 4 mg  
Heating rate: 5 °C/min  
Decomposition temperature: about 160 °C

Table 6

Substance	% loss in weight
ICRS 191152	5.2
WHO 2nd Biol.	4.8
EPCRS, Batch 1	4.8
ICRS 191152, 98% RH 14 days, 20 °C	7.3
ICRS 191152, 105 °C 20 hours	4.7
ICRS 191152, day-light 18 days, 20 °C	5.9

The substance is hygroscopic when exposed to high humidity (98%) or kept in open container in day-light.

Concluding table

Table 7

Substance	HPLC assay, expressed as % of ICRS	% impurities expressed as area %	Microbiological assay IU/mg	% of ICRS	UV 304 nm	TG % loss in weight
ICRS 191152	100.0	7.9	4968	100.0	100.0	5.2
WHO 2nd Biol.	86.5	12.1	declared 4855	97.7	97.1	4.8
EPCRS, Batch 1	79.3	11.1	4221 (declared 4980)	85.0	86.4	4.8
ICRS 191152, 98% RH 14 days, 20 °C	95.9	7.9	4605	92.7	94.9	7.3
ICRS 191152, 105 °C 20 hours	40.8	14.9	1930	38.8	63.0	4.7
ICRS 191152, day-light 18 days, 20 °C	88.3	10.1	4074	82.0	-	5.9

Conclusion:

The agreement between the HPLC assay and the microbiological assay is acceptable, with one exception, the WHO 2nd Biological standard which has a high microbiological activity, although it is chemically of lower quality than the ICRS. One possible explanation is that the late eluting impurity (cf Fig 2) at 33 min, which is dominating in the biological standard, has a microbiological activity similar to nystatin A<sub>1</sub>. Attempts will be made to identify it with LC-MS.

There is no perfect agreement between the HPLC assay values and the amount of impurities found. This could be due to the fact that peak area measurements had to be used for the estimation of impurities, since no reference substances for them were available. The highest amounts of impurities were however found in the most degraded sample.

It should also be noted that data from simple UV measurements at 304 nm showed surprisingly good agreement with the results obtained by other methods.

APPENDIX 8

## INTERNATIONAL CHEMICAL REFERENCE SUBSTANCES - PROJECT LIST 1994

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

Volume 3

Calcium folinate (*)	Nifurtimox
Doxorubicin hydrochloride	Noroxymorphone hydrochloride (*)
Fludrocortisone acetate	(impurity in Naloxone hydrochloride)
Gentamicin sulfate (*)	Paromomycin sulfate
Hydrocortisone sodium succinate (*)	Praziquantel
Levonorgestrel (*)	Prednisolone sodium phosphate
Loperamide hydrochloride (*)	Sulfacetamide
Methotrexate (*)	Testosterone enantate (*)

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

Volume 4

Amidotrizoic acid	Norethisterone enantate
3-Amino-2,4,6-triiodobenzoic acid	Paracetamol
Betamethasone sodium phosphate	Pentamidine isetionate
Chloroquine sulfate	Piperazine adipate
Cisplatin	Piperazine citrate
Dactinomycin	Prednisolone sodium phosphate
Ephedrine sulfate	Prednisolone succinate
Flucloxacillin sodium	Primaquine diphosphate
Iohexol	Pyrazinamide
Iopanoic acid	Sodium amidotrizoate
Iotroxic acid	Streptomycin sulfate
Kanamycin monosulfate	Tamoxifen citrate
Ketamine hydrochloride	Tamoxifen citrate E-isomer
Mebendazole	Thiopental sodium
Medroxyprogesterone acetate	Timolol maleate
Neomycin sulfate	Toluene-2-sulfonamide
Niclosamide	Vinblastine sulfate
Nitrofurantoin	

**APPENDIX 9****4-EPITETRACYCLINE HYDROCHLORIDE**

Control No 293098

Analytical Report

**INTENDED USE**

The stock of the current batch of the International Chemical Reference Substance of 4-epitetracycline ammonium salt, Control No 180098, is depleted and has to be replaced.

The monograph for Tetracycline hydrochloride in the International Pharmacopoeia 3rd Ed. Vol 2 requires the use of a reference substance of 4-epitetracycline hydrochloride in the thin-layer chromatographic test for related substances.

**MATERIAL**

About 5 g of the sample were received at the WHO Centre in December 1993. The material is being stored in tightly closed containers at + 5 °C, protected from light. This ICRS is from the same batch as the Ph Eur CRS Batch 3.

**ANALYTICAL DATA**

Description: A yellow powder.

**EVIDENCE OF CHEMICAL STRUCTURE****Infrared spectrum**

An infrared spectrum of 4-epitetracycline hydrochloride is given in Figure 1 (No W 293098 A). The spectrum is concordant with the spectrum of the USP reference standard lot F.

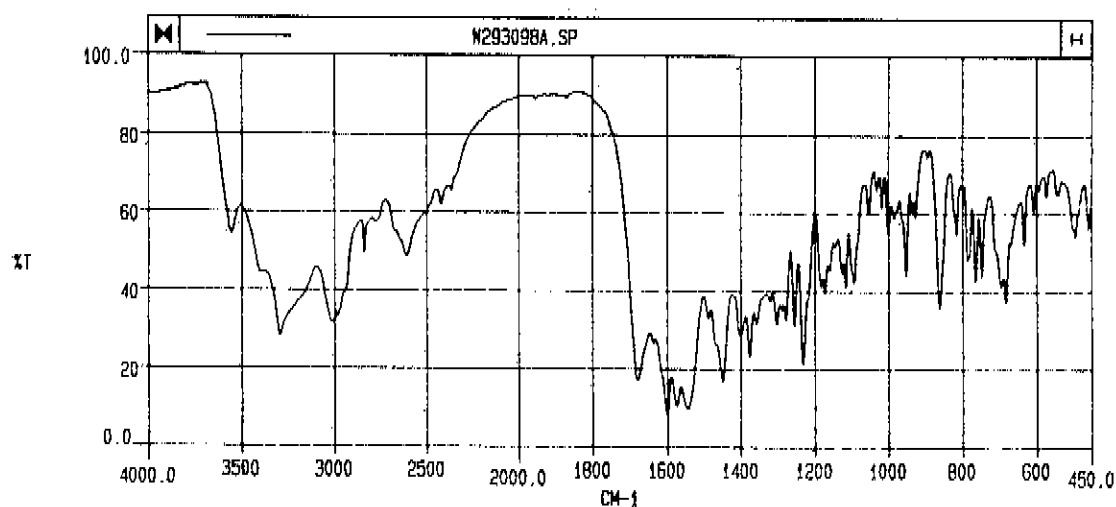


Figure 1. IR-spectrum of 1.1 mg of 4-epitetracycline hydrochloride Control No 293098 in 300 mg KBr recorded against a KBr disc.

Instrument: Perkin -Elmer 1600 FTIR.

### UV-spectrum

A UV-spectrum in 0.01 M HCl is given in Figure 2. UV-maxima are observed at 218 nm, 256 nm and 358 nm.

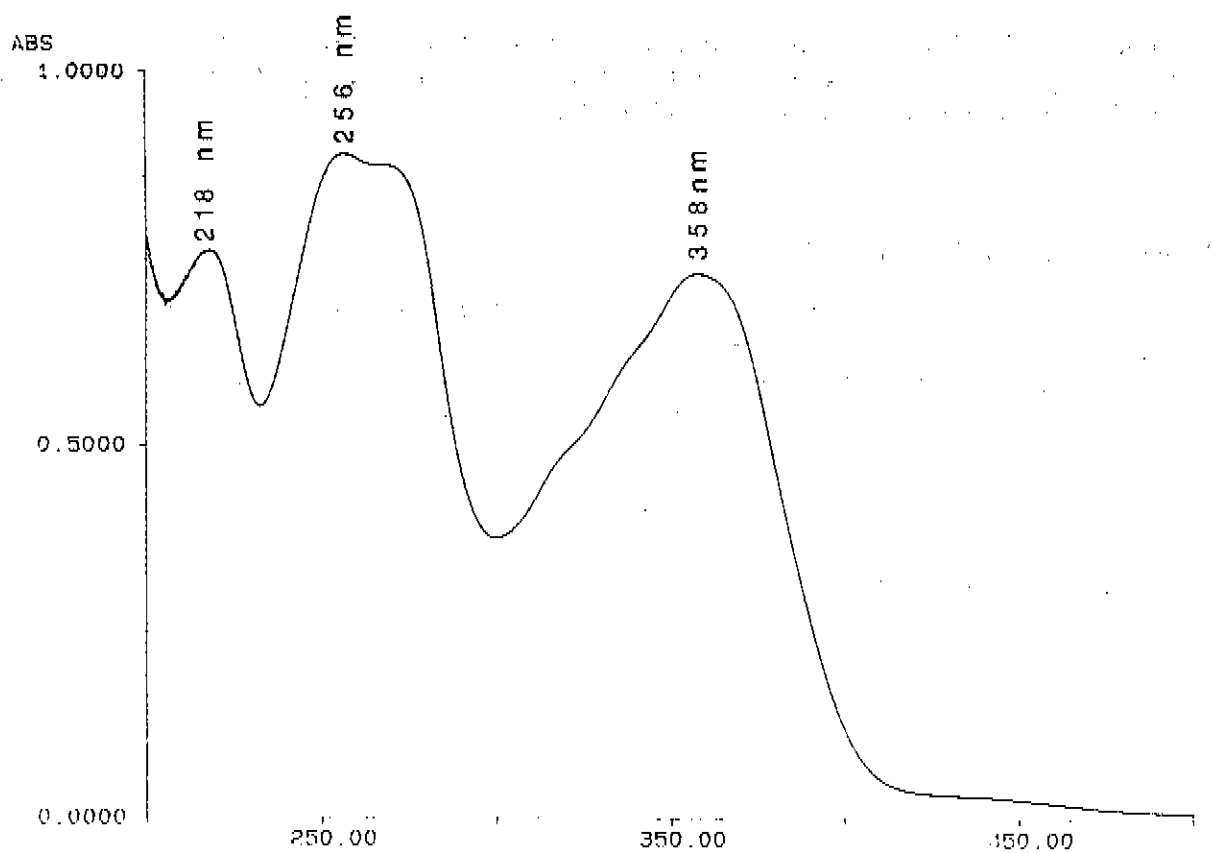


Figure 2. *UV-spectrum of 4-epitetracycline hydrochloride Control No 293098.*

**HPLC identity test:** The main peak obtained with 4-epitetracycline hydrochloride Control No 293098 in the chromatographic system used under assay and purity corresponds in retention time to that obtained with the USP reference standard Lot F.

### ASSAY

**Liquid chromatographic assay:** 93.3% ( $n = 6$ , RSD = 1.8%) when determined against the USP reference standard for 4-epitetracycline hydrochloride Lot F, regarded as 100%. The chromatographic system used was the same as described under purity, except for the eluent that was changed to 80 /20 (buffer:acetonitrile). The calculations were performed on the dried substances.

When assayed against the old ICRS for 4-epitetracycline  $\text{NH}_4$ -salt, 180098, regarded as 100%, the content was estimated to be 92.2%.

**Spectrophotometric assay:** 94.9% ( $n = 2$ ) when determined in 0.01 M HCl at 218 and 256 nm against the USP reference standard for 4-epitetracycline Lot F, regarded as 100%. The calculations were performed on the dried substances.

**Thermogravimetric analysis:** When the substance was heated to 105 °C for 16 hours a loss of weight of 4.7% was observed.

Instrument: Perkin Elmer TGA 7 Thermogravimetric analyzer.  
Sample weight: about 2 mg  
Heating rate: multiramp, 0.2 °C/min  
Melting point: about 120 °C with decomposition

As the values from the Karl Fischer titration differ between manufacturer and collaborating laboratories, we rely more on the thermogravimetric analysis, which was performed with multiramp technique for 16 hours. Water and solvents are codetermined with thermogravimetric analysis.

## PURITY

### High performance liquid chromatography

The total amount of impurities was estimated by using external standards for tetracycline and 4-epianhydrotetracycline hydrochloride to be 6.2%. The peak eluting at 7.4 minutes was identified as tetracycline present at 0.3% when the corresponding reference substance was used. The peak eluting at 46.4 minutes was identified as 4-epianhydrotetracycline, present at 5.9% when the corresponding reference substance was used. Peak area normalisation gave far too high values for 4-epianhydrotetracycline (about 12%) due to differences in molar absorptivity. Anhydrotetracycline hydrochloride which was eluted when the amount of acetonitrile was increased to 18% was not found in the ICRS.

A chromatogram is shown in Figure 3.

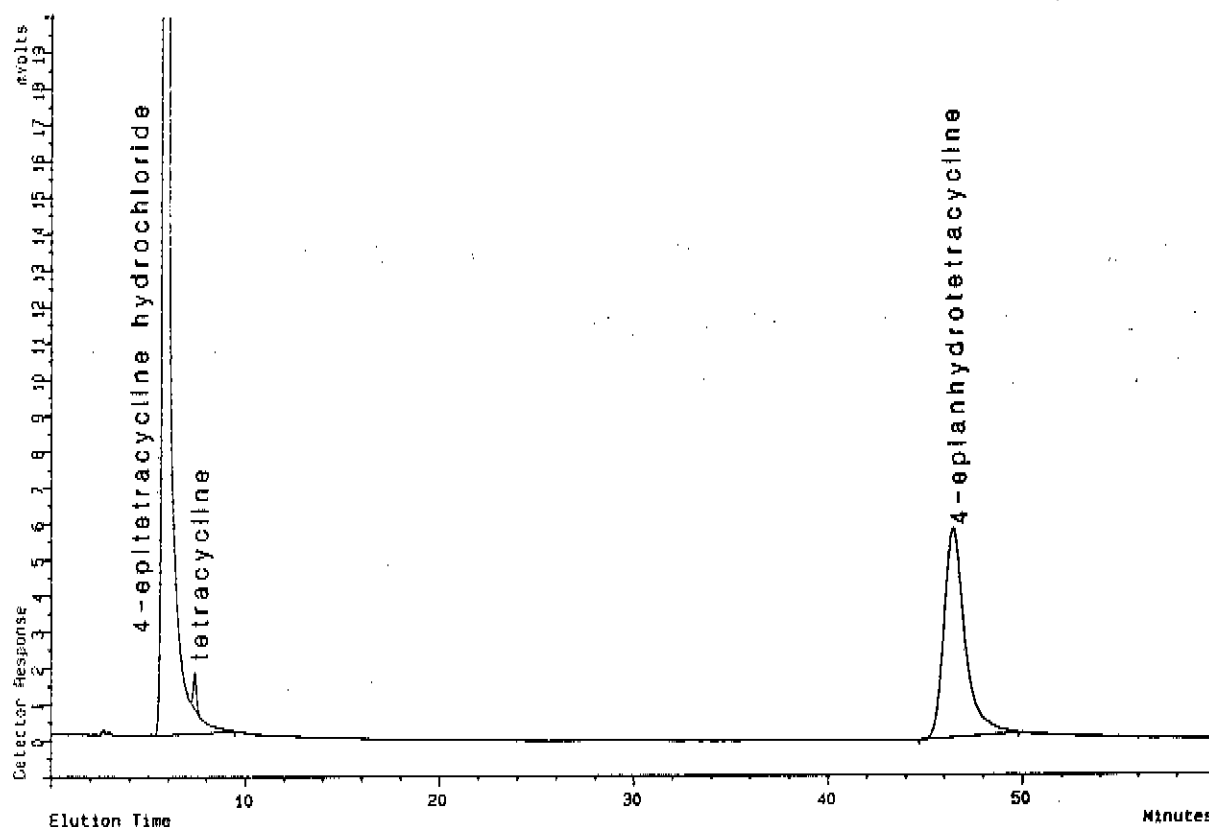


Figure 3. Chromatogram of 4-epitetracycline hydrochloride, Control No 293098.

The following conditions were used:

Eluent: 0.1M sodium phosphate buffer pH 2.4:Acetonitrile (85:15)

Column: Vydac C18, 218 TP54

Detector: Varian 100 operated at 271 nm.

Pump: Varian 5500

Integrator: PeakPro (Beckman)

Sample: 0.5 mg/ml dissolved in the eluent. The sample must be freshly prepared.  
20 µl corresponding to 10 µg were injected.

#### DATA GIVEN BY THE MANUFACTURER

Description: Yellow powder

Identification: IR spectrum attached

Identification: Sulphuric acid gives a violet colour, by addition of water it becomes yellow.

Identification: Chlorides positive reaction

HPLC: 4% impurities (tetracycline and 4-epianhydrotetracycline)

Water by KF: 3.4% (n = 4, RSD = 3.9%)

Assay (non-aqueous titration): 102.9% (n = 4, RSD = 0.6%). High titration value is due to the presence of some ammonium chloride.

#### DATA GIVEN BY COLLABORATING LABORATORIES

EPCRS Batch 3, results from 1993.

Description: Yellow powder

Identification: IR complies with CRS 1

TLC: Complies with CRS 1

Water by KF: 2.4% (n = 2)

#### STABILITY

Stability studies were not performed as it was considered that this substance, based on the experience of the previous lot, was stable and showed no signs of degradation when stored for 12 years at +5 °C. Regular re-examinations of the ICRS will be performed.

#### CONCLUSION

4-Epitetracycline hydrochloride, Control No 293098, can be considered suitable as International Chemical Reference Substance for the intended purpose.

(-)-3-(4-HYDROXY-3-METHOXYPHENYL)-2-HYDRAZINO-2-METHYLALANINE  
(3-O-METHYLCARBIDOPA)

Control No 193180

Analytical Report

INTENDED USE

The monograph for Carbidopa in the International Pharmacopoeia 3rd Ed. Vol 3 requires a reference substance for 3-O-methylcarbidopa to be used for a liquid chromatographic purity determination.

MATERIAL

About 5 g of the sample (manufacturers batch no L-588,581-000H006) were received at the WHO Centre in November 1992. The material is being stored in tightly closed containers at + 5 °C, protected from light.

ANALYTICAL DATA

Description: A white to pale yellow, crystalline powder.

EVIDENCE OF CHEMICAL STRUCTURE

Infrared spectrum

An infrared spectrum is given in Figure 1 (No 193180). The spectrum is concordant with the spectrum of the Ph Eur CRS Batch 1 for methylcarbidopa.

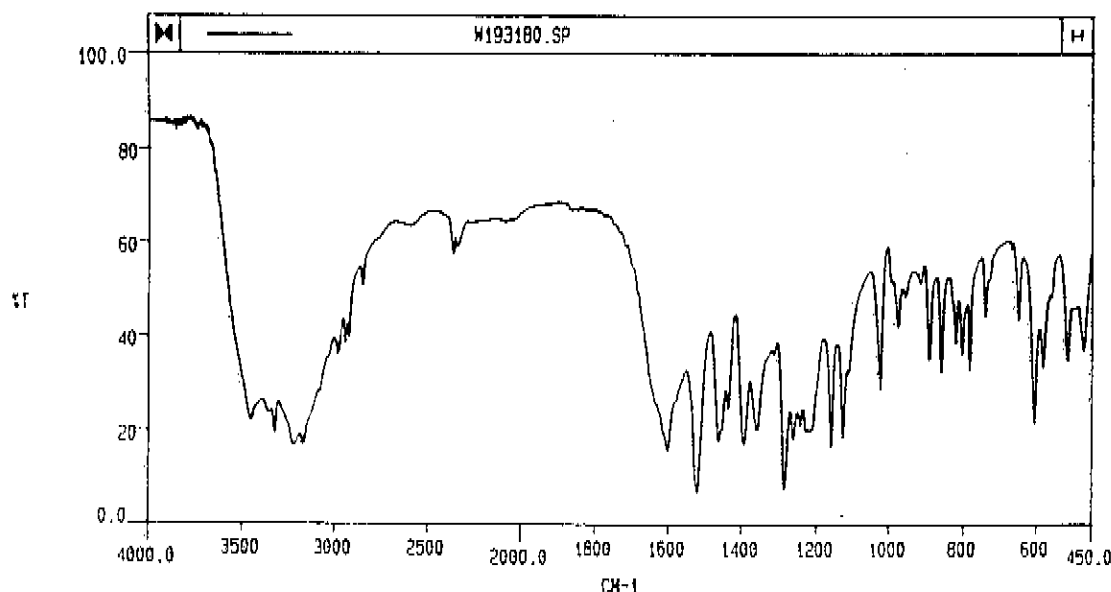


Figure 1. IR-spectrum of 1.1 mg of 3-O-methylcarbidopa Control No 193180 in 300 mg KBr recorded against a KBr disc.

Instrument: Perkin-Elmer 1600 FTIR.

UV-spectrum: See under diode array.

HPLC: The ICRS showed the same retention time as the Ph Eur CRS lot 1 for methylcarbidopa in the chromatographic system described under purity.

## ASSAY

Thermogravimetric analysis: When the substance was heated to 160 °C a loss of 3.5% of weight was observed.

Instrument: Perkin Elmer TGA 7 Thermogravimetric analyzer.  
Sample weight: 3 mg  
Heating rate: 5 °C /min  
Melting point: about 200 °C

## PURITY

### High performance liquid chromatography

About 1% impurities were found (n = 9, RSD = 0.2%). The same result was observed at 210 nm and 282 nm. Two impurities were detected. Impurity 1 eluting at 5.5 minutes was estimated to about 0.3% by peak area normalization. It was shown to be carbidopa and estimated using an external standard at about 0.4%. Impurity 2 eluting at 8.6 minutes was estimated to be about 0.7% by peak area normalisation and identified as 3-O-methylmethyldopa when compared to ICRS 179085 and by using an external standard of 3-O-methylmethyldopa it was estimated to be about 0.8%. Methyldopa with a retention time of 4.4 minutes was not detected in the sample.

A chromatogram is shown in Figure 2.

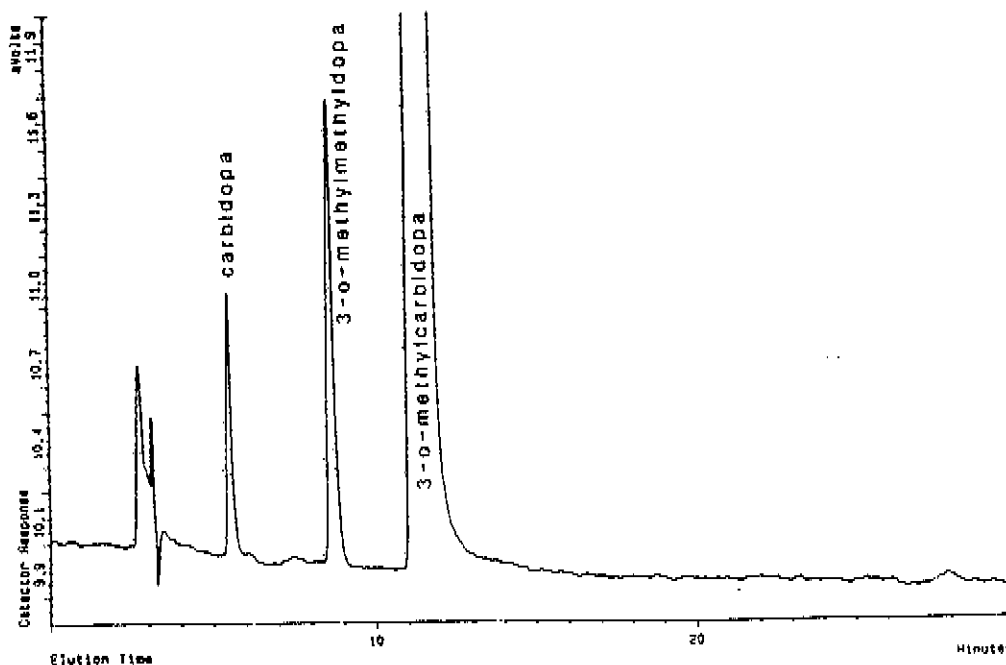


Figure 2. Chromatogram of 3-O-methylcarbidopa Control No 193 180 monitored at 282 nm.

The following conditions, which is a modified version of the system described in Ph Int, were used:

Eluent: Methanol: 0.1 M NaH<sub>2</sub>PO<sub>4</sub> buffer pH = 2.9 (15:85)

Column: Chromasil 100 C8, 5 µm (HiChrom)

Detector: Varian 9065 Polychrom at 282 nm and 210 nm.

Pump: Varian 9012 operated at a flow rate of 1.0 ml/min.

Integrator: PeakPro (Beckman)

Sample: 0.9 mg/ml dissolved in the eluent. No degradation was found in a sample stored for 24 hours in the eluent.

20 µl corresponding to 18 µg were injected.

The detection limit for 3-O-methylcarbidopa was 0.001 mg/ml (0.05%).

The Ph Eur CRS lot 1 of methylcarbidopa was also shown to contain about 1% impurities, with a similar impurity pattern as the ICRS.

#### Diode-array detection

The chromatographic system described above was also evaluated with a Varian 9065 Polychrom detector. UV-maxima for 3-O-methylcarbidopa were found to be at 200 nm, 224 nm and 278 nm when recorded in the eluent. UV maxima for the two impurities were at 195 nm and 283 nm for carbidopa and at 195 nm, 224 nm and 278 nm for 3-O-methylmethyldopa. The UV-spectrum of 3-O-methylcarbidopa in the eluent is given in Figure 3.

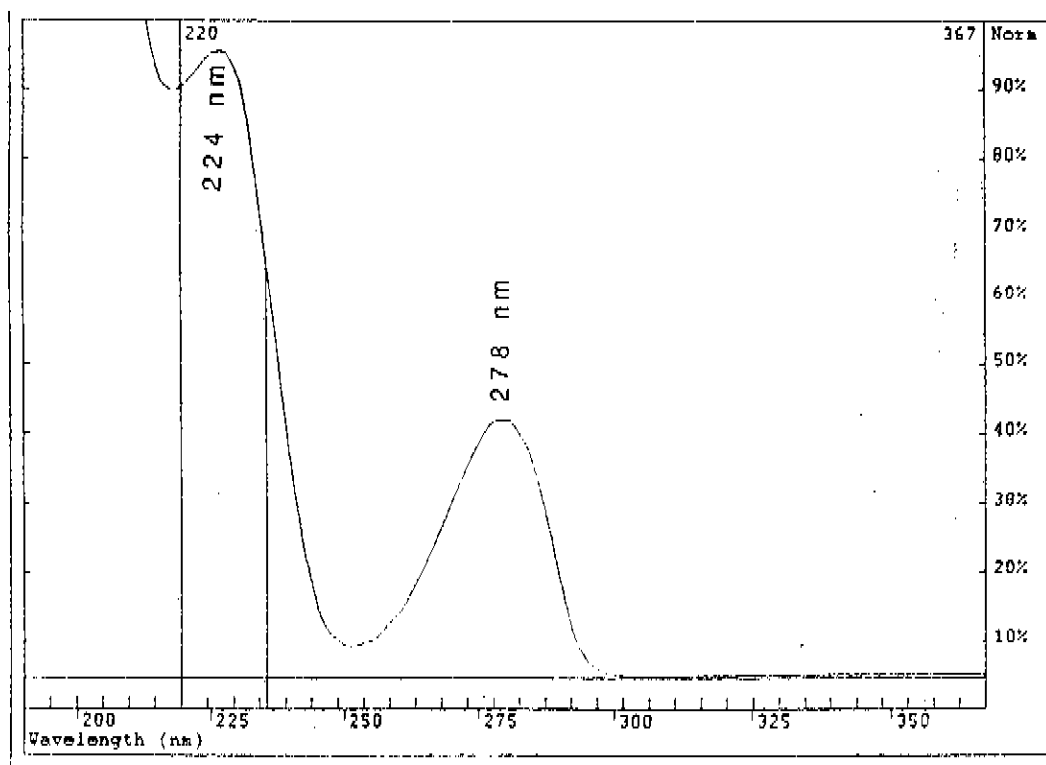


Figure 3. UV-spectra for 3-O-methylcarbidopa recorded by the diode array detector.

**DATA GIVEN BY THE MANUFACTURER**

Infrared spectrum: conforms

UV: A (1%, 1 cm) = 123 at 282 nm.

A (1%, 1 cm) = 285 at 232 nm.

Loss on drying: 3.8%

Liquid chromatography: 99.3%

**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**

(-)-3-(4-Hydroxy-3-methoxyphenyl)-2-hydrazino-2-methylalanine (3-O-methylcarbidopa), Control No 193180, can be considered suitable as International Chemical Reference Substance for the intended purpose.

**LIOTHYRONINE SODIUM**

Control No 193179

Analytical Report

INTENDED USE

The monograph for Levothyroxine sodium in the International Pharmacopoeia 3rd Ed. Vol 3 requires a reference substance of liothyronine sodium to be used in the thin-layer chromatographic test for liothyronine.

MATERIAL

About 25 g of the sample (manufacturers batch no 395/z) were received at the WHO Centre in June 1993. The material is being stored in tightly closed containers at + 5 °C, protected from light.

ANALYTICAL DATA

Description: Almost white slightly brownish coloured powder.

EVIDENCE OF CHEMICAL STRUCTURE

Infrared spectrum

The infrared spectrum is given in Figure 1 (No 193179 A). The spectrum is concordant with the spectrum of Ph Eur CRS batch 2 of liothyronine sodium.

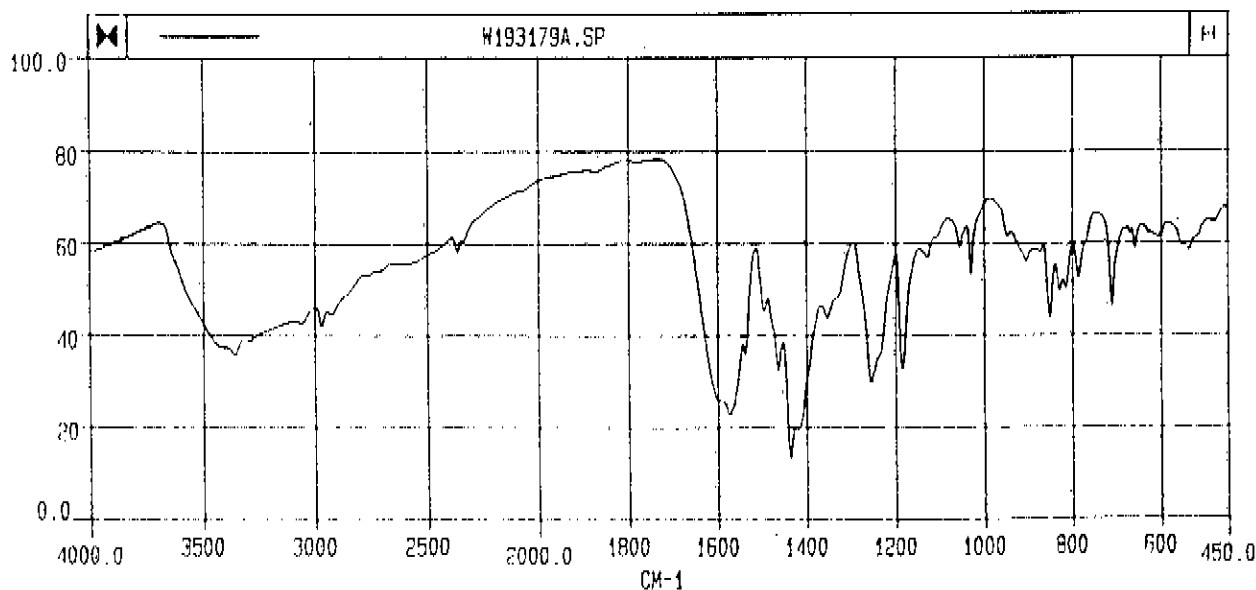


Figure 1. IR-spectrum of 1.9 mg of liothyronine sodium Control No 193179 in 300 mg KBr recorded against a KBr disc.

Instrument: Perkin-Elmer 1600 FTIR.

UV-spectrum: See below under diode array.

Sodium: 4.3%, determined by atomic adsorption spectroscopy.

Specific optical rotation: + 21.0° determined on the dried substance according to BP (limits +18.0° to +22.0°)

#### ASSAY

Assay: See data given by the manufacturer.

Thermogravimetric analysis: When the substance was heated to 105 °C for 2 hours a loss of weight of 3.5% was observed.

Instrument: Perkin Elmer TGA 7 Thermogravimetric analyzer.  
Sample weight: 6 mg  
Heating rate: multiramp  
Melting point: about 236 °C with decomposition.

Loss on drying: 3.0%, when dried to constant weight at 105 °C.

Water: 2.6%, determined by Karl Fischer titration.

#### PURITY

##### Thin-layer chromatography

The total amount of impurities present was estimated to be approximately 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 (Merck).

Eluent: Ammonia conc: 2-propanol: ethylacetate (20:35:55)

Sample: 100 µg were applied. The sample was dissolved in methanol:ammonia (14:1).

Visualization: Evaluation under UV-light of 254 nm and scanning by densitometry at 225 nm with a Desaga CD 60 Scanner. Spraying with ferric dichloride -ferricyanide -arsenite solution and visualization in day-light.

Four secondary spots were detected visually at 254 nm. When evaluated by densitometry four secondary spots were detected and estimated to be about 1%. One of the impurities was identical to levothyroxine, it was estimated to be present at approximately 0.3%. The detection limit of the system was about 0.2 µg (0.2%) at 225 nm. After spraying the same four secondary spots were detected.

R<sub>f</sub> (liothyronine sodium) = 0.13

R<sub>f</sub> (levothyroxine sodium) = 0.11

In the Ph Eur CRS batch 2 approximately 5% impurities were detected. The amount of impurities found in the USP reference standard Lot I was approximately 0.5%.

High performance liquid chromatography

The total amount of impurities present was estimated by peak area normalisation to be 0.8%. The peak eluting at 12.5-13 minutes was identified as levothyroxine and estimated at 0.2% both by peak area normalisation and by external standard measurement.

A chromatogram is shown in Figure 2.

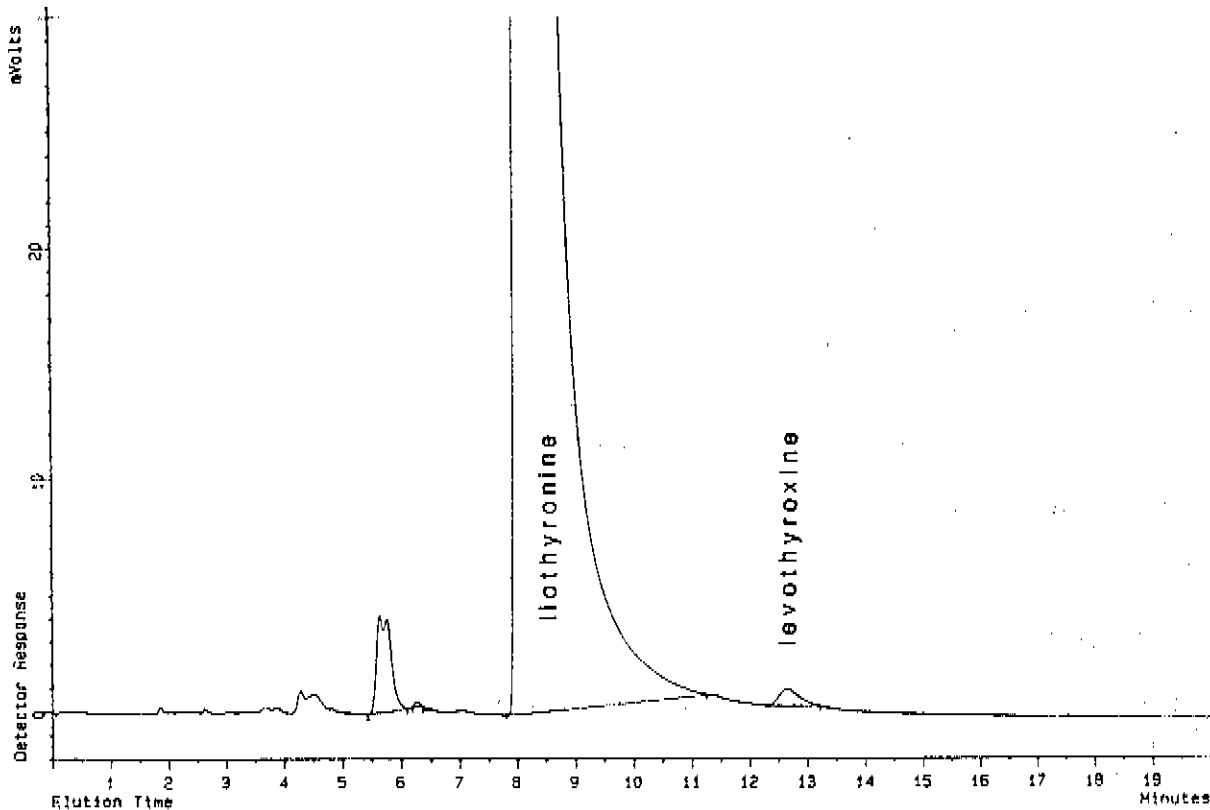


Figure 2. *Chromatogram of liothyronine sodium Control No 193179.*

The following conditions were used:

Eluent: Acetonitrile:water containing 0.02 M sodium dodecylsulfate and 0.02 M  $H_3PO_4$  (55:45)

Column: Spheri- 5 OD-5A RP 18 (Brownlee)

Detector: Varian UV-100 at 225 nm

Pump: Varian 5500 operated at 1ml/min

Integrator: PeakPro (Beckman)

Sample: 1 mg/ml dissolved in the eluent. The sample must be freshly prepared.  
20  $\mu$ l corresponding to 20  $\mu$ g were injected.

Liothyronine USP reference substance and the Ph Eur CRS batch 2 were also tested and about 0.5% impurities and 5% impurities were found respectively.

### Diode-array detection

The chromatographic system described above was also evaluated with a Varian 9065 Polychrom detector. The same chromatographic system as described above was used. UV-maxima were recorded for liothyronine and four extra peaks. UV-maximum were found to be at 224 nm for all investigated peaks. A UV-spectrum in the eluent is given for the liothyronine peak in Figure 3.

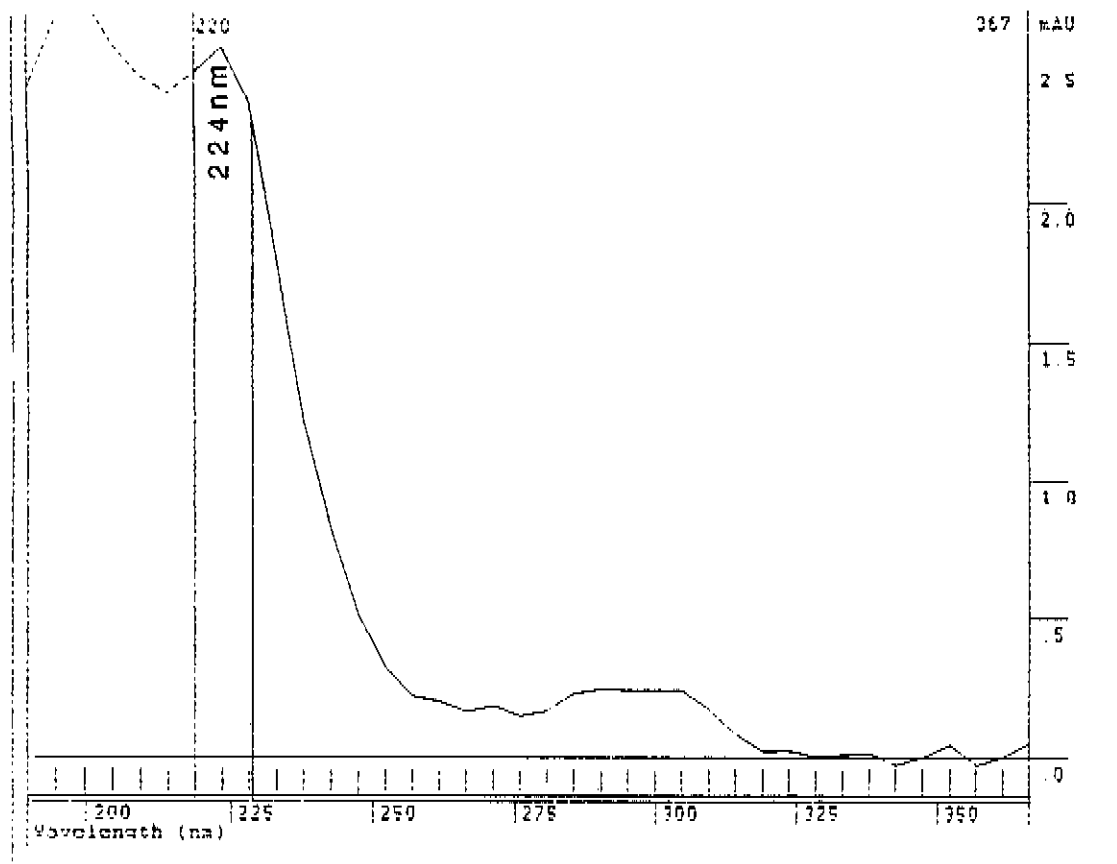


Figure 3. *UV-spectrum for liothyronine recorded by the diode array detector.*

### DATA GIVEN BY THE MANUFACTURER

There is no monograph for Liothyronine sodium in the International Pharmacopoeia. Limits from USP and BP are given.

Description: Cream powder

Identification: Conforms

Loss on drying: 3.1% (limits <4%, USP, BP)

Specific rotation: + 21.9° (limits +18.0° - +22.0°, USP, BP)

Assay: 96.9%

Sodium: 3.2% (limits 2.9 - 4.0%, USP)

L-dioiodothyronine sodium: < 0.5%

L-thyroxine sodium: < 2% (limits <2%, BP, <5% USP)

Chloride: < 0.02%

Inorganic iodide: <0.08%

Heavy metals: < 20 ppm

Colour of solution: pass test

STABILITY

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

CONCLUSION

Liothyronine sodium, Control No 193179, can be considered suitable as International Chemical Reference Substance for the intended purpose.

APPENDIX 12NEAMINE HYDROCHLORIDE  
(NEOMYCIN A)  
Control No 193177

## Analytical Report

INTENDED USE

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

MATERIAL

About 50 mg of the sample (Ph Eur CRS Lot 1) were received at the WHO Centre in October 1987. The material is being stored in tightly closed ampoules at + 5 °C, protected from light.

No other reference material of neamine is available which limits the number of tests that can be performed.

ANALYTICAL DATA

Description: A fluffy white powder.

EVIDENCE OF CHEMICAL STRUCTUREInfrared spectrum

An infrared spectrum is given in Figure 1, No W 193177 A.

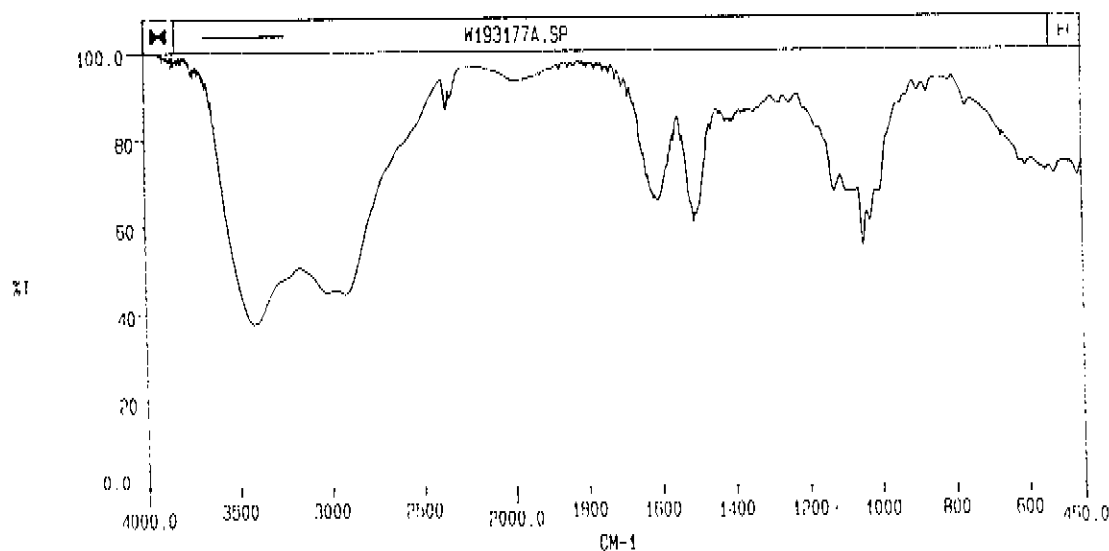


Figure 1. IR-spectrum of 0.4 mg of neamine hydrochloride Control No 193177 in 300 mg KBr recorded against a KBr disc.

Instrument: Perkin-Elmer 1600 FTIR.

UV-spectrum

There was no significant absorbance of a 0.07 mg/ml solution in water in the range 200 nm to 500 nm.  
A (1%, 1 cm) = 30 at 200 nm.

LC-MS: A spectrum was recorded by electrospray LC-MS for the main peak (neamine). The molecular weight obtained 322.3 supports the structure for neamine. The chromatographic system used was a Brownlee RP18 column, and an eluent consisting of 10% Methanol and 90% buffer containing 0.11 M TFA, with pH adjusted to 3.6 with NH<sub>4</sub>OH.

NMR: <sup>1</sup>H-NMR spectrum was recorded in D<sub>2</sub>O, it supports the structure for neamine.

Chlorides: Positive identity test, according to Ph Eur.

ASSAY

Thermogravimetric analysis: When the substance was heated to 140 °C a loss of 8.6% of weight was observed.

Instrument: Perkin Elmer TGA 7 Thermogravimetric analyzer.  
Sample weight: 0.5 mg  
Heating rate: 2 °C/min

PURITY

Thin-layer chromatography

Two different chromatographic systems were tested.

*System 1*

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

Thin-layer: Silica gel 60 F-254, HPTLC (Merck)  
Eluent: 40g ammonium acetate / l in water, freshly prepared.  
Sample: 40 µg of neomycin B sulfate and 0.5 and 1 µg of neamine hydrochloride dissolved in water were applied.  
Visualization: Spraying with ninhydrin, 0.1 g /100ml butanol.

No separation of neomycin B sulfate and neamine was observed. All spots were retained at the application spot.

*System 2*

The following thin-layer chromatographic system according to the International Pharmacopoeia 3rd Ed. Vol 3 described under test for neamine test was used.

Thin-layer: Laboratory made silica gel H, carbomer pH 7, layer thickness 0.75 mm.  
Eluent: 100 g potassium dihydrogen phosphate / l in water.  
Sample: 25 and 50 µg of neomycin B sulfate and 0.2, 0.5 and 1 µg of neamine hydrochloride dissolved in water were applied.  
Visualization: Spraying with ninhydrin + stannous chloride according to Ph.Int.

No neamine was found in the neomycin B sulfate ICRS, which means less than 1%. (0.5 µg of neamine).

The spot obtained for neomycin B exhibited tailing.

Rf (neomycin B sulfate) = 0.35

Rf (neamine) = 0.42

#### High performance liquid chromatography

As neomycin and neamine has practically no UV -absorbance other detection principles was investigated. Electrochemical detection gave insufficient response but a pre-column derivatization step with 2,4-dinitrofluorobenzene, based on the method in USP XXII, ninth supplement for tobramycin, proved to be satisfactory.

Three unknown impurities were found, estimated to be about 4% by peak area normalisation. Lack of reference materials of the corresponding impurities precluded the study of their behaviour in the derivatization step.

A chromatogram is shown in Figure 2.

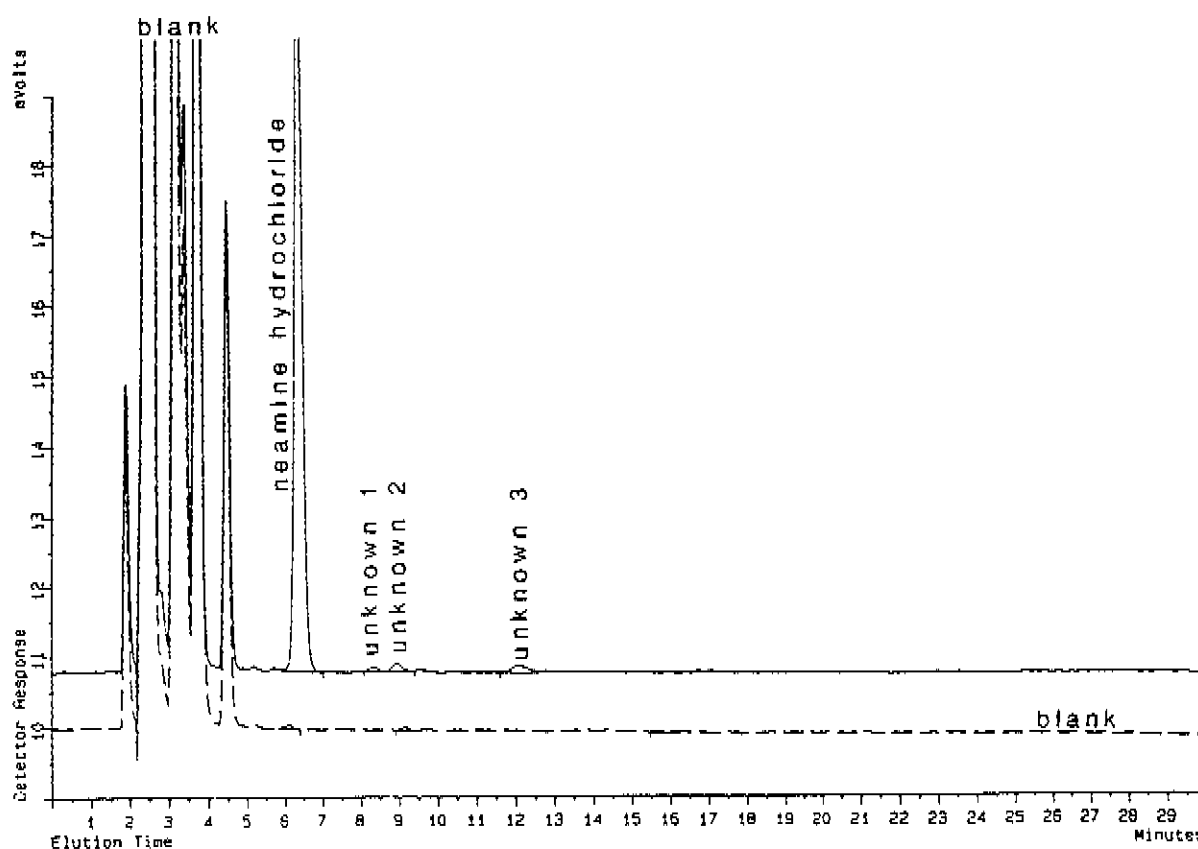


Figure 2. Chromatogram of neamine Control No 193177 as 2,4-dinitrofluorobenzene derivative monitored at 367 nm. (Eluent 65/35)

The following conditions were used:

Eluent: Acetonitrile: buffer, pH 2.6, consisting of 2 g tris (hydroxymethyl)aminomethane and 10 ml 1 M sulfuric acid dissolved in water and diluted to 1000 ml (65/35)

Column: Brownlee Labs RP -18 OD -5A at 40 °C.

Detector: Varian 9065 Polychrom operated at 367 nm.

Pump: Varian 9010 operated at a flow rate of 1 ml/min.

Integrator: PeakPro (Beckman)

Derivatization reagents:

2,4-dinitrofluorobenzene reagent: 10 mg 2,4-dinitrofluorobenzene per ml in ethanol.

Tris (hydroxymethyl)aminomethane reagent: 20 ml of a solution containing 15 mg tris (hydroxymethyl)aminomethane per ml in water is transferred into a 100 ml volumetric flask and diluted to volume with dimethyl sulfoxide, freshly prepared.

Derivatization: A sample solution containing 5 mg accurately weighed into a 10 ml volumetric flask is diluted to volume with water in a 25 ml volumetric flask. 2.00 ml of this sample solution is mixed with 2.00 ml of 2,4-dinitrofluorobenzene reagent and 2.00 ml of tris (hydroxymethyl)aminomethane reagent and heated for 50 minutes in a water bath at 60 °C. Allow to stand in room temperature for 10 minutes. Dilute to volume with acetonitrile.

NB dilute carefully, do not add all acetonitrile until room temperature is reached in the mixture since cooling occurs on mixing.

Sample: 20 µl of the above mentioned solution corresponding to 0.8 µg neomycin B sulfate were injected. A blank is also prepared in the same manner with 2.00 ml of water instead of sample solution.

#### DATA GIVEN BY COLLABORATING LABORATORIES

Ph Eur CRS Lot 1  
TLC reported

#### STABILITY

Regular reexaminations of the ICRS will be performed.

#### CONCLUSION

Neamine hydrochloride, Control No 193177, can be considered suitable as International Chemical Reference Substance for the intended purpose.

APPENDIX 13

NEOMYCIN B SULFATE  
(FRAMYCETIN SULFATE)  
Control No 193178

Analytical Report

INTENDED USE

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

MATERIAL

About 50 g of the sample (manufacturers batch no OG 1046) were received at the WHO Centre in January 1993. 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 193178 A). The spectrum is concordant with the spectra of the USP reference standard for neomycin sulfate Lot K-3 and the International Biological 1st Reference Preparation for neomycin B (WHO, 1970). It is also concordant with the spectrum obtained from the 2nd International reference preparation for neomycin (WHO, 1974). The spectrum for ICRS 193178 differs from those for neomycin C and for neamine (neomycin A) given in Analytical Profiles, Florey, Vol 8.

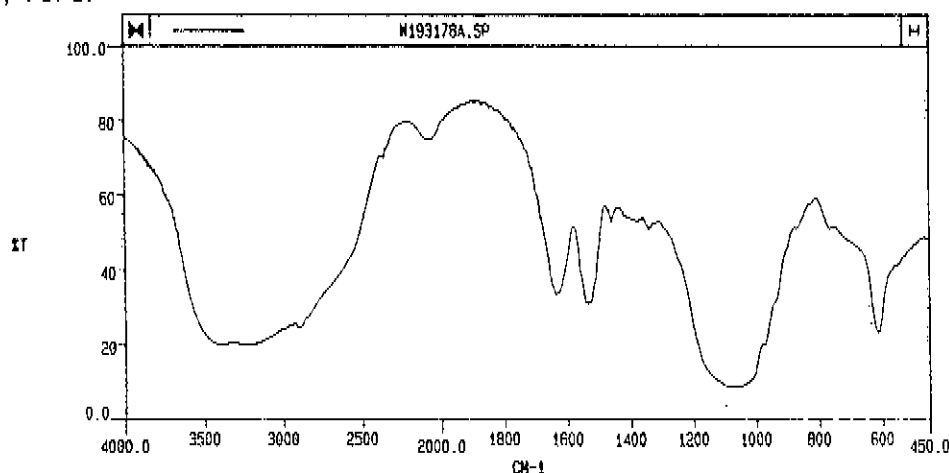


Figure 1. IR-spectrum of 1.7mg of neomycin B sulfate Control No 193178 in 300 mg KBr recorded against a KBr disc.

Instrument: Perkin-Elmer 1600 FTIR.

Thin-layer chromatography: See purity.

Liquid chromatography: See purity.

UV-spectrum

There was no significant absorbance of a 0.8 mg/ml solution in water in the range 200 nm to 500 nm.  
 $A(1\%, 1\text{ cm}) = 0.14$  at 228 nm.

Sulfate: 28.5% determined by ion chromatography on the dried substance.

ASSAY

Liquid chromatographic assay: Taking the proposed ICRS as 100% ( $n = 6$ ,  $RSD = 1.3\%$ ) the calculated value for the International Biological 1st Reference Preparation for neomycin B (sulfate) was 97.8% ( $n = 6$ ,  $RSD = 0.5\%$ ). The difference between the proposed ICRS and the biological reference preparation is statistically significant at the 95% confidence level when using the unpaired t-test. The determination was performed by the liquid chromatographic method described under purity.

Microbiological assay: 637 IU/mg "as is" with limits of error 95-105%. The International Biological 1st Reference Preparation for neomycin B(670 IU/mg "as is") was used as the standard. This corresponds to 723 IU/mg when calculated on the dried substance.

Thermogravimetric analysis: When the substance was heated to 175 °C, a loss of 11.9% of weight was observed.

Instrument: Perkin Elmer TGA 7 Thermogravimetric analyzer.  
Sample weight: 6 mg  
Heating rate: 2 °C/min  
Melting point: decomposes at about 180 °C

The corresponding results for WHO International Biological 1st Reference Preparation was 10.8%.

Water: 7.8% w/w ( $n = 3$ ) determined by Karl Fischer titration. This method is not recommended for this type of substance.

Loss on drying: 10.8% (60 °C, reduced pressure, diphosphorus pentoxide)  
11.5% (105 °C, reduced pressure, diphosphorus pentoxide)

Gaschromatography: No alcohols were detected (less than 0.1% of methanol, ethanol, propanol and butanol).

PURITY

Thin-layer chromatography

Two different chromatographic systems were employed.

*System 1*

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

Thin-layer: Silica gel 60 F-254, HPTLC ( Merck)  
Eluent: 40g ammonium acetate / l in water ,freshly prepared.  
Sample: 40 µg of neomycin B sulfate dissolved in water were applied.  
Visualization: Spraying with ninhydrin, 0.1 g /100ml butanol.

No or very small migration of the spots  $R_f = 0$  for neomycin B, neomycin C and neamine (neomycin A). To perform a reliable identification between different qualities of neomycines it is necessary to perform high performance liquid chromatography as given below.

### System 2

The following thin-layer chromatographic system according to the International Pharmacopoeia 3rd Ed. Vol 3 described under test for neamine test was used.

Thin-layer: Laboratory made silica gel H, carbomer pH 7, layer thickness 0.75 mm.  
Eluent: 100g potassium dihydrogen phosphate / l in water.  
Sample: 25 µg of neomycin B sulfate and 0.1 -1 µg dissolved in water were applied.  
Visualization: Spraying with ninhydrin + stannous chloride according to Ph.Int.

Neamine was not detected in the proposed ICRS of neomycin B sulfate, which means less than 1% (detection limit for neamine).

The spot obtained for neomycin B sulfate was tailing.

$R_f$  (neomycin B sulfate) = 0.27

$R_f$  (neamine) = 0.47

$R_f$  (neomycin C) = 0.27

No separation between neomycin B and C is observed. To be able to distinguish between different qualities of neomycines as eg. farmycetin it is necessary to perform high performance liquid chromatography as given below.

### High performance liquid chromatography-determination of neamine (neomycin A) and neomycin C.

As neomycin has practically no UV-absorbance other detection principles had to be chosen. Electrochemical detection was investigated but the response was too weak. Pre-column derivatization using 2,4-dinitrofluorobenzene as denoted in the monograph for tobramycin of the USP XXII, was found to be satisfactory for its detection.

0.12% of neamine (neomycin A), 1.6% of neomycin C sulfate and 0.4% of paromomycins I+II were found by peak area normalization. Neamine was determined against an external standard and was estimated to be present at 0.12%.

A chromatogram is shown in Figure 2.

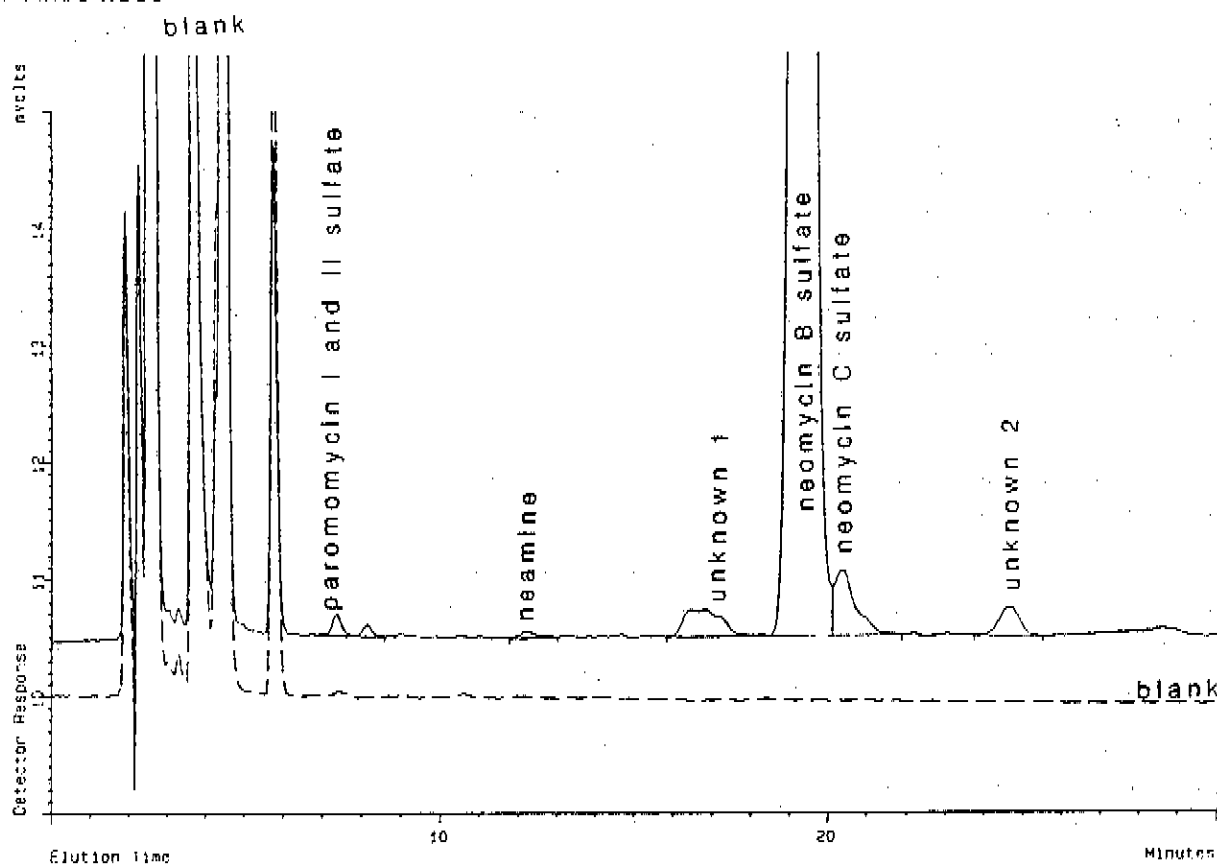


Figure 2. Chromatogram of neomycin B sulfate Control No 193178 as 2,4-dinitrofluorobenzene derivative monitored at 367 nm. (Eluent 58:42)

The following conditions were used:

Eluent: Acetonitrile: buffer, pH 2.6, consisting of 2 g tris (hydroxymethyl)aminomethane and 10 ml 1 M sulfuric acid dissolved in water and diluted to 1000 ml (58:42)

Column: Brownlee Labs RP -18 OD -5A at 40 °C.

Detector: Varian 9065 Polychrom operated at 367 nm.

Pump: Varian 9010 operated at a flow rate of 1 ml/min.

Integrator: PeakPro (Beckman)

Derivatization reagents:

2,4-dinitrofluorobenzene reagent: 10 mg 2,4-dinitrofluorobenzene per ml ethanol.

Tris (hydroxymethyl)aminomethane reagent: 20 ml of a solution containing 15 mg tris (hydroxymethyl)aminomethane per ml water is transferred into a 100 ml volumetric flask and diluted to volume with dimethyl sulfoxide freshly prepared.

Derivatization: A sample solution containing 5 mg accurately weighed in a 10 ml volumetric flask diluted to volume with water. In a 25 ml volumetric flask 2.00 ml of this solution is mixed with 2.00 ml of 2,4-dinitrofluorobenzene reagent and 2.00 ml of tris (hydroxymethyl)aminomethane reagent and heated for 50 minutes in a water bath at 60 °C. Allow to stand in room temperature for 10 minutes. Dilute to volume with acetonitrile.

NB: dilute carefully, do not add all acetonitrile until room temperature is reached in the mixture, since cooling occurs on mixing.

Sample: 20  $\mu$ l of the above mentioned solution corresponding to 0.8  $\mu$ g neomycin B sulfate were injected. A blank is also prepared in the same manner with 2.00 ml of water instead of sample solution.

A comparison of results from different neomycin B sulfate reference materials is given in Table 1.

Table 1

Reference material	neamine (neomycin A) %	neomycin C %
Neomycin B sulfate International Biological 1st Reference Preparation	0.25	traces
USP neomycin sulfate lot K-3	0.11	0.36
BPCRS Lot 1507 Framycetin sulfate	0.16	2.5
ICRS 193178 Neomycin B sulfate (framycetin sulfate)	0.12	1.6

Total "apparent" purity by liquid chromatography

In the system described above the major part of the impurities were retained on the column. In order to elute them the eluent was changed as follows.

Eluent: Acetonitrile: buffer, pH 2.6, consisting of 2 g tris (hydroxymethyl)aminomethane and 10 ml 1 M sulfuric acid dissolved in water and diluted to 1000 ml (65:35)

All other parameters are the same as described above. A typical chromatogram is given in Figure 3.

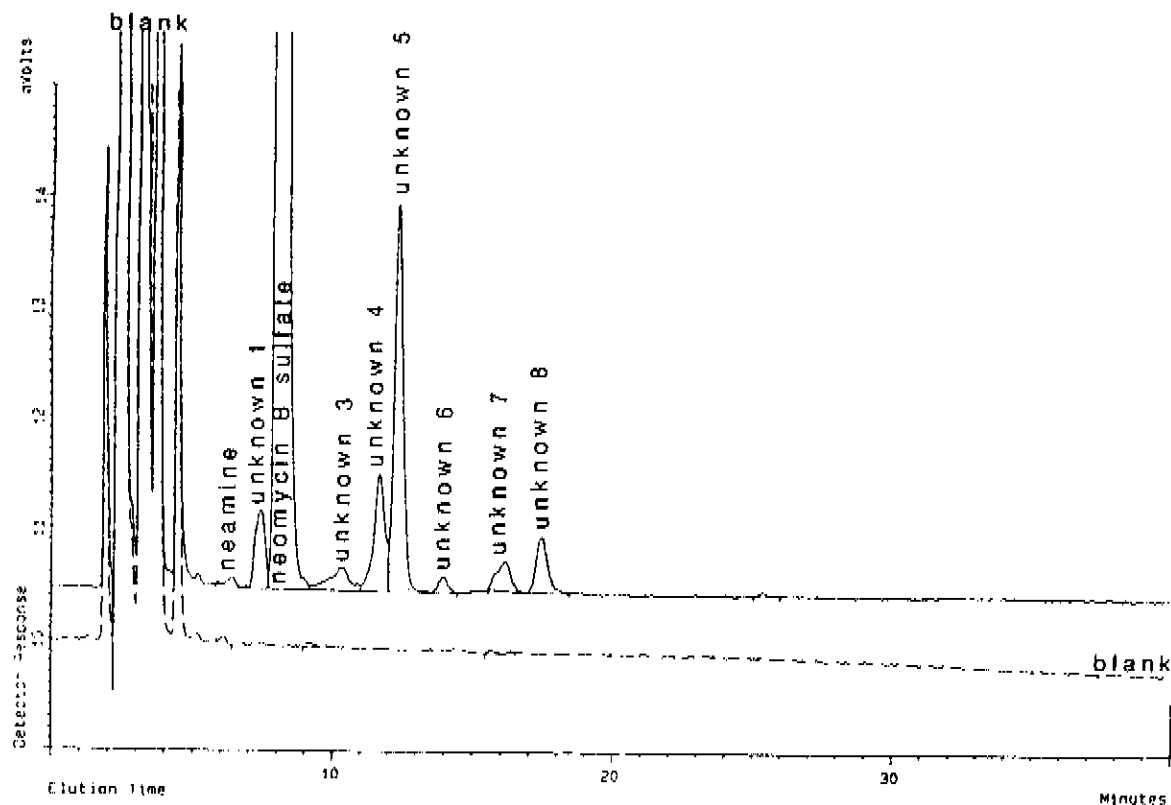


Figure 3. Chromatogram of neomycin B sulfate Control No 193178 as 2,4-dinitrofluorobenzene derivative monitored at 367 nm. (Eluent 65:35)

When the results from those two systems are combined eight unknown impurities as well as neamine, neomycin C and the paromomycines were found. The total "apparent" purity was estimated to be about 86.2%. The purity is referred to as "apparent" as we do not have the corresponding impurities as references and thus had no possibility to study their behaviour in the derivatization step.

The total purity for the International Biological 1st Reference Preparation for neomycin B (sulfate) was 87.3%, for the USP reference standard for neomycin sulfate lot K-3 86.5% and for BPCRS framycetin sulfate lot 1507 it was found to be 83.8%.

#### DATA GIVEN BY THE MANUFACTURER

There is no specific monograph for neomycin B sulfate (framycetin) in the International Pharmacopoeia. Limits from Ph Eur are given.

Description: satisfactory

Identification tests: satisfactory

pH: 6.0

(limit 6.0 - 7.0)

Specific optical rotation(on dry substance): +54 °

(limit +52.5 ° - +55.5 °)

Alcohols (in methanol): less than 1g /100 g

(limit < 2%)

Neamine: satisfactory

(limit < 1%)

Neomycin C: 1.5 g/100 g

(limit < 3%)

Loss on drying: 4.8 g/100 g

(limit < 8%)

Sulphated ash: less than 0.1 g/100 g

(limit < 1%)

Sulfates (on dry substance):29.8 g/100 g

(limit 27-31%)

Assay (on dry substance): 676 IU/mg

(limit > 630 IU/mg)

STABILITY

Neomycin B sulfate is hygroscopic. Regular reexaminations of the ICRS will be performed.

CONCLUSION

Neomycin B sulfate, Control No 193178, can be considered suitable as International Chemical Reference Substance for the intended purpose.

SPECTINOMYCIN HYDROCHLORIDE  
(SPECTINOMYCIN DIHYDROCHLORIDE PENTAHYDRATE)

Control No 193176

Analytical Report

INTENDED USE

The monograph for Spectinomycin hydrochloride in the International Pharmacopoeia 3rd Ed. Vol 3 requires a reference substance of spectinomycin hydrochloride to be used in the infrared spectrophotometric test for identity and in the gas-chromatographic assay.

MATERIAL

About 100 g of the sample (manufacturers batch no 17160) were received at the WHO Centre in July 1990. The material is being stored in tightly closed containers at + 5 °C, protected from light.

This reference substance has been evaluated in collaboration between the WHO Centre in Stockholm and the National Biological Standards Laboratory, Canberra, Australia. Results reported from NSBL are indicated with an asterisk(\*) .

ANALYTICAL DATA

Description: A white crystalline powder.

EVIDENCE OF CHEMICAL STRUCTURE

Infrared spectrum

An infrared spectrum is given in Figure 1 (No W 193176). The spectrum is concordant with the spectra of the USP reference standard (Lot F-1) and the International Biological 1st Reference Preparation (WHO).

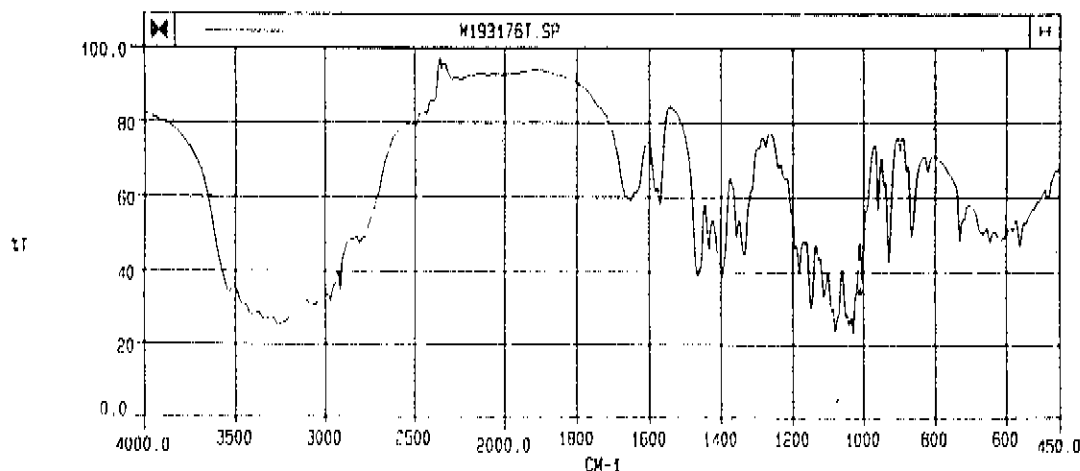


Figure 1. IR-spectrum of 1.4 mg of spectinomycin hydrochloride Control No 193176 in 300 mg KBr recorded against a KBr disc.  
Instrument: Perkin-Elmer 1600 FTIR.

(\*)Infrared spectrum

An infrared spectrum of the material, using the KBr disc technique was recorded on a BioRad FTS-7 Fourier transform Infrared Spectrophotometer. The spectrum was concordant with the spectrum of the International Biological 1st Reference Preparation as well as with a published spectrum from BP 88.

(\*)Test for chloride: Positive according to BP 88.

(\*)Acidity: pH of a 10% w/v solution was 4.0.

(\*)Specific optical rotation: + 19.1 °, determined on a 10% w/v solution.

UV-spectrum

There was no significant absorbance of a 0.5 mg/ml solution in water in the range 200 nm to 500 nm. When a spectrum was taken of a 5mg/ml solution in the mobile phase used for liquid chromatography, a weak maximum was found at 225 nm.

(\*)UV-spectrum

There was no significant absorbance of a 20.72 mg/100 ml solution in water in the range 200 - 800 nm.

(\*)GC-MS

A HP 5988 A GC/MS system was used. A TMS derivative of spectinomycin hydrochloride was prepared. M+ = 548 mass units of the TMS derivative. There was no evidence of significant amounts of actinamine TMS derivative in the trace of the TMS derivative of spectinomycin.

ASSAY

Liquid chromatographic assay: Taking the proposed ICRS as 100% (n = 10, RSD = 3.4%) the calculated value for the International Biological 1st Reference Preparation was 98.7% and for the USP reference substance 100.9%. The difference between the proposed ICRS and the other two reference materials is not statistically significant at the 95% confidence level using unpaired t-test. The determination was performed with the liquid chromatographic method with electrochemical detection described below under purity.

Microbiological assay: 680 IU/mg "as is" with limits of error 96-104%. The 1st International Biological Preparation (671 IU/mg "as is") was used as standard.

(\*)Microbiological assay: 663 IU/mg "as is" with fiducial limits of error (P = 0.95) of 667 and 659 IU/mg. 812 IU/mg as anhydrous substance with fiducial limits of error (P = 0.95) of 817 and 807 IU/mg. The first International Preparation of spectinomycin hydrochloride (1975) was used as standard.

Gas chromatographic assay: Taking the proposed ICRS as 100% the USP Reference substance Lot F-1 (671 µg/mg) and the International Biological 1st Preparation (671 IU/mg) were estimated to be 95.5% and 98.3% respectively when determined with the gas chromatographic method described in Ph. Int. Due to disturbances in the silanization step caused by the high water content, a double peak was formed. The direct liquid chromatographic method with electrochemical detection and no derivatization step described below is preferred for assay purposes.

(\*)Gas chromatographic assay: 98% if the International Biological 1st Reference Preparation (1975) was used as standard. The gas chromatographic method described in USP 85 was used.

Thermogravimetric analysis: When the substance was heated to 169 °C, a loss of 18.1% of weight was observed.

Instrument: Perkin Elmer TGA 7 Thermogravimetric analyzer.

Sample weight: 3 mg

Heating rate: 5 °C/min

Melting point: about 184-194 °C

The corresponding results for USP Lot F-1 was 18.5% and for WHO International Biological 1st Reference Preparation 18.0%.

(\*)Water: 18.4% w/w (n = 3, RSD = 0.07%) determined by Karl Fischer titration.

#### PURITY

##### (\*)Thin-layer chromatography

No secondary spots were detected. The thin-layer chromatographic system described in BP 88, which is the same as in the International Pharmacopoeia, was used. Rf for spectinomycin hydrochloride is 0.3-0.4. This Rf -value corresponds to that of the International Biological 1st Reference Preparation (1975) of spectinomycin dihydrochloride. The degradation product actinamine was shown to be well separated from spectinomycin in this system, with a lower Rf-value than 0.3, and it was not detected in the proposed ICRS.

##### Thin-layer chromatography

No 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 F-254 ( Merck)

Eluent: 1-propanol:water: glacial acetic acid:pyridine (10:8:1:1)

Sample: 200 and 300 µg of spectinomycin hydrochloride dissolved in water were applied.

Visualization: Visualization in day-light after spraying with potassium permanganate (25 g/l).

No secondary spots were detected after spraying. An attempt was made to scan the plate at 210 nm before spraying. However the result was unsuccessful, no spots at all were visible.

The detection limit of the system was about 5 µg (1.6%) after spraying.

Rf (spectinomycin hydrochloride) = 0.4

The same results were obtained for the USP reference substance Lot F-1 and the WHO International Biological 1st Reference Preparation.

##### High performance liquid chromatography

A liquid chromatographic system with electrochemical detection according to Elrod et al. Pharmaceutical Research, Vol.5, No 10, 1988 page 664 was used.

A chromatogram is shown in Figure 2.

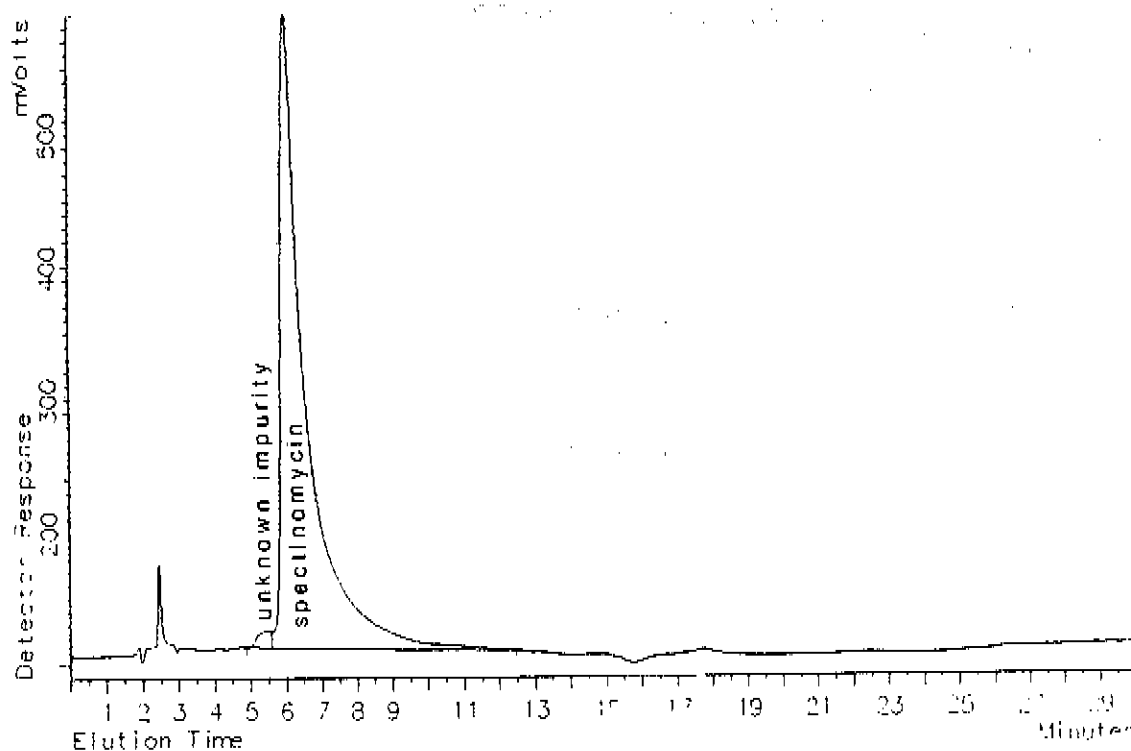


Figure 2. *Chromatogram of spectinomycin hydrochloride Control No 193176 monitored by electrochemical detection.*

The following conditions were used:

Eluent: Acetonitrile: Aqueous buffer containing 0.02 M sodium citrate and 0.0015 M octyl sodium sulfate, pH was adjusted to 6.1 with 70% perchloric acid (11.5 : 88.5)

Column: Brownlee Labs RP -18 OD -5A

Detector: ESA, Coulochem II Guard cell :0.95 V Single porous working electrode:850 mV  
Background current:10  $\mu$ A

Pump: Varian 5500 operated at a flow rate of 1ml/min.

Integrator: PeakPro (Beckman)

Sample: 0.02-0.03 mg/ml dissolved in the eluent. A 0.2 mg/ml solution is too concentrated for the use of this detector 20  $\mu$ l corresponding to 0.4  $\mu$ g were injected.

As can be seen from figure 2 one possible impurity is observed at about 5.4 minutes. It was estimated to be about 1.5% in the proposed ICRS and in the USP (Lot F-1), and to 1.6% in the International Biological 1st Reference Preparation. 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 normalisation when working with electrochemical detection, the necessity for impurity reference substances being obvious. The peak at 2.5 minutes originates from the blank.

DATA GIVEN BY THE MANUFACTURER

Spectinomycin hydrochloride = spectinomycin dihydrochloride pentahydrate  
Purity: 67.1% "as is" as the anhydrous base determined by GC and micro assay  
GC = 67.2% as is (n = 9, RSD = 0.4%)  
HPLC = 66.4% as is (n = 9, RSD = 1.6%)  
Micro assay: 66.6%  
Description: White to pale buff crystalline powder  
Identity by GC: Complies  
Identity by IR: Complies  
Water KF: 18.2%  
Acetone: 0.1%  
Hygroscopicity: Slightly hygroscopic (0.3% at 90%RH for 24 hours)

STABILITY

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

CONCLUSION

Spectinomycin hydrochloride, Control No 193176, can be considered suitable as International Chemical Reference Substance for the intended purpose. When used in chemical assays the content of spectinomycin hydrochloride ( $C_{14}H_{24}N_2O_7 \cdot 2HCl$ ) is taken to be 81.9% on the "as is" basis corresponding to 100% on the dried basis. This corresponds to 67.2% of spectinomycin anhydrous base ( $C_{14}H_{24}N_2O_7$ ). The result from microbiological assay was 680 IU/mg when determined "as is".

APPENDIX 15

VINCRIStINE SULFATE

Control No 193181

Analytical Report

INTENDED USE

The monograph for Vincristine sulfate in the International Pharmacopoeia 3rd Ed. Vol 3 requires a reference substance for vincristine sulfate to be used for the infrared spectrophotometric identity test and in a thin-layer chromatographic test for related substances.

MATERIAL

About 70 x 10 mg of freeze-dried material obtained from the European Pharmacopoeia Commission were received at the WHO Centre in July 1992. The material is being stored in tightly closed containers at - 20 °C, protected from light. After analysis and labelling they will be stored at +5 °C, protected from light.

*CAUTION:* As vincristine sulfate is a cytotoxic drug it should be handled with care. Avoid contact with the skin and inhalation of airborne particles. Do not open the ampoules for weighing. After releasing the vacuum by piercing the rubber stopper with a hypodermic needle, inject the solvent eg. water or methanol directly through the rubber stopper and dissolve the freeze-dried material in the ampoule and on the stopper while shaking the ampoule with the solvent. Each ampoule contains approximately 9.7 mg of vincristine sulfate.

The lyophilized samples are very static and as such difficult to weigh.

ANALYTICAL DATA

Description: White crystals.

EVIDENCE OF CHEMICAL STRUCTURE

Infrared spectrum

An infrared spectrum is given in Figure 1 (No W 193181 W). The spectrum is concordant with the spectrum of vincristine sulfate no 968 published in Dibbern.

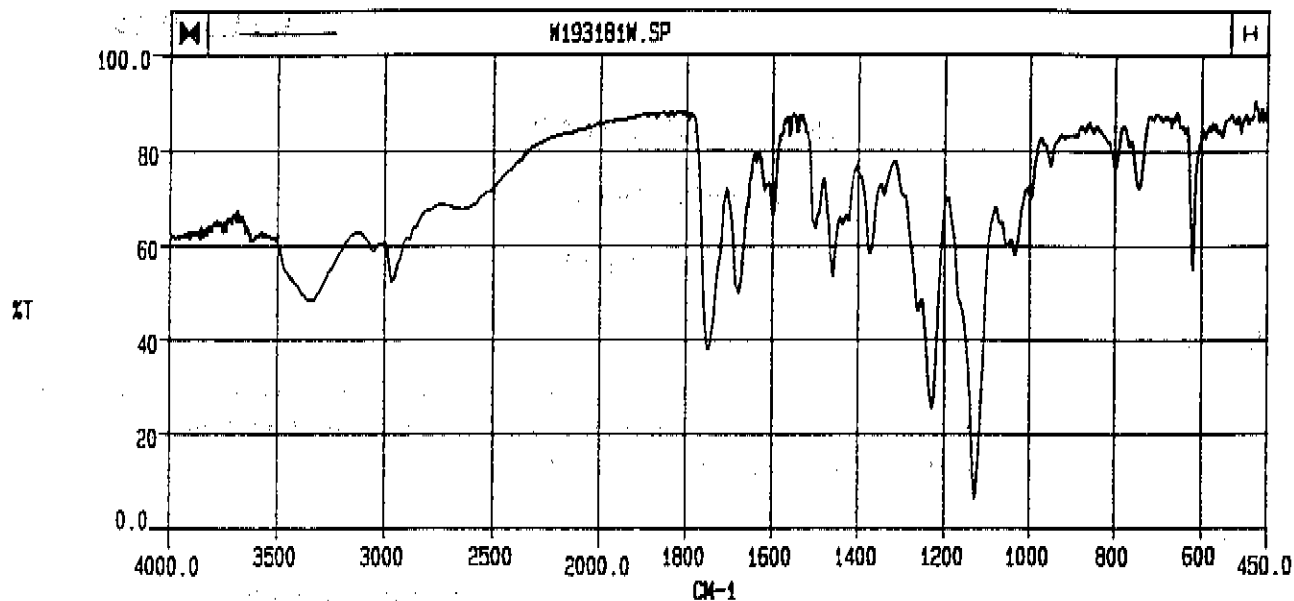


Figure 1. IR-spectrum of vincristine sulfate Control No 193181. A solution of vincristine sulfate in methanol was dropped onto a KBr disc and dried to a film for five minutes in 105 °C. Instrument: Perkin-Elmer 1600 FTIR.

UV-spectrum: A UV-spectrum of a 20 µg/ml solution in methanol is given in Figure 2.

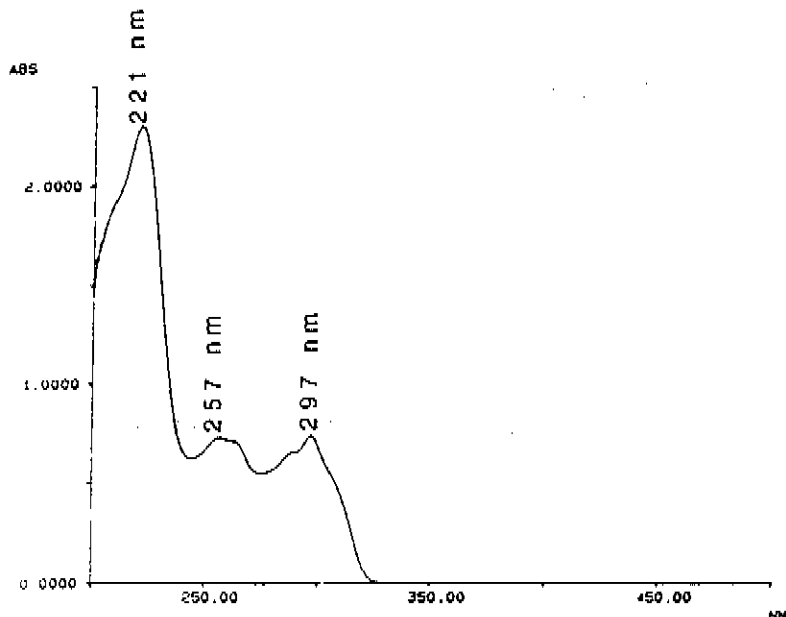


Figure 2. UV-spectrum of vincristine sulfate Control No 193181 20 µg/ml in methanol.

HPLC: The ICRS showed the same retention time as the USP reference standard lot L for vincristine sulfate in the chromatographic system described under assay and purity.

## ASSAY

Liquid chromatographic assay: 9.3 mg of vincristine sulfate per ampoule (n = 3, RSD = 1.4%).

The bulk substance of vincristine sulfate (540 mg 19.2.90) before freeze-drying was used as standard, which according to thermogravimetric analysis showed a 7.2% loss in weight.

The liquid chromatographic system used is described under purity. The total amount in each ampoule was dissolved in 5.00 ml of 0.9% Na Cl.

Spectrophotometric assay: 9.7 mg of vincristine sulfate per ampoule (n = 6, RSD = 2.1%) determined according to the spectrophotometric assay described in BP 88 and Ph. Int 3rd Ed. Vol 3. According to the Pharmacopoeia the calculations were performed with  $A(1\%, 1\text{ cm}) = 177$ . The sample was dissolved in methanol injected directly into the vial and further diluted to 0.02 mg/ml. The higher result obtained with this method could be due to the calculation with a theoretical value for  $A(1\%, 1\text{ cm})$ .

Thermogravimetric analysis: When one ampoule of the lyophilized substance was heated to 250 °C a loss of about 8% of weight was observed. The lyophilized samples are very static and difficult to weigh.

Instrument: Perkin Elmer TGA 7 Thermogravimetric analyzer.  
Sample weight: 3 mg  
Heating rate: 5 °C /min

## PURITY

### Thin-layer chromatography

The total amount of impurities was estimated to about 0.3%.

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: Toluene:chloroform:diethylamine (40:20:3)  
Sample: 50 µg and 100 µg of vincristine sulfate were applied.  
The sample was dissolved in methanol.  
Visualization: Evaluation under UV-light of 254 nm and scanning by densitometry at 297 nm, with a Desaga CD 60 Scanner.

Two secondary spots were detected visually at 254 nm. When evaluated by densitometry at 297 nm two secondary spots were detected. The total amount was estimated to about 0.3% at 297 nm. The detection limit of the system was about 0.1 µg (0.1%) at 297 nm.

R<sub>f</sub> (vincristine sulfate) = 0.15

### High performance liquid chromatography

About 1.2% of impurities were found. Four unknown impurities were detected at 297 nm.

A chromatogram is shown in Figure 3.

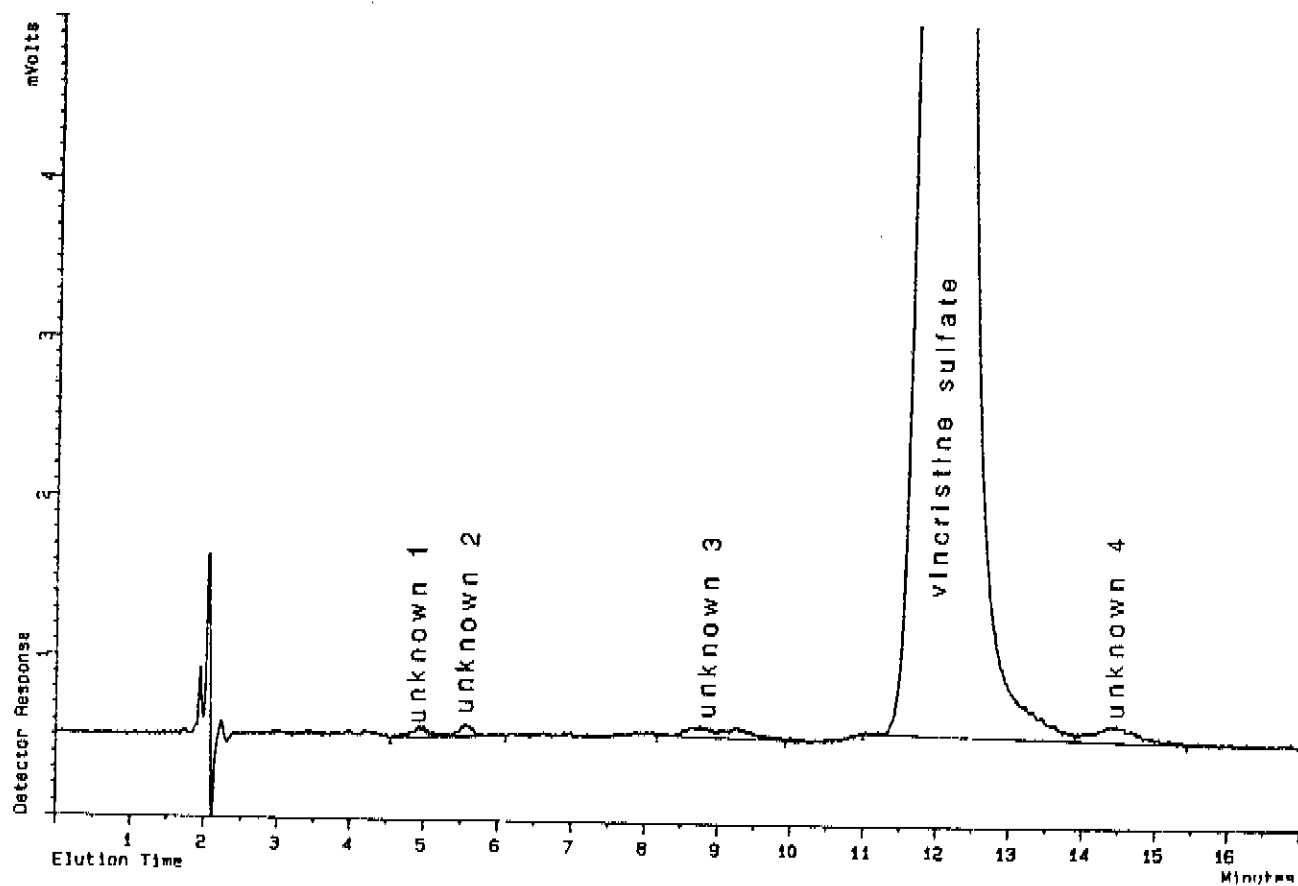


Figure 3. Chromatogram of vincristine sulfate Control No 193 181 monitored at 297 nm.

The following conditions were used:

Eluent: Ammonium hydrogenphosphate 0.01 M, pH 4.5:Acetonitrile:Methanol (120:40:40).  
The eluent also contained 0.01 M sodium heptane sulphonate.

Column: Nucleosil 100 -5 CN

Detector: Shimadzu SPD -6 AV monitored at 297 nm.

Pump: LDC Constrametric III operated at a flow rate of 1.0 ml/min.

Integrator: PeakPro (Beckman)

Sample: 40 µg/ml dissolved in 0.9% NaCl.  
25 µl corresponding to 1µg were injected.

A comparison was also made with USP lot L for vincristine sulfate which was shown to contain about 1% impurities, predominantly as unknown 4.

DATA GIVEN BY COLLABORATING LABORATORIES

Ph Eur CRS  
Infrared: conforms  
HPLC purity: 99.5%  
TG: 7.4%

STABILITY

Stability studies were not performed as this substance was not suspected to degrade easily in dry state at +5 °C. However this substance is very hygroscopic and degrades rapidly at room temperature. Regular re-examinations of the ICRS will be performed.

CONCLUSION

Vincristine sulfate, Control No 193181, can be considered suitable as International Chemical Reference Substance for the intended purpose.

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