



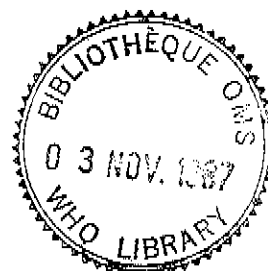
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WHO COLLABORATING CENTRE FOR CHEMICAL REFERENCE SUBSTANCES

Report on the work in 1986

by M. Westermark

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### Distribution of reference substances in 1986

During 1986 the total number of International Chemical Reference Substances distributed from the Centre were 2239 and 25 sets of Melting Point Reference Substances. The substances were distributed to drug control laboratories in 46 different countries. Compared to the figures for 1985 this corresponds to an increase of 1.4 per cent. The five most frequently requested substances during 1986 were Ampicillin Trihydrate, Ampicillin Sodium, Benzylpenicillin Sodium, Tetracycline Hydrochloride and Dicloxacillin Sodium. Detailed figures for the distribution of the individual substances are given in Appendix 1.

### Establishment of reference substances in 1986

In accordance with the procedure recommended by the WHO Expert Committee on Specifications for Pharmaceutical Preparations in its Twenty-fifth report (Technical Report Series No. 567), 6 International Chemical Reference Substances were established in 1986. The substances are listed in Appendix 2 to this report. Ergotamine Tartrate is a replacement batch as the former stock was depleted during 1986.

A complete list of all the International Chemical Reference Substances available from the Centre in January 1987, with information about package sizes and control numbers for the current batches, is given in Appendix 3 to this report. The list also includes 9 substances mentioned below, which are expected to be formally adopted during the first half of 1987.

### Work on new reference substances completed in 1986

Work is being continued on new reference substances required to support specifications in the third edition of the International Pharmacopoeia. During 1986 the following new reference substances were examined: Acetazolamide, 2-Amino-5-nitrothiazole, Niridazole, Niridazole-chlorethylcarboxamide, Norethisterone and Reserpine. The analytical reports for these materials are given in Appendices 6, 7, 10, 11, 12 and 13. All these substances were considered suitable for their intended uses and were proposed for adoption as International Chemical Reference Substances.

The following three stocks of International Chemical Reference Substances were depleted and have been replaced by new batches during 1986. Chloramphenicol No 379004 was replaced by No 486004, Chloramphenicol Palmitate No 175072 was replaced by No 286072 and Vitamine A Acetate No 581038 which was replaced by No 686038. The analytical results are given in Appendices 8, 9 and 14 to this report.

### Stability testing

Each year a number of the International Chemical Reference Substances held in stock at the Centre are being reexamined to control their storage stability. During 1986 the re-examination was performed on twelve substances.

The selection of analytical methods to be used for the stability monitoring requires careful reflection. The choice of method, is of course, much depending on the nature of the substance concerned. However, a generally applicable guiding principle is to use methods of high reproducibility and to adhere as closely as possible to the same methods and the same experimental conditions for the reexamination of a reference material as were used in the initial analysis. This will reduce the influence of analytical errors and facilitate early detection of onset of degradation of the material. It is, however, also prudent to consider from time to time the progress of analytical chemistry and to introduce new methods if they are considered to be more informative and/or more convenient.

The results obtained in the reexamination together with the results from earlier studies are summarized in Appendix 4 to this report. Details about the methods used can be obtained from the Centre.

### Work in progress and future work

Work on the establishment of new chemical reference substances is being continued. There are still 3 substances left to support the monographs in volume 2 of the International Pharmacopoeia. For two of these the work will be finished during 1987. To support the monographs in volume 3 there is today a need for 45 new reference substances. Eleven of these are already in work at the Centre. Older batches have also to be replaced because the stocks are depleted. At present three substances have to be replaced during 1987 but this figure may increase depending on the distribution. A great deal of the work load originates from the increasing demand for regular reexamination of already existing reference substances. Some substances are very old and the increasing total amount of reference substances results in still more work. The reference substances the Centre has to establish are listed in Appendix 5 to this report. The substances that are already in work are indicated with an asterix.

During 1986 computerization of the activities concerning the work on reference substances has started. The system consists of an IBM XT Personal Computer. Today information about bulk ordering, analytical schemes, dispensing worksheets and a plan for regular reexamination are available in the computer. Plans for computerized orders and an inventory of the stock of existing reference substances are in progress. Collaboration with other laboratories to decrease the workload on the Centre in Stockholm has also started. Hopefully this will facilitate the preparation of the new reference substances for volume 3.

### Administrative and financial matters

The financial situation of the Centre remains unsatisfactory. The total cost for running the Centre in 1986 was estimated at 207.000 US\$. The income from sales of reference substances to industrial laboratories was about 19.000 US\$ and the contribution received from the WHO Headquarters was 16.000 US\$, which leaves a deficit of 172.000 US\$. The management board of the National Corporation of Swedish Pharmacies has agreed to support the continued operation of the Centre at an unchanged level, provided all possibilities to reduce the deficit would be investigated.

The fee for the substances has been kept unchanged at US\$ 25 per package during 1986. However, from January 1987 the fee has been increased to US\$ 40 per package and a freight and handling charge of US\$ 10 will also be added to each order.

In order to alleviate the financial deficit of the Centre, National Research Centres have been requested to offer analytical support and WHO Regional Offices have been approached for financial assistance.

### Acknowledgements

As usual the Centre has reason to express the most sincere thanks to Dr C. A. Johnson, Scientific Director and Secretary to the British Pharmacopoeia Commission, and a member of the WHO Expert Advisory Panel on the International Pharmacopoeia and Pharmaceutical Preparations, for his never-failing interest in our work and extremely valuable help as counsellor to the Centre in all matters concerning the establishment of reference substances. The Centre would also like to express its sincere gratitude to all pharmaceutical industries who have assisted the Centre by provision of candidate reference materials as well as by participation in the analytical testing. This year we particularly wish to thank Ercopharm in Vedbaeck, Denmark, CIBA-GEIGY AG in Basle, Switzerland, Farmitalia Carlo Erba in Milano, Italy, SYNTEX in Palo Alto, USA and the U.S. Pharmacopoeial Convention Inc., USA.

## APPENDIX 1

## DISTRIBUTION OF CHEMICAL REFERENCE SUBSTANCES IN 1986

Aceclidine Salicylate	4 items	Ethambutol Hydrochloride	12 items
p-Acetamidobenzalazine	3 "	Ethinylestradiol	32 "
Allopurinol	2 "	Ethisterone	10 "
3-Aminopyrazole-4-carboxamide		Ethosuximide	5 "
Hemisulfate	7 "	Etocarlide	3 "
Amitriptyline Hydrochloride	24 "	Flucytosine	6 "
Ampicillin	54 "	Fluorouracil	19 "
Ampicillin Sodium	82 "	Fluphenazine Decanoate	
Ampicillin Trihydrate	84 "	Dihydrochloride	11 "
Anhydrotetracycline Hydrochloride	46 "	Fluphenazine Enantate	
Atropine Sulfate	24 "	Dihydrochloride	10 "
Azathioprine	4 "	Fluphenazine Hydrochloride	13 "
Bendazol Hydrochloride	4 "	Folic Acid	36 "
Benzobarbital	8 "	Furosemide	15 "
Benzylamine Sulfate	4 "	Griseofulvin	22 "
Benzylpenicillin Potassium	50 "	Haloperidol	13 "
Benzylpenicillin Sodium	70 "	Hydrochlorothiazide	13 "
Bephenium Hydroxynaphthoate	10 "	Hydrocortisone	39 "
Betamethasone	21 "	Hydrocortisone Acetate	35 "
Betanidine Sulfate	4 "	(-)-3-(4-Hydroxy-3-methoxyphenyl)-	
Bupivacaine Hydrochloride	5 "	2-methylalanine	3 "
Caffeine	17 "	Ibuprofen	19 "
Carbenicillin Monosodium	25 "	Imipramine Hydrochloride	12 "
Chloramphenicol	36 "	Indometacin	23 "
Chloramphenicol Palmitate	18 "	o-Iodhippuric Acid	4 "
Chloramphenicol Palmitate		Isoniazid	14 "
(Polymorph A)	40 "	Lanatoside C	17 "
5-Chloro-2-methylaminobenzophenone	6 "	Levodopa	5 "
2-(4-Chloro-3-sulfamoylbenzoyl)		Lidocaine	16 "
benzoic Acid	11 "	Lidocaine Hydrochloride	29 "
Chlorphenamine Hydrogen Maleate	8 "	Mefenamic Acid	4 "
Chlorpromazine Hydrochloride	21 "	Melting Point Reference Substances	25 "
Chlortalidone	5 "	(set of 13 substances)	
Cloxacillin Sodium	33 "	Metazide	3 "
Cortisone Acetate	26 "	Methaqualone	8 "
Dapsone	13 "	Methylodopa	11 "
Desoxycortone Acetate	9 "	Methyltestosterone	10 "
Dexamethasone	36 "	Meticillin Sodium	16 "
Dexamethasone Acetate	11 "	Metronidazole	22 "
Diazepam	23 "	Nafcillin Sodium	7 "
Diazoxide	6 "	Nicotinamide	30 "
Dicloxacillin Sodium	57 "	Nicotinic Acid	17 "
Dicolinium Iodide	3 "	Norethisterone Acetate	5 "
Dicoumarol	8 "	Quabain	6 "
Diethylcarbamazine Dihydrogen		Oxacillin Sodium	39 "
Citrate	3 "	Papaverine Hydrochloride	6 "
Digitoxin	20 "	Phenethicillin Potassium	7 "
Digoxin	38 "	Phenoxymethylpenicillin	32 "
NN'-Di-(2,3-xyllyl)anthranilamide	4 "	Phenoxymethylpenicillin Calcium	8 "
4-Epianhydrotetracycline		Phenoxymethylpenicillin Potassium	34 "
Hydrochloride	35 "	Phenytoin	9 "
4-Epitetracycline Ammonium Salt	31 "	Prednisolone	39 "
Ergometrine Hydrogen Maleate	17 "	Prednisolone Acetate	15 "
Ergotamine Tartrate	20 "	Prednisone	25 "
Estradiol Benzoate	10 "	Prednisone Acetate	12 "
Estrone	8 "	Procaine Hydrochloride	14 "
Etacrynic Acid	4 "	Procarbazine Hydrochloride	7 "

Progesterone	18	items
Propicillin Potassium	19	"
Propylthiouracil	2	"
Pyridostigmine Bromide	9	"
Riboflavin	25	"
Rose Bengal Sodium	3	"
Sulfamethoxazole	33	"
Sulfamethoxypyridazine	10	"
Sulfanilamide	12	"
Testosterone Propionate	16	"
Tetracycline Hydrochloride	59	"
Thioacetazone	4	"
4,4'-Thiodianiline	7	"
Tolbutamide	6	"
Tolnaftate	7	"
Trimethoprim	31	"
Trimethylguanidine Sulfate	3	"
Tubocurarine Chloride	3	"
Vitamin A Acetate (solution)	26	"
Warfarin	12	"
Total	2 264	items

APPENDIX 2

INTERNATIONAL CHEMICAL REFERENCE SUBSTANCES ESTABLISHED IN 1986

Reference Substance	Control number	Analytical Report	Remarks
Ergotamine Tartrate	385013	WHO/PHARM/86.527 Appendix 6	Replaces No 276013
Isoniazid	185124	WHO/PHARM/86.527 Appendix 7	
Norethisterone Acetate	185123	WHO/PHARM/86.527 Appendix 8	
Papaverine Hydrochloride	185127	WHO/PHARM/86.527 Appendix 9	
Propylthiouracil	185126	WHO/PHARM/86.527 Appendix 10	
Trimethadione	185125	WHO/PHARM/86.527 Appendix 11	

APPENDIX 3

LIST OF AVAILABLE INTERNATIONAL CHEMICAL REFERENCE SUBSTANCES

1987

General information

International Chemical Reference Substances are established upon 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.

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 these substances to be used.

Directions for use and analytical data as required for the use intended in the relevant specifications of 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.

It is generally recommended that the substances should 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 kept at the Collaborating Centre is monitored by regular reexamination and deteriorated materials are replaced by new batches when 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 the 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)

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

<u>Reference substance</u>	<u>Package size</u>	<u>Control number for current batch</u>
Aceclidine Salicylate	100 mg	172048
p-Acetamidobenzalazine	100 mg	171042
Acetazolamide	100 mg	186128
Allopurinol	100 mg	172049
2-Amino-5-nitrothiazole	25 mg	186131
3-Aminopyrazole-4-carboxamide Hemisulfate	100 mg	172050
Amitriptyline Hydrochloride	100 mg	181101
Ampicillin	200 mg	274001
Ampicillin Sodium	200 mg	274002
Ampicillin Trihydrate	200 mg	274003
Anhydrotetracycline Hydrochloride	25 mg	180096
Atropine Sulfate	100 mg	183111
Azathioprine	100 mg	172060
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
Betanidine Sulfate	100 mg	172053
Bupivacaine Hydrochloride	100 mg	172054
Caffeine	100 mg	181102
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
Cloxacillin Sodium	200 mg	274005
Cortisone Acetate	100 mg	167006
Dapsone	100 mg	183115
Desoxycortone Acetate	100 mg	167007
Dexamethasone	100 mg	279008
Dexamethasone Acetate	100 mg	168009
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	377011
NN'-Di-(2,3-xyllyl)anthranilamide	50 mg	173067
4-Epianhydrotetracycline Hydrochloride	25 mg	180097
4-Epitetracycline Ammonium Salt	25 mg	180098
Ergometrine Hydrogen Maleate	50 mg	277012
Ergotamine Tartrate	50 mg	385013
Estradiol Benzoate	100 mg	167014
Estrone	100 mg	279015
Etacrynic Acid	100 mg	281056
Ethambutol Hydrochloride	100 mg	179081
Ethinylestradiol	100 mg	167016
Ethisterone	100 mg	167017
Ethosuximide	100 mg	179088
Etocarlide	100 mg	172057
Flucytosine	100 mg	184121

<u>Reference substance</u>	<u>Package size</u>	<u>Control number for current batch</u>
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	277019
Furosemide	100 mg	171044
Griseofulvin	200 mg	280040
Haloperidol	100 mg	172063
Hydrochlorothiazide	100 mg	179087
Hydrocortisone	100 mg	283020
Hydrocortisone Acetate	100 mg	280021
(-)-3-(4-Hydroxy-3-methoxyphenyl)- 2-methylalanine	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
Levodopa	100 mg	172065
Lidocaine	100 mg	181104
Lidocaine Hydrochloride	100 mg	181105
Mefenamic Acid	100 mg	173068
Melting Point Reference Substances (set of 13 substances with melting temper- atures ranging from +69 °C to +263 °C)	13x4 g	
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
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
Ouabain	100 mg	283026
Oxacillin Sodium	200 mg	382027
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
Prednisolone	100 mg	283029
Prednisolone Acetate	100 mg	167030
Prednisone	100 mg	167031
Prednisone Acetate	100 mg	169032
Procaine Hydrochloride	100 mg	183119
Procarbazine Hydrochloride	100 mg	184120
Progesterone	100 mg	167033
Propicillin Potassium	200 mg	274034
Propylthiouracil	100 mg	185126
Pyridostigmine Bromide	100 mg	182110
Reserpine	100 mg	186133
Riboflavin	250 mg	382035
Sulfamethoxazole	100 mg	179092

<u>Reference substance</u>	<u>Package size</u>	<u>Control number for current batch</u>
Sulfamethoxypyridazine	100 mg	178079
Sulfanilamide	100 mg	179094
Testosterone Propionate	100 mg	167036
Tetracycline Hydrochloride	200 mg	180095
Thioacetazone	100 mg	171046
4,4'-Thiodianiline	50 mg	183116
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)	5 caps. (*)	686038
Warfarin	100 mg	168041

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

APPENDIX 4

STABILITY TESTING

The storage stability of the International Chemical Reference Substances is monitored by regular reexamination of the substances held in stock at the Centre. The results obtained for the substances reexamined in 1986 are summarized below. For comparison results obtained at earlier occasions are included in the summaries. The substances have been stored at +5° C. The following abbreviations are used in the tables:

DTA      Differential Thermal Analysis  
HPLC     High Performance Liquid Chromatography  
TLC      Thin-layer Chromatography  
PSA      Phase Solubility Analysis  
KF       Karl Fischer titration  
IR       Infrared Spectrophotometry

The estimates of total solid impurities by HPLC and TLC are expressed as area per cent if otherwise not stated, by DTA as mol per cent, and by PSA as weight per cent. Assay values are calculated with reference to the dried or anhydrous substance.

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

Benzobarbital, Control No 172051

Initial analytical report: WHO/PHARM/72.471, Appendix 13

Examination year:	1972	1977	1981	1987
Light absorption, 250 nm	0.495	0.499	0.495	0.486
TLC	no sec. spots	one sec. spot	two sec. spots (<1%)	4-5 sec. spots by scanning about 0.5%
IR	conforms	-	-	conforms
DTA, %	-	-	1.7	about 1
HPLC, %	-	-	-	0.3
Loss on drying, %	0.4	0.02	0.1	0.1
Assay (potentiometric)	100.0	100.2	-	100.3

Digitoxin, Control No 277010

Initial analytical report: WHO/PHARM/78.494, Appendix 7

Examination year:	1977	1987
TLC	5 faint sec. spots	no additional spots
IR	conforms	conforms
HPLC, %	no contaminants	about 0.1
Loss on drying, %	0.6	0.6
Assay, % (colorimetric)	99.7	100.7

Hydrochlorothiazide, Control No 179087

Initial analytical report: WHO/PHARM/80.504, Appendix 8

Examination year:	1979	1987
IR	conforms	conforms
DTA, %	0.4	0.7
HPLC, %	0.4	0.4
Loss on drying, %	0.0	0.2

Lanatoside C, Control No 281022

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

Examination year:	1981	1987
TLC	5 sec. spots	5 sec. spots
IR	conforms	conforms
HPLC, % (0.4% of this is Lanatoside B)	0.8	1.0
Loss on drying, %	7.2	7.2
Assay, %	99.9	99.9

Nicotinamide, Control No 179090

Initial analytical report: WHO/PHARM/80.504, Appendix 11

Examination year:	1979	1987
Light absorption, 263 nm	0.59	0.59
TLC	2 sec. spots	2 sec. spots (by scanning)
DTA, %	about 0.1	0.04
IR	conforms	conforms
Loss on drying, %	0.3	0.0
Assay, % (potentiometric)	100.0	99.8

Nicotinic Acid, Control No 179091

Initial analytical report: WHO/PHARM/80.504, Appendix 12

Examination year:	1979	1987
Light absorption, 263 nm	0.57	0.59
TLC	no sec. spots	no sec. spots
DTA, %	0.1	0.1
IR	conforms	conforms
Loss on drying, %	0.05	about 0.2
Assay, % (potentiometric)	99.8	99.9

Ouabain, Control No 283026

Initial analytical report: WHO/PHARM/84.513, Appendix 14

Examination year:	1983	1987
LOD, %	20.0	19.9
HPLC, %	0.4	0.7
TLC	0.2	0.3
Assay, % (Baljet reaction)	100.1	100.0

Prednisolone, Control No 283029

Initial analytical report: WHO/PHARM/84.513, Appendix 15

Examination year:	1983	1987
Light absorption, 263 nm	0.417	0.416
TLC, %	1.6	about 2.5
	2 sec. spots	2 sec. spots
LOD, %	0.08	-
KF (water), %	-	0.2
IR	conforms	conforms
HPLC, %	1.4	2.1
Assay, % (spectrophotometric)	100.0	99.9

Prednisolone Acetate, Control No 167030

Initial analytical report: WHO/PHARM/66.431, Appendix 7

Examination year:	1966	1975	1984	1987
UV-absorption 242 nm, E(1%, 1 cm)	382	377	377	376
Loss on drying, %	0.0	0.2	-	0.0
TLC	2 sec. spots	1 sec. spots	3 sec. spots	3 sec. spots
IR	conforms			
HPLC, %	-	-	1.8	2.2
PSA, %	0.5	-	-	-

Riboflavin, Control No 382035

Initial analytical report: WHO/PHARM/83.510, Appendix 10

Examination year:	1982	1987
IR	conforms	conforms
Loss on drying, %	0.3	0.4
HPLC, %	< 1	0.6
Assay, % (spectrophotometric)	99.5	100.0

Sulfamethoxypyridazine, Control No 178079

Initial analytical report: WHO/PHARM/79.499, Appendix 11

Examination year:	1978	1987
IR	conforms	conforms
DTA, %	0.2	0.3
HPLC, %	-	0.22
Loss on drying, %	0.0	0.2
Assay, % (potentiometric)	99.8	100.4

Tolbutamide, Control No 179086

Initial analytical report: WHO/PHARM/80.504, Appendix 16

Examination year:	1979	1987
TLC, %	no sec. spot	no sec. spot
IR	conforms	
DTA, %	0.2	0.2
LOD, %	0.1	0
HPLC, %	0.02	0.01

APPENDIX 5

INTERNATIONAL CHEMICAL REFERENCE SUBSTANCES - PROJECT LIST 1987

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

Volume 2

Chlortetracycline Hydrochloride (\*)  
Colecalciferol  
Propranolol Hydrochloride (\*)

Volume 3

Amodiaquine Hydrochloride	Liothyronine
Amphotericin B (*)	(impurity in Levothyroxine Sodium)
Bacitracin Zinc	Loperamide Hydrochloride
Beclomethasone Dipropionate	Methotrexate
Betamethasone Valerate	Neamine
Calcium Folate	(impurity in Neomycin Sulfate)
Carbamazepine (*)	Neomycin B Sulfate
Cimetidine	(impurity in Neomycin Sulfate)
Clomifene Citrate (*)	Neostigmine Methylsulfate (*)
Clomifene Citrate Z-isomer (*)	Nifurtimox
Dexamethasone Sodium Phosphate	Noroxymorphone Hydrochloride
Dopamine Hydrochloride	(impurity in Naloxone Hydrochloride)
Doxorubicin Hydrochloride	Nystatin
Emetine Hydrochloride (*)	Oxytetracycline Dihydrate (*)
Ergocalciferol	Oxytetracycline Hydrochloride (*)
Fludrocortisone Acetate	Paromomycin Sulfate
3-Formylrifamycin SV	Praziquantel
(impurity in Rifampicin)	Prednisolone Sodium Phosphate
Gentamicin Sulfate	Pyrantel Embonate (*)
Hydrocortisone Sodium Succinate	Probenecid (*)
(-)-3-(4-Hydroxy-3-methoxyphenyl)-2-hydrazino- 2-methylalanine (impurity in Carbidopa)	Rifampicin quinone
Levonorgestrel	(impurity in Rifampicin)
Levothyroxine Sodium	Salazosulfapyridine
	Sodium Cromoglicate
	Spectinomycin Hydrochloride
	Sulfacetamide
	Testosterone Enantate
	Vincristine Sulfate

Replacements

The following existing International Chemical Reference Substances should be replaced by new batches in 1987

Allopurinol (\*)  
Digoxin (\*)

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

ACETAZOLAMIDE

Control No 186128

Analytical Report

The monograph for acetazolamide in the International Pharmacopoeia Ed. III, Vol 2 requires a reference substance to be used in the infrared spectrophotometric identity test.

MATERIAL

About 100 g of the sample (manufacturers lot no R 6233) were received at the WHO Centre in November 1984. The material is being stored protected from light in tightly closed containers at +5 °C.

ANALYTICAL DATA

Description: A white, crystalline powder.

EVIDENCE OF CHEMICAL STRUCTURE

Infrared spectrum

An infrared spectrum is given in Figure 1 (no 186128). The spectrum is concordant with the spectrum obtained from the USP reference substance Lot I.

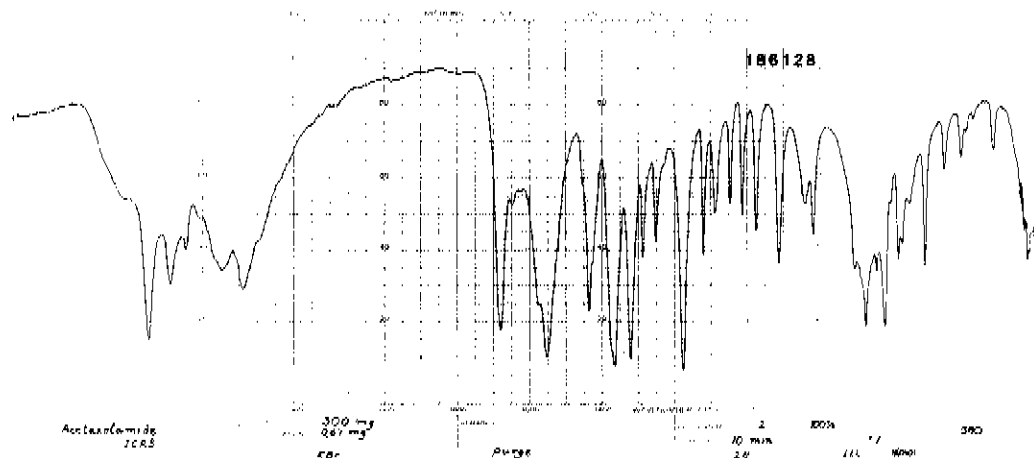


Figure 1. IR-spectrum of 0.7 mg of acetazolamide in 300 mg KBr, recorded against a KBr reference disc. Instrument: Perkin Elmer 580.

Elemental analysis

	C(%)	H(%)	N(%)
Theoretical	21.6	2.7	25.2
Found	21.7	2.7	25.2

The analysis was performed at Mikro Kemi AB, Uppsala.

UV-spectrum

A UV-spectrum in 0.1 M sodium hydroxide is given in Figure 2.  
 $\lambda$  max in 0.1 M sodium hydroxide = 291 nm.  $E(1\%, 1\text{ cm}) = 601$  ( $n=8$ ). Spectra were also recorded in ethanol, methanol and acetonitrile  $\lambda$  max were 264 nm, 264 nm and 263 nm, respectively. However, irregular E-values were obtained due to solubility problems.

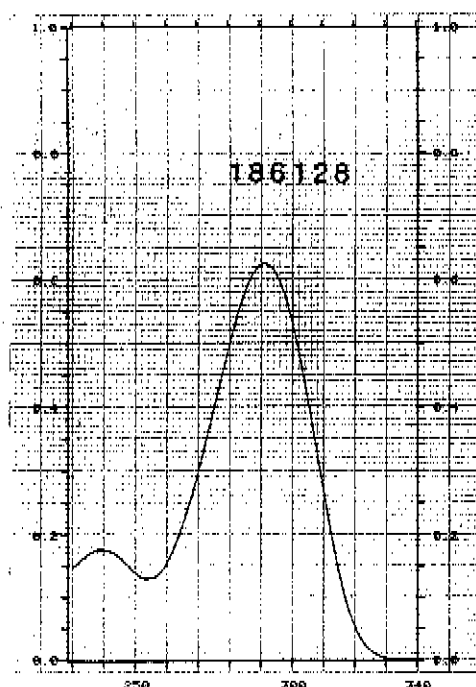


Figure 2. UV-spectrum of acetazolamide 10.4  $\mu\text{g/ml}$  in 0.1 M sodium hydroxide.

ASSAY

99.7% ( $n=7$ ). Determined by potentiometric titration with 0.1 M tetrabutylammonium hydroxide (solution in methanol/toluene) according to Ph. Int. Ed. III, Vol 2.

Loss on drying

0.15% (105 °C)

PURITYTotal solid impurities

Differential thermal analysis (DTA): It was not possible to estimate the purity by this method as the substance melts with decomposition.

Thin-layer chromatography

The following thin-layer chromatography systems were used.

I. Thin-layer: Silica gel 60, F-254 (Merck)

Eluent: Ethyl acetate:acetone:chloroform:methanol (5 + 5 + 5 + 1)

Sample: 100  $\mu\text{g}$  of acetazolamide were applied. 20 mg of the substance was dissolved in 0.5 ml of DMF followed by the addition of 1.5 ml of acetone.

Visualization: UV-light at 254 nm.

$R_f$  (acetazolamide) = 0.4

Result: No extra spots were detected. The detection limit for the system was less than 0.1 µg (0.1%). The chromatogram was evaluated using a Zeiss KM3 Chromatogram Spectrophotometer operated in the reflectance mode at 264 nm.

A comparison was made with USP reference substance Lot I which contained two impurities estimated to about 0.5%.

II. This system was used in order to look for hydrazine.

Thin-layer: Silica gel 60, F-254 (Merck)

Eluent: Acetone:water (98 + 2)

Sample: 1000 µg of acetazolamide were applied. The substance was dissolved as above. As reference 0.1 µg hydrazine hydrate was applied.

Visualization: Spraying with 4-dimethylaminobenzaldehyde and examination in daylight.

Result: No extra spots were detected.

#### High performance liquid chromatography

No impurities were found. A chromatogram is shown in Figure 3.

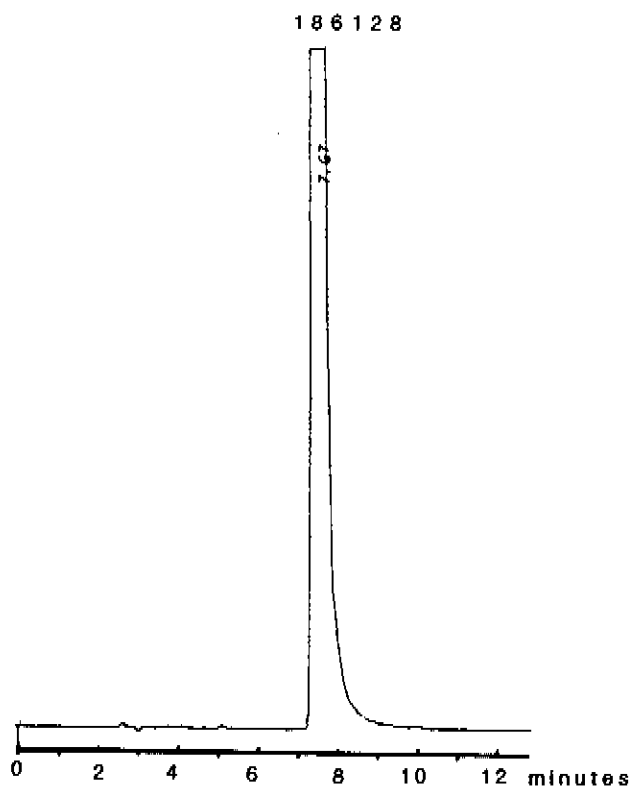


Figure 3. A chromatogram of acetazolamide Control No 186128. In USP reference substance Lot I about 0.9% impurities were found.

The following conditions were used:

Eluent: Acetonitrile/Acetate buffer pH 4.0 (10:90)

Column: RP-18, Spheri-5 (Brownlee Labs)

Detector: Varian UV-200 operated at 254 nm

Pump: Varian 5560 operated at a flow rate of 1.0 ml/min

Integrator: Varian 4270 Attenuation: 1

Sample: 1 mg/ml. First 25 µg were dissolved in 2.5 ml of acetonitrile, thereafter 2.5 ml of eluent were added and the final dilution was performed with eluent. 10 µl corresponding to 10 µg were injected.

DIODE ARRAY DETECTION

The chromatogram was also evaluated with a LKB 2140 Rapid Diode Array Detector. The same chromatographic system as described above was used, except for the injection volume which was changed to 100  $\mu$ l. An isogram is given in Figure 4.

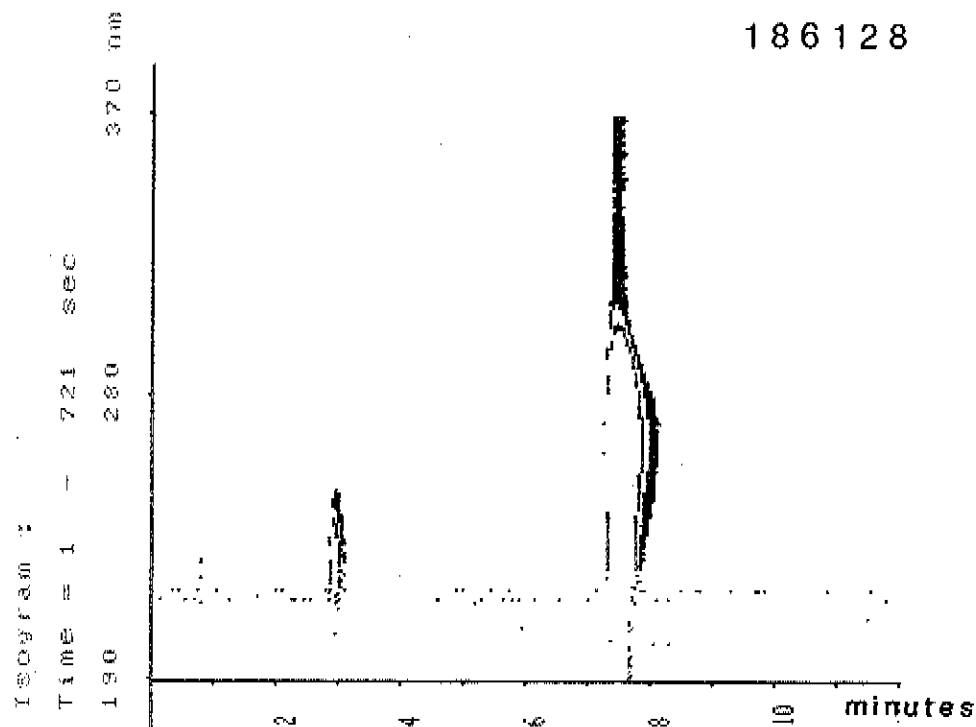


Figure 4. Isogram of acetazolamide Control No 186128. Sensitivity: 0.005

As seen from the figure no impurities are observed at any wavelength. Acetazolamide eluting after about 7.5 minutes has a maximum absorbance at 265 nm. The peak purity was tested by recording spectra at the up- and downslope of the peak. They were all identical with maxima at 265 nm. The small spot at about 3 minutes originates from the blank.

STABILITY

Acetazolamide was exposed to air of different relative humidity at room temperature (about 20° C) for a period of 6 weeks as described in WHO/PHARM/82.509. All samples were unchanged at visual inspection and no weight changes were noted. No signs of degradation were observed when selected samples were analyzed by the liquid chromatographic method described above.

DATA GIVEN BY THE MANUFACTURER

Description	A white crystalline powder, odourless.
Identification	Conforms
Clarity and colour of solution	Conforms
Acidity or alkalinity	Conforms
Sulfate	<0.5 ‰
Heavy metals	<10 ppm
Sulfated ash	0.0%
Assay	99.5%

CONCLUSION

Acetazolamide Control No 186128 can be considered suitable as International Chemical Reference Substance for the intended purpose.

APPENDIX 7

## 2 - A M I N O - 5 - N I T R O T H I A Z O L E

Control No 186131

Analytical Report

The International Chemical Reference Substance for 2-amino-5-nitrothiazole is intended to be used in the thin-layer chromatographic test for related substances according to the monograph for niridazole in the International Pharmacopoeia, Ed. III, Vol 3.

MATERIAL

About 25 g of the sample (manufacturers batch no Sch-1695-1C) were received at the WHO Centre in December 1984. The material is being stored protected from light in a tightly closed container at +5 °C.

ANALYTICAL DATA

Description: A yellow powder, almost odourless.

EVIDENCE OF CHEMICAL STRUCTUREInfrared spectrum

An infrared spectrum is given in Figure 1 (no 186131). The spectrum is concordant with a reference spectrum received from the manufacturer.

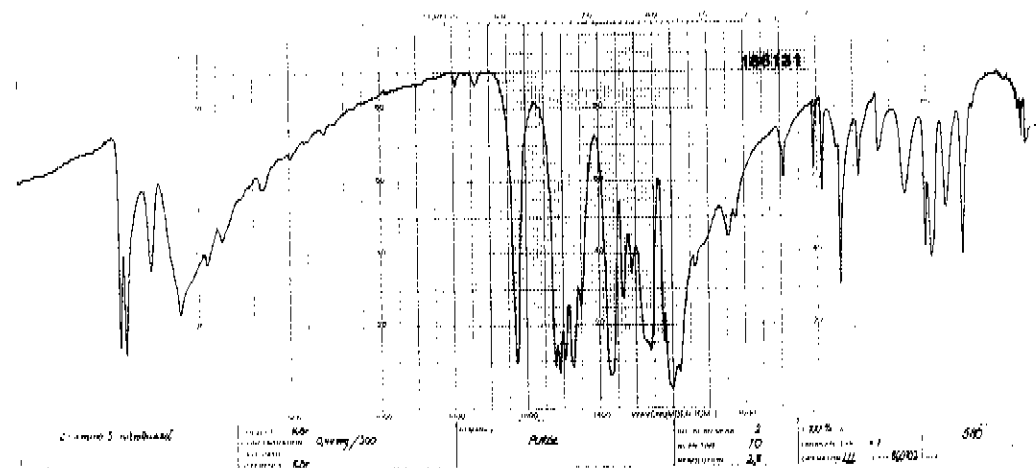


Figure 1. IR-spectrum of 0.5 mg of 2-amino-5-nitrothiazole in 300 mg KBr recorded against a KBr disc. Instrument: Perkin Elmer 580.

Melting temperature

About 195-200 °C with decomposition, determined by the capillary method of Ph. Int. Ed. III. According to Merck Index, Ed 9, it decomposes at 202 °C.

Elemental analysis: The analysis was performed at Mikro Kemi AB, Uppsala.

	C (%)	H (%)	N (%)
Theoretical	24.8	2.1	29.0
Found	24.8	1.9	28.5

### UV-spectrum

A UV-spectrum in 0.1% dimethylformamide in ethanol is given in Figure 2. The substance is first dissolved in dimethylformamide and then diluted with ethanol.

$\lambda$  max in ethanol = 379 nm  
E (1%, 1cm) = 1037 (n= 5)

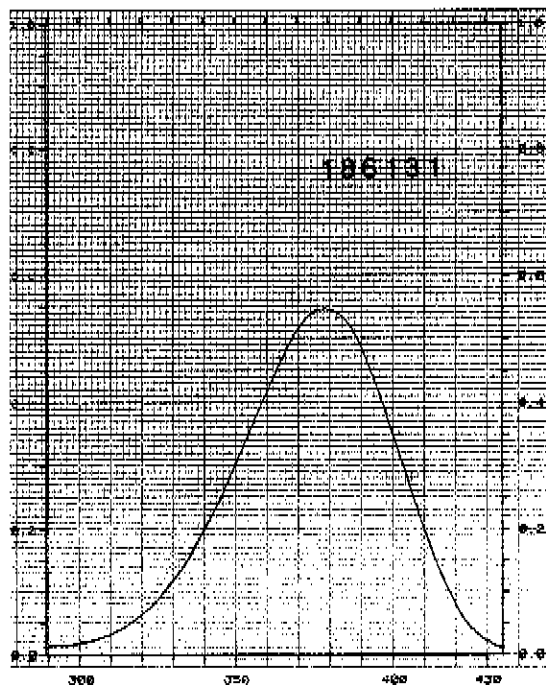


Figure 2. UV-spectrum of 2-amino-5-nitrothiazole 5  $\mu$ g/ml in ethanol.

### Loss on drying

0.5% (100 °C, reduced pressure)

### PURITY

#### Total solid impurities

Differential thermal analysis (DTA): It was not possible to estimate the purity by this method as the substance melts with decomposition.

#### Thin-layer chromatography

The following TLC systems were used.

Thin-layer: Silica Gel 60, F-254 (Merck)

Eluent: Toluene: Acetone: Acetic acid: Isobutanol (12 + 8 + 3 + 8)

Sample: 100  $\mu$ g of 2-amino-5-nitrothiazole were applied.

Visualization: UV-light of 254 nm and 365 nm visually and 379 nm by scanning. Rf (niridazole)= 0.48; Rf (2-amino-5-nitrothiazole)= 0.55; Rf (niridazole-chlorethyl-carboxamide)= 0.58. The detection limit for 2-amino-5-nitrothiazole was 0.006  $\mu$ g (0.006%) when scanned at 379 nm.

Result: No impurities were found.

The thin-layer chromatographic system described in Ph. Int. Ed. III, Vol. 3 with toluene:acetone (12+8) as eluent was tested but 2-amino-5-nitrothiazole did not separate from niridazole. No impurities were found.

High performance liquid chromatography

The total amount of impurities was estimated by peak area measurement to about 0.1%. A chromatogram is shown in Figure 3.

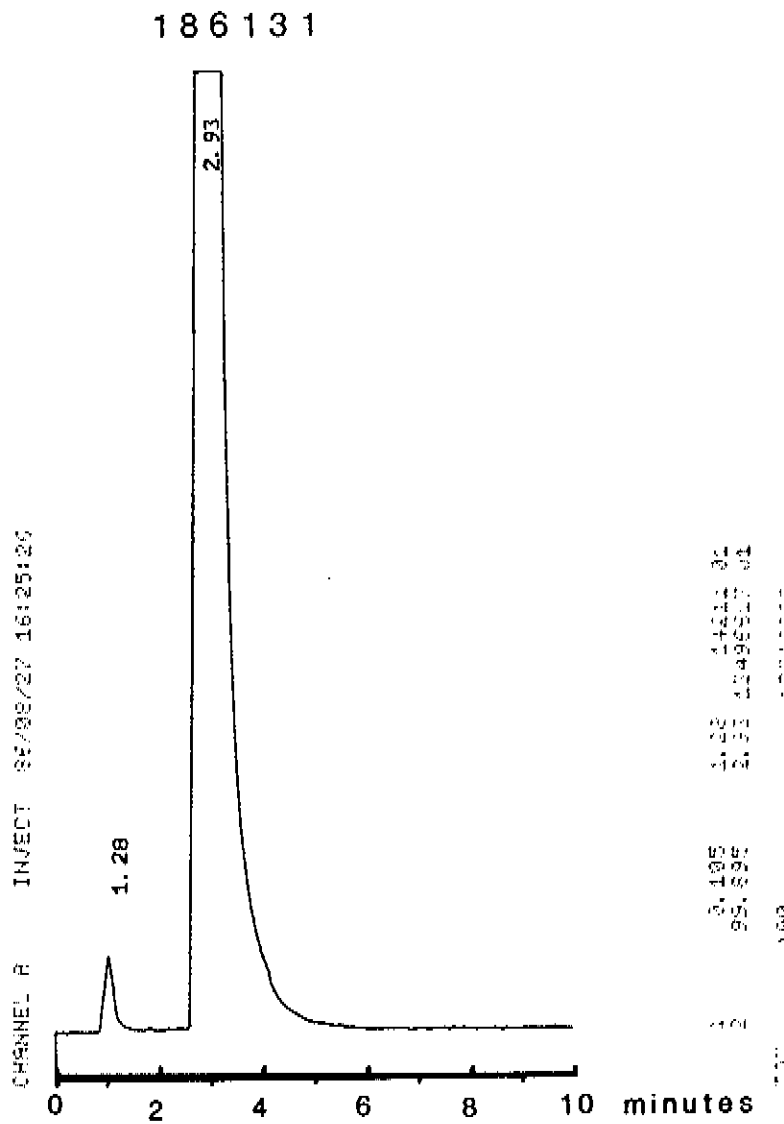


Figure 3. A chromatogram of 2-amino-5-nitrothiazole No 186131.

The following conditions were used:

- Eluent: Acetonitrile / Water (55 + 45)
- Column: RP-18, Spheri-5 (Brownlee)
- Detector: Varian UV 200 operated at 379 nm
- Pump: Varian 5560 operated at a flow rate of 1 ml/min.
- Integrator: Varian 4270 Attenuation: 8
- Sample: 0.6 mg/ml dissolved in acetonitrile with additional adjustment to eluent composition. 10  $\mu$ l corresponding to 6  $\mu$ g were injected.

## DIODE-ARRAY DETECTION

The chromatogram was also evaluated with a LKB 2140 Rapid Diode Array Detector. The same chromatographic system as described above was used, except for the injection volume that was increased to 100  $\mu$ l to get maximum sensitivity. An isogram is given in Figure 4.

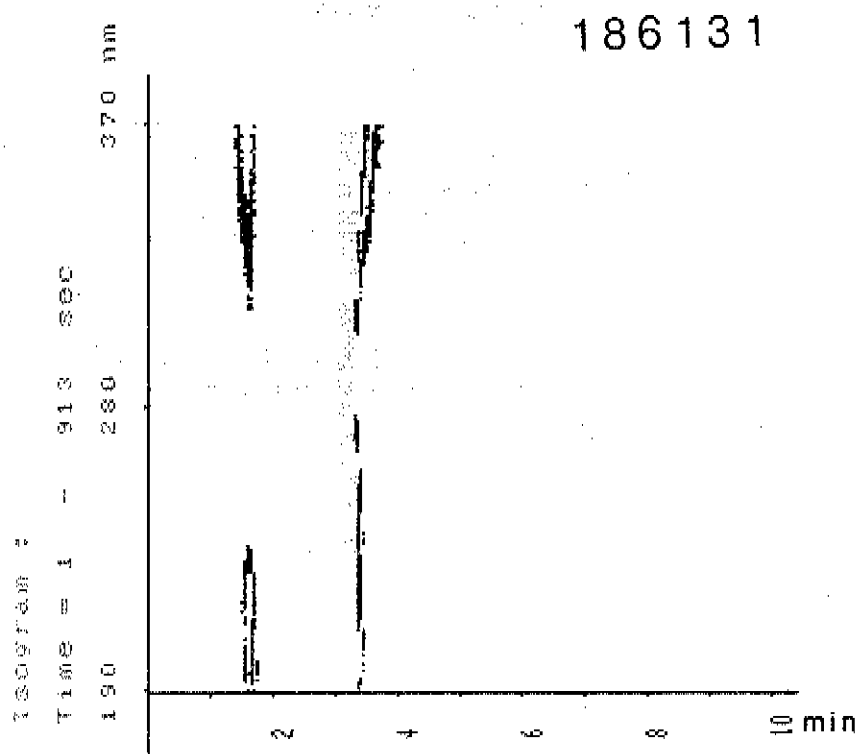


Figure 4. Isogram of 2-amino-5-nitrothiazole, Control No 186131. Sensitivity: 0.005

As seen from the figure the impurity eluting after 1.5 minutes exhibits UV-maxima at 210 nm and 360 nm, respectively. The results from peak area measurements at these wavelengths were compared to the result at 379 nm which is chosen in the method described above. At 379 nm 0.1% impurities were detected compared to 0.1% at 360 nm and 0.2%-0.3% at 210 nm. The higher value at 210 nm is due to the fact that 2-amino-5-nitrothiazole has lower UV-absorption at this wavelength than at 379 nm.

## STABILITY

2-Amino-5-nitrothiazole was exposed to air of different relative humidity at room temperature (about 20<sup>o</sup> C) for a period of eight weeks as described in WHO/PHARM/82.509. All samples were unchanged at visual inspection and no weight changes were noted. No signs of degradation were observed when selected samples were analyzed by the liquid chromatographic method described above.

## DATA GIVEN BY THE MANUFACTURER

IR spectrum	conforms
Proton NMR spectrum	conforms
Elemental analysis	C (25.16%) H (2.08%) N (28.97%)
Melting point	195-200 <sup>o</sup> C
TLC	more than 95% pure

## CONCLUSION

2-Amino-5-nitrothiazole No 186131 can be considered suitable as International Chemical Reference Substance for the intended purpose.

CHLORAMPHENICOL

Control No 486004

Analytical Report

The stock of the current batch of the International Chemical Reference Substance for chloramphenicol, control no 379004, is depleted and has to be replaced. The monograph for chloramphenicol in the International Pharmacopoeia Ed. III, Vol 2 requires a reference substance to be used in the infrared spectrophotometric and thin-layer chromatographic identity tests. The reference substance is also to be used in the spectrophotometric assay.

MATERIAL

About 200 g of the sample (manufacturers lot no AR15A001) were received at the WHO Centre in July 1985. The material is being stored protected from light in tightly closed containers at +5 °C.

ANALYTICAL DATA

Description: A greyish-white, crystalline powder.

EVIDENCE OF CHEMICAL STRUCTURE

Infrared spectrum

An infrared spectrum is given in Figure 1 (no 486004). The spectrum is concordant with the spectrum obtained from the ICRS control no 379004.

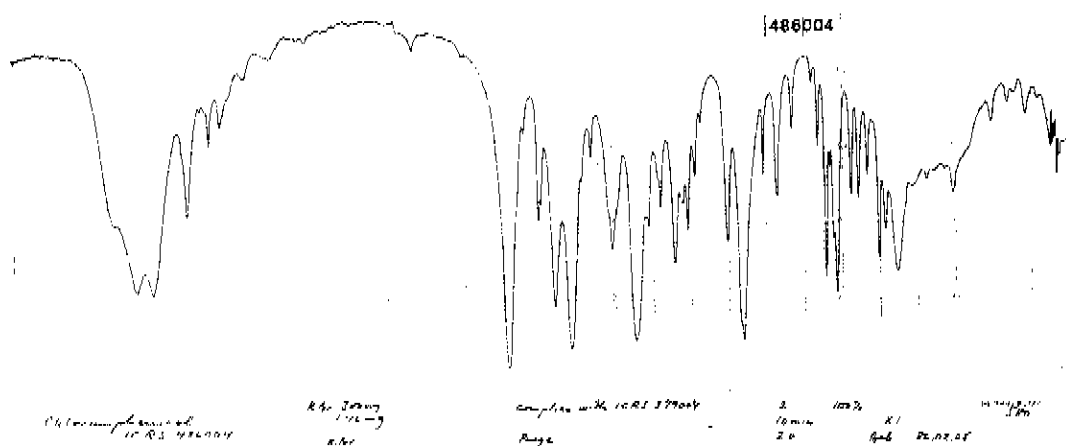


Figure 1. IR-spectrum of 1.46 mg of chloramphenicol in 300 mg KBr recorded against a KBr reference disc. Instrument: Perkin Elmer 580.

UV-spectrum

A UV-spectrum in water is given in Figure 2.  
 $\lambda$  max in water = 277.6 nm  
 $E(1\%, 1 \text{ cm}) = 295 (n=5)$

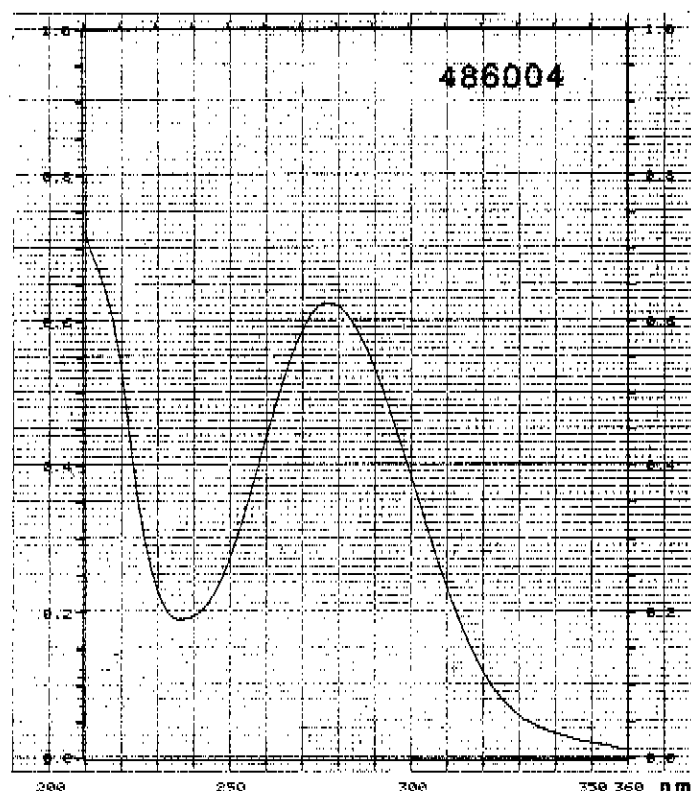


Figure 2. UV-spectrum of chloramphenicol 20 µg/ml in water.

Test for free chlorides

According to the monograph not more than 0.5 mg/g (500 ppm).  
Result: Conforms (less than 250 ppm).

Melting range: 151-152 °C, determined by the capillary method of Ph Int III.

Specific optical rotation:  $[\alpha]_D^{20} = +20.1^\circ$  (n= 2), determined in a 50 mg/ml solution in dehydrated ethanol R.

ASSAY

The spectrophotometric assay described in the International Pharmacopoeia Volume 3 was used. The ICRS for chloramphenicol (Control No 379004) was used as standard and regarded as 100%. The result is calculated with reference to the dried substance.  
Result: 99.8% (n= 5)

Loss on drying

0.05% (105 °C to constant weight).

PURITY

Total solid impurities

1) Differential thermal analysis (DTA): About 0.2 mol % (n= 4). The determination was carried out on about 3 mg using a heating rate of 2 °C per minute. Melting temperature: 150.4 °C  
Instrument: Mettler TA 2000 system, operated on line with a Hewlett-Packard calculator 9815 A. Calculation: By the Mettler standard computer program for purity analysis.

### Thin-layer chromatography

The total amount of impurities was estimated to about 0.2%.  
The following thin-layer chromatographic systems were used.

Thin-layer: Silica gel 60, F-254 (Merck) and HPTLC, silica gel 60, F-254 (Merck)  
Eluent: Chloroform /Methanol (80 + 20)  
Sample: 100 µg and 400 µg of chloramphenicol, 50 mg/ml in ethanol (750 g/l).  
Visualization: UV-light of 254 nm after heating at 105 °C for 5 minutes. The chromatogram was evaluated using a Zeiss PMQ3/MQ3 Chromatogram Spectrophotometer operated in the reflectance mode at 278 nm.

Result: One very faint secondary spot with  $R_f = 0.41$  was noted when 100 µg were applied (the amount prescribed in the monograph). The  $R_f$ -value for chloramphenicol was 0.47 and the detection limit for the system was about 0.2 µg. Three very faint secondary spots were noted when 400 µg were applied. They were estimated to about 0.2% when evaluated by densitometry at 278 nm.

The TLC system described above was also applied to a HPTLC-plate. The impurities were about the same but the spots were more distinct and the detection limit was 0.1 µg.

### High performance liquid chromatography

The total amount of impurities was estimated by peak area measurement to about 0.2%. A chromatogram is shown in Figure 3.

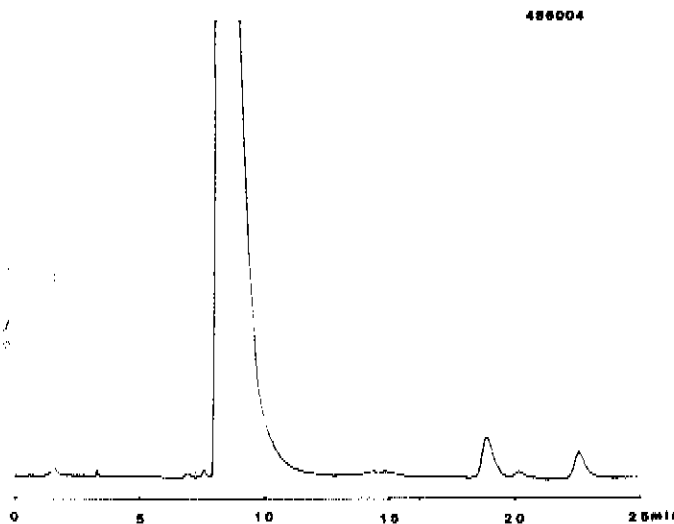


Figure 3. Chromatogram of chloramphenicol Control No. 486004

The following conditions were used:

Eluent: Acetonitrile/Water (30:70)  
Column: Spheri 5, RP18, Brownlee (300 x 4.6 mm)  
Detector: Varian UV 200 operated at 280 nm  
Pump: Varian 5560  
Integrator: Varian 4270 Attenuation: 1  
Sample: 5 mg/ml dissolved in the eluent.  
10 µl corresponding to 50 µg were injected.

## DIODE ARRAY DETECTION

The chromatogram was also evaluated with a LKB 2140 Rapid Diode Array Detector. The same chromatographic system as described above was used, except for the injection volume that was increased to 100  $\mu$ l to get maximum sensitivity.

An isogram is given in Figure 4.

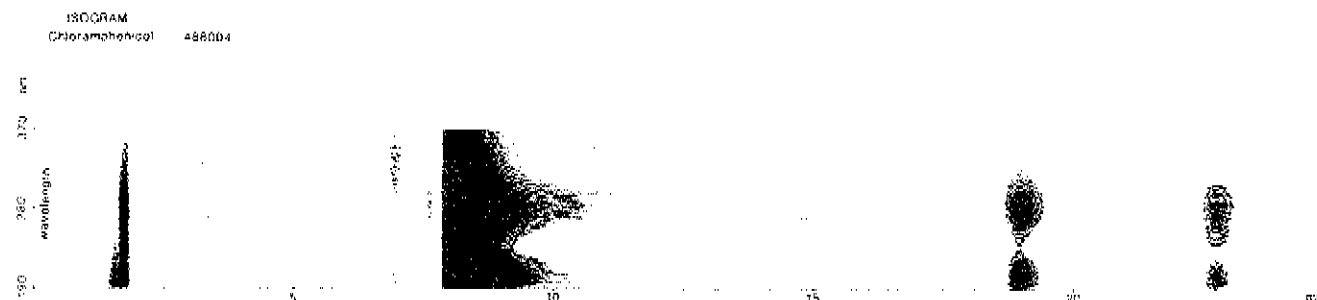


Figure 4. Isogram of chloramphenicol Control No 486004.

As seen from the figure the impurities eluting after 1.5, 18.9 and 22.6 minutes, respectively, are all detected at the same wavelengths i.e. with maxima at 280 nm and 200 nm. Chromatograms recorded simultaneously at these wavelengths were identical.

## STABILITY

No special stability studies were performed as we have good experience of the stability of this substance from earlier batches. Chloramphenicol ICRS 370994 showed no tendency of degradation when stored for seven years at +5 °C at our Centre.

## DATA GIVEN BY THE MANUFACTURER

Description:	White or yellowish white, crystalline powder.
Melting point:	151 °C
Optical rotation:	20.21°
Loss on drying:	0.06%
Sulfated ash:	0.04%
Chlorides:	<100 ppm
Related compounds:	passes test
Assay	99.8% (spectrophotometrically on dried substance)

## CONCLUSION

Chloramphenicol No 486004 can be considered suitable as International Chemical Reference Substance for the intended purpose. On the basis of the results obtained the content of chloramphenicol when used in the spectrophotometric assay is taken to be 99.8% calculated with reference to the dried substance.

## CHLORAMPHENICOL PALMITATE

Control No 286072

Analytical Report

The stock of the current batch of the International Chemical Reference substance for chloramphenicol palmitate, Control No 175072 is depleted and has to be replaced. The monograph for chloramphenicol palmitate in the International Pharmacopoeia Ed. III, Vol. 3 requires a reference substance to be used in the thin-layer chromatographic identity test. The reference substance may also be used in the infrared spectrophotometric limit test for polymorph A in chloramphenicol palmitate mixture.

MATERIAL

About 200 g of the sample (manufacturers lot no AR15D014) were received at the WHO Centre in July 1985. The material is being stored protected from light in tightly closed containers at +5 °C.

ANALYTICAL DATA

Description: A white, unctuous powder.

## EVIDENCE OF CHEMICAL STRUCTURE

Infrared spectrum

An infrared spectrum is given in Figure 1 (no 286072). The spectrum is concordant with the spectrum obtained from the ICRS control no 175072.

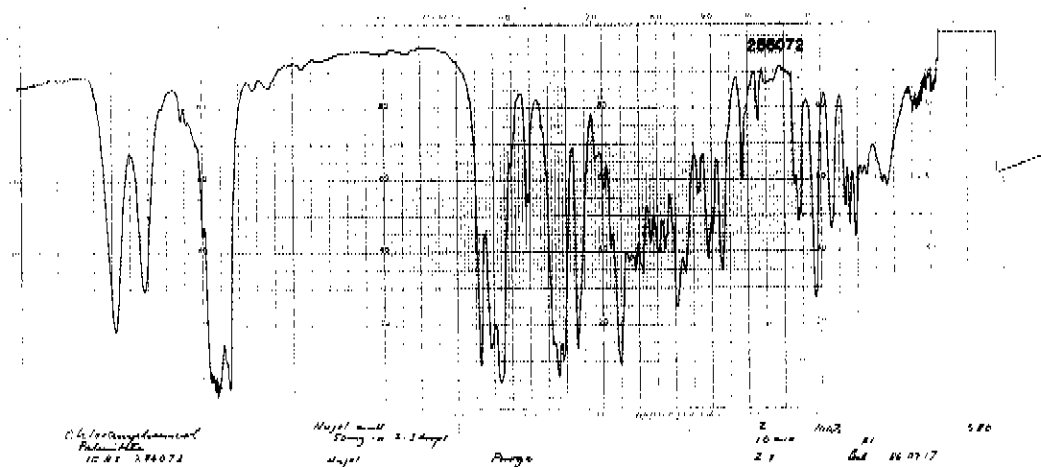


Figure 1. IR-spectrum of about 50 mg of chloramphenicol palmitate in a liquid paraffin mull. Instrument: Perkin Elmer 580.

Note: The International Chemical Reference Substance for chloramphenicol palmitate may also be used in the limit test for biologically inactive chloramphenicol palmitate (polymorph A) in chloramphenicol palmitate mixture. A suitable infrared spectrophotometric method is described in the British Pharmacopoeia 1980, Vol. II, page 688 (B.P. 80).

Polymorph A: Not detected by infrared spectrophotometry in ICRS 286072.

Infrared absorption spectra of mulls prepared as directed in B.P. 80 are shown in Figure 2, a-d.

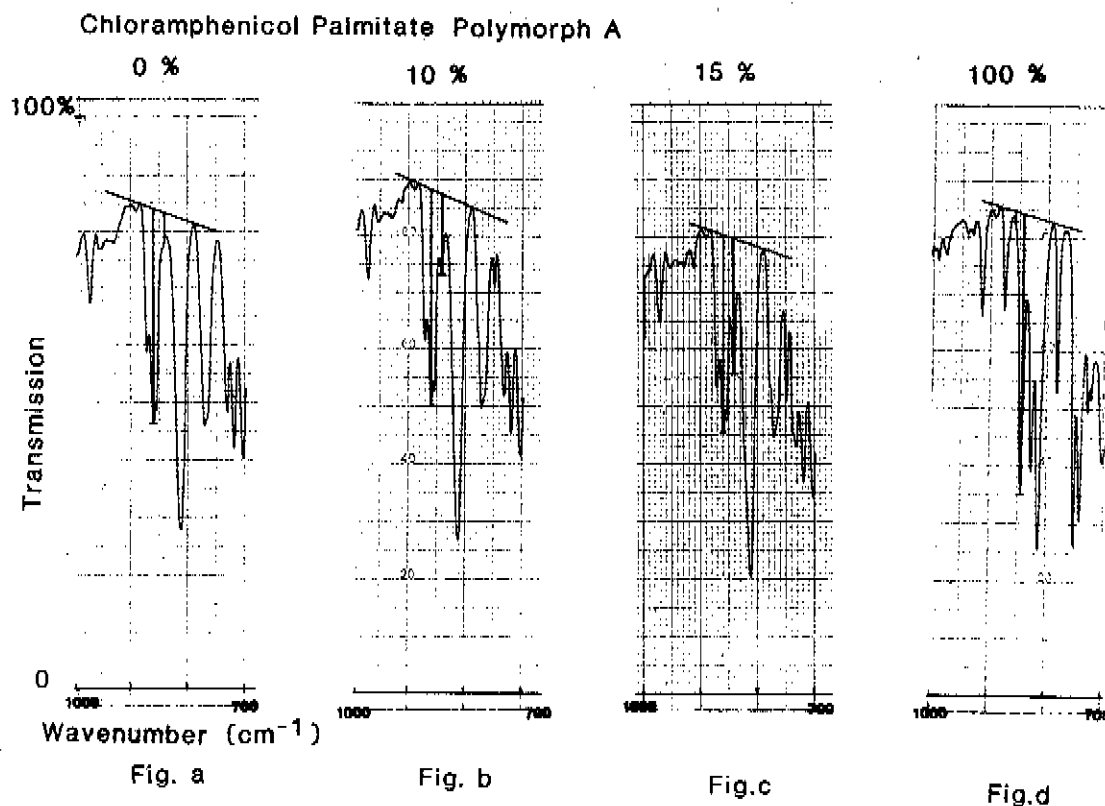


Figure 2. Infrared absorption spectra of chloramphenicol palmitate with increasing amount of chloramphenicol palmitate polymorph A added.

Figure a) shows the spectrum obtained with the International Chemical Reference Substance for chloramphenicol palmitate. The ratio of the peak height at about 858 cm<sup>-1</sup> to that at about 840 cm<sup>-1</sup> is about 7.6.

Figure b) shows the spectrum obtained with the ICRS for chloramphenicol palmitate to which 10% of ICRS chloramphenicol palmitate (polymorph A) has been added. The ratio is about 2.9.

Figure c) and d) show how the peak at about 840 cm<sup>-1</sup> increases with increasing content of chloramphenicol palmitate (polymorph A).

Specific optical rotation:  $[\alpha]_D^{20} = +24.3^{\circ}$  (n= 3), determined in a 50 mg/ml solution in dehydrated ethanol.

#### UV-spectrum

A UV-spectrum in dehydrated ethanol is given in Figure 3.

$\lambda$  max in dehydrated ethanol = 270.8 nm  
E (1%, 1 cm) = 180 (n= 5)

The absorbance of a 30  $\mu$ g/ml solution was 0.57.

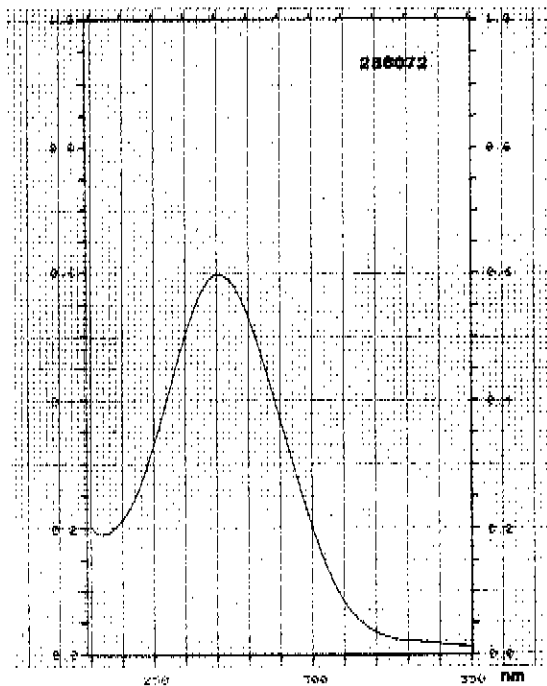


Figure 3. UV-spectrum of chloramphenicol palmitate in dehydrated ethanol, 31 µg/ml.

Thin-layer chromatography: The system described in Ph. Eur., Ed. II where chloramphenicol palmitate is partly hydrolyzed to chloramphenicol and palmitic acid was used. Both hydrolyzed products were found and no other extra spots were observed. See figure 4.

Chlor- amph- nicol	Palmi- tic acid	Hydrolyzed chlorampheni- col palmitate	Chloram- phenicol palmitate
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286072

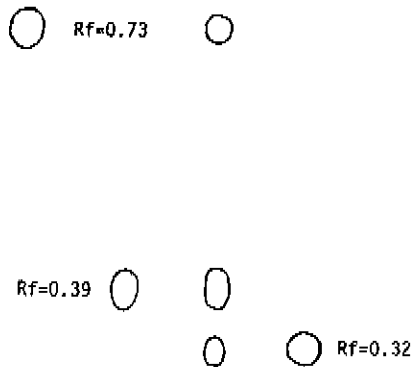


Figure 4. Thin-layer chromatogram of hydrolyzed chloramphenicol palmitate.

## ASSAY

The spectrophotometric assay described in the International Pharmacopoeia, Volume 3 was used. The ICRS for chloramphenicol palmitate (Control No 175072) was used as reference substance and regarded as 100%. The result is calculated with reference to the dried substance. Result: 100.2% (n= 5).

When calculated according to the International Pharmacopoeia, Ed. III, Volume 3, E(1%, 1 cm)=178, the result was 101.2%.

### Loss on drying

0% (60 °C to constant weight under reduced pressure over phosphorous pentoxide R).

### Water

0.18%, determined by Karl Fischer titration.

### Thermogravimetric analysis

0.1% loss in weight.

## PURITY

### Total solid impurities

1) Differential thermal analysis (DTA): About 2.7 mol % (n= 5). The determination was carried out on 4.3 mg using a heating rate of 2 °C per minute.

Melting temperature: 89.1 °C

Instrument: Mettler TA 2000 system, operated on line with a Hewlett-Packard calculator 9815 A

Calculation: By the Mettler standard computer program for purity analysis.

2) Differential scanning calorimetry (DSC): About 2.7 mol % (n= 2). The determination was carried out on 1.6 mg using a heating rate of 2 °C per minute.

Instrument: Perkin Elmer DSC-4

Polymorph A: Not detected in ICRS 286072 by DTA and DSC. The minimum amount that could be detected is about 0.1%.

### Free chloramphenicol

Determined according to Ph. Int. Ed. III, Vol. 3.

Result: 0.085 mg/g (85 ppm)

### Thin-layer chromatography

The total amount of impurities was estimated to about 2.7% when compared to corresponding impurity references. The following thin-layer chromatographic system was used.

Thin-layer: Silica gel 60. F-254 (Merck) and HPTLC silica gel 60 F-254 (Merck)

Eluent: Chloroform/Methanol/Water (90 + 10 + 1)

Sample: 100 µg of chloramphenicol palmitate (10 mg/ml) in acetone

Visualization: UV-light of 254 nm.

The chromatograms were evaluated using a CAMAG TLC Scanner II with integrator SP 4290 as well as with a Zeiss PMQ 3 Scanner, both operated in the reflectance mode at 271 nm.

Result: Two secondary spots with  $R_f = 0.58$  (chloramphenicol palmitate isomer) and  $R_f = 0.79$  (chloramphenicol dipalmitate) were noted when 100  $\mu\text{g}$  was applied (the amount prescribed in the monograph). The  $R_f$ -value for chloramphenicol palmitate was 0.65 and the detection limit for the system was about 0.5  $\mu\text{g}$ . No further secondary spots were noted when 250  $\mu\text{g}$  were applied. A chromatogram is shown in Figure 5.

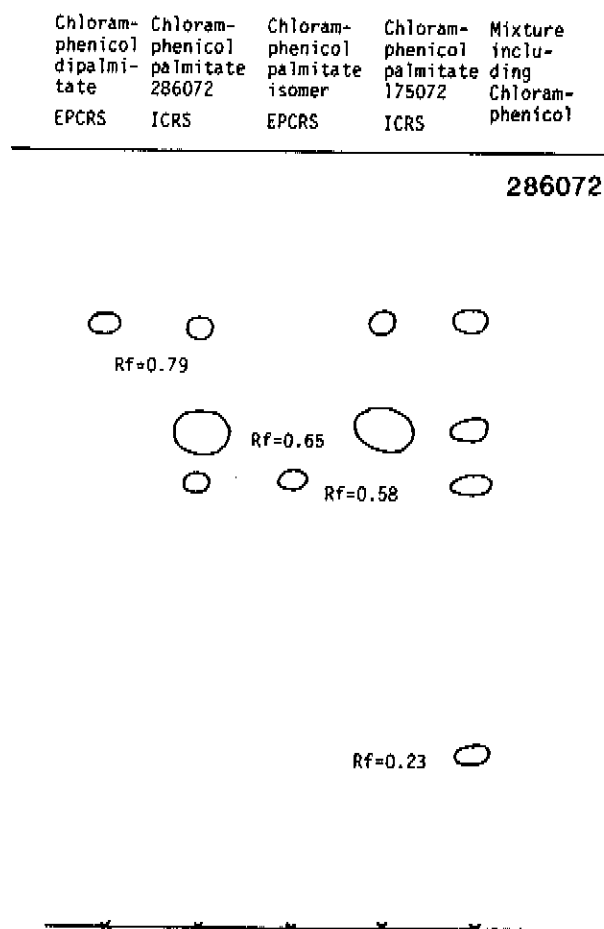


Figure 5. Thin-layer chromatogram of chloramphenicol palmitate, Control No 286072.

In the new ICRS 286072 1.5% of the chloramphenicol palmitate isomer and 1.2% of chloramphenicol dipalmitate were found by comparison with the corresponding EPCRS.

The thin-layer chromatographic system for related substances described in Ph. Eur. Ed. II was also tested. One of the two impurities observed was found to be identical with chloramphenicol palmitate isomer (EPCRS). It was estimated to about 1.5%.  $R_f = 0.25$  (corresponding to the spot with  $R_f = 0.58$  in the Ph. Int. system). The chromatogram was evaluated using a Zeiss PMQ 3 Scanner operated in the reflectance mode at 271 nm.

#### High performance liquid chromatography

The total amount of impurities (except chloramphenicol dipalmitate) was estimated by peak area measurements to about 3.1%. A chromatogram is shown in Figure 6.

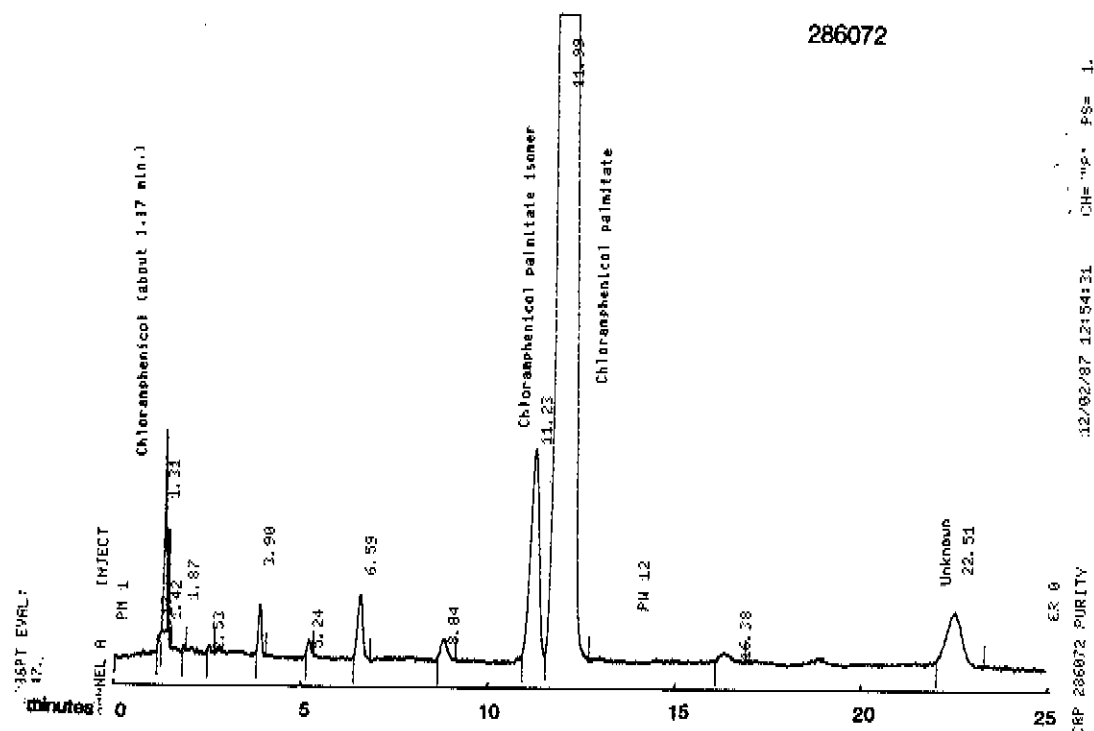


Figure 6. A chromatogram of chloramphenicol palmitate No 286072.

The following conditions were used (according to a modified method from USP XXI):

Eluent: Acetonitrile/water/glacial acetic acid (86:13.5:0.5)  
 Column: Spheri-5, RP-18, Brownlee, (250 x 4.6 mm) 5  $\mu$ m particles  
 Detector: Varian Vista 2500 operated at 271 nm  
 Pump: Varian 5560  
 Integrator: Varian 4270 Attenuation: 2  
 Sample: 0.52 mg/ml dissolved in the eluent. 10  $\mu$ l corresponding to 5.2  $\mu$ g were injected

As seen from the figure at least seven impurities are observed. The major impurities are chloramphenicol palmitate isomer eluting after 11 minutes (1.4%) and one unknown eluting after 22 minutes (0.7%). When the former ICRS 175072 was subjected to chromatography in this system the result was about 1.8% impurities compared to 3.1% in ICRS 286072.

However, it was not possible to elute chloramphenicol dipalmitate in the above mentioned system. By changing the eluent to 100% acetonitrile the dipalmitate was eluted after 29-30 minutes and the amount was estimated to about 1.2% against the EPCRS. Chloramphenicol palmitate elutes after 4 minutes with this eluent.

#### DIODE ARRAY DETECTION

The chromatogram was also evaluated with a LKB 2140 Rapid Diode Array Detector. The same chromatographic system as described above was used, except for the injection volume that was increased to 100  $\mu$ l to get maximum sensitivity. An isogram is given in Figure 7.

286072

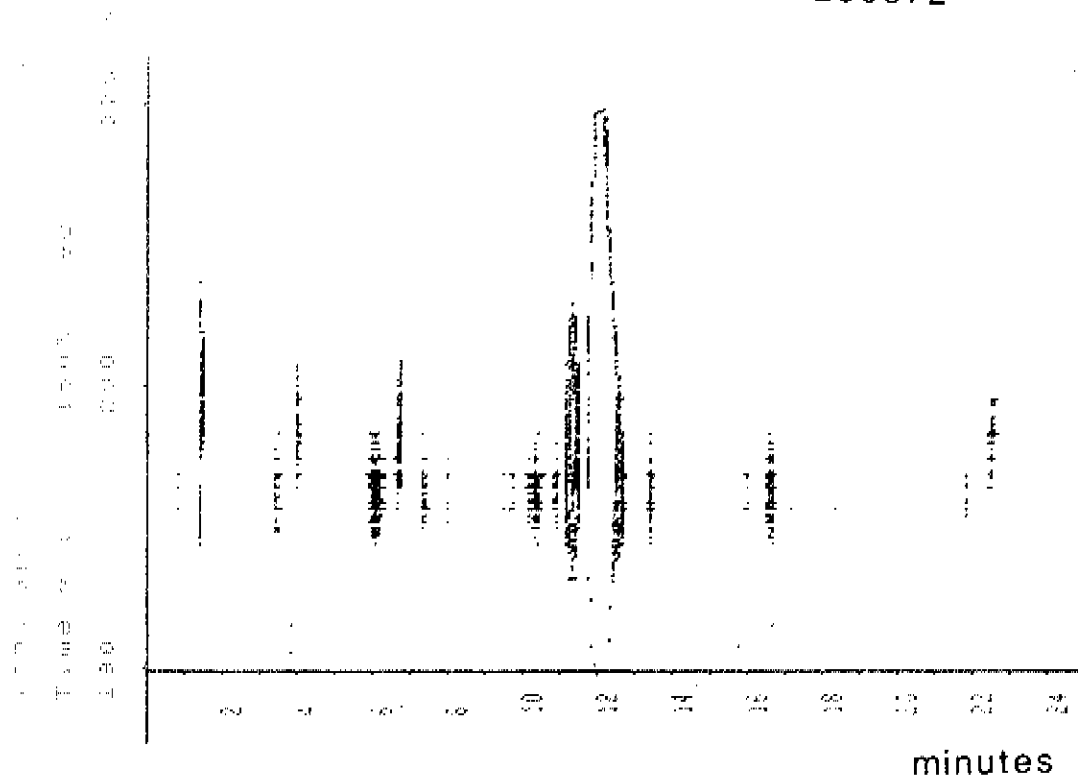


Figure 7. Isogram of chloramphenicol palmitate, Control No 286072. Sensitivity: 0.002

As seen from the figure chloramphenicol palmitate has an absorbance maximum at 271 nm and the observed impurities are all visible at this wavelength.

#### STABILITY

No special stability studies were performed as we have good experience of the stability of this substance from the earlier batch. Chloramphenicol palmitate ICRS 175072 showed no tendency of degradation when stored for ten years at +5 °C at our Centre.

#### DATA GIVEN BY THE MANUFACTURER

Description:	White or almost white powder
Melting point:	89.5 °C
Specific optical rotation:	+24.16°
Acidity:	0.24%
(palmitic acid)	
Loss on drying:	0.07%
Sulphated ash:	<0.1%
Free chloramphenicol:	95 ppm
Related compounds:	passes test
Assay:	101.74% (spectrophotometrically on dried substance)
Non Polymorphous "A":	100%
(=Polymorph B)	

#### CONCLUSION

Chloramphenicol palmitate No 286072 can be considered suitable as International Chemical Reference Substance for the intended purpose.

# N I R I D A Z O L E

Control No 186129

Analytical Report

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

## MATERIAL

About 200 g of the sample (manufacturers lot Prod. Stand. 85) were received at the WHO Centre in June 1985. The material is being stored protected from light in tightly closed containers at +5 °C.

## ANALYTICAL DATA

Description: A yellow, crystalline powder: odourless or almost odourless.

## EVIDENCE OF CHEMICAL STRUCTURE

### Infrared spectrum

An infrared spectrum is given in Figure 1 (No 186129). The spectrum is concordant with the reference spectrum from the manufacturer.

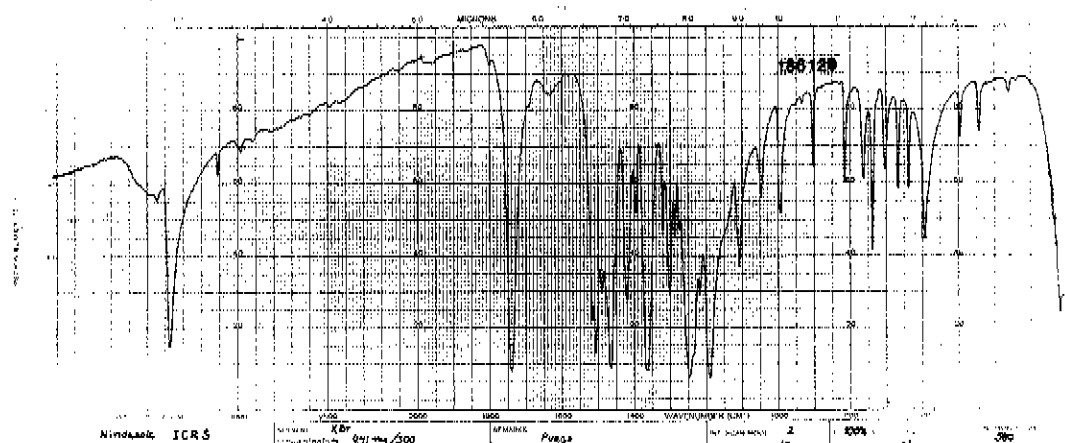


Figure 1. IR-spectrum of 0.4 mg of Niridazole in 300 mg KBr recorded against a KBr disc. Instrument: Perkin Elmer 580.

### Melting temperature

About 264 °C with decomposition, determined by the capillary method of Ph. Int. Ed. III.

### Elemental analysis

	C (%)	H (%)	N (%)
Theoretical	33.6	2.8	26.2
Found	33.4	2.6	26.0

The analysis was performed at Mikro Kemi AB, Uppsala.

UV-spectrum

A UV-spectrum in 0.1% dimethylformamide in ethanol is given in Figure 2. The substance is first dissolved in dimethylformamide and then diluted with ethanol.

$\lambda$  max in methanol = 358 nm

E (1%, 1 cm) = 704 (n= 6)

The absorbance of a 10  $\mu$ g/ml solution was 0.70

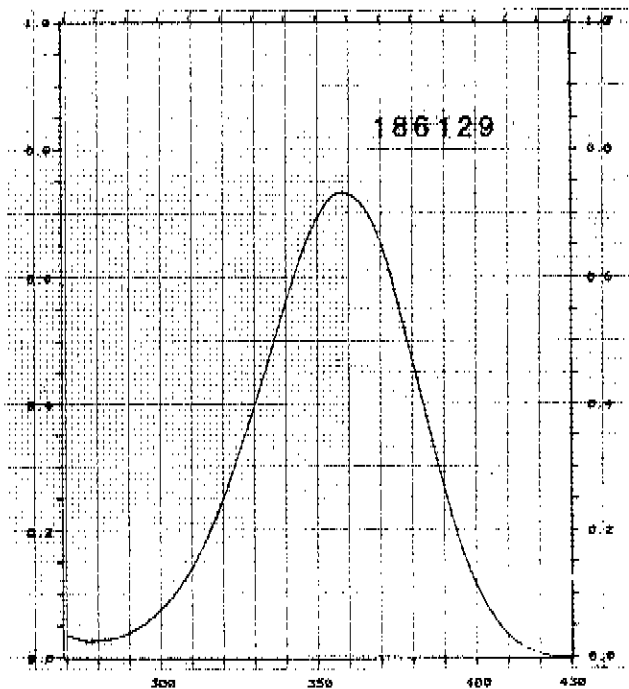


Figure 2. UV-spectrum of niridazole 10.4  $\mu$ g/ml

ASSAY

When used in the spectrophotometric assay according to Ph. Int. Ed. III, Vol 3, the content is 100.0% with reference to the dried substance. See results under UV-spectrum.

Loss on drying

0.2% (100 °C, reduced pressure)

PURITYTotal solid impurities

1) Differential thermal analysis (DTA): It was not possible to estimate the purity by this method as the substance melts with decomposition.

2) Phase solubility analysis: About 0.2%. When calculated with the method of least squares with 95 per cent confidence intervals as described in WHO/PHARM/66.431:  $0.17 \pm 0.34$ .

Solvent: Pyridine:Ethanol (750 g/l), 60 + 40

Equilibration: Vibro-mixer at 28.0 °C for 45 hours.

Thin-layer chromatography

The following thin-layer chromatographic systems were used:

Thin-layer: Silica gel 60, F-254 (Merck)  
Eluent: Toluene: Acetone: Acetic acid: Isobutanol  
 (12 + 8 + 3 + 8)  
Sample: 100  $\mu$ g of Niridazole were applied

Visualization: UV-light of 254 nm and 365 nm visually and by scanning. Rf (niridazole)= 0.48; Rf (2-amino-nitrothiazole)= 0.55; Rf (niridazole-chlorethylcarboxamide)= 0.58. The detection limit for niridazole was 0.01 ug (0.01%) when scanned at 365 nm. By visual detection the detection limit was only 0.1%.

Result: Only one impurity was found and it was estimated to about 0.03%. This impurity was only observed when the chromatogram was evaluated using a Zeiss KM3 Chromatogram Spectrophotometer operated in the reflectance mode at 365 nm. The impurity was identical with niridazole-chlorethylcarboxamide.

The thin-layer chromatographic system described in Ph. Int. Ed. III, Vol 3 with toluene: acetone (12 + 8) as eluent was tested but 2-amino-5-nitrothiazole did not separate from niridazole. Rf (niridazole)= 0.38, Rf (2-amino-5-nitrothiazole)= 0.39 and Rf (niridazole-chlorethylcarboxamide)= 0.48. One weak impurity with Rf= 0.48 was observed.

#### High performance liquid chromatography

The total amount of impurities was estimated by peak area measurement to about 0.1%. A chromatogram is shown in Figure 3. The peak eluting after 5.62 minutes corresponds to niridazole-chlorethylcarboxamide it was estimated to 0.04%. 2-amino-5-nitrothiazole elutes after 2.87 minutes in negligible amounts.

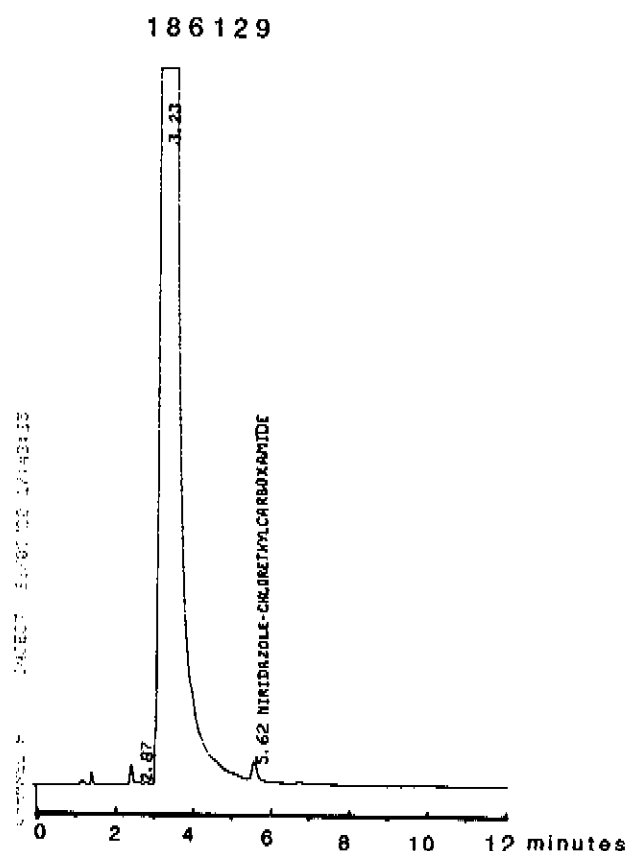


Figure 3. Chromatogram of niridazole No 186129.

The following conditions were used:

Eluent: Acetonitrile/Water (55 + 45)  
Column: RP-18, Spheri-5 (Brownlee)  
Detector: Varian UV 200 operated at 215 nm.  
Pump: Varian 5560 operated at a flow rate of 1 ml/min.  
Integrator: Varian 4270 Attenuation: 16  
Sample: 1 mg/ml dissolved in the acetonitrile with additional adjustment to eluent composition. 10  $\mu$ l corresponding to 10  $\mu$ g were injected.

The two potential impurities were also estimated against corresponding reference substances which gave the same result as the peak area measurements.

#### DIODE-ARRAY DETECTION

The chromatogram was also evaluated with a LKB 2140 Rapid Diode Array Detector. The same chromatographic system as described above was used, except for the injection volume that was increased to 100  $\mu$ l to get maximum sensitivity. An isogram is given in Figure 4.

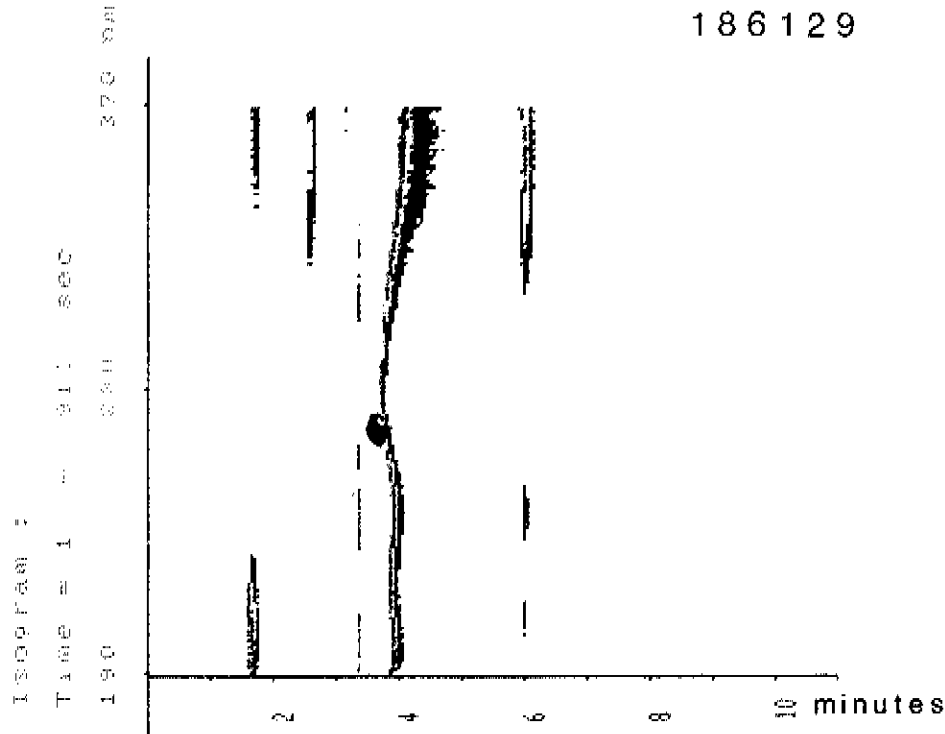


Figure 4. Isogram of niridazole, Control No 186129. Sensitivity: 0.005

As can be seen from the figure the impurities eluting after 1.4, 2.3 and 6.0 minutes, respectively are all visible at the wavelength chosen in the method i.e. 359 nm.

#### STABILITY

Niridazole was exposed to air of different relative humidity at room temperature (about 20 °C) for a period of 8 weeks as described in WHO/PHARM/82.509. All samples were unchanged at visual inspection and no weight changes were noted. No signs of degradation were observed when samples were analyzed by the liquid chromatographic method described above.

#### DATA GIVEN BY THE MANUFACTURER

Loss on drying:	<0.05%
Assay:	100.0%
TLC:	on by-product
Total by-products:	0.1%
Sulfate:	<200 ppm
Lead:	<4 ppm
Arsenic:	<2 ppm
Copper:	<6 ppm
Sulfated ash:	<0.05%

#### CONCLUSION

Niridazole No 186129 can be considered suitable as International Chemical Reference Substance for the intended purpose. The content of niridazole when used in the spectrophotometric assay is taken to be 100.0% calculated with reference to the dried substance.

APPENDIX 11

NIRIDAZOLE -  
CHLOROETHYL CARBOXAMIDE

Control No 186130

Analytical Report

The International Chemical Reference Substance for niridazole-chlorethylcarboxamide is intended to be used in the thin-layer chromatographic test for related substances according to the monograph for niridazole in the International Pharmacopoeia Ed. III, Vol. 3.

MATERIAL

About 25 g of the sample (manufacturers lot no C-44'132-Ba/II) were received at the WHO Centre in December 1984. The material is being stored protected from light in a tightly closed container at +5 °C.

ANALYTICAL DATA

Description: A light yellow, crystalline powder, odourless.

EVIDENCE OF CHEMICAL STRUCTURE

Infrared spectrum

An infrared spectrum is given in Figure 1 (No 186130). The spectrum is concordant with a spectrum from the manufacturer.

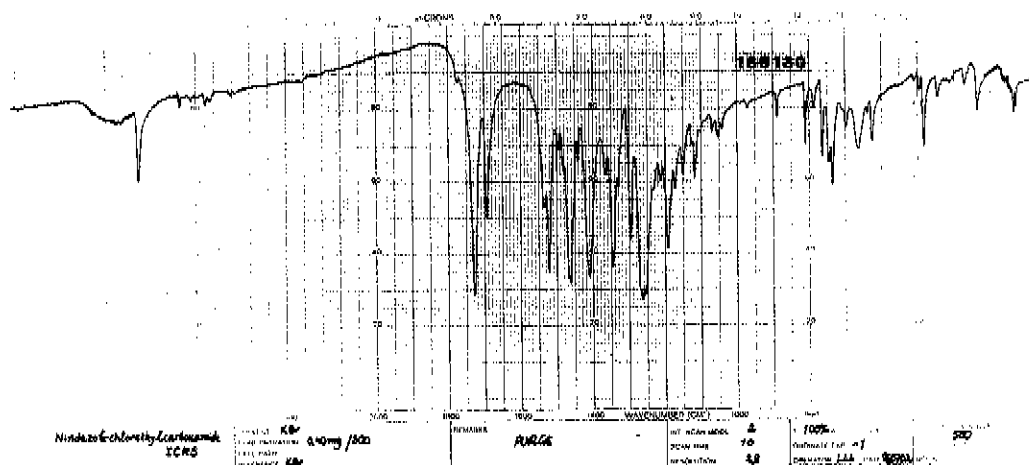


Figure 1. IR-spectrum of 0.4 mg of niridazole-chlorethylcarboxamide in 300 mg KBr recorded against a KBr disc. Instrument: Perkin Elmer 580.

Melting range: About 195.6-196.1 °C determined by the capillary method of Ph. Int. Ed. III.

Elemental analysis

	C (%)	H (%)	N (%)
Theoretical	33.8	3.2	21.9
Found	33.7	3.0	21.8

The analysis was performed at Mikro Kemi AB, Uppsala.

UV-spectrum

A UV-spectrum in 0.1% dimethylformamide in ethanol is given in Figure 2. The substance is first dissolved in dimethylformamide and then diluted with ethanol.

$\lambda$  max in water = 346 nm  
E (1%, 1 cm) = 485 (n= 4)

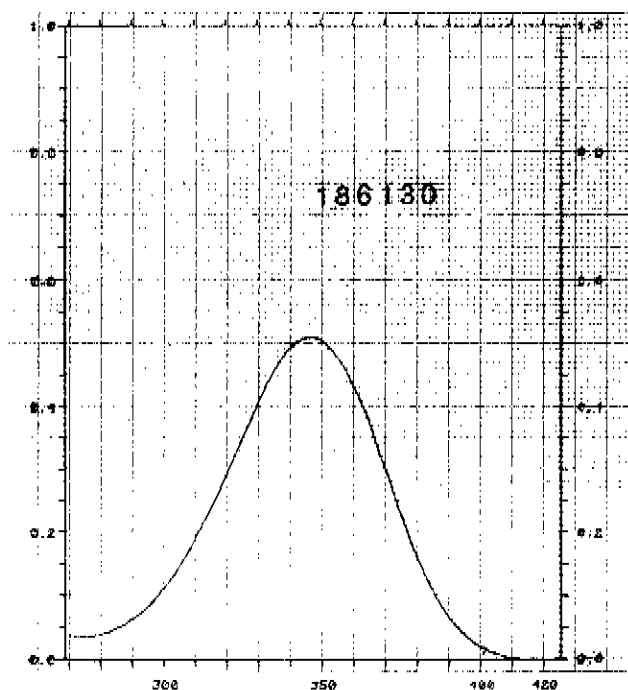


Figure 2. UV-spectrum of niridazole-chlorethylcarboxamide 10.5  $\mu$ g/ml.

Loss on drying

0.1% (100 °C, reduced pressure)

## PURITY

Total solid impurities

1) Differential thermal analysis (DTA):

About 0.1 mol per cent (n= 5). The determination was carried out on about 3 mg using a heating rate of 2 °C per minute.

Melting temperature: 195.0 °C

Instrument: Mettler TA 2000 system, operated on-line with a Hewlett Packard calculator (9815 A)

Calculation: By the Mettler standard computer program for purity analysis.

Thin-layer chromatography

The following TLC systems were used:

Thin-layer: Silica gel 60, F-254 (Merck)

Eluent: Toluene: Acetone: Acetic acid: Isobutanol (12 + 8 + 3 + 8)

Sample: 100  $\mu$ g of niridazole-chlorethylcarboxamide were applied

Visualization: UV-light of 254 nm and 365 nm visually and at 346 nm by scanning

R<sub>f</sub> (niridazole)= 0.48; R<sub>f</sub> (2-amino-5-nitrothiazole)= 0.55; R<sub>f</sub> (niridazole-chlorethylcarboxamide)= 0.58. The detection limit for niridazole-chlorethylcarboxamide was 0.05  $\mu$ g (0.05%) when scanned at 346 nm.

Result: Only one impurity was found and it was estimated to about 0.1%. This impurity was only observed when the chromatogram was evaluated using a Zeiss KM3 Chromatogram Spectrophotometer operated in the reflectance mode at 346 nm. The impurity was identical to niridazole.

The thin-layer chromatographic system described in Ph. Int. Ed. III, Vol 3 with toluene:acetone (12 + 8) as eluent was tested but 2-amino-5-nitrothiazole did not separate from niridazole.

Rf (niridazole)= 0.38, Rf (2-amino-5-nitrothiazole)= 0.39 and Rf (niridazole-chlorethylcarboxamide)= 0.48. This system was the best to separate the two impurities from niridazole-chlorethylcarboxamide. The amount of niridazole was estimated to about 0.08% and 2-amino-5-nitrothiazole to about 0.05%.

#### High performance liquid chromatography

The total amount of impurities was estimated by peak area measurement to about 0.1%. A chromatogram is shown in Figure 3. The peak eluting after 2.93 minutes corresponds to 2-amino-5-nitrothiazole, it was estimated to 0.05%. Niridazole elutes after 3.24 minutes and estimated to 0.03%. The same results were obtained by peak area measurements as when compared with external standards of the two impurities.

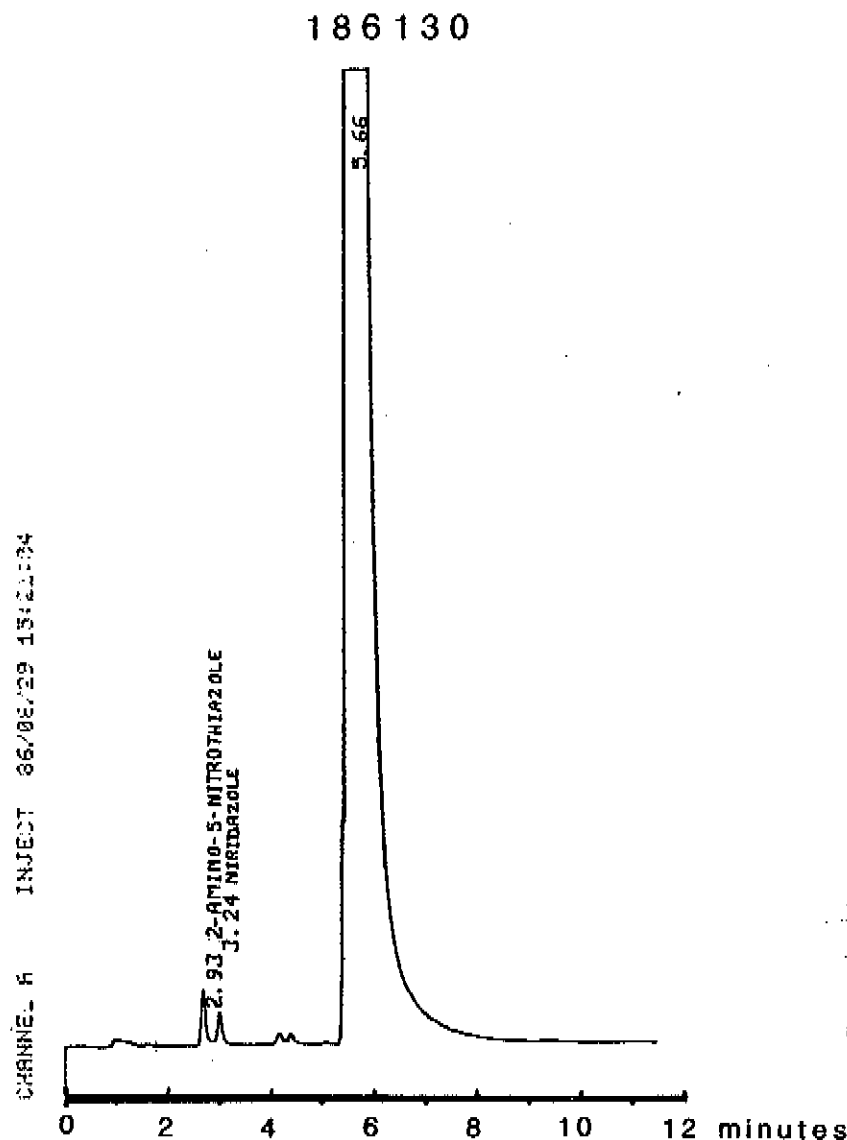


Figure 3. A chromatogram of niridazole-chlorethylcarboxamide No 186130.

The following conditions were used:

Eluent: Acetonitrile/Water (55:45)  
Column: RP-18, Spheri-5 (Brownlee)  
Detector: Varian UV 200 operated at 346 nm  
Pump: Varian 5560 operated at a flow rate of 1 ml/min  
Integrator: Varian 4270 Attenuation: 8  
Sample: 0.6 mg/ml dissolved in acetonitrile with additional adjustment to eluent composition.  
10  $\mu$ l corresponding to 6  $\mu$ g were injected

#### DIODE-ARRAY DETECTION

The chromatogram was also evaluated with a LKB 2140 Rapid Diode Array Detector. The same chromatographic system as described above was used except for the injection volume that was increased to 100  $\mu$ l to get maximum sensitivity. An isogram is given in Figure 4.

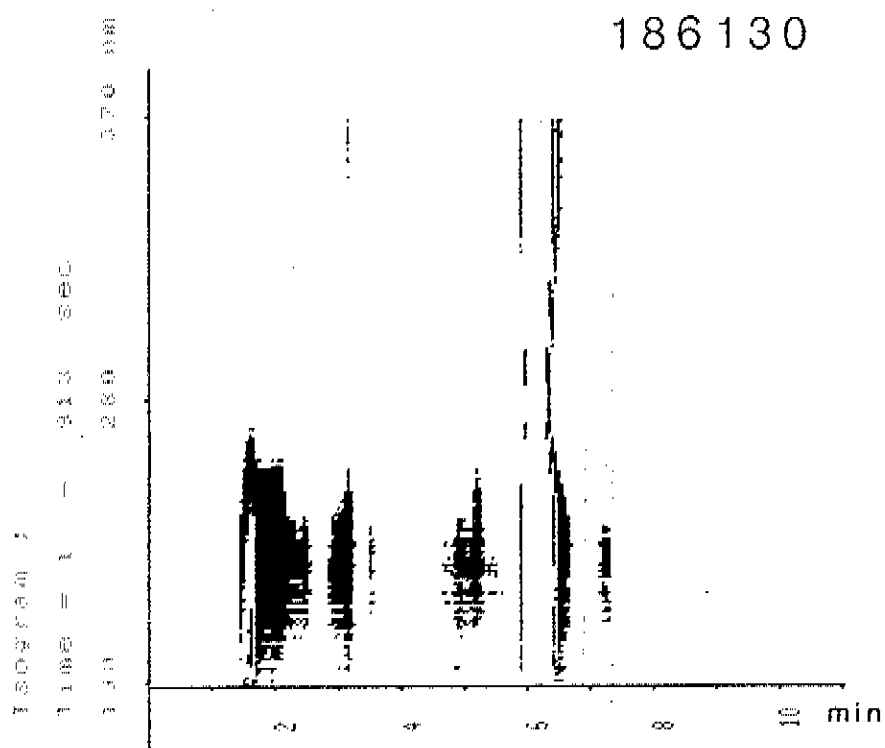


Figure 4. Isogram of niridazole-chlorethylcarboxamide, Control No 186130. Sensitivity: 0.005

As seen from the figure impurities are visible mainly at 227 nm but traces are also observed at higher wavelengths and 346 nm which was chosen in the method described above. The results from peak area measurements at these wavelengths were compared and it was found that at 227 nm 0.5% impurities were detected compared to 0.1% at 346 nm. The difference is mainly due to the fact that the UV-absorption for niridazole-chlorethylcarboxamide is higher at 346 nm.

#### STABILITY

Niridazole-chlorethylcarboxamide was exposed to air of different relative humidity at room temperature (about 20 °C) for a period of 8 weeks as described in WHO/PHARM/ 82.509. All samples were unchanged at visual inspection and no weight changes were noted. No signs of degradation were observed when selected samples were analyzed by the liquid chromatographic method described above.

## DATA GIVEN BY THE MANUFACTURER

Melting point: 196.0 °C capillary method  
196.2 °C Mettler FP 5  
IR spectrum: conforms  
Proton NMR: conforms  
Elemental analysis: C(33.9%) H(3.3%) N(21.96%) O(20.2%) S (9.81%) Cl(11.09%)  
TLC: more than 95% pure

## CONCLUSION

Niridazole-chlorethylcarboxamide No 186130 can be considered suitable as International Chemical Reference Substance for the intended purpose.

## N O R E T H I S T E R O N E

Control No 186132

Analytical Report

The monograph for norethisterone in the International Pharmacopoeia Ed. III, Vol 2 requires a reference substance to be used in the infrared spectrophotometric and thin-layer chromatographic identity tests and in the spectrophotometric assay.

MATERIAL

About 100 g of the sample (manufacturers batch No 50-0074) were received at the WHO Centre in August 1985. The material is being stored protected from light in tightly closed containers at +5 °C.

ANALYTICAL DATA

Description: A white, crystalline powder.

EVIDENCE OF CHEMICAL STRUCTUREInfrared spectrum

An infrared spectrum is given in Figure 1 (No 186132). The spectrum is concordant with the spectrum obtained from the USP reference substance Lot H.

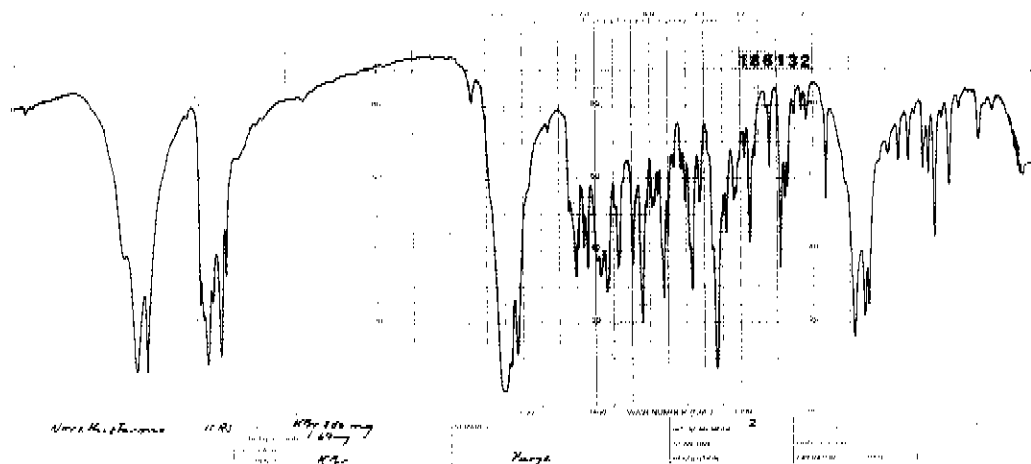


Figure 1. IR-spectrum of 1.7 mg of norethisterone in 300 mg KBr recorded against a KBr reference disc. Instrument: Perkin Elmer 580.

Melting range: 202.5-206.8 °C (n= 3) determined by the capillary method of Ph. Int. Ed. III.

Specific optical rotation:  $[\alpha]_D^{20} = -24^{\circ}$  (n= 3), determined in chloroform at a concentration of 10 mg/ml.

UV-spectrum

A UV-spectrum in ethanol (750 g/l) is given in Figure 2.

$\lambda$  max in ethanol = 240 nm

E(1%, 1 cm) = 576 (n= 5)

The absorbance of a 10 µg/ml solution was 0.58

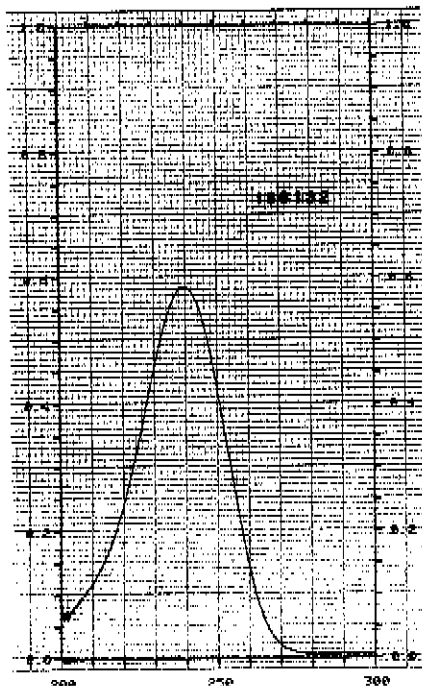


Figure 2. UV-spectrum of norethisterone 10.1 µg/ml in ethanol.

ASSAY

Loss on drying:

0% (105 °C) (n= 3)

Spectrophotometric assay: 99.8% (n= 5) determined according to Ph. Int. Ed. III, Vol. 2. USP reference substance Lot H was used as reference and regarded as 100%.

PURITY

Total solid impurities

1) Differential thermal analysis (DTA): It was not possible to estimate the purity by this method as the substance melts with decomposition.

2) Phase solubility analysis: About 0.7%. When calculated with the method of least squares with 95 per cent confidence intervals as described in WHO/PHARM/66.431:  $0.7 \pm 0.18$ .

Solvent: Ethanol (750 g/l)/Water (85 + 15)  
Equilibration: Vibro-mixer at 27.0 °C for 120 hours.

Thin-layer chromatography

The following thin-layer chromatographic systems were used.

Thin-layer: HPTLC, Silica gel 60, F-254 (Merck)  
Eluent: Chloroform: methanol (95 + 5)  
Sample: 100-200 µg of norethisterone were applied  
Visualization: UV-light of 254 nm, scanning at 240 nm and spraying with sulfuric acid/ethanol TS followed by heating to 105 °C and examination in day-light.

Result: The thin-layer plate is shown in Figure 3.

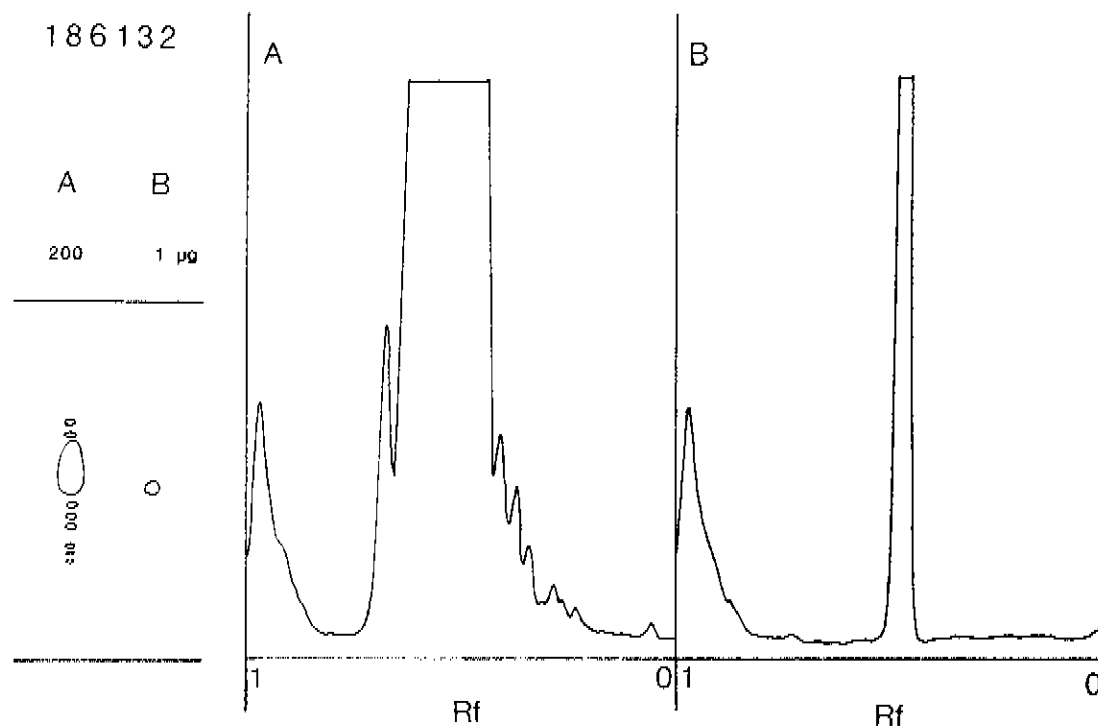


Figure 3. High performance thin-layer chromatogram of norethisterone No 186132.

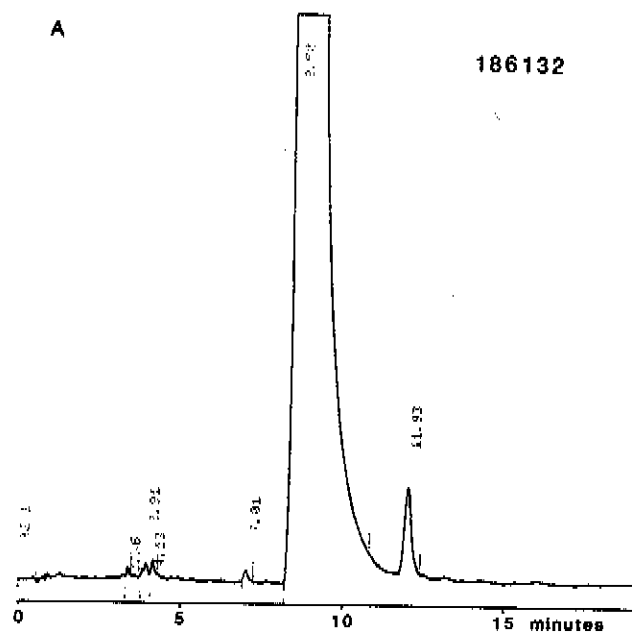
As seen from the figure two dominating extra spots are observed above the norethisterone spot. Six faint additional spots below the main spot are also observed. The total amount of impurities was roughly estimated to about 0.7% after spraying. When the chromatogram was evaluated using a CAMAG TLC scanner with the scanning wavelength set to 240 nm the total amount of impurities was estimated to 0.4%. The system separates norethisterone acetate but not ethisterone. In the USP reference substance Lot H six weak spots were observed but the total amount of impurities was less than in the proposed International Chemical Reference Substance.

As described in Ph. Int. Ed. III, Vol. 2 an ordinary thin-layer plate was also used. This gave similar results but the impurities were more distinctly separated on the HPTLC-plate. The strongest spots were estimated to 0.3% and 0.2%, respectively, all the others were significantly weaker.

#### High performance liquid chromatography

The total amount of impurities was estimated by peak area measurement to about 0.3%. Two chromatograms are shown in Figure 4 A + B. As seen from the figure, 80% acetonitrile is required to elute the most lipophilic impurities. However, to check the presence of impurities with similar structure to norethisterone a system with 50% acetonitrile was conveniently chosen.

Figure 4 A + B. Chromatogram of norethisterone Control No. 186132 eluted with 50% acetonitrile (A) and 80% acetonitrile (B).



Integrator: Varian 4270 Attenuation: 1  
Sample: 1 mg/ml dissolved in the eluent.  
10  $\mu$ l corresponding to 10  $\mu$ g were injected.

Ethisterone: The norethisterone sample was spiked with 10% of ethisterone. When running the system with 50% acetonitrile a distinct peak elutes after 10.15 minutes (ethisterone) while norethisterone elutes after 8.5 minutes. However, to detect minor amounts of ethisterone a system with better selectivity is necessary.

#### DIODE ARRAY DETECTION

The chromatogram was also evaluated with a LKB 2140 Rapid Diode Array Detector. The same chromatographic system as described above was used, except for the injection volume that was increased to 100  $\mu$ l and the concentration to 2 mg/ml to get maximum sensitivity.

An isogram is given in Figure 5.

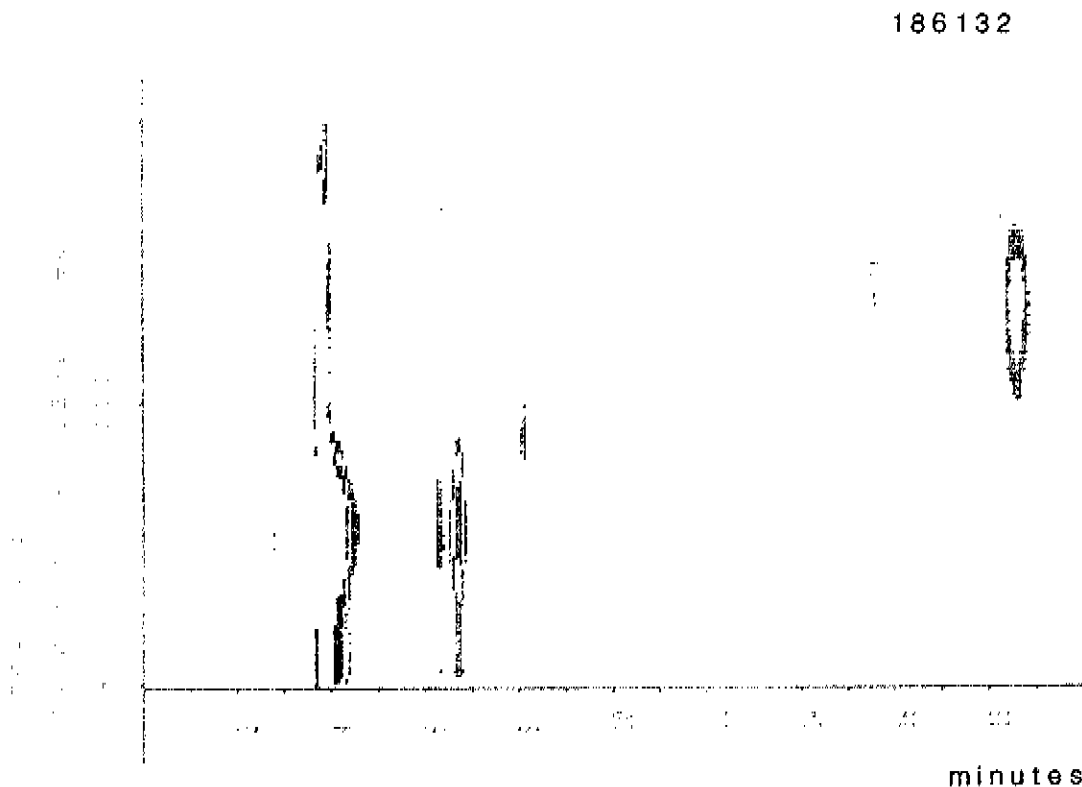


Figure 5. Isogram of norethisterone Control No 186132. Eluent 80% acetonitrile. Sensitivity: 0.004.

As seen from the figure the major impurity eluting after about 6.5 minutes exhibits a UV-maximum at 240 nm. One small impurity with a UV-maximum at 284 nm can also be observed, hidden in the front of the main peak. The impurities eluting at 15.6 and 18.6 minutes have their maxima at 310 - 320 nm. The results from peak area measurements at these wavelengths were compared to the result at 240 nm, which is chosen in the method described above. At 315 nm 17% impurities were detected, compared to 2.3% impurities at 284 nm and 0.26% at 240 nm. The higher values at 315 nm and 284 nm are due to the fact that norethisterone has lower UV-absorption at these wavelengths than at 240 nm. Based on the results from other analytical techniques 240 nm seems to be the best wavelength for the estimation of impurities.

## STABILITY

Norethisterone was exposed to air of different relative humidity at room temperature (about 20 °C) for a period of 8 weeks as described in WHO/PHARM/82.509. All samples were unchanged at visual inspection and no weight changes were noted. No signs of degradation were observed when the samples were analyzed by the liquid chromatographic method described above.

## CONCLUSION

Norethisterone No 186132 can be considered suitable as International Chemical Reference Substance for the intended purpose. The content of norethisterone when used in the spectrophotometric assay is taken to be 99.8% calculated with reference to the dried substance.

R E S E R P I N E

Control No 186133

Analytical Report

The monograph for reserpine in the International Pharmacopoeia Ed. III, Vol 2 requires a reference substance to be used in the infrared spectrophotometric test for identity and in a colorimetric assay.

MATERIAL

About 175 g of the sample (manufacturers batch No 000283) were received at the WHO Centre in September 1985. The material is being stored protected from light in tightly closed containers at +5 °C.

ANALYTICAL DATA

Description: Faintly pale beige powder.

EVIDENCE OF CHEMICAL STRUCTURE

Infrared spectrum

An infrared spectrum is given in Figure 1 (No 186133). The spectrum is concordant with the spectrum obtained from the USP reference substance Lot K.

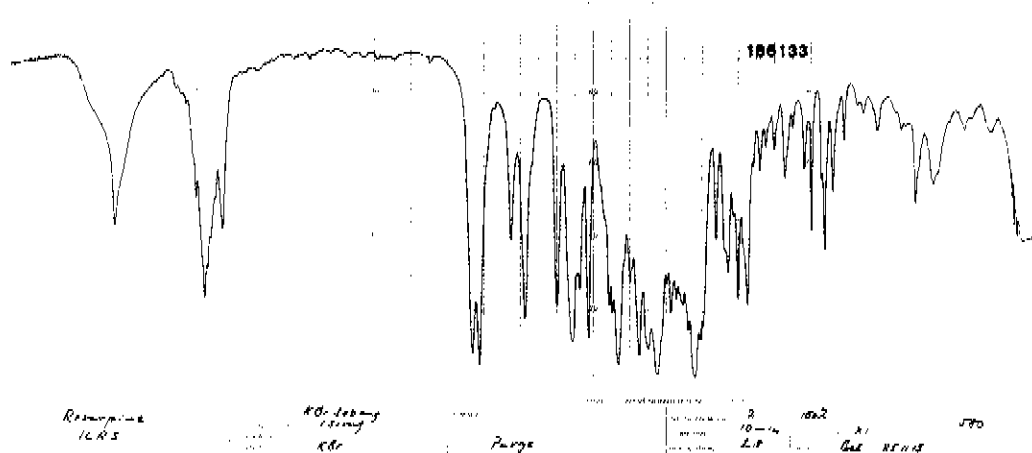


Figure 1. IR-spectrum of 1.3 mg of reserpine in 300 mg KBr recorded against a KBr reference disc. Instrument: Perkin Elmer 580.

Melting range: 260.4-265.6 °C with decomposition determined by the capillary method of Ph. Int. III.

Optical rotation:  $[\alpha]_D^{20} = -122^{\circ}$  determined on a 10 mg/ml solution in chloroform according to Ph. Int. Ed. III.

UV-spectrum

A UV-spectrum in ethanol (750 g/l) is given in Figure 2. The sample was first dissolved in chloroform and then diluted with ethanol.

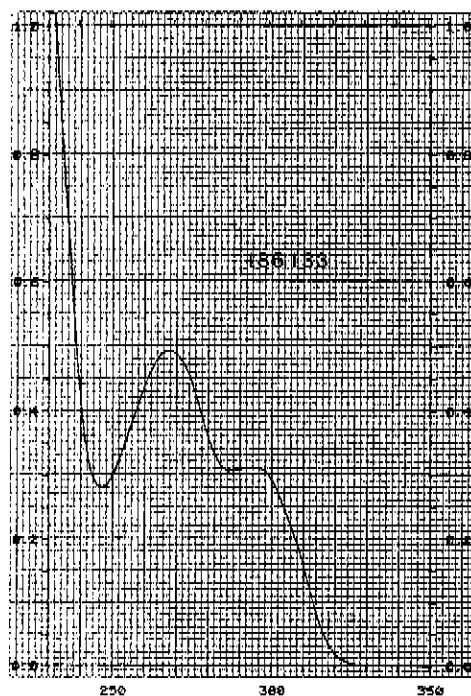


Figure 2. UV-spectrum of reserpine 18 µg/ml in ethanol.

$\lambda$  max in ethanol = 268 nm and 295 nm

E (1%, 1 cm) was determined at three wavelengths i.e. 247, 268 and 295 nm. The results are given in Table 1.

Table 1

Wavelength	247 nm	268 nm	295 nm
E (1%, 1 cm)	155	273	173
n	4	4	4
s rel %	0.73	0.70	0.70

Oxidation products, absorbance at 388 nm: 0.0, 0.2 mg/ml of reserpine was dissolved in glacial acetic acid. The UV-absorbance was measured at 388 nm.

#### ASSAY

##### Thermogravimetric analysis

0.2% loss in weight

##### Water

0.14% determined by Karl Fischer titration

##### Colorimetric assay

Determined by the colorimetric assay according to Ph. Int. Ed. III, Vol. 2. The USP reference substance Lot M was used as reference with the content taken to be 100%.

#### Result:

Content of proposed ICRS	Reference substance	s rel % (n= 5)
99.8%	USP Lot M	0.59

The absorbance of the reference solution was 0.42 for the proposed International Chemical Reference Substance.

#### Titrimetric assay

99.4% (n= 9), S rel % = 0.59, determined by titration with perchloric acid according to Ph. Int. Ed. III, Vol. 3. The calculations are performed with reference to the anhydrous substance.

#### PURITY

#### Thin-layer chromatography

The following thin-layer chromatographic system was used:

Thin-layer: HPTLC, Silica gel 60, F-254 (Merck)  
Eluent: Chloroform: diethylamine:cyclohexane (40:10:50)  
Sample: 100 µg of reserpine were applied  
The following possible impurities were also applied:  
1. Reserpic acid  
2. Methylreserpate  
3. 3,4,5-Trimethoxybenzoic acid  
4. 3,4-Dehydroreserpine  
5. 3-Isoreserpine

Visualization: UV-light of 254 nm and 365 nm.

Result: The thin-layer plate is shown in Figure 3.

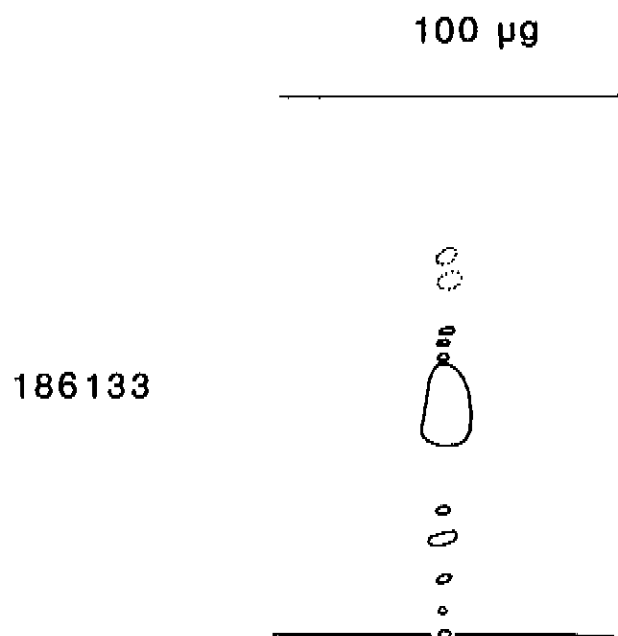


Figure 3. High performance thin-layer chromatogram of reserpine No 186133.

As seen from the figure about ten weak extra spots are observed, five above the main spot and five below. Rf (reserpine) is equal to 0.44. The dominating extra spot has Rf= 0.67 which corresponds to 3,4-dehydroreserpine. Reserpic acid with Rf= 0 as well as 3-isoreserpine with Rf= 0.54 were also detected. 3,4-Dehydroreserpine and 3-isoreserpine were probably formed on the plate or during the run. This results in that the thin-layer chromatographic method is not suitable for a quantitative evaluation. USP reference substance Lot L was also applied to the plate and showed several extra spots as well.

High performance liquid chromatography

The total amount of impurities was estimated by peak area measurement to about 0.8%. A chromatogram is shown in Figure 4.

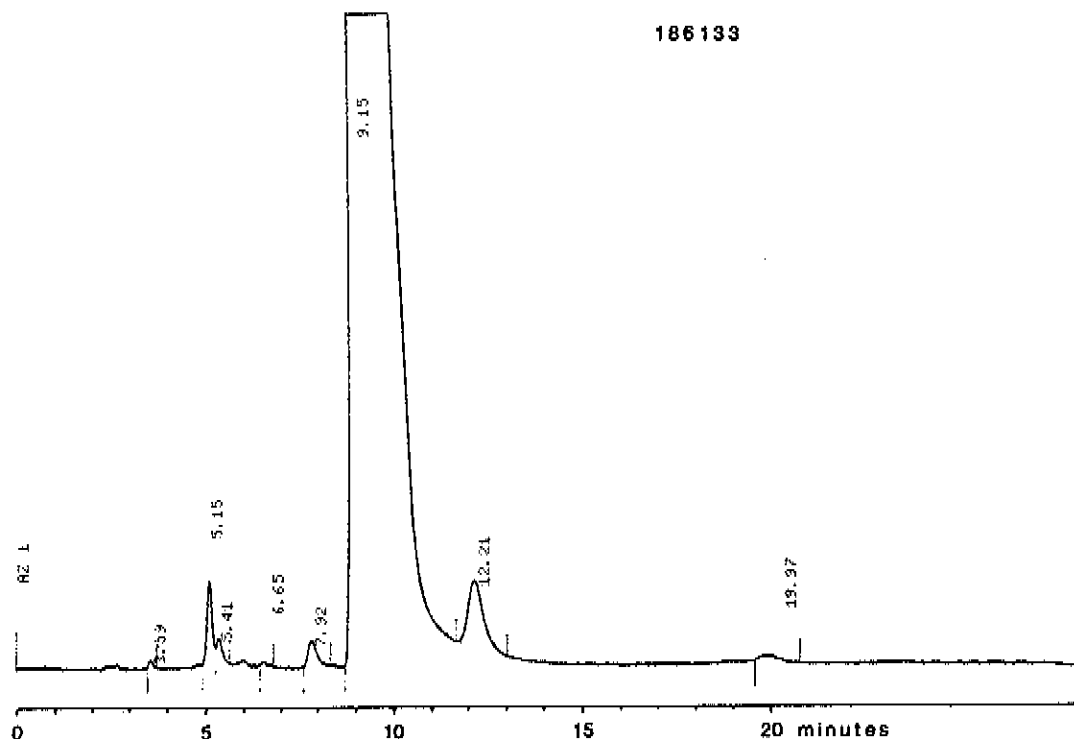


Figure 4. Chromatogram of reserpine Control No. 186133.

The following conditions were used:

Eluent: Acetonitrile/Phosphate pH= 4.5 (35:65)  
Column: Vydac 218 TP  
Detector: Varian UV 200 operated at 220 and 254 nm  
Pump: Varian 5560 operated at a flow rate of 1 ml/min  
Integrator: Varian 4270 Attenuation:  
Sample: 1 mg/ml dissolved in the eluent.  
10  $\mu$ l corresponding to 10  $\mu$ g were injected.

The International Chemical Reference Substance with Control No. 186133 was spiked with the impurities used in the TLC system. The major impurity determined by peak area measurement was estimated to 0.4%. It elutes after 12.2 min and is identical to 3,4-dehydroreserpine. After 5.1 min one unknown impurity estimated to 0.2% was observed. Reserpic acid elutes after 2.6 min and was estimated to 0.02%. 3-Isoreserpine was not found in the sample. 3,4-Dehydroreserpine was also quantified against an external standard and estimated to 0.5%. In the USP reference substance Lot M about 1% impurities were found. A chromatogram with the potential impurities and degradation products in the proposed International Chemical Reference Substance is shown in Figure 5.

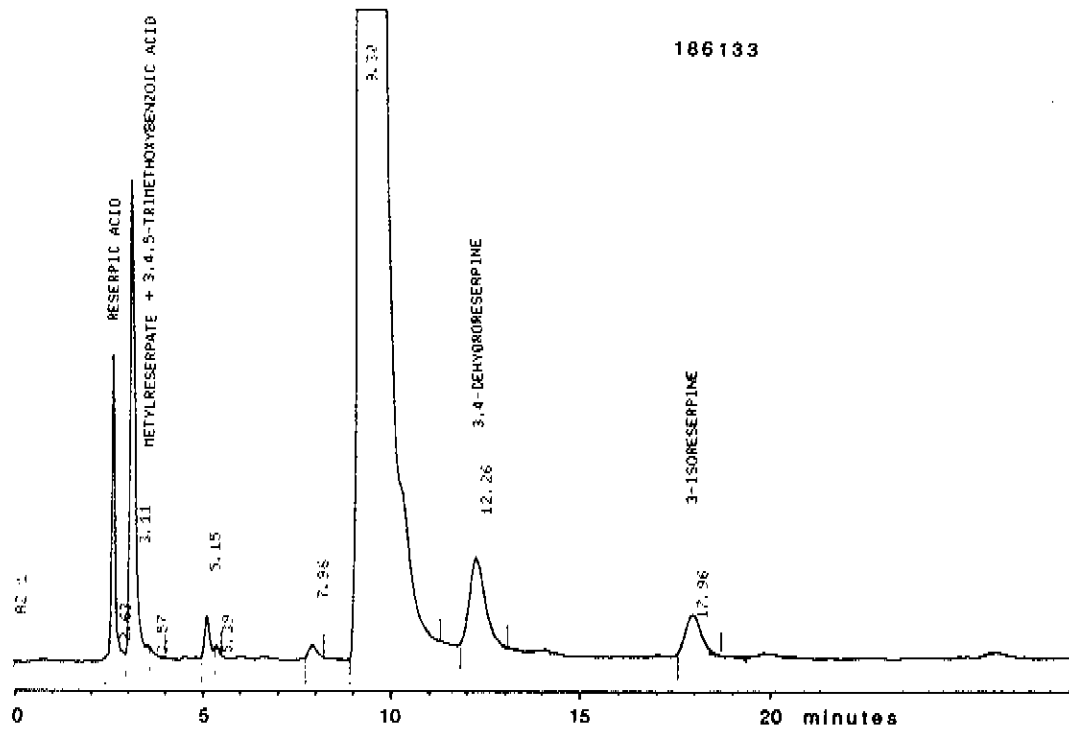


Figure 5. Chromatogram of reserpine spiked with potential impurities and degradation products (1% of each).

DIODE ARRAY DETECTION

The chromatogram was also evaluated with a LKB 2140 Rapid Diode Array Detector. The same chromatographic system as described above was used, except for the injection volume that was increased to 100  $\mu$ l to get maximum sensitivity. An isogram is given in Figure 6.

186133

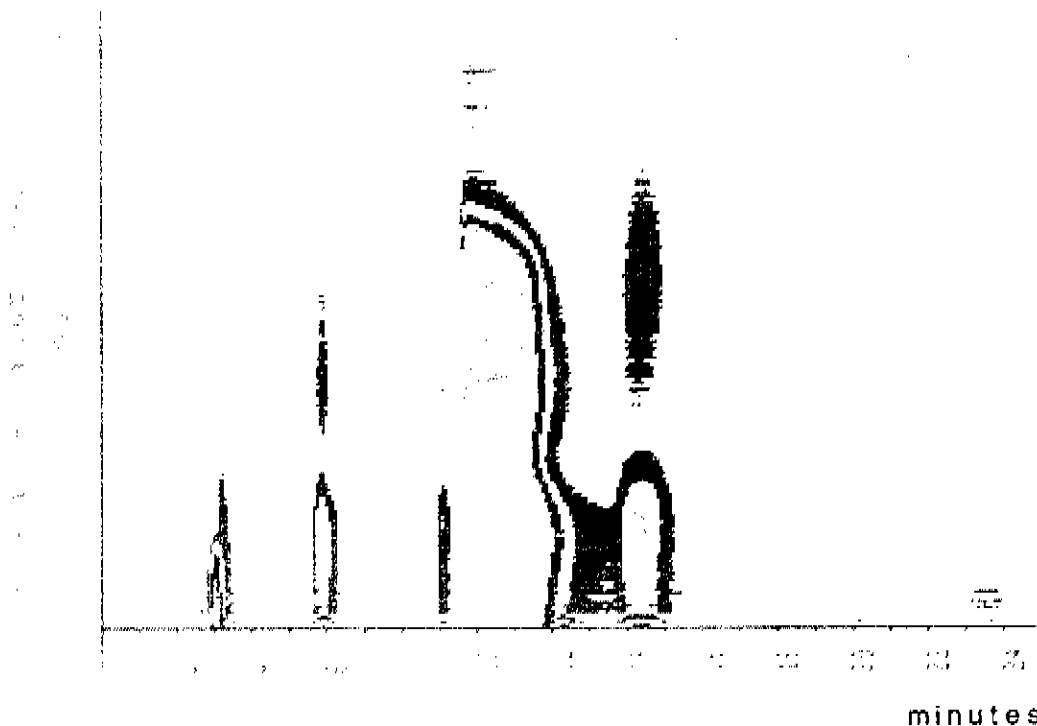


Figure 6. Isogram of reserpine Control No 186133. Sensitivity: 0.01

As can be seen from the figure the major impurities as well as reserpine have their absorbance maximum at 220 nm.

#### STABILITY

Reserpine was exposed to air of different relative humidity at room temperature (about 20 °C) for a period of 8 weeks as described in WHO/PHARM/82.509. All samples were unchanged at visual inspection and no weight changes were noted. No signs of degradation were observed when selected samples were analyzed by the liquid chromatographic method described above.

#### DATA GIVEN BY THE MANUFACTURER

Appearance:	Faintly pale, beige powder
Specific rotation:	-129.4° in dioxan
Absorbance 296 nm:	175.9
Absorbance 268 nm:	276.0
Absorbance 247 nm:	154.8
Identity:	IR, TLC
Absorbance 388 nm:	0.022
Loss on drying:	0.0%
Sulfated ash:	0.10%
Thin-layer chromatography:	0.1% of reserpine acid
Additional by-products:	each of them less than 1%
Assay acidimetric:	100.2%
Assay photometric:	100.8%

#### CONCLUSION

Reserpine No 186133 can be considered suitable as International Chemical Reference Substance for the intended purpose. The content of reserpine when used in the colorimetric assay is taken to be 99.8% calculated with reference to the dried substance, which corresponds to 99.6% calculated on the "as is" basis.

V I T A M I N A A C E T A T E  
R E T I N O L A C E T A T E

(solution)

Control No 686038

Analytical Report

The International Chemical Reference Substance for vitamin A acetate is intended to be used in validation of UV spectrophotometric assay procedures. The preparation is a solution in cottonseed oil of crystalline all-trans retinol acetate, dispensed in gelatin capsules, each containing approximately 9 mg in 250 mg of oil per capsule.

MATERIAL

The material proposed as a new International Chemical Reference Substance for vitamin A acetate has generously been donated by the U.S. Pharmacopeial Convention Inc. and is of the same batch as the U.S.P. Reference Standard Lot S.

The content of vitamin A acetate in the material has been determined in a collaborative study organized by the USP. The following result was obtained:

34.7 mg vitamin A acetate per g solution (equivalent to 30.3 mg of retinol)

The determination was carried out as a direct spectrophotometric measurement of the absorption in 2-propanol. The value given is an average of the results obtained in 6 different laboratories.

ASSAY

34.4 mg of vitamin A acetate per g of solution (equivalent to 30.0 mg of retinol and to about 100,000 IU of vitamin A per g of solution).

Method: Direct spectrophotometric measurement of the absorption (a) at the maximum at 325 nm of a 100 µg/ml solution in 2-propanol. The result was calculated using the following formula.

$a (\lambda \text{ max , caps.}) \times 1000$

----- = mg vitamin A acetate/g

$a (\lambda \text{ max , theoretical value for vitamin A acetate})$

Theoretical absorption for vitamin A acetate= 155.7

Duplicate determinations were carried out on 4 capsules (n= 7, relative standard deviation 1%)

In the calculations the following conversion factors were used:

1 mg of vitamin A (retinol) is equivalent to 1.1468 mg of vitamin A acetate.

1 IU of vitamin A is equivalent to 0.000344 mg of vitamin A acetate.

PURITY

Thin-layer chromatography

The following chromatographic systems were used to check the identity of vitamin A acetate.

Thin-layer: Silica gel 60, F-254 (Merck) and HPTLC silica gel 60 F-254 (Merck)  
Eluent: Cyclohexane/diethyl ether (4:1)  
Sample: 200 µg oil which corresponds to about 7 µg vitamin A acetate.  
Visualization: UV-light of 254 nm and 325 nm and iodine vapor.

Result: Rf of vitamin A acetate= 0.4 corresponding to the retention of a crystalline sample. It was not possible to estimate the purity by this method as vitamin A acetate degrades on the plate (checked by two-dimensional TLC), besides the cottonseed oil gave rise to disturbing spots.

ADDITIONAL DATA

Assay, vitamin A acetate HPLC: 101900 IU/g. As a standard USP lot R with a potency of 85600 IU/g was used.  
Assay vitamin A acetate USP: 100600 IU/g  
Vitamin A acetate crystals prior to encapsulation.  
Vitamin A acetate HPLC: 0.08% cis-A acetate  
99.77% trans A acetate

The results were obtained through the U.S.P.

CONCLUSION

Vitamin A acetate No 686038 can be considered suitable as International Chemical Reference Substance for the intended purpose. The content of vitamin A acetate is taken to be 34.4 mg per g of solution, corresponding to 30.0 mg of retinol.

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