

**Analytical Profiles  
of  
Drug Substances**

**Volume 13**

*Edited by*

**Klaus Florey**

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of  
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Volume 13

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# Analytical Profiles of Drug Substances

Volume 13

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**Klaus Florey**

The Squibb Institute for Medical Research  
New Brunswick, New Jersey

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## PREFACE

The compilation of *Analytical Profiles of Drug Substances* to supplement the information contained in the official compendia is now a well-established activity.

That we are able to publish one volume per year is a tribute to the diligence of the editors to solicit articles and even more so to the enthusiastic response of our authors, an international group associated with pharmaceutical firms, academic institutions, and compendial authorities. I would like to express my sincere gratitude to them for making this venture possible.

Over the years, we have had queries concerning our publication policy. Our goal is to cover all drug substances of medical value, and therefore, we have welcomed any papers of interest to an individual contributor. We also have endeavored to solicit profiles of the most useful and used medicines, but many in this category still need to be profiled.

In the preface to the eleventh volume, I announced that we would try to supplement previously published profiles with new data. Unfortunately, most of the original contributors are no longer available to undertake this task, and it has proven difficult to find other volunteers. We shall continue to pursue the updating program, but it will not be as comprehensive as originally envisioned.

Again, I would like to request those who have found these profiles useful to contribute papers of their own. We, the editors, stand ready to receive such contributions.



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# ATENOLOL

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<sup>1</sup>*Correspondence.*

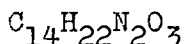
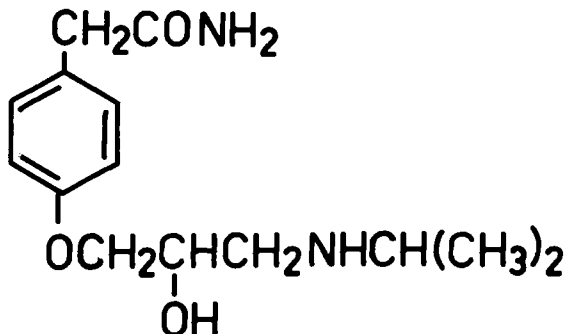
## 1. Foreword, History, Therapeutic Category

Atenolol is a  $\beta$ -adrenolytic, cardioselective drug, having no intrinsic sympathomimetic activity.  $\beta$ -Receptor blocking drugs were introduced in 1966, to treat cardiovascular disorders. These drugs are especially efficient in cases of coronary failure (angina pectoris), arterial hypertension and cardiac arrhythmia. The synthesis of atenolol was first reported in 1970, (1) and the first pharmacological and clinical studies were made in 1973. and 1974. (2-4).

## 2. Description

### 2.1. Name, Formula, Molecular Weight

Atenolol is 4-(2-hydroxy-3-(1-methylethyl)amino)-2-propoxy-benzenacetamide and is also known as 2-(p-2-hydroxy-3-(isopropylamino)propoxy)phenyl/acetamide or 1-p-carbamoylmethylphenoxy-3-isopropylamino-2-propanol.



Mol. wt. 266.33

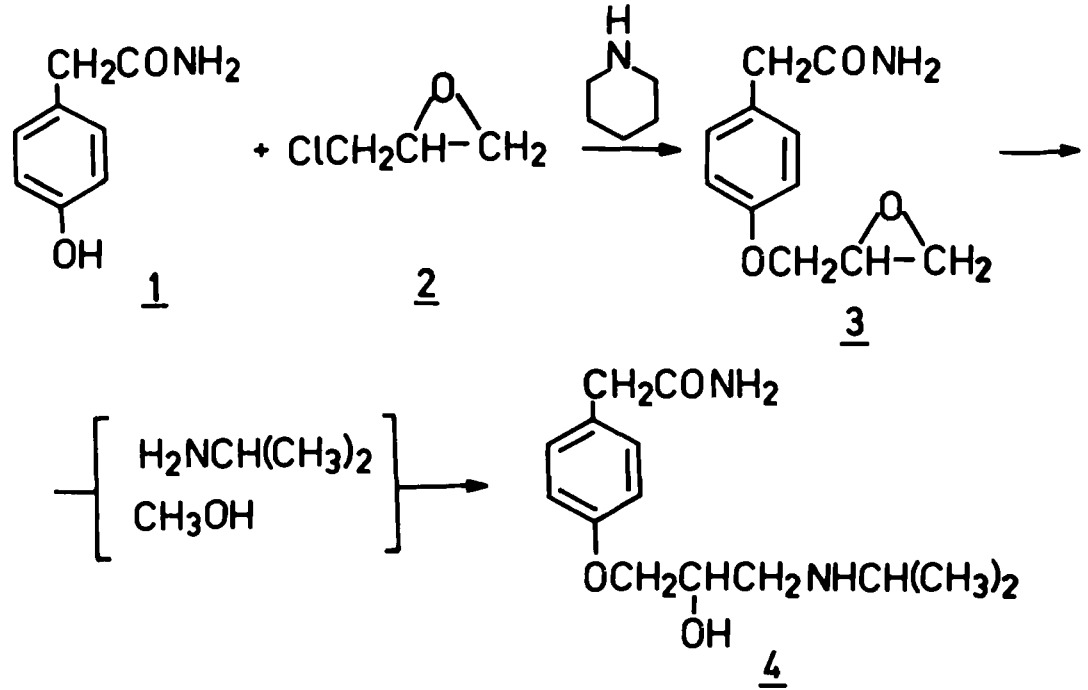
### 2.2. Appearance, Color, Odor, Taste

Atenolol is a white powder. It is odorless and has a slightly bitter taste.

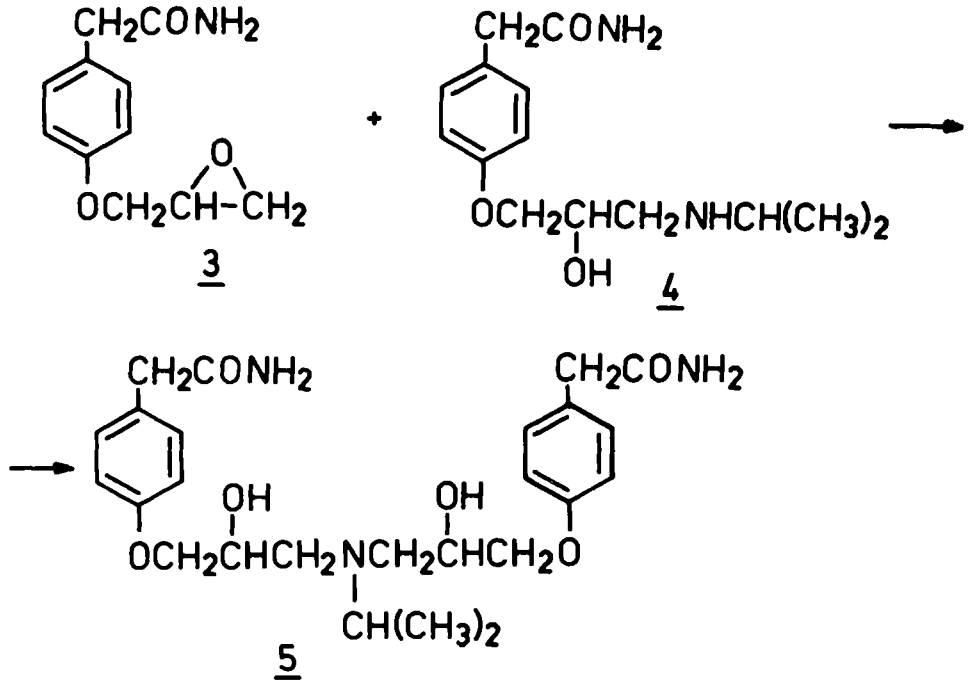
## 3. Synthesis

Atenolol is synthesized starting from commercially available 4-hydroxyphenylacetamide (1) and epichlorohydrine (2) in 15-fold molar excess, (1) with piperidine as a catalyst (see Scheme 1.). The reaction mixture is stirred 4-6 hours at 95-100°C, then allowed to cool to room temperature and left

SCHEME 1.



SCHEME 2.



standing overnight. The precipitate is collected by filtration and thoroughly washed with methanol to remove unreacted epichlorohydrine. The product, 1-(4-carbamoylmethylphenoxy)2,3-epoxy-propane (3), may be recrystallized from methanol, water or dioxane. The recrystallized compound 3 is reacted with isopropylamine (24-fold molar excess), in methanol as solvent, at the boiling point, for 5-30 minutes. The solvent and excess of isopropylamine are distilled off, and the crude atenolol recrystallized from water. The use of isopropylamine in the equimolar amount, or in a small excess, resulted in formation of an impurity, namely the product of a reaction of atenolol 4 and its precursor, 3, which had the structure of di-/3-(4'-carbamoylmethylphenoxy)-2-hydroxy-propyl/-isopropylamine (5) (Scheme 2.).

#### 4. Physical Properties

##### 4.1. Spectra

##### 4.1.1. Infrared

The infrared spectrum of atenolol, presented in Fig. 1., was recorded with a KBr pellet. The bands are assigned as follows: 3340 and 3160  $\text{cm}^{-1}$  (-CO-NH), 2940 (=CH), 1625 (-C=O, amide I), 1500 (-N-C=O, amide II), 1400 ( $\text{H}_2\text{N-CO-}$ ), 1385 (i-Pr), 1390, 1235 (arylether), 1176 (i-Pr), 1105, 1080, 1030, 910, 880, 810, 790, 700, 660. (5) The ir spectrum of the "dimeric" compound 5 exhibits the same bands with the same intensities in the region 4000-1100  $\text{cm}^{-1}$  as does compound 4. However, the bands at 1085, 910 and 880  $\text{cm}^{-1}$  (weak to medium) present in the spectrum of atenolol are lacking in the spectrum of compound 5.

##### 4.1.2. Ultraviolet

The ultraviolet spectrum of atenolol is presented in Fig. 2. It was recorded with a methanolic solution at the concentration  $10^{-2}$  g/L. The  $\lambda_{\text{max}}$  values of bands of atenolol and compound 5 lie at the same wavelenths (i.e. 225, 275 and 283 nm), but their intensities differ slightly.

##### 4.1.3. Proton Magnetic Resonance

The proton magnetic resonance spectrum of atenolol in  $\text{CD}_3\text{OD}$  is presented in Fig. 3. The spec-



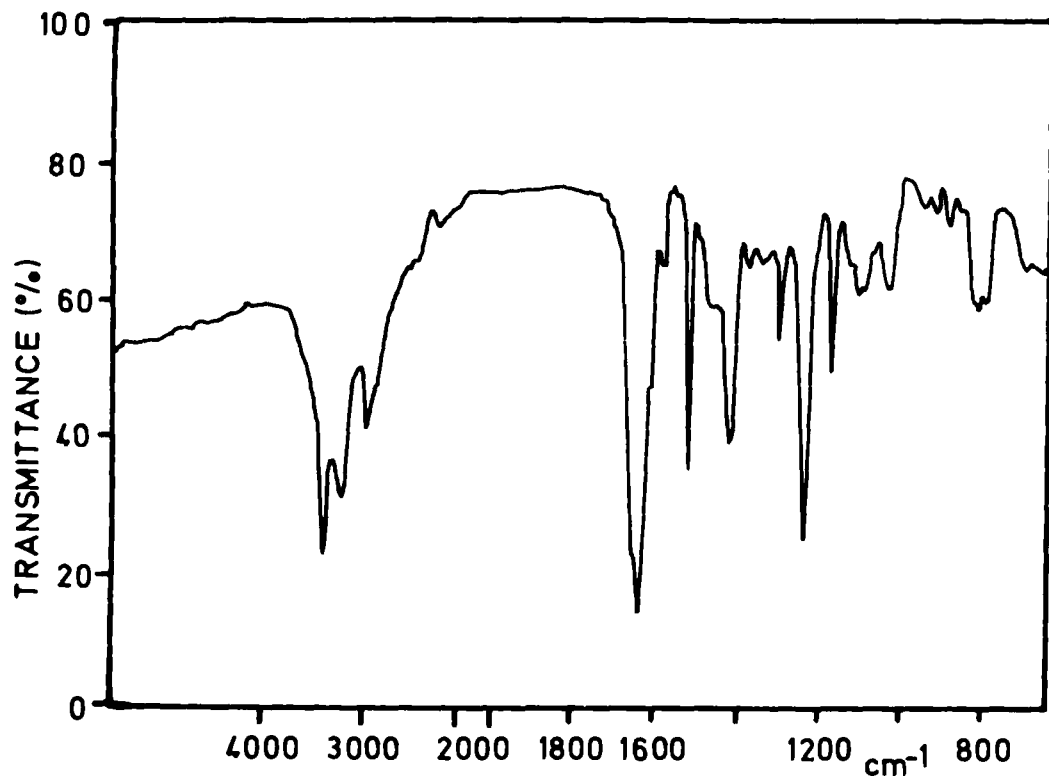


Fig. 1. Infrared spectrum of atenolol (KBr pellet).  
Instrument: Pye Unicam SP3-200.

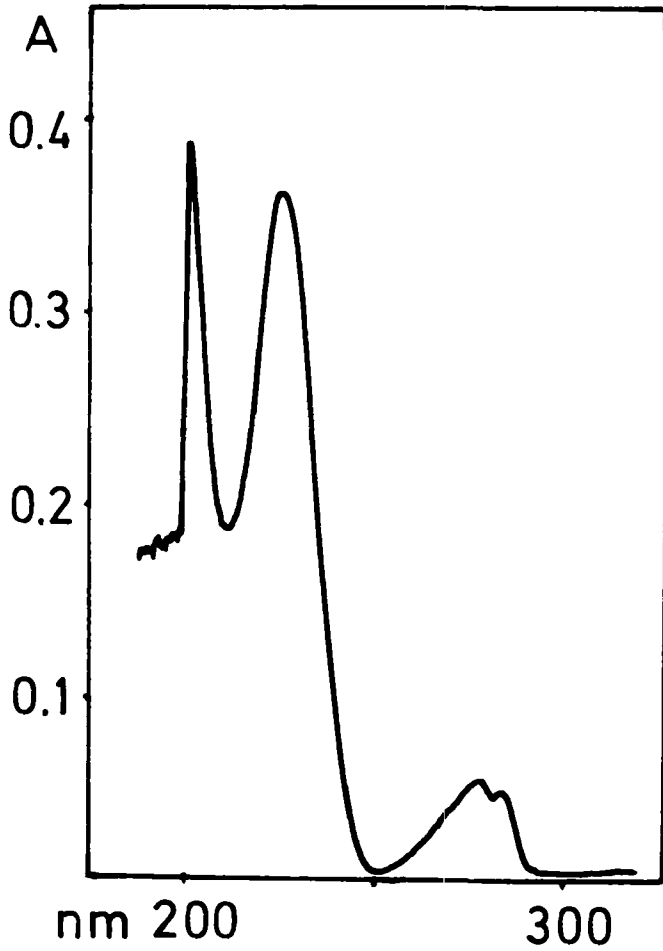


Fig. 2. Ultraviolet spectrum of atenolol. Instrument: Pye Unicam SP8-100 UV-VIS

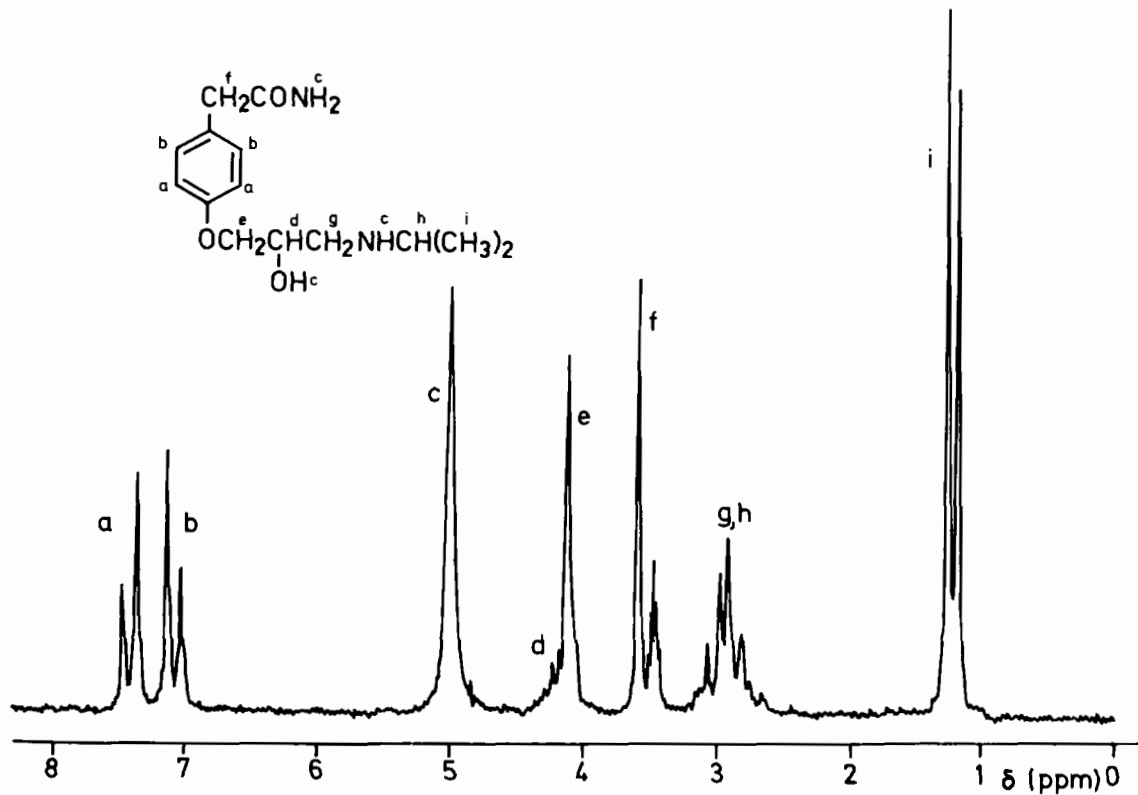
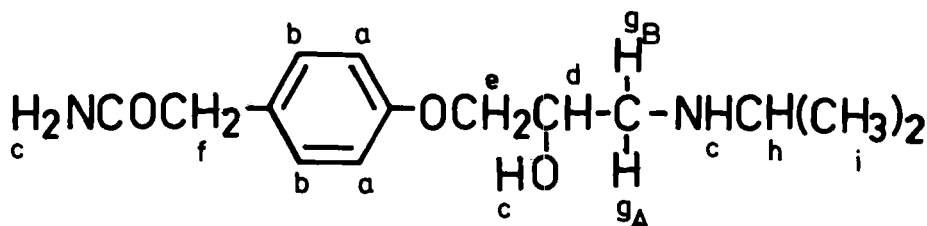


Fig. 3. 80 MHz proton MR spectrum of atenolol. Instrument: Bruker WP 80 DS at 80 MHz (8 K points).

Table 1. Assignment of proton MR signals in the spectrum of atenolol



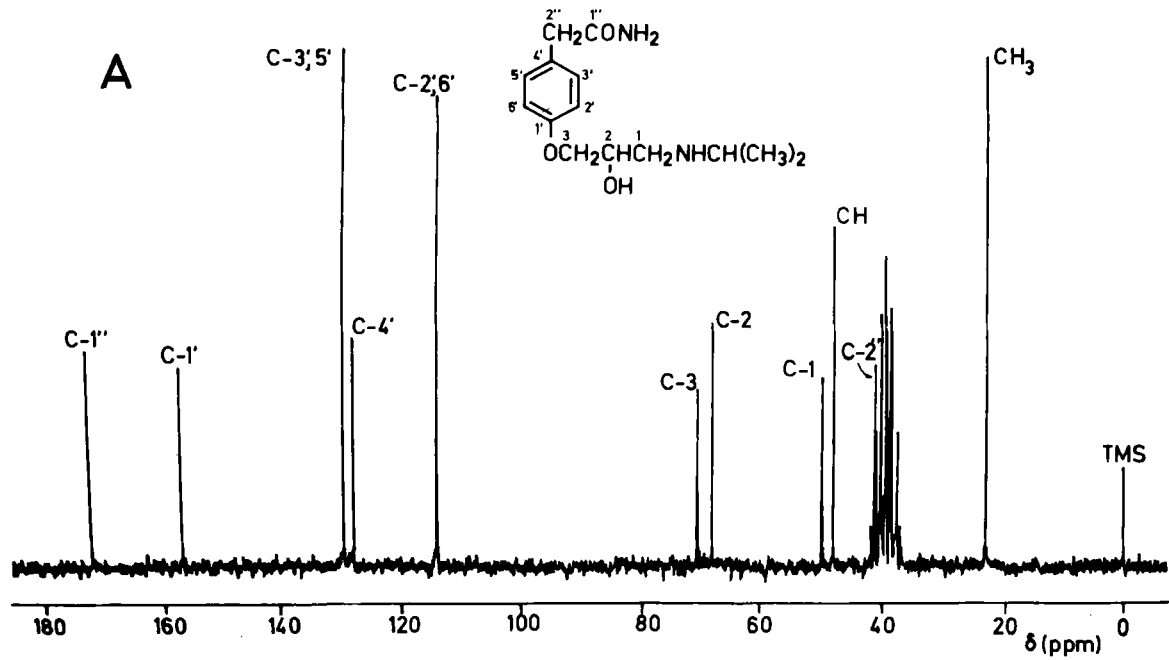
Protons	Chemical shift $\sigma$ (ppm)	Type	Inten- sity	Coupling	const. J (Hz)
$\text{H}_a$	7.38	d(AB)	2 H	$J_{a,b}$	8.755
$\text{H}_b$	7.05	d(AB)	2 H	$J_{b,a}$	8.755
$\text{H}_c$	4.99	s	2+1+1 H		-
$\text{H}_d$	4.28-4.10	m	1 H		-
$\text{H}_e$	4.12	s	2 H		-
$\text{H}_f$	3.59	s	2 H		-
$\text{H}_{gA}$	3.03	dd	1 H	$J_{gA,gB}$ $J_{gA,d}$	12.5 5.00
$\text{H}_{gB}$	2.81	dd	1 H	$J_{gB,gA}$ $J_{gB,d}$	12.5 5.00
$\text{H}_h$	3.14-2.65	m	1 H		-
$\text{H}_i$	1.25	d	6 H	$J_{i,h}$	6.254

tral assignments are shown in Table 1. (5) Characteristic peaks of the proton MR spectrum of compound 5 (which may be present as an impurity in crude atenolol) is presented in Table 2.

4.1.4.  $^{13}\text{C}$ -NMR

$^{13}\text{C}$ -NMR spectra of atenolol and compound 5 (presented in Fig. 4.) were run in pulse FT mode on a Jeol FX-100 spectrometer operating at 25.05 MHz. The samples were measured in 5 mm tubes with TMS as an internal standard. An 8K computer memory and internal deuterium lock were used. (5)

The broad-band  $^{13}\text{C}$  NMR spectra of DMSO- $d_6$  solutions of atenolol and its "dimer" 5 are shown in



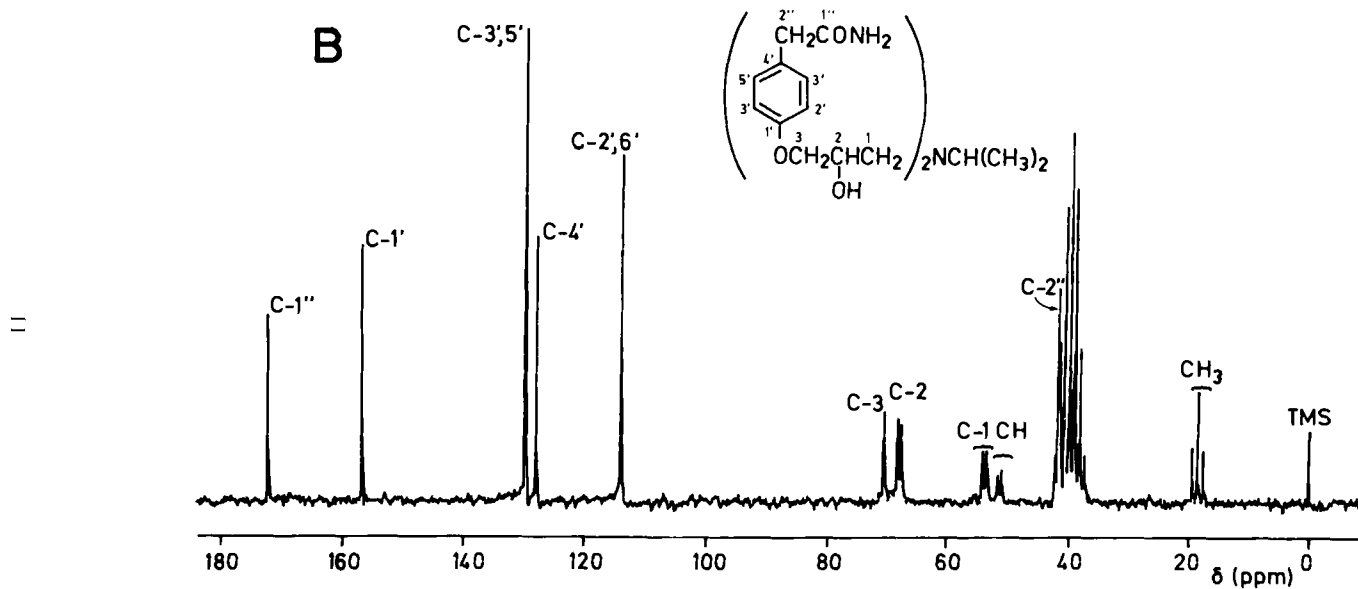
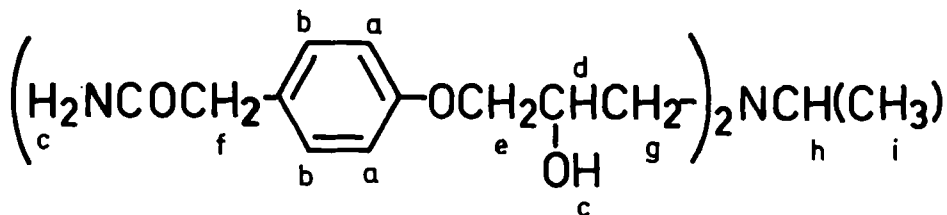


Fig. 4.  $^{13}\text{C}$  NMR spectra of atenolol (A) and compound 5, (B) in  $\text{DMSO-d}_6$ . Instrument: Jeol FX-100 spectrometer at 25.05 MHz.



Table 2. Assignment of proton MR signals in the spectrum of compound 5



Protons	Chemical shift $\delta$ (ppm)	Type	Inten- sity	Coupling J (Hz)	const.
H <sub>a</sub>	7.36	d	4 H	J <sub>a,b</sub>	8.755
H <sub>b</sub>	7.02	dd	4 H	J <sub>b,a</sub>	8.755
H <sub>c</sub>	4.98	s	6 H	-	-
H <sub>d</sub>	4.2-4.0	m	2 H	-	-
H <sub>e</sub>	4.13	s	4 H	-	-
H <sub>f</sub>	3.59	s	4 H	-	-
H <sub>gA</sub>	2.875	d	2 H	J <sub>gA,gB</sub>	5.0
H <sub>gB</sub>	2.98	d	2 H	J <sub>gB,gA</sub>	5.0
H <sub>h</sub>	2.8-3.0	m	1 H	-	-
H <sub>i</sub>	1.20	d	6 H	J	6.504

Fig. 4. Since the solvent peaks cover one carbon signal, the complementary CD<sub>3</sub>OD solutions were also used in making the complete assignment. In this way an unambiguous assignment was achieved, applying off-resonance and gated decoupling (NOE) spectra, as well as tabulated data of chemical shifts for related constitutional parts contained in the atenolol molecule. (6)

The <sup>13</sup>C NMR spectra of the CD<sub>3</sub>OD solutions reveal two signals belonging to nonequivalent methyl carbons of the isopropyl group. In the NOE spectrum only one complex quartet was produced correspondingly, so that only one first order C-H coupling constant was determined, the other one was only estimated, as being higher by about 4 Hz (see Table 3.). The other multiplets were also very complex, due to several long-range couplings and overlaps, with both the solvents used. However, a rather complete analysis of the coupled <sup>13</sup>C NMR spectrum was made with numerous first and higher C-H coupling

Table 3.  $^{13}\text{C}$  Chemical shifts<sup>a</sup> and  $^{13}\text{C}$ - $^1\text{H}$  coupling constants<sup>b</sup> for atenolol dissolved in  $\text{CD}_3\text{OD}$

C-atom	$\delta$ (ppm)	$^1J_{\text{CH}}$	$^2J_{\text{CH}}$	$^3J_{\text{CH}}$
C-1 <sup>c</sup>	50.75 (t)	132.6	2.0;1.5	-
C-2	69.59 (d)	142.6	2.4;2.4	-
C-3	71.78 (t)	144.0	2.4	-
$\text{CH}_2$	22.61 (q)	129 <sup>d</sup>	-	-
$\text{CH}_2$	22.42 (q)	125.5	5.4	2.0
$\text{CH}^e$	49.68 (d)	131 <sup>e</sup>	-	-
C-1'	158.86 (s)	-	2	9.7
C-2',6'	115.36 (d)	160.1	4.9	-
C-3',5'	130.84 (d)	158.7	2.4	7.1;4.9
C-4'	128.83 (s)	-	5.7	7.8
C-1''	176.80 (s)	-	6.6;4.4	-
C-2''	42.41 (t)	128.4	-	3.9

<sup>a</sup>  $\delta$  in ppm downfield from internal TMS; accuracy  $\pm 0.02$  ppm; off-resonance multiplets are given in parentheses

<sup>b</sup>  $^nJ_{\text{CH}}$  in Hz; accuracy  $\pm 0.5$  Hz

<sup>c</sup>  $^nJ_{\text{CH}}$  values determined from the  $\text{DMSO-d}_6$  solution

<sup>d</sup> estimated to  $\pm 1$  Hz

<sup>e</sup> estimated to  $\pm 2$  Hz; the long range coupling constants can not be determined due to very complex multiplets

constants which may be useful for a further structural study. The complete data are listed in Table 3.

In the  $\text{DMSO-d}_6$  solution only one  $\text{CH}_2$ -signal was found in the spectrum of atenolol, and the solvent caused a slight downfield effect upon the chemical shift. On the other hand, all other  $^{13}\text{C}$  chemical shifts were moved substantially upfield, the changes ranging from 0.60 for C-4' to 4.27 ppm for the C=O group (Table 4.). The NOE spectrum shows rather smeared multiplets which were not suitable for precise determination of coupling constants, except in very few cases.

The broad band  $^{13}\text{C}$  NMR spectrum of the compound 2 dissolved in  $\text{DMSO-d}_6$  (Fig. 4B) shows the doubling of the signals around the chiral centre (Table 4.), which demonstrates the presence of diastereomers. In comparison with the atenolol carbon

Table 4.  $^{13}\text{C}$  Chemical shifts<sup>a</sup> for atenolol and compound 5 dissolved in  $\text{DMSO-d}_6$ 

C-atom	Type	Atenolol	Compound <u>5</u>
C-1	t	49.92	53.94 53.16
C-2	d	68.25	68.13 67.94
C-3	t	70.71	70.32 <sup>b</sup> 70.32 <sup>b</sup>
$\text{CH}_3$	q	22.83	18.66 17.77 <sup>c</sup>
$\text{CH}_3$	q	22.83	17.77 <sup>c</sup> 16.76
CH	d	48.12	51.41 50.78
C-1'	s	157.15	157.25
C-2',6'	d	114.08	114.08
C-3',5'	d	129.87	129.91
C-4'	s	128.23	128.16
C-1''	s	172.53	172.90
C-2''	t	41.27	41.27

<sup>a</sup>  $\delta$  in ppm downfield from internal TMS; accuracy  $\pm 0.02$  ppm; off-resonance multiplets are given

<sup>b,c</sup> broadened signal indicates to chemical shifts separated by less than 0.02 ppm

chemical shifts, the methyl signals are significantly moved upfield, and those belonging to isopropyl CH and C-1 are moved downfield. All other signals are close to those of atenolol.

#### 4.1.5. Mass

The mass spectra of atenolol and compound 5 were obtained by direct insertion of the sample into CEC 21-110 B mass spectrometer. Characteristics of these mass spectra are summarized in Tables 5. and 6, and Fig. 5. The ion source temperature was  $150^\circ\text{C}$  and  $200\text{-}250^\circ\text{C}$ , respectively, and the ionizing electron beam energy was 70 eV.

Atenolol gave a molecular ion, with very low intensity, at  $m/e$  266. Other significant peaks are due to the following fragments: isopropyl group (M-43), carboxamide group (M-44) and carboxamidomethylene group (M-56). The base peak in the atenolol

Table 5. Peak assignments in the mass spectrum of atenolol

$\%$	m/e	fragment
1.2	266	$M^+$
24.5	222	$M^+ - 44 (-CONH_2)$
3.9	223	$M^+ - 43 [-CH(CH_3)_2]$
12.9	116	$-CH_2\overset{\text{OH}}{\underset{ }{\text{C}}}\text{HCH}_2\text{NHCH(CH}_3)_2$
43.1	107	$\left[ \text{CH}_2 - \text{C}_6\text{H}_4 = \text{O} \right]^{+\bullet}$
14.3	78	107-CHO
3.7	151	$\text{O} = \text{C}_6\text{H}_4 = \text{CH}_2\text{CONH}_2$
100	72	$-CH_2\text{NHCH(CH}_3)_2$
23.5	73	$-\text{HCH}\overset{\text{OH}}{\underset{ }{\text{C}}}\text{HCH}_2\text{NH}-$

spectrum is due to isopropylaminomethylene group (m/e 72). The base peak in the spectrum of compound 5 corresponds to radical-ion of protonated p-methylenequinone (m/e 107).

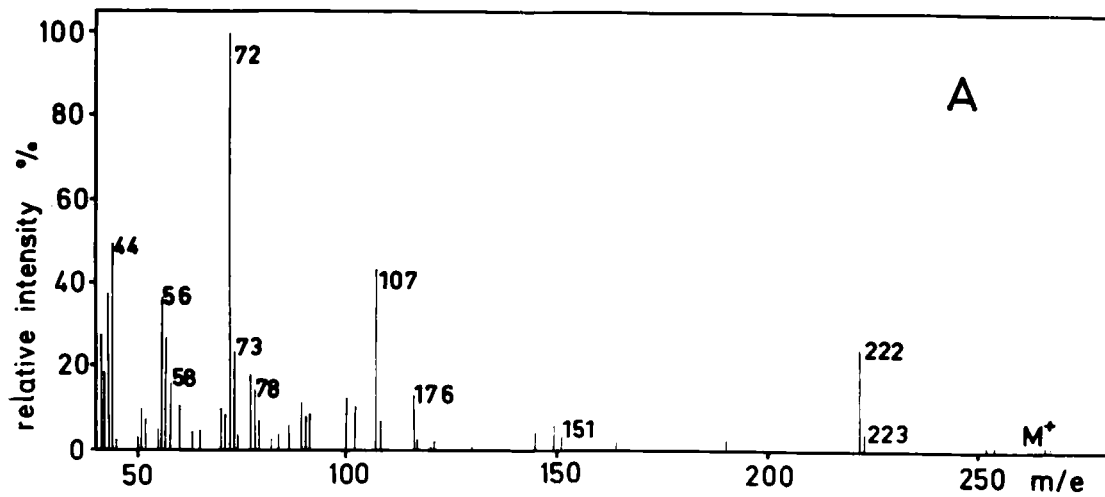
4.2. Solid Properties

4.2.1. Melting Range

Atenolol melts between 150° and 152°C; compound 5 melts between 154° and 156°.

4.2.2. Crystal Properties

X-Ray diffraction data of atenolol are presented in Table 7.(5) The diffraction spectrum was produced by monochromatic radiation from the CuK line (1.542 Å) which was obtained by excitation at 35 kV and 20 mA. Recording conditions were as follows. Optics: detector slit 0.2°; M.R. soller slit, 3°; beam slit, 0.0007''; Ni filter, 3° take off angle. Goniometer: scan at 2°, 20/min. Detector: amplifier gain 16 coarse, 9.1 fine. Scintillation co-



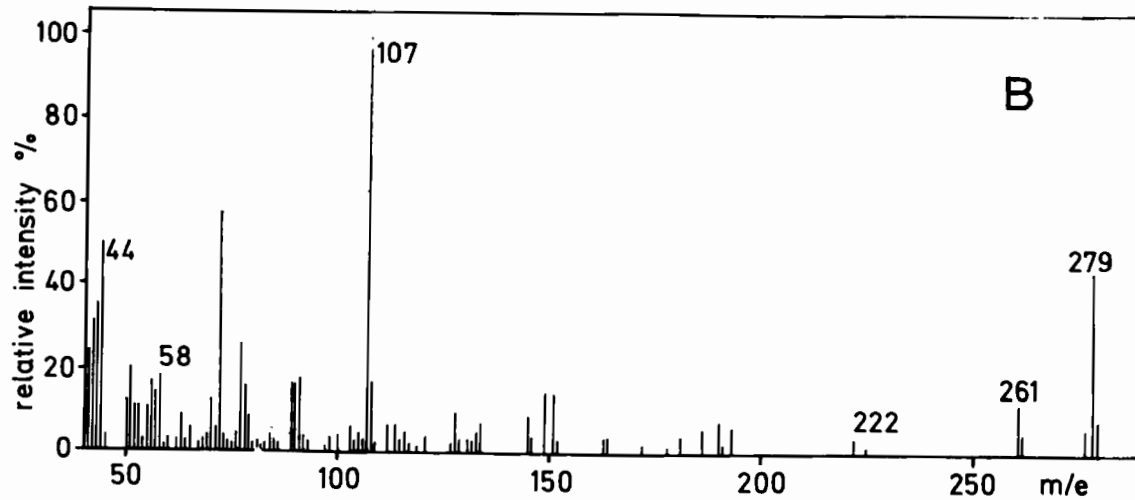


Fig. 5. Mass spectra of atenolol at 150°C (A) and compound 5 at 200-250°C (B). Instrument: CEC 21-110 B.



Table 6. Peak assignments in the mass spectrum of compound **2**

%	m/e	fragment
0.055	473	M <sup>+</sup>
3.3	222	
100	107	
42.6	279	
12	261	
50	44	-CONH <sub>2</sub>
35.2	43	-CH(CH <sub>3</sub> ) <sub>2</sub>

Table 7. X-Ray diffraction data of atenolol crystal

2 $\theta$	I <sup>a</sup>	d <sup>b</sup> (interplanar distance)
9.62	43	9.1936
12.84	6	6.8943
16.04	20	5.5254
17.74	54	4.9996
18.22	61	4.8689
19.22	74	4.6178
20.60	100	4.3115
22.24	91	3.9971
23.80	81	3.7385
24.36	72	3.6538
26.40	92	3.3759
29.10	11	3.0686
31.84	42	2.8105
34.58	14	2.5938
35.94	17	2.4987
40.46	15	2.2296

<sup>a</sup> based on the highest intensity which is selected as unity

<sup>b</sup>  $d = n\lambda / 2 \sin \theta$

unter tube and DC voltage at plateau. Pulse light selection  $E_L$  9V,  $E_n$  out. Rate meter: T.C. 1.0, 5000 cps.(5)

4.3. Solution Properties

4.3.1. Solubility

All solubilities were determined at room temperature and are presented in Table 8. The solubilities are reported in accordance with U.S.P.(XX<sup>th</sup> ed.) definitions.(5)

Table 8. Solubilities of atenolol

Solvent	Solubility
methanol	freely soluble
acetic acid	soluble
dimethylsulfoxide	soluble
96% ethanol	sparingly soluble
water	slightly soluble
isopropanol	slightly soluble
acetone	very slightly soluble
dioxane	very slightly soluble
acetonitrile	insoluble
ethylacetate	insoluble
chloroform	insoluble

4.3.2. Acidity ( $pK_a$ )

The negative logarithm of the proton dissociation constant for atenolol was determined as  $pK_a = 9.6$ .(7)

4.3.3. Partition Coefficient

Atenolol has a low partition coefficient for n-octanol-phosphate buffer (0.16 M). Its values were 0.008 at pH 7.0 and 0.052 at pH 8.0.(7)

4.3.4. Dipole Moment

The dipole moment of atenolol was determined in propionic acid solutions, at 20°C, using a Dipolmeter DM 01 (Wiss.-Techn. Werkstätten, D 812 Weilheim). The value found was 5.71±0.20 D.(5)

#### 4.3.5. Optical Rotation

The atenolol molecule contains an asymmetric carbon atom. The commercial product, however, is a racemic mixture and its resolution has not been reported so far.

### 5. Methods of Analysis

#### 5.1. Elemental Analysis

Atenolol  $C_{14}H_{22}N_2O_3$  (266.33) calc.: C 63.13%  
 H 8.33%  
 N 10.52%  
 O 18.02%  
 100.00%

#### 5.2. Titrimetric Determination - Electrochemical

Atenolol may be assayed in glacial acetic acid/acetic anhydride 15:2 by titration with 0.1 M perchloric acid. The endpoint may be determined potentiometrically using a glass/calomel electrode pair.(5)

### 5.3. Chromatographic Methods

#### 5.3.1. Thin Layer

Thin-layer chromatography was performed using silica gel 60 F<sub>254</sub> plates (Merck); the solvent system was dioxane-acetonitrile-methanol-conc. ammonia (25%) (60:36:5:4). One hundred mg of the sample was dissolved in 10 ml of methanol, the solution spotted on the plate and subjected to ascending chromatography. After a front migration for at least 16 cm the plate was air-dried and sprayed with Dragendorff's reagent. Atenolol and compound 2 appear as orange-brown spots, on a yellow background, at  $R_f$  0.3 and 0.6, respectively.(5)

High performance thin-layer chromatography (HPTLC) on silica gel plates was used for the separation  $\beta$ -adrenoceptor blocking drugs. The detection limit for atenolol was 25.0 ng, and the absorption wavelength selected for its determination was 205 nm.(8)

#### 5.3.2. Gas-Liquid

For gas-liquid chromatographic determinations a 1 m x 4 mm (I.D.) glass column packed with 3% OV-101 on 80-100 mesh Gas Chrom Q was used.(5) The in-

jector temperature was set at 300°C, oven temperature at 190°C, and detector (flame ionization) temperature at 300°C. The sample was pretreated with trifluoroacetic acid anhydride as follows: to 20 mg of atenolol in 10-ml volumetric flask was added 5 ml of dioxane and 0.5 ml of trifluoroacetic acid anhydride; the mixture was heated to 40°C and kept 5 min. at this temperature. The flask was then cooled to room temperature and the contents made up to 10 ml with dioxane. One ml of this solution was mixed with 0.2 ml of internal standard solution (2 mg/ml of methyl ester of margaric acid in dioxane) and 1  $\mu$ l of the mixture forced into the injection block. The relative weight response of the detector was calculated according to equation (1):

$$RWR = \frac{A_A \times C_S}{A_S \times C_A} \quad (1)$$

in which symbols have the following meanings:

$A_A$  = atenolol peak area of standard

$A_S$  = internal standard peak area

$C_A$  = concentration of atenolol (mg/ml)

$C_S$  = concentration of methyl margarinate (mg/ml)

In actual analyses the content of atenolol is calculated from the areas of atenolol and internal standard according to eq. (2):

$$\text{mg of atenolol} = \frac{A_A \times C_S \times 1000}{A_S \times RWR \times W} \quad (2)$$

in which the meanings of symbols are following:

$A_A$  = atenolol peak area of sample

$A_S$  = internal standard peak area

$C_S$  = concentration of methyl margarinate (mg/ml)

RWR = relative weight response

W = weight of the sample (mg)

### 5.3.3. High Pressure Liquid

The apparatus used for high pressure liquid chromatography was by Pye-Unicam (Cambridge), PU-4011 equipped with a PU-4020 UV detector. The co-

lumn was prepacked with Partisil 10  $\mu\text{m}$  ODS (25 cm; 4.6 mm ID, 6 mm OD). The mobile phase was acetonitrile-dist. water-orthophosphoric acid (35:100:0.1) having a pH of 3.5. In some analyses benzimidazole was used as an internal standard. The flow rate was 1.0 ml/min. and the detector monochromator was adjusted at 222 nm; the amplifier gain was set at 0.08 A.U.F.S. The retention time of atenolol was 5.48.(5) In another case, a LiChrosorb E (RD-2, ethylsilanized) column and a mobile phase consisting of 35% MeOH, and 65% of an aqueous solution 0.0005 M in HCl and 0.05 M in NaCl were used. The analyte was detected at its peak absorption 220 nm, using a variable-wavelength detector.(9)

#### 5.4. Determination of Impurities

The "dimeric" compound 5, which may be present in atenolol can be detected by all chromatographic methods and determined by gas-liquid and HPL chromatography.

#### 6. Stability - Degradation

Atenolol, as the powder and in the form of tablets was kept at 50-60% relative humidity and 45-50°C for seven days. No changes were observed in HPL chromatogram, neither did the color and appearance of the material show change.

#### 7. Drug Metabolism, Pharmacokinetics, Bioavailability

Owing to low hydrophilicity only a small part of atenolol is metabolised (about 10% of a dose). The drug is also poorly bound to plasma protein (less 5% of the amount in blood).(10) Mostly of the drug is eliminated, in its unchanged form, by several routes, but prevailing via the kidney.(11) After oral administration atenolol is excreted with urine to the extent of about 40%; (12-14), after intravenous administration to the total urinary excretion encompasses 75-100% of the dose (about 10-14% appeared in the form of catabolites).(12,15, 16) Urinary pH variations do not change the extent of excretion with the urine.(17) Evidences was put forward to show that atenolol is not eliminated exclusively by glomerular filtration.(11) Atenolol is also excreted with the faeces. After intravenous

administration, about 10% of the dose excreted unchanged; after oral administration, 40-50% of the dose appears in faeces, of which 6-8% accounts for catabolites.(12)

Atenolol is characterized by a low hepatic clearance; for the most part of body clearance is extrahepatic.(18) The hepatic biotransformation of atenolol gives rise to the pharmaceutically inactive catabolites, such as compound hydroxylated at the methylene group attached to the benzene ring, and compounds conjugated with glucuronic acid.(7)

Only a small fraction of a dose of atenolol reaches the brain and there is ratio of 2:10 between the brain and plasma concentration. Atenolol does not accumulate in tissues, such as lungs, heart and others.(7)

The pharmacokinetics of atenolol was studied in young and old subjects: no age-related differences were found with respect to volume of distribution, and bioavailability.(11) The absolute bioavailability (F), defined as the fraction of an oral dose which reaches the systemic circulation in unchanged form, was 0.55-0.56.(11) Other authors report bioavailabilities of 50-63% for oral dosage forms.(15,16,19-22) However, a given oral preparation possesses uniform bioavailability, and exhibits but little variation in peak plasma concentration.(18) Postprandial intake reduces the bioavailability of atenolol.(19) Several investigators reported less than 100% bioavailabilities of injectable preparations: with intravenously administered dosage forms, bioavailabilities between 85-98% were obtained.(15,16,21,22)

### 8. Identification and Determination in Body Fluids and Tissues

The concentrations of atenolol in various biological material was determined by gas chromatography.(23-25) Another method, HPLC, was used for determination in whole blood,(26) plasma, (27,28) and urine.(28)

### 9. Determination in Pharmaceuticals

In tablets atenolol is determined titrimetrically as follows: A sufficient number of tablets are powdered in a mortar, and a powder portions corresponding to one-half of average tablet mass

are transferred to the titration vessel containing 15 ml of glacial acetic acid. Two ml of acetic acid anhydride and 2 ml of a saturated solution of mercury acetate in acetic acid are added per vessel, and the resulting suspension is titrated with 0.1 M  $\text{HClO}_4$  using a glass electrode as endpoint indicator (vs. a satd. calomel reference electrode). The atenolol content in one tablet is calculated by using equation (3). One ml of 0.1 M  $\text{HClO}_4$  corresponds to 26.63 mg of atenolol.

$$\text{mg of atenolol in one tablet} = \frac{V \times f \times 26.63 \times W_A}{W} \quad (3)$$

where V = volume of 0.1 M  $\text{HClO}_4$  consumed,  
 f = normality factor,  
 $W_A$  = average mass of a tablet,  
 W = mass of the powder sample.

Acknowledgement. The authors are thankful to dr. Z. Meić, "Ruđer Bošković" Institute, Zagreb, Yugoslavia, for his help in interpretation of  $^{13}\text{C}$  NMR spectra.

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# CAMPHOR

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## 1. DESCRIPTION

### 1.1. Nomenclature

#### 1.11 Chemical Names

- a) 2-Oxo-1,7,7-trimethyl bicyclo (2,2,1) heptan.
- b) 1,7,7-Trimethylbicyclo(2,2,1) heptan-2-one.
- c) 2-Keto-1,7,7-trimethyl norcamphane.
- d) Bicyclo (2,2,1) heptan -2-one,1,7,7-trimethyl.
- e) (4,7,7-Trimethyl bicyclo 1,4) heptan -5-one.
- f) 2-Bornanone.
- g) d-2-Camphanone.(1,2)

#### 1.12 Generic Names

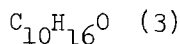
Camphor.

#### 1.13 Trade Names

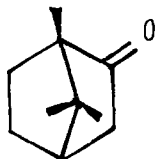
- a) Formosa camphor
- b) Laurel camphor
- c) Alcanfor
- d) Camphre du Japon (Natural)
- e) Camphre Droit (Natural)
- f) Gum camphor.

## 1.2 Formulae

### 1.21 Empirical



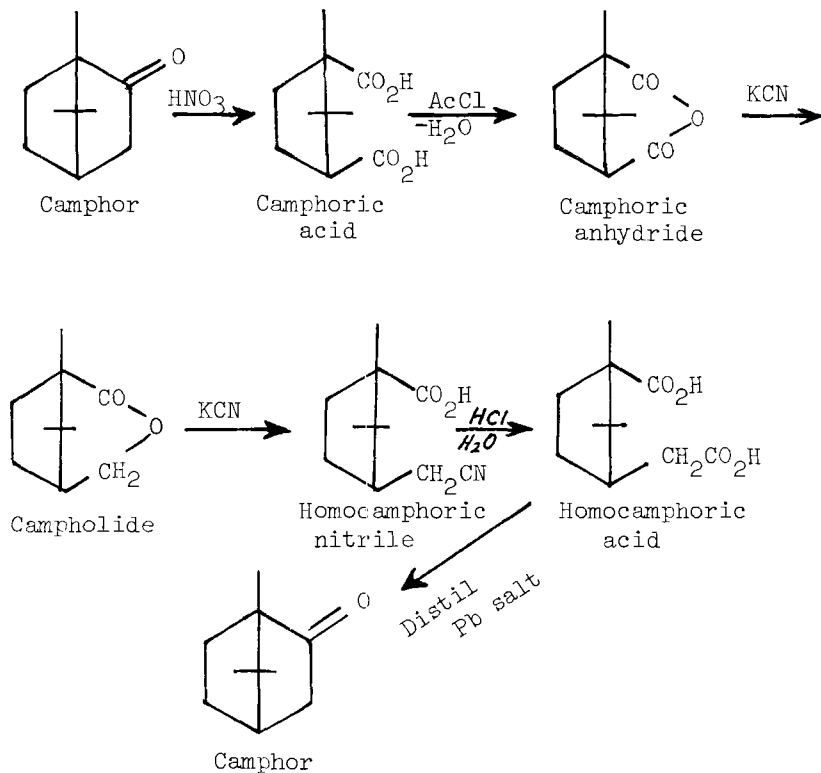
### 1.22 Structural



Camphor is a saturated ketone,  $C_{10}H_{16}O$ , which on reduction yields the corresponding hydrocarbon camphane,  $C_{10}H_{18}$ . Camphor must therefore be

bicyclic. The carbonyl group is flanked by only one reactive  $\text{CH}_2$  group, since camphor forms a monobenzylidene derivative only, in reaction with benzaldehyde. The presence of a  $-\text{CO}\cdot\text{CH}_2-$  group is confirmed by the formation of an isonitroso-derivative with nitrous acid. On distillation with phosphorus pentoxide camphor yields p-cymene. The formation of which suggests that the basic skeleton of camphor is a six membered carbon-ring, substituted in the 1- and 4- positions, and since camphor is bicyclic it is probable that this 1:4 substitution takes the form of a bridge (5,6).

A tremendous amount of work was done before the structure of camphor was successfully elucidated. Bredt (1893) was the first to assign the correct formula to camphor. The structure was confirmed by total synthesis, which was achieved by several authors. The most important aspects of this work are summarised (5) below:



1.23 CAS Registry Number

- a) Natural camphor [76-22-2]  
 b) Synthetic camphor [76-22-2] (2)

1.24 Wiswesser Line Notation

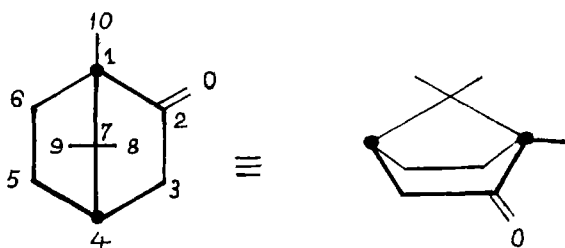
- |                      |            |               |     |
|----------------------|------------|---------------|-----|
| a) Natural camphor   | L55A<br>AB | CVTJA<br>DX   |     |
| b) Synthetic camphor | L55A<br>AB | CVTJA<br>DXLV | (7) |

1.25 Stereochemistry

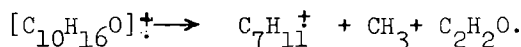
The stereochemistry of camphor is well summarised by Finar and Pinder (5,8,9).

Camphor has two dissimilar chiral centres (C-1 and C-4) and therefore four enantiomorphs might be expected. But only one pair of enantiomers is known. This is due to the fact that only the *cis*-form is possible; *trans* fusion of the *gem*-dimethylmethylene bridge to cyclohexane ring is impossible. Thus only the enantiomers of *cis*-isomer are known.

Camphor and its derivatives exist in the boat conformation. Since the *gem*-dimethyl bridge must be *cis*, the cyclohexane ring must have the boat form.



The mass spectrum of camphor shows the common peaks of isoprene :  $m/e$  27, 29, 41, 53, 67, 68. There are also the molecular ion ( $M^+$  152) and the base peak  $m/e$  95 ( $C_7H_{11}^+$ ). The base peak is probably formed as follows:



### 1.3 Molecular Weight

- a) Natural camphor 152.24
- b) Synthetic camphor 152.24 (10)

### 1.4 Elemental Composition

- C, 78.89%; H, 10.60%;
- O, 10.51%. (2)

### 1.5 Appearance, Colour, Odour and Taste.

Colourless, transparent crystals, crystalline masses, blocks of tough consistence or pulverulent masses known as 'Flowers of Camphor'; translucent mass with crystalline fracture, Rhombohedral crystals from alcohol, cubic crystals by melting and chilling; odour, fragrant, penetrating and characteristic; taste, pungent, aromatic, slightly bitter followed by a sensation of cold (2,4).

## 2. PHYSICAL PROPERTIES

### 2.1 Melting Range

- a) Natural Camphor 179.8°C Sub. 204°C
- b) Synthetic Camphor 178°C. (10,11)

### 2.2 Solubility

Soluble in 700 of water (giving colloidal solution), 1 in 0.25 of chloroform, 1 in 1 of alcohol (95%). 1 in 1 of ether, 1 in 0.4 of benzene, 1 in 0.4 of acetone, 1 in 1.5 of turpentine oil and 1 in 4 of olive oil, very soluble in carbon disulphide, light petroleum ether, fixed and volatile oils. Also soluble in concentrated mineral acids, in phenol, in liquid ammonia, and in liquid sulphur dioxide. It is insoluble in glycerin. It liquifies when triturated with chloral hydrate, menthol, resorcinol, salol,  $\beta$ -naphthol; thymol, phenol and urethane (2,12-15).

### 2.3 Non Volatile Matter

When distilled at 105°, leaves not more than 0.1% of residue (4).

## 2.4 Optical Rotation

Camphor occurs in nature is optically active (*d* and *l* forms), whereas the synthetic camphor is in racemic form. The following optical rotations were reported, for (+)-Camphor.

$[\alpha]_D^\circ$	Solvent	Ref.
$[\alpha]_D^{25} + 41$ to $43^\circ$	95% Alcohol (C=1.0 in alcohol)	(4)
+ $42.20^\circ$	90% Alcohol ( C=5 in 100 ml).	(16)
+ $42.12^\circ$	Benzene	(17)
$[\alpha]_D^{20} + 43.8^\circ$	Absolute alcohol (C=7.5 in 100 ml)	(2)

The specific rotation of camphor as 5% solution in chloroform has been determined by using a Perkin Elmer Polaromatic Model 241 MC and found to be :

$$[\alpha]_D^{25} = + 43.4^\circ$$

## 2.5 Crystal Structure

Allen and Rogers (18) have redetermined the crystal structure of (+)-3-bromocamphor to elucidate its absolute stereochemistry and which is known to have the same stereochemistry of (+)-camphor. The determination was achieved by the use of new three dimensional x-ray data.

The crystals of (+)-3-bromo camphor  $C_{10}H_{15}O$  Br, are monoclinic, space group  $PZ_1$ , with  $a = 7.36$ ,  $b = 7.59$ ,  $c = 9.12 \text{ \AA}$ ;  $\beta = 94.1$ ;  $Z = 2$ . This work has unambiguously defined the absolute stereochemistry of (+)-3-bromo camphor and hence the (+)-camphor. The latter may be represented as [1] with the *gem*-dimethyl bridge below the plane. The substitution of bromine at (C-3) in (+)-camphor gives rise to a third asymmetric centre. The [100]-projection of the structure in Fig.1 shows that bromine is *trans* to the *gem*-dimethyl bridge (*endo*-configuration) as it is in the (+)-3-bromo camphor-9- $\Pi$ -sulphonate anion [2] (19) and in bromoisofenchone (20). This contrasts with the *cis*-

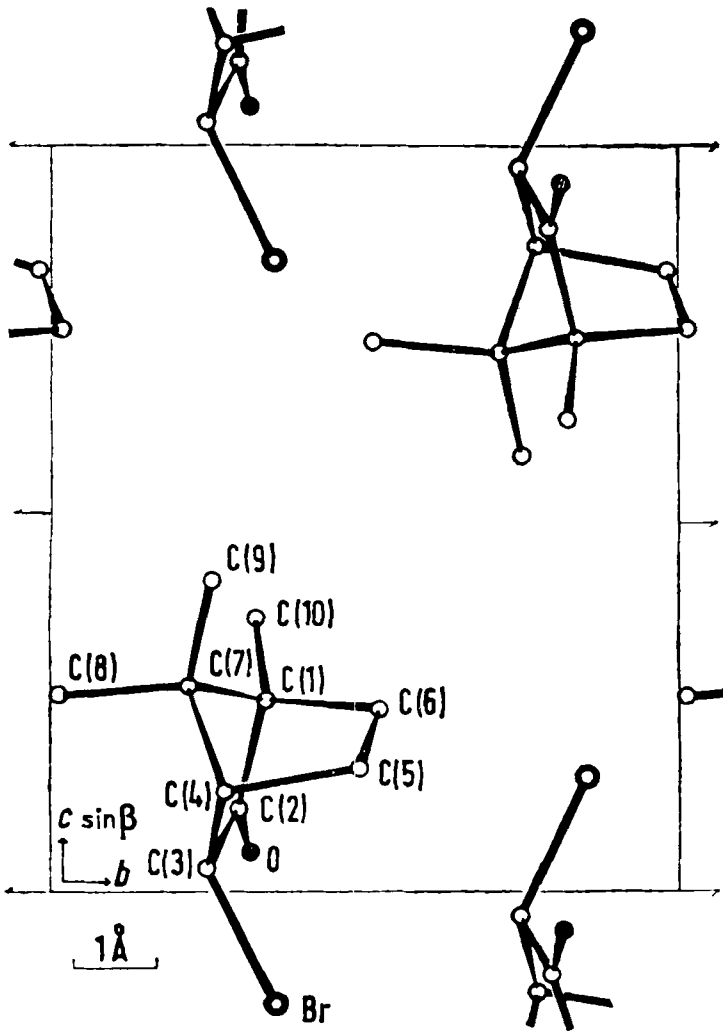
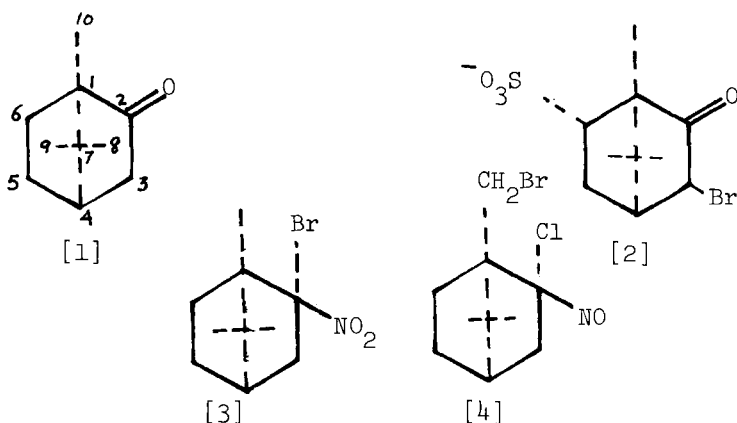


Fig. 1. A view of the structure down the  $\alpha$ -axis.



position (*exo*-configuration) adopted by the bulky halogen atom in (-)-2 bromo-2-nitrobornane [3] (21) and (+)-10-bromo-2-chloro-2-nitrosobornane [4] (22).



Bond lengths and valence angles are also given in Tables 1 and 2.

Table 1

Bond lengths/Å, estimated standard deviations in parentheses refer to the least significant figure(s):

C(1)-C(2)	1.54(2)	C(5)-C(6)	1.56(3)
C(1)-C(6)	1.49(3)	C(7)-C(8)	1.54(3)
C(1)-C(7)	1.53(3)	C(7)-C(9)	1.55(2)
C(1)-C(10)	1.52(2)		
C(2)-C(3)	1.54(2)	C(3)-H(31)	1.0(2)
C(2)-O	1.20(1)	C(4)-H(41)	1.0(2)
C(3)-C(4)	1.46(2)	C(5)-H(51)	1.1(1)
C(3)-Br.	1.924(14)	C(5)-H(52)	0.8(2)
C(4)-C(5)	1.57(3)	C(6)-H(61)	1.0(2)
C(4)-C(7)	1.54(2)	C(6)-H(62)	1.2(2)

## 2.6 Spectral Properties

### 2.61 Ultraviolet Spectrum

The UV spectrum of camphor in chloroform (Fig.2) was scanned from 200 to 400 nm using DMS 90 Varian spectrophotometer. It exhibited a  $\lambda_{max}$  at 290 nm. Also it was scanned in methanol from 200 to 400 nm using Varian Carry 219, and exhibited a  $\lambda_{max}$  at 289 nm. Other reported UV spectral data

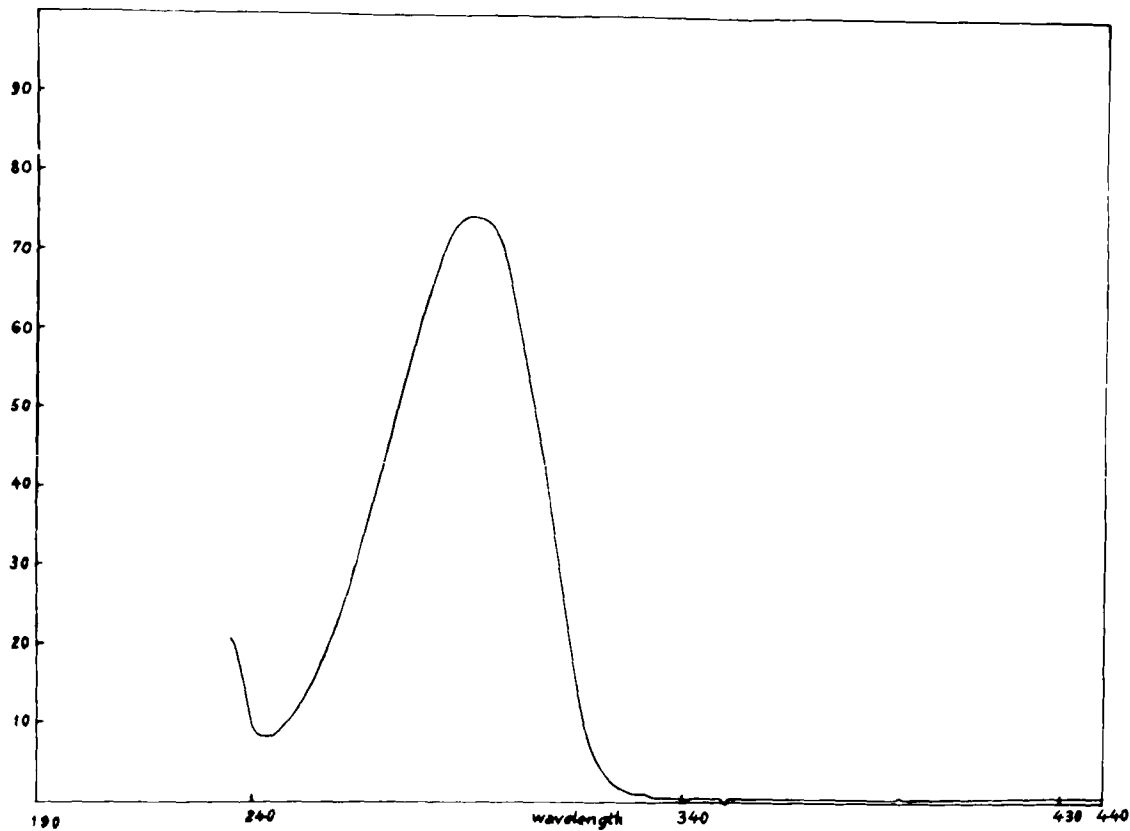


Fig. 2. UV spectrum of camphor in chloroform.

Table 2: Valency angles/ $^{\circ}$ : estimated standard deviations in parentheses refer to the least significant figure(s).

---

C(2)-C(1)-C(6)	102.8(1.4)	C(2)-C(3)-H(31)	113(8)
C(2)-C(1)-C(7)	100.8(1.4)	C(4)-C(3)-H(31)	124(8)
C(2)-C(1)-C(10)	112.3(1.2)	Br-C(3)-H(31)	86(9)
C(6)-C(1)-C(7)	102.6(1.4)	C(3)-C(4)-H(41)	111(10)
C(6)-C(1)-C(10)	115.7(2.1)	C(5)-C(4)-H(41)	128(11)
C(7)-C(1)-C(10)	120.5(1.8)	C(7)-C(4)-H(41)	99(10)
C(1)-C(2)-C(3)	105.6(1.0)	C(4)-C(5)-H(51)	101(9)
C(1)-C(2)-O	127.7(1.2)	C(4)-C(5)-H(52)	111(14)
C(3)-C(2)-O	126.6(1.2)	C(6)-C(5)-H(51)	122(7)
C(2)-C(3)-C(4)	102.0(1.1)	C(6)-C(5)-H(52)	98(11)
C(2)-C(3)-Br	112.6(1.1)	H(51)-C(5)-H(52)	120(14)
C(4)-C(3)-Br	119.1(1.3)	C(1)-C(6)-H(61)	125(11)
C(3)/C(4)-C(5)	111.4(1.6)	C(1)-C(6)-H(62)	112(8)
C(3)-C(4)-C(7)	103.2(1.2)	C(5)-C(6)-H(61)	106(10)
C(5)-C(4)-C(7)	98.3(1.4)	C(5)-C(6)-H(62)	133(7)
C(4)-C(5)-C(6)	102.3(1.6)	H(61)-C(6)-H(62)	77(13)
C(1)-C(6)-C(5)	104.1(1.6)		
C(1)-C(7)-C(4)	94.9(1.3)		
C(1)-C(7)-C(8)	112.3(1.4)		
C(1)-C(7)-C(9)	112.2(1.5)		
C(4)-C(7)-C(8)	112.6(1.5)		
C(4)-C(7)-C(9)	113.8(1.3)		
C(8)-C(7)-C(9)	110.3(1.5)		

---

for camphor in methanol (7) :  $\lambda_{\max}$ , at 289 nm;  
 camphor in ethanol (1) :  $\lambda_{\max}$ , at 289 nm (E1%,  
 1 cm<sup>2</sup>); as 0.25 percent w/v solution in alcohol  
 (95 percent) exhibits a  $\lambda_{\max}$  at 289 nm (E 1.06)  
 (4) and in chloroform  $\lambda_{\max}$ , at 292 nm (2).

## 2.62 Infrared Spectrum

The IR spectrum of camphor as KBr disc and Nujol mull were recorded on a Perkin Elmer 580 B Infrared spectrophotometer to which Infrared data station is attached (Fig. 3). The structural assignments have been correlated with the following frequencies (Table 3).

Table 3: IR Characteristics of Camphor.

<u>Frequency cm<sup>-1</sup></u>	<u>Assignment</u>
3450 b	OH or H <sub>2</sub> O
1744 s	C = O Stretch.

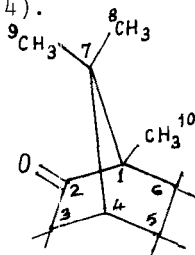
Other characteristic bands are:

2959, 2873, 1471, 1451, 1418, 1391, 1372, 1324, 1278, 1094, 1046, 1021, 751, and 521. The Infrared data for (+), (-) and (±)-Camphor are also reported (7,23). The reported data are in agreement with our data except for the hydroxyl band at 3450 cm<sup>-1</sup>.

## 2.63 Nuclear Magnetic Resonance Spectra

### 2.631 Proton Spectrum

The PMR spectrum of camphor in CDCl<sub>3</sub> was recorded on a Varian T-60A, 60 MHz NMR spectrometer using TMS (Tetramethylsilane) as an internal reference (Fig. 4). The following structural assignments have been made (Table 4).



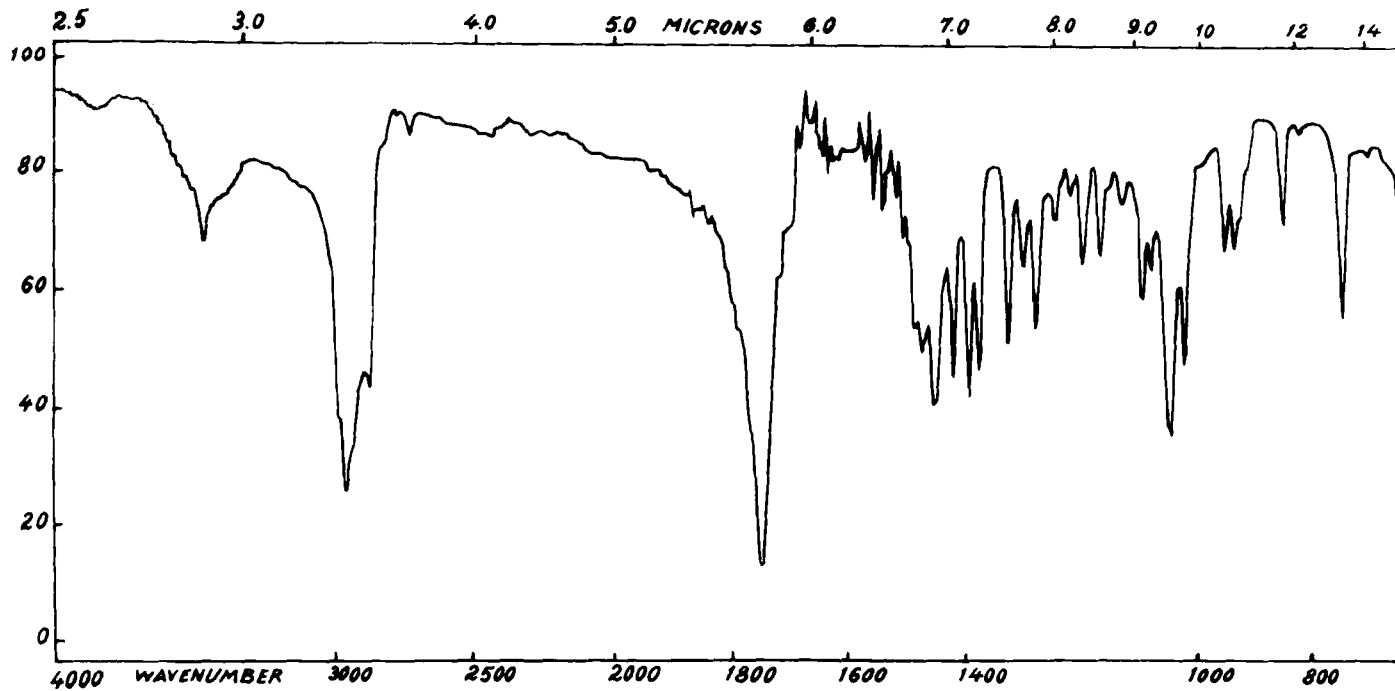


Fig. 3. IR Spectrum of camphor as K Br disc.

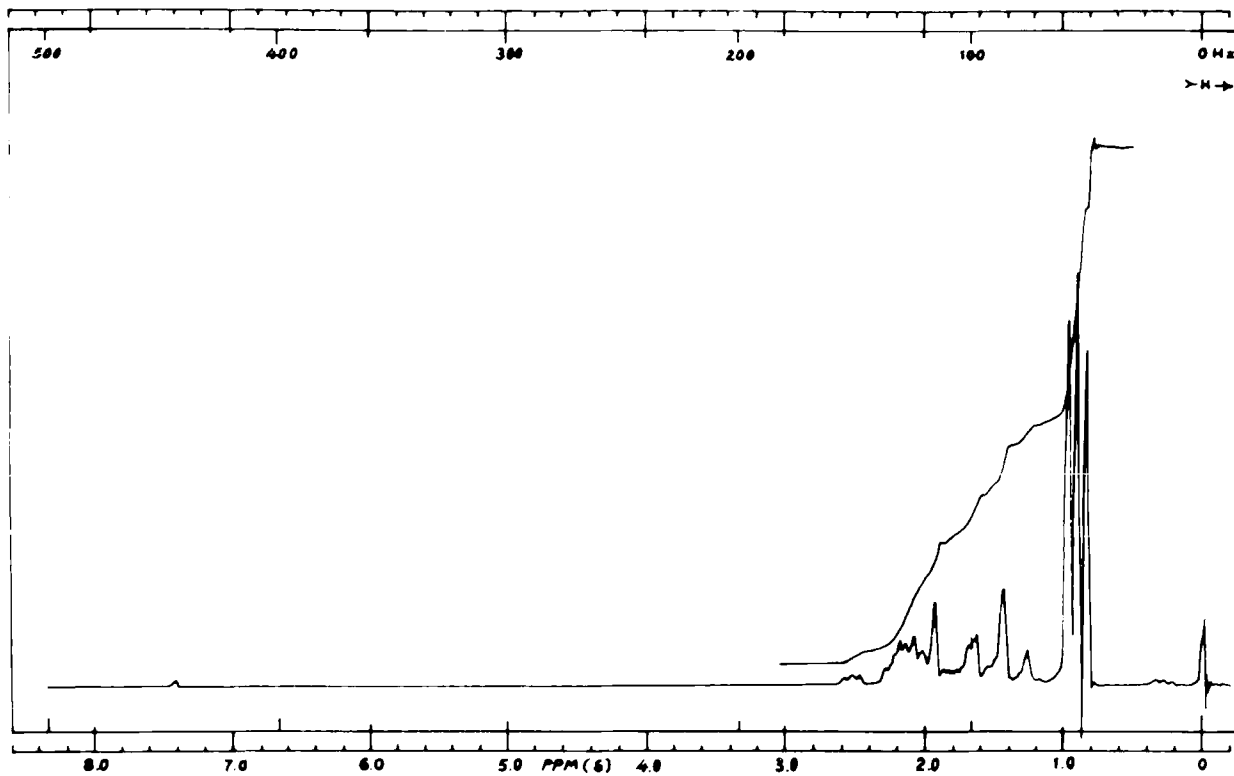


Fig. 4. PMR spectrum of camphor in  $\text{CDCl}_3$ .

Table 4. PMR Characteristics of Camphor

Group	Chemical shift (ppm)
Methylene at (C-2, C-4 and C-5) .	2.5 m 2.07 m
and Methine at C-3 protons.	1.43 bt
C-9 Me	0.93 s
C-8 Me	0.88 s
C-10 Me	0.83 s

s = singlet; t = triplet; bt = broad triplet; m = multiplet.

Our assignment of the methyl protons singlets is based on the anisotropy of the carbonyl function of camphor. The 9-methyl protons are falling within the deshielding zone of the carbonyl group and is expected to be the most lower field while C-10 methyl protons are away from the carbonyl function and is expected to suffer no deshielding effect. Our Model is shown in Fig. 5. In contrast C-8 methyl protons will have an intermediate chemical shift value (24).

Other reported data (25-29) are in agreement with our data except for the assignments of C-8 and C-10 methyl protons resonances and which are listed below:

	C-8	C-9	C-10
Our values.....	0.88	0.93	<u>0.83</u>
Ref. 7 and 8.....	<u>0.85</u>	0.92	0.98
Ref. 9 (deuterium substitution).....	<u>0.85</u>	0.98	0.92
Ref. 10 (using metal Reagent shifts)....	<u>0.83</u>	0.95	0.86

#### 2.632 <sup>13</sup>C-NMR Spectra

The <sup>13</sup>C-NMR noise-decoupled and off-resonance spectra (Fig. 6 and 7), were recorded over 5000 Hz width in CDCl<sub>3</sub> on Varian FT-80, 80 MHz NMR spectrometer, using a 10 mm sample tube and TMS as a reference standard at 20°.

The carbon chemical shifts are assigned on the basis of the off-resonance splitting pattern and additivity principles and is

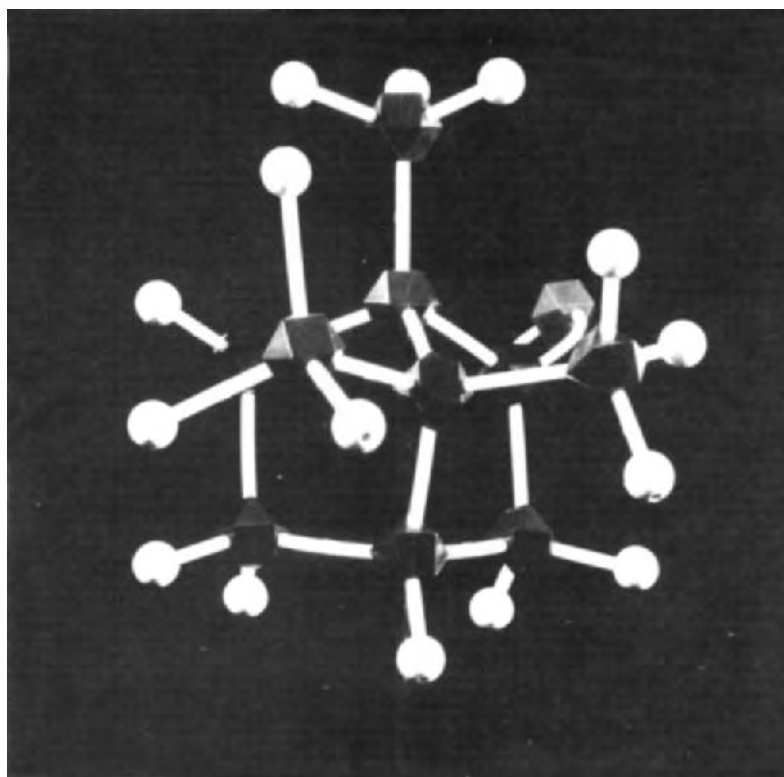


Fig. 5. Camphor model.



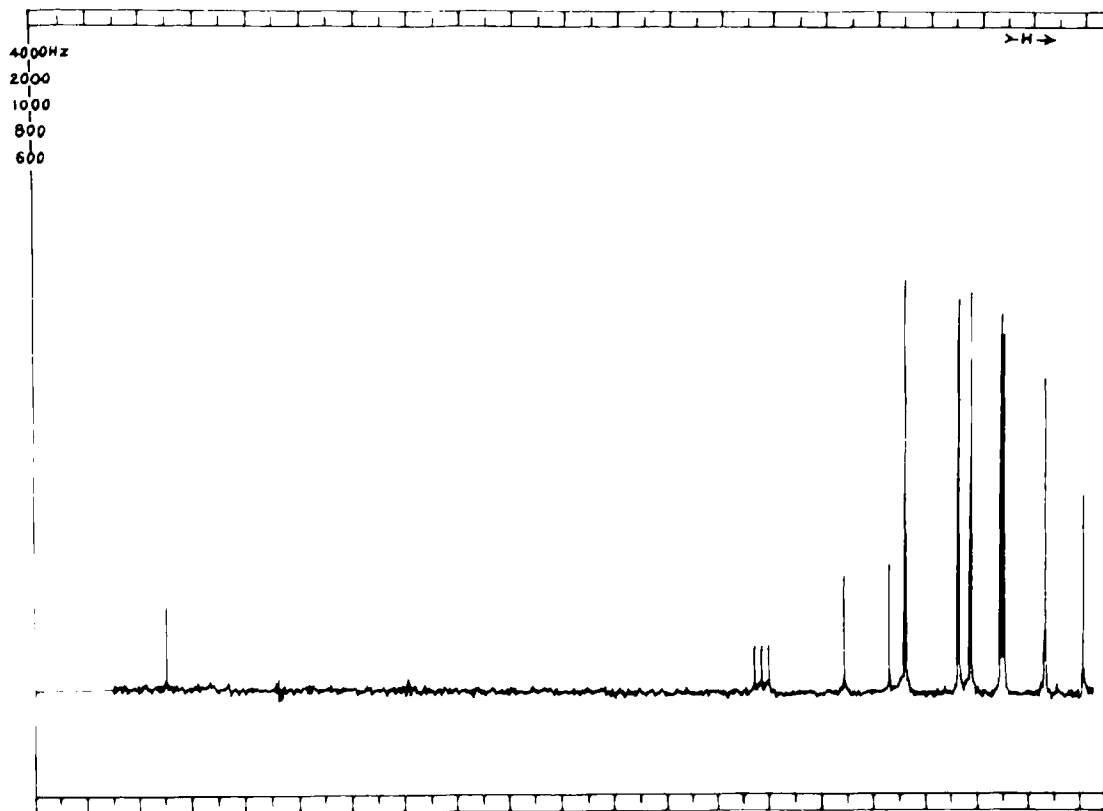


Fig. 6.  $^{13}\text{C}$ -NMR noise-decoupled spectrum of camphor in  $\text{CDCl}_3$ .

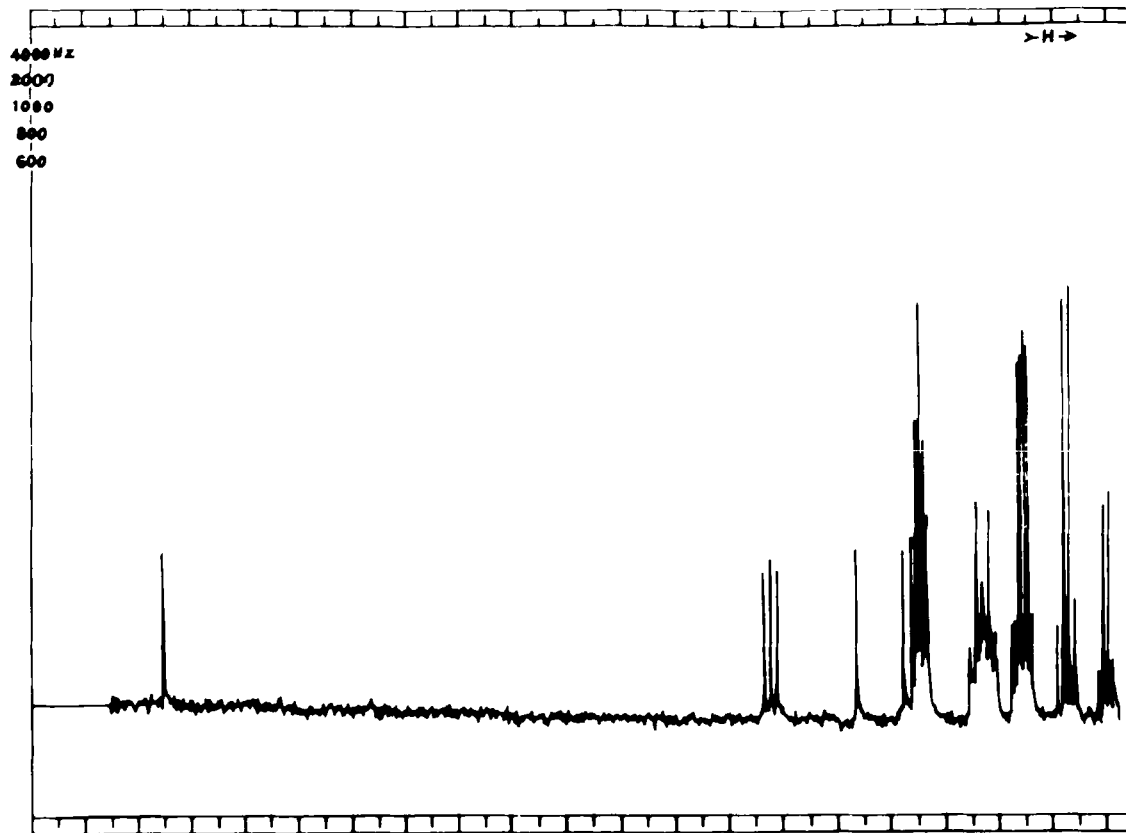
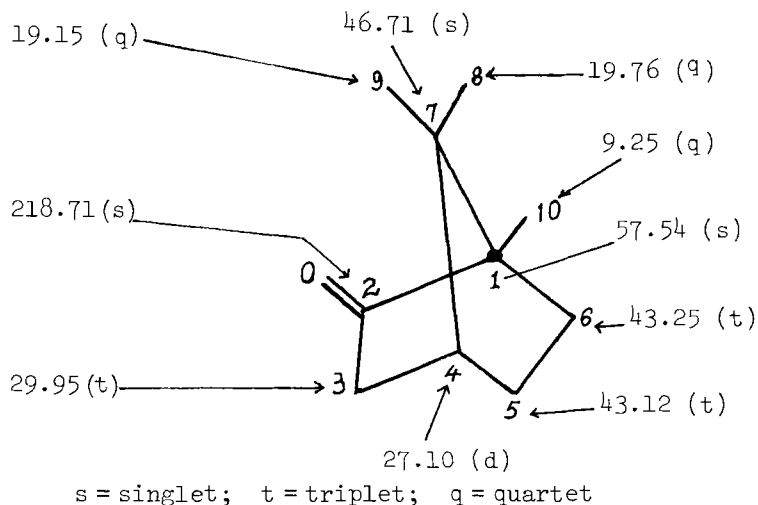


Fig. 7.  $^{13}\text{C}$ -NMR off-resonance spectrum of camphor in  $\text{CDCl}_3$ .

shown in the structure below. Other reported data are also available (7,23,30).



### 2.64 Mass Spectrum

The mass spectrum of camphor obtained by electron impact ionization (Fig. 8) which was recorded on a Varian Mat 112S mass spectrometer. The spectrum was scanned from 40" up to 250 a.m.u. Electron energy was 70eV. The mass spectral data are shown in Table 5. (31-33).

Table 5: The Most Prominent Fragments of Camphor

<u>m/e</u>	<u>Relative Intensity.</u>	<u>Fragment</u>
95	100	Base peak (M-57)(C <sub>7</sub> H <sub>11</sub> ) <sup>+</sup>
152	18	(M <sup>+</sup> )
137	2	(M-CH <sub>3</sub> )
110	11	(M-CH <sub>2</sub> = C = O)
109	28	(C <sub>8</sub> H <sub>13</sub> <sup>+</sup> )
108	38	(C <sub>8</sub> H <sub>12</sub> <sup>+</sup> )
83	43	(C <sub>6</sub> H <sub>11</sub> <sup>+</sup> )
81	71	(C <sub>6</sub> H <sub>9</sub> <sup>+</sup> )

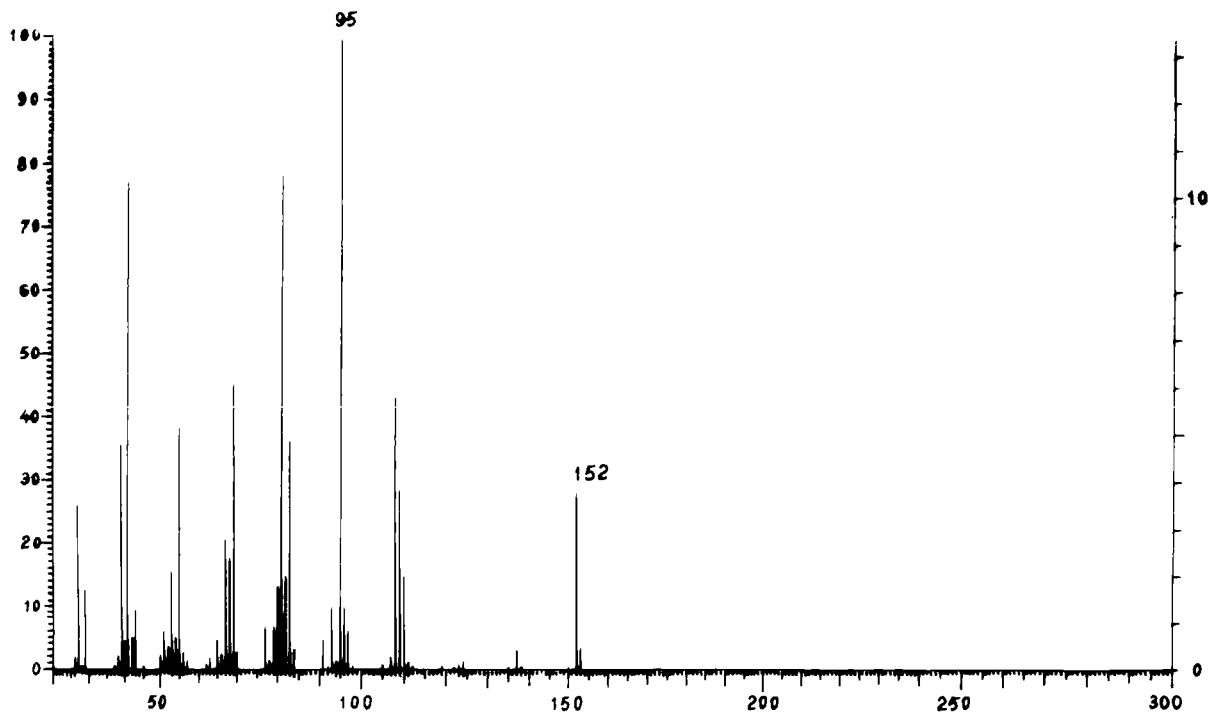
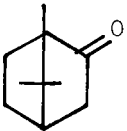
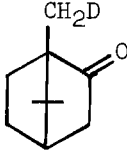
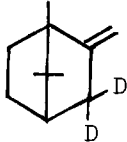
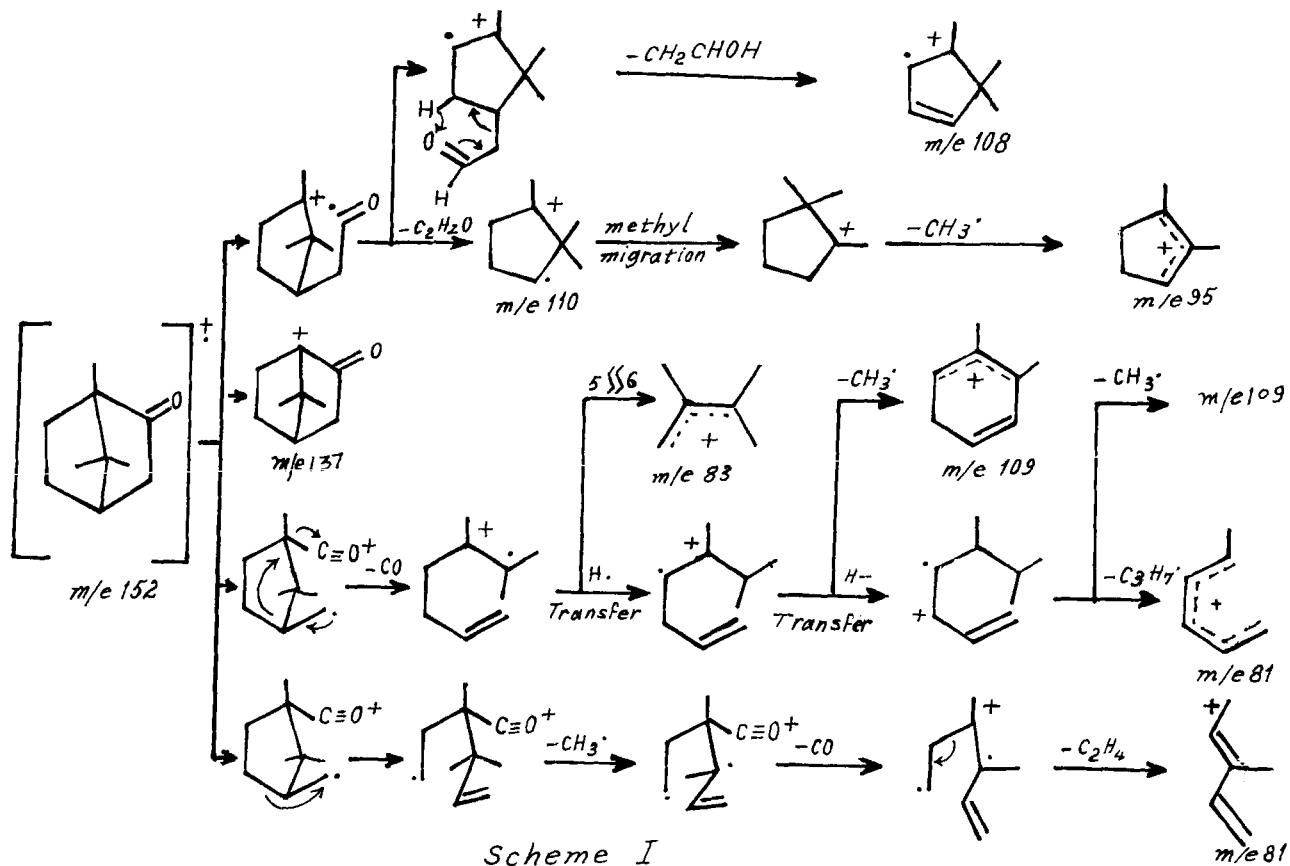


Fig. 8. EI-mass spectrum of camphor.

Table 6: Principal Mass Spectra Peaks of Camphor and Deuterated Analogs.

Compound	Isotopic purity, %	m/e (% shift)						
		M - 15	M - 42	M - 43	M - 57	M - 57	M - 69	M - 71
		137	110	109	108	95	83	81
	92 d <sub>1</sub>	138(66)	111(q)	109(35)	109(q)	95(44)	84(q)	82(93)
	8 d <sub>0</sub>	137(33)		110(65)		96(56)		
	95 d <sub>2</sub>	139(q)	110(q)	109(12)	108(q)	95(q)	83(q)	83(80-90)
	5 d <sub>1</sub>			111(88)				

The symbol q refers to a quantitative transfer (i.e., > 95%).



Reed (34,35) and Von Sydow (36) have examined recently the mass spectrum of Camphor and offered a highly speculative explanation of the fragmentation of camphor on electron impact. Weinberg (37) have studied the mass fragmentation of camphor and its deuterated derivatives and proposed fragmentation reactions (Table 6 and Scheme 1) based on labeling and high-resolution results.

### 3. PREPARATION OF CAMPHOR

Camphor is the main constituent of camphor oil which is obtained from the wood and the leaf of camphor tree, *Cinnamomum camphora* (Linne) Nees et Ebermaier, family Lauraceae. The plant grows well in Japan, China, Formosa, India, Burma and Malaysia. Camphor also occurs in certain species of *Artemisia*, (*Compositae*) *chrysanthemum* (*compositae*), *Salvia* (*Labiatae*), *Ocimum* (*Labiatae*); *Lavander* (*Labiatae*), *Pinus* (*Pinaceae*). It is also present in *Rosemarinus officinalis* (*Labiatae*), *Aristolochia indica* (*Aristolochiaceae*), *Blumea balsamifera* (*Camphreaceae*), *Prunella vulgaris* (*Labiatae*), *Cinnamomum glanduliferum* (*Lauraceae*) etc (38-55).

Commercially camphor is produced from *cinnamomum camphora*. The production of camphor was started much earlier, probably at the end of 13<sup>th</sup> century. In the middle of the 15<sup>th</sup> century, numerous camphor industries were established in China, Japan and Formosa. Any how the major production of camphor was obtained from Japan and Formosa. The production of camphor from the natural source is well summarised below:

The trees over 40 years old are felled, their roots are dug out. The roots and the trunks of the camphor trees are brought to the factory where they are reduced to small chips. The camphor oil is then distilled with steam in special, rather wooden still (Fig. 9). The camphor oil which accumulates in the condensing vessels is usually emptied once a month. About 1/3 of the pure camphor, present in the oil is crystalized out and it is separated from the liquid by straining. The camphor remaining in the oil is recovered either by fractional distillation or by freezing out or by the formation of complexes with the strong acids.

The camphor thus obtained is further purified by mixing with soda lime, sand, charcoal and subjected to sublimation

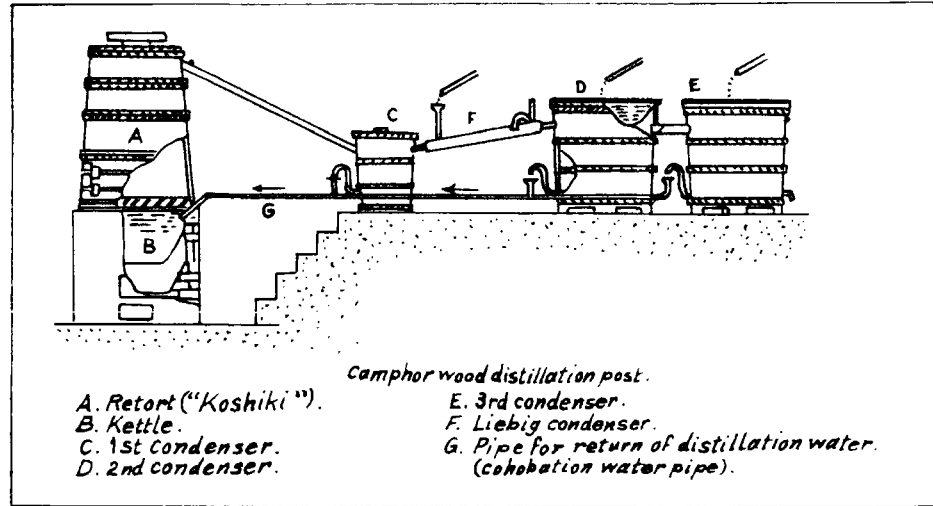


Figure 9.



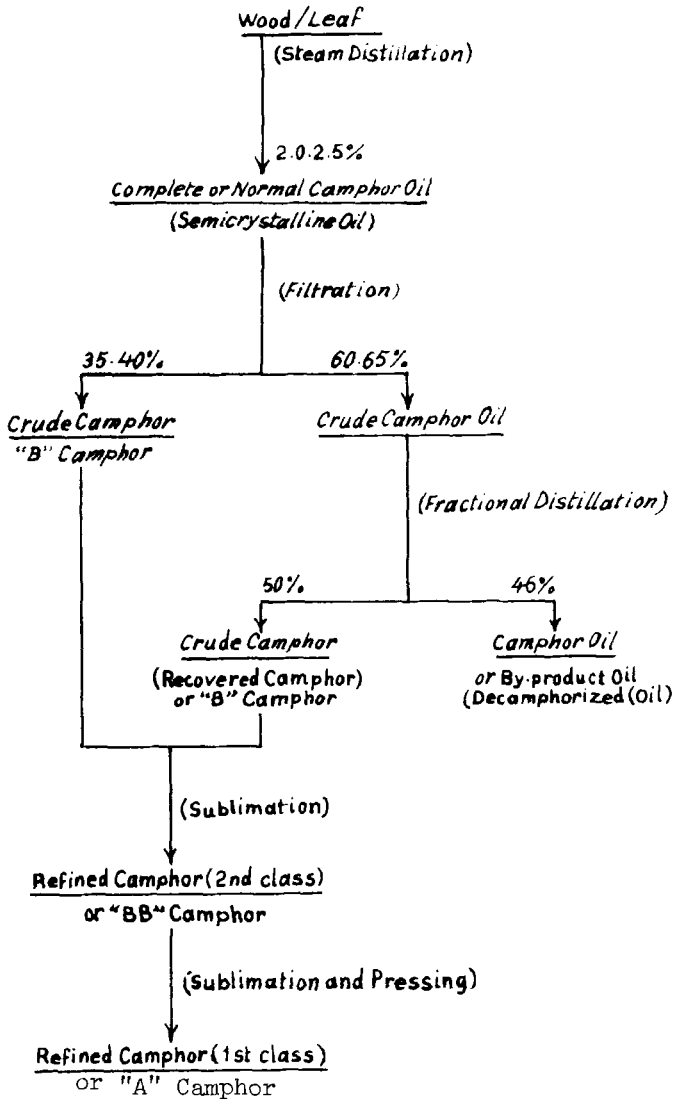


Figure 10.

where camphor will sublime in pure state (56,57).  
The procedure outline is presented in Figure 10.

#### 4. SYNTHESIS OF CAMPHOR

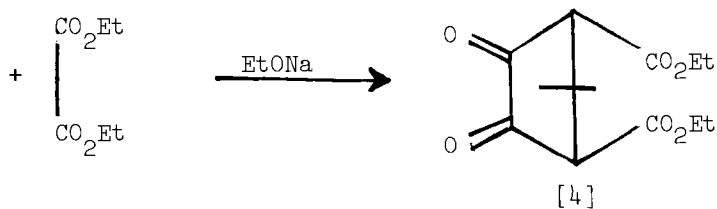
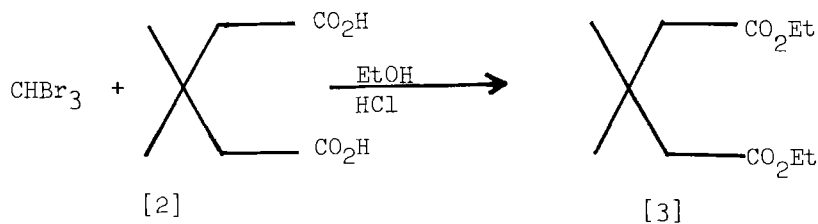
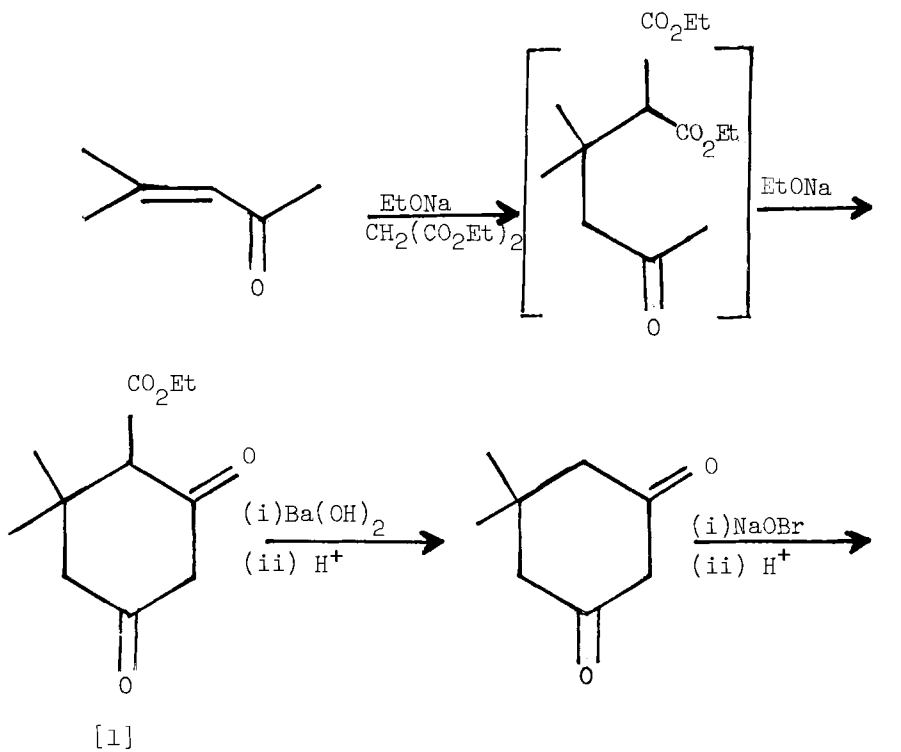
Many methods of preparing camphor are described in technical journals and in the patent literatures(5,8,58-62).

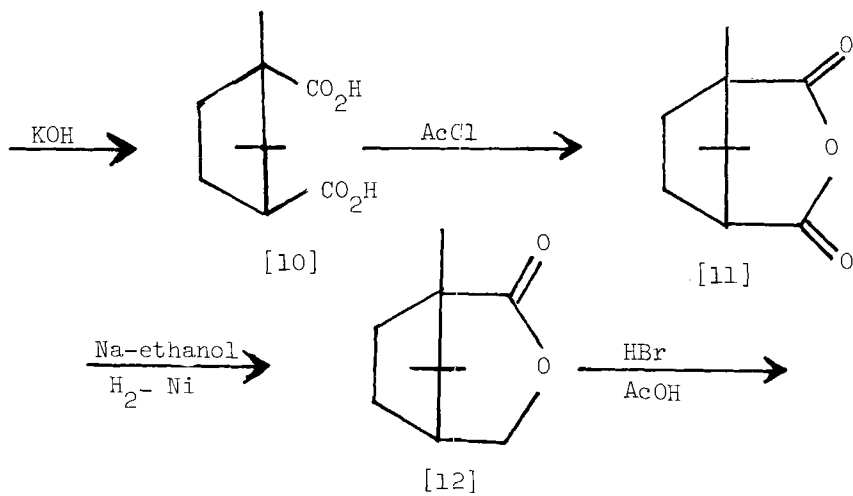
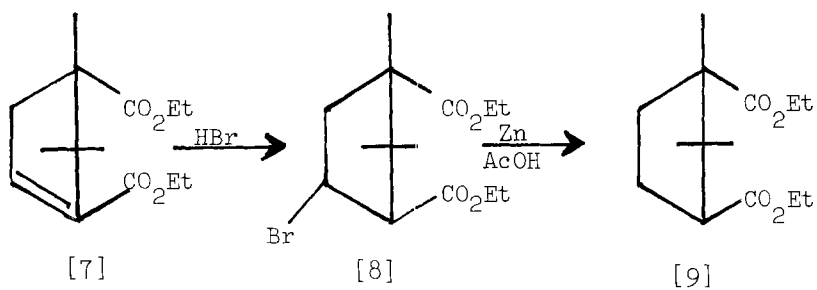
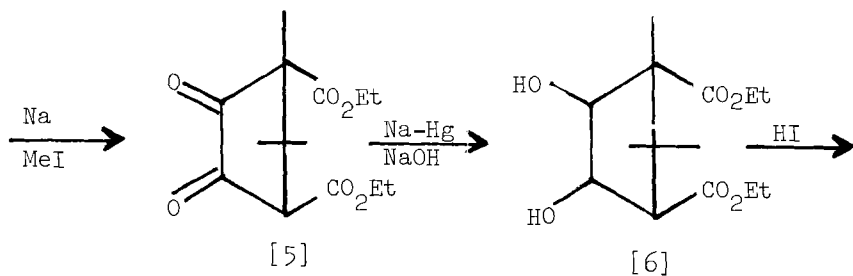
##### 4.1 Total Synthesis

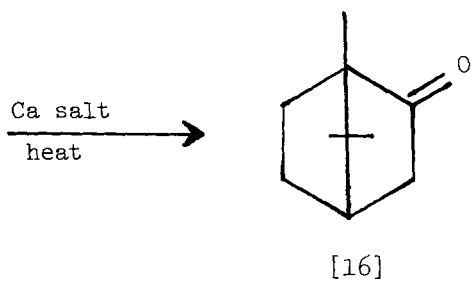
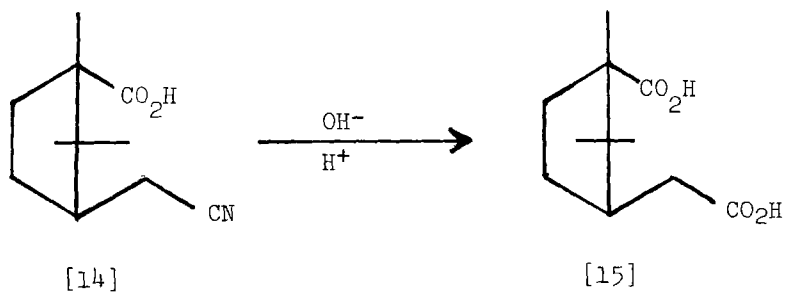
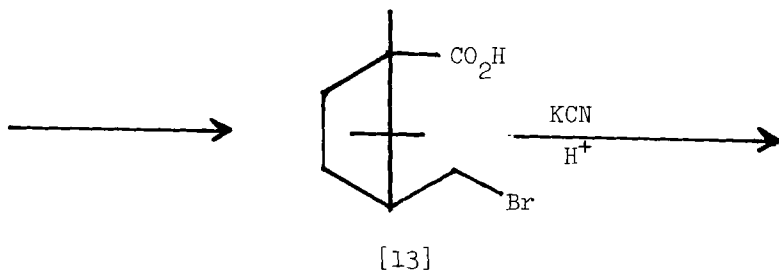
Route I: The bicyclic ketone structure of camphor proposed by Bredt in 1893 was confirmed by total synthesis (58,59) as follows:

Komppa (1903) first synthesised (+)-Camphoric acid followed by synthesis of (+) camphor in 1908.

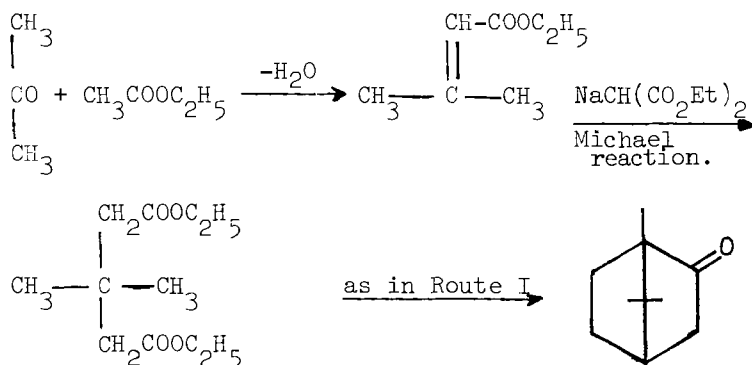
Komppa (1889) first synthesised 3,3-dimethylglutaric ester starting with mesityl oxide and ethyl malonate. The product obtained was 6,6-dimethylcyclohexane-2,4-dione-1-carboxylic ester [1]. (This is produced first by a Michael condensation, followed by a Dieckmann reaction). On hydrolysis followed by oxidation with sodium hypobromite, 3,3 dimethylglutaric acid [2] was obtained. Esterification of which with ethanol and HCl afforded diethyl  $\beta\beta$ -dimethylglutarate [3]. Diethyl oxalate and diethyl  $\beta\beta$ -dimethylglutarate condense to yield the cyclopentanedione [4], which being a  $\beta$ -Ketoester, can be C-methylated to diketocamphoric ester [5]. Sodium amalgam reduction converts the diketone [5] to the glycol [6]. Treatment with hydroiodic acid effects a reduction and dehydration to cyclopentene diester [7]. The bromo derivative [8] of which yields diethylcamphorate [9] on debromination. A final hydrolysis affords (+)-camphoric acid [10] as a mixture of *cis*- and *trans*-forms. The *cis*-form is separated by conversion to its anhydride [11], dl-camphoric anhydride is reduced by sodium and absolute ethanol or hydrogen and nickel to dl-campholide [12], which on treatment with HBr and AcOH gives dl-bromcampholic acid [13]. The camphohalide and KCN form dl-cyanocampholic acid [14]. [14] when boiled with KOH it is transformed into dl-homocamphoric acid [15]. Distillation of (15) with  $\text{Ca}(\text{OH})_2$ , gives dl-camphor [16].

Route I





Route II: By Perkin and Thorpe (60,61).  
as follows:



diethyl  $\beta$ -dimethylglutarate.

Route III: More recently a neat alternative synthesis of camphoric acid has been described by Alder (62). 1:1:2-Trimethylcyclopentadiene [1] and diethyl-acetylenedicarboxylate [2] undergo a diene addition reaction to give the diester [3]. Partial reduction yields [4], which is oxidised to the bis-glyoxalate [5] by nitric acid. The latter on decarbonylation yields diethyl camphorate [6] which on hydrolysis affords camphoric acid [7]. Camphoric acid is converted to camphor as described in Route I.

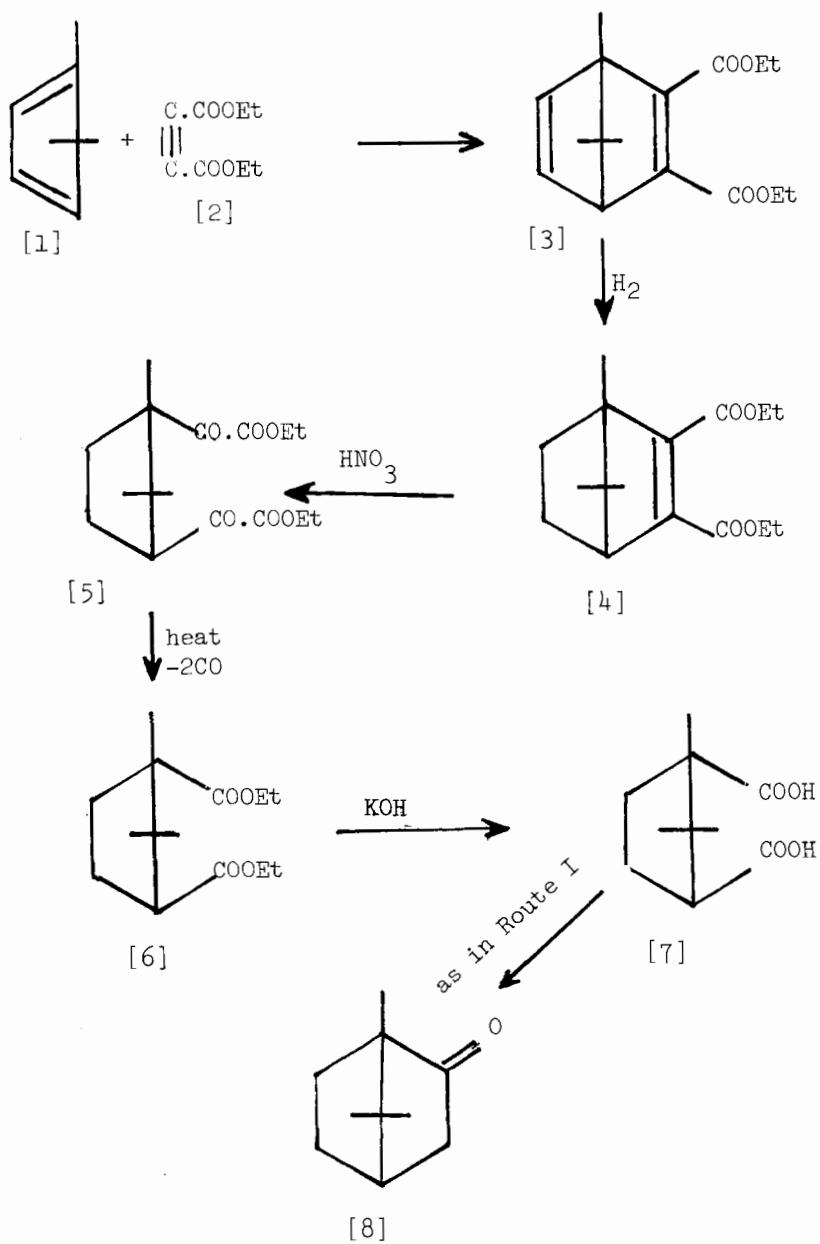
#### 4.2 Partial Synthesis

Many routes for the partial synthesis of camphor are described (5,8, 63,64).

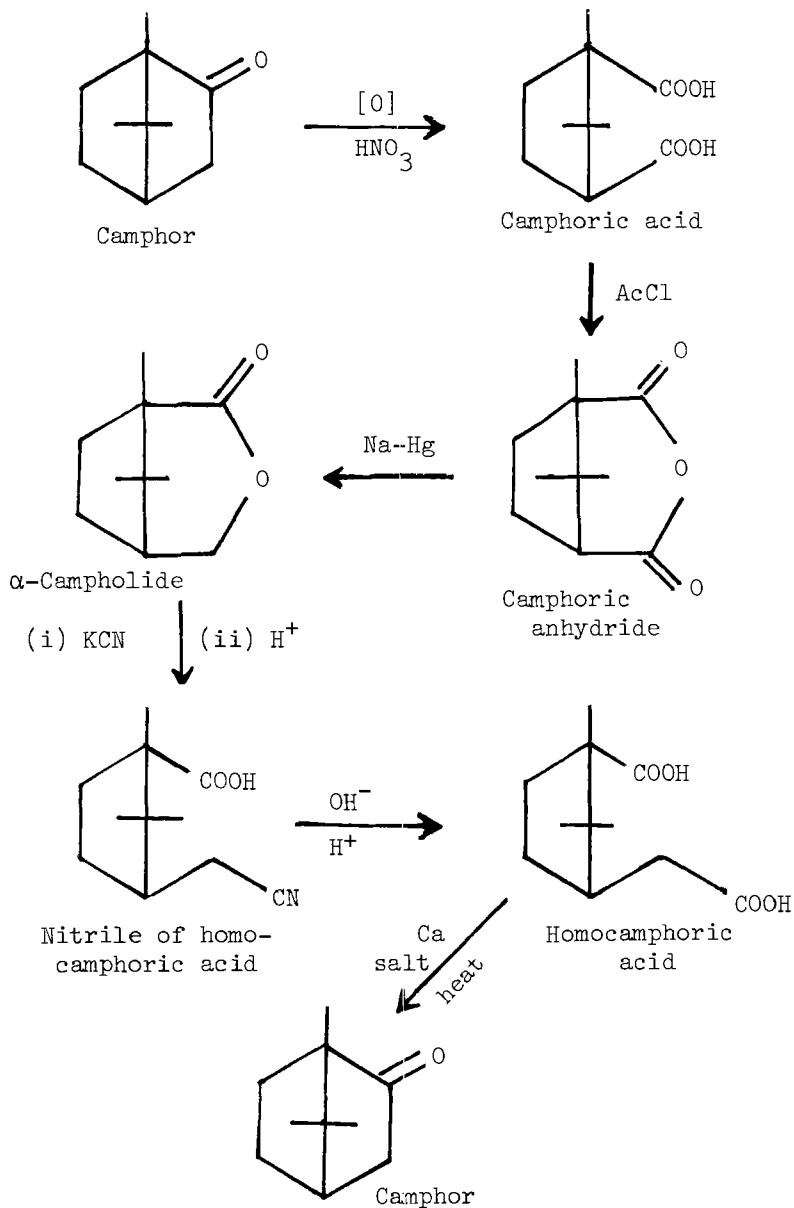
Haller, 1896 started with camphoric acid prepared by the oxidation of camphor as illustrated in Scheme II.

This is not unambiguous synthesis, since the campholide obtained might have had the structure of the  $\beta$ -campholide [1] which in turn will give homo-camphoric acid [2] and this would have given camphor with structures [3] which was rejected (Scheme IIa).

Sauers (1959) has now oxidised camphor directly to the  $\alpha$ -campholide by means of peracetic acid. It is also of interest to note that Otvos *et al* (1960) have shown, using labelled -  $\text{CH}_2\text{C}^*\text{O}_2\text{H}$  ( $^{14}\text{C}$ ), that in the pyrolysis of the calcium salt of homocamphoric acid to camphor, it is the labelled carboxyl group that is lost.

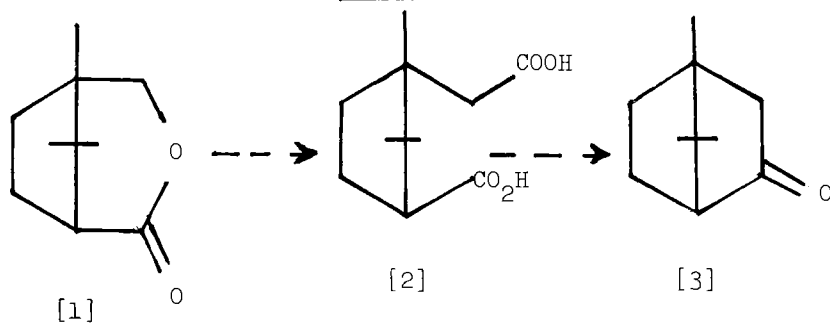
Route III

Scheme II

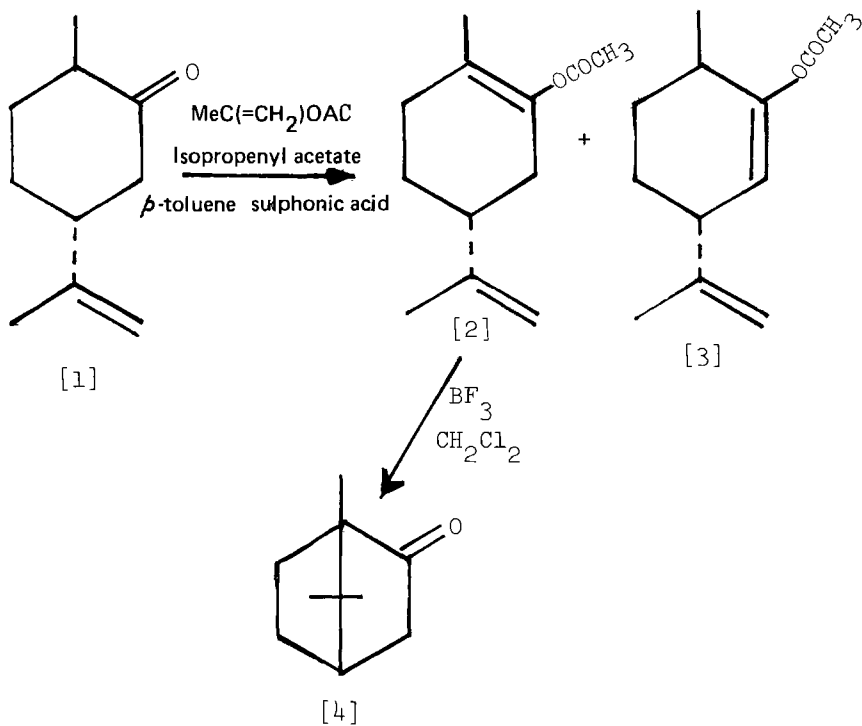




Scheme IIa.



Scheme III

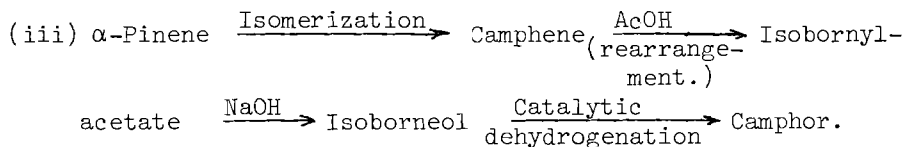
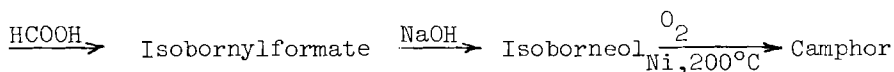
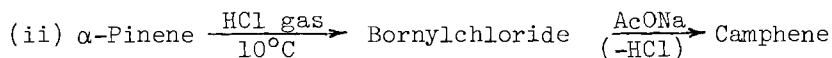
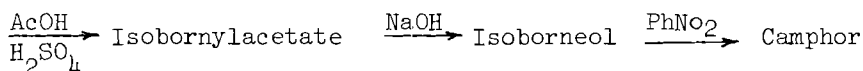
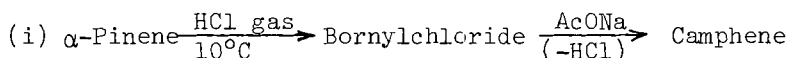


Money *et al* (1969) (65) have now carried out a two step synthesis of ( $\pm$ )-camphor from dihydrocarvone. (Scheme III).

Dihydrocarvone [1] was treated with isopropenyl acetate in the presence of *p*-toluene sulphonic acid and converted into a mixture of enol acetates [2] and [3], separated by GLC. Treatment of [2] with boron trifluoride in methylene chloride at room temperature for 10 minutes gave (+) Camphor [4]. This synthesis is particularly interesting in that it is a chemical analogy for the biosynthesis conversion of a monocyclic into a bicyclic monoterpenoid.

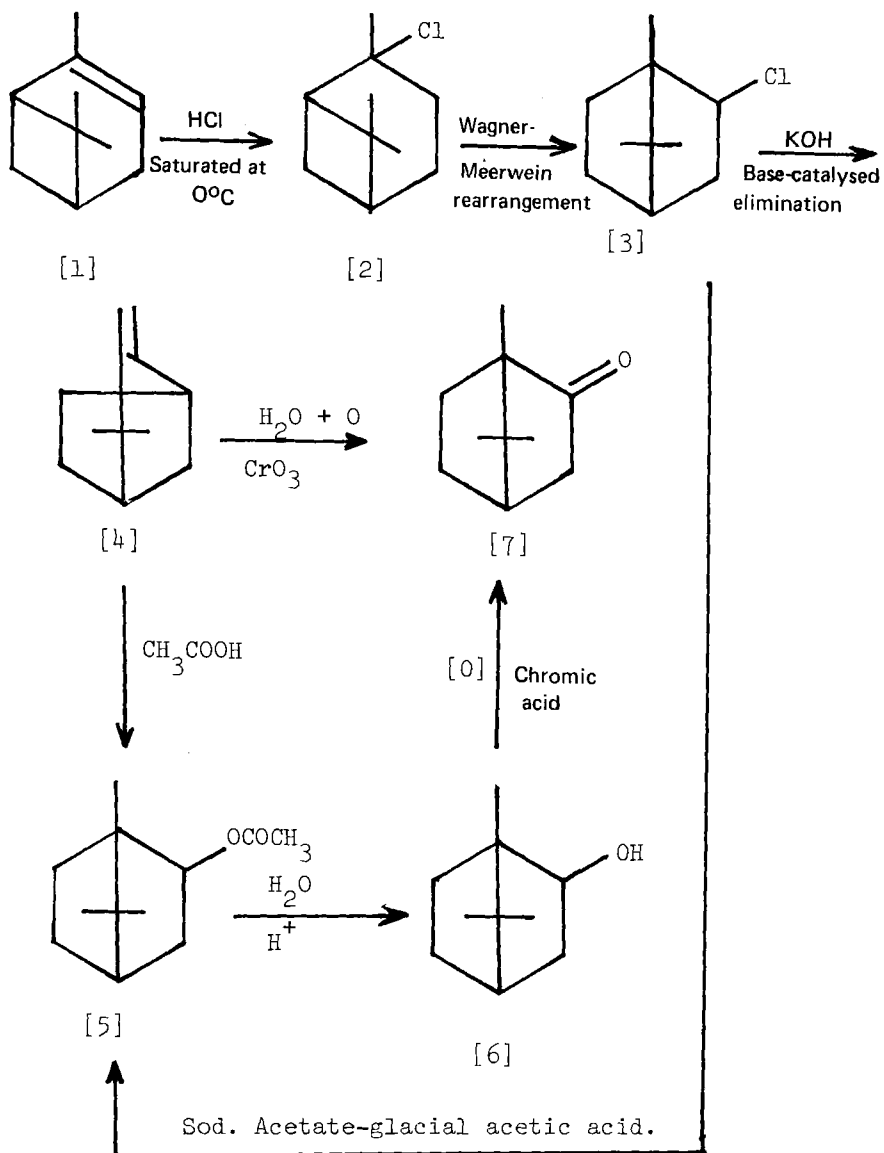
#### 4.3 Commercial Synthesis

Camphor is of considerable importance technically, being used in the manufacture of celluloid and medicinal products. It is manufactured industrially from  $\alpha$ -pinene, obtained from turpentine, by several processes (66-107) which differ mainly in detail. Synthetic camphor is usually obtained as the racemic modification. The formation of camphor involves the Wagner-Meerwein rearrangements, e.g.:



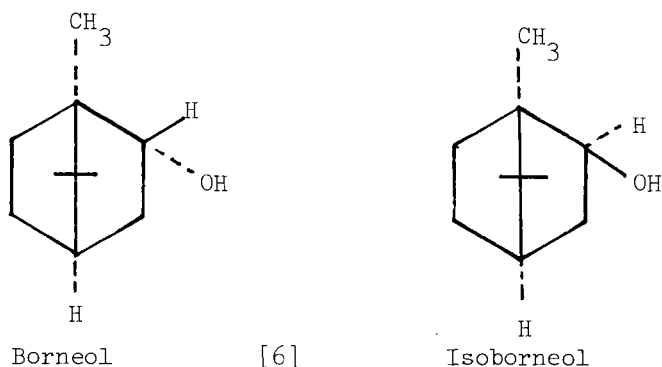
One such process is described in detail in Scheme IV.

Scheme IV



The most straightforward method involves the conversion of pinene [1] into pinene hydrochloride [2], bornylchloride [3] and hence into borneol [6] (or isoborneol) from which camphor [7] is obtained by oxidation. Also bornylchloride [3] is converted to isobornylacetate [5] upon heating with sodium acetate and glacial acetic acid. Alternatively, camphene [4] is oxidatively hydrated to camphor [7].

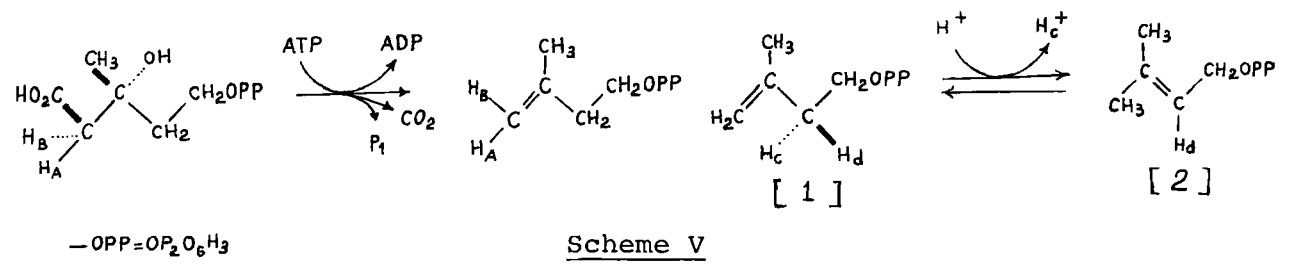
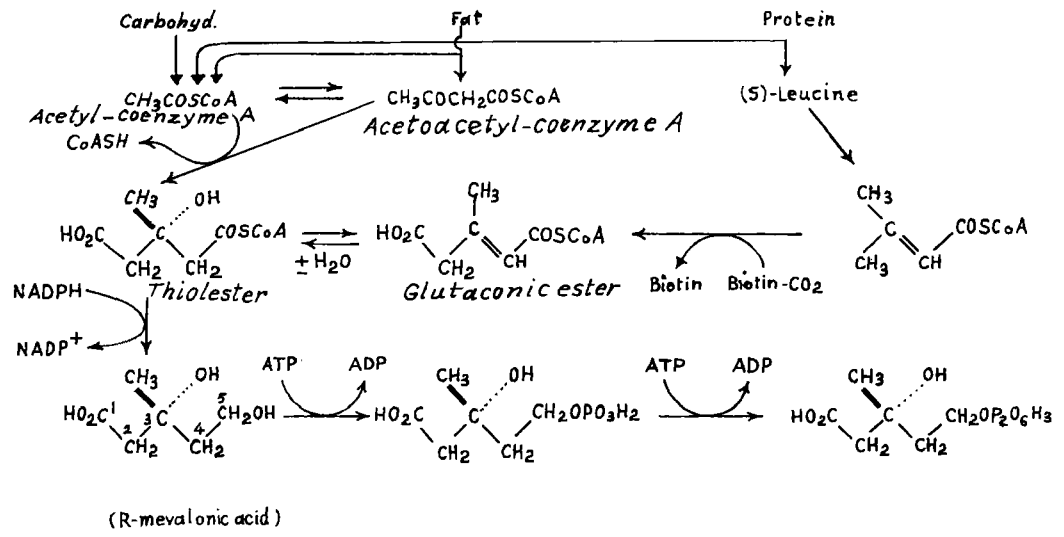
By starting with optically active  $\alpha$ -pinene, optically active camphor can be synthesised. Commercial camphor is best purified by sublimation.



## 5. BIOSYNTHESIS OF CAMPHOR

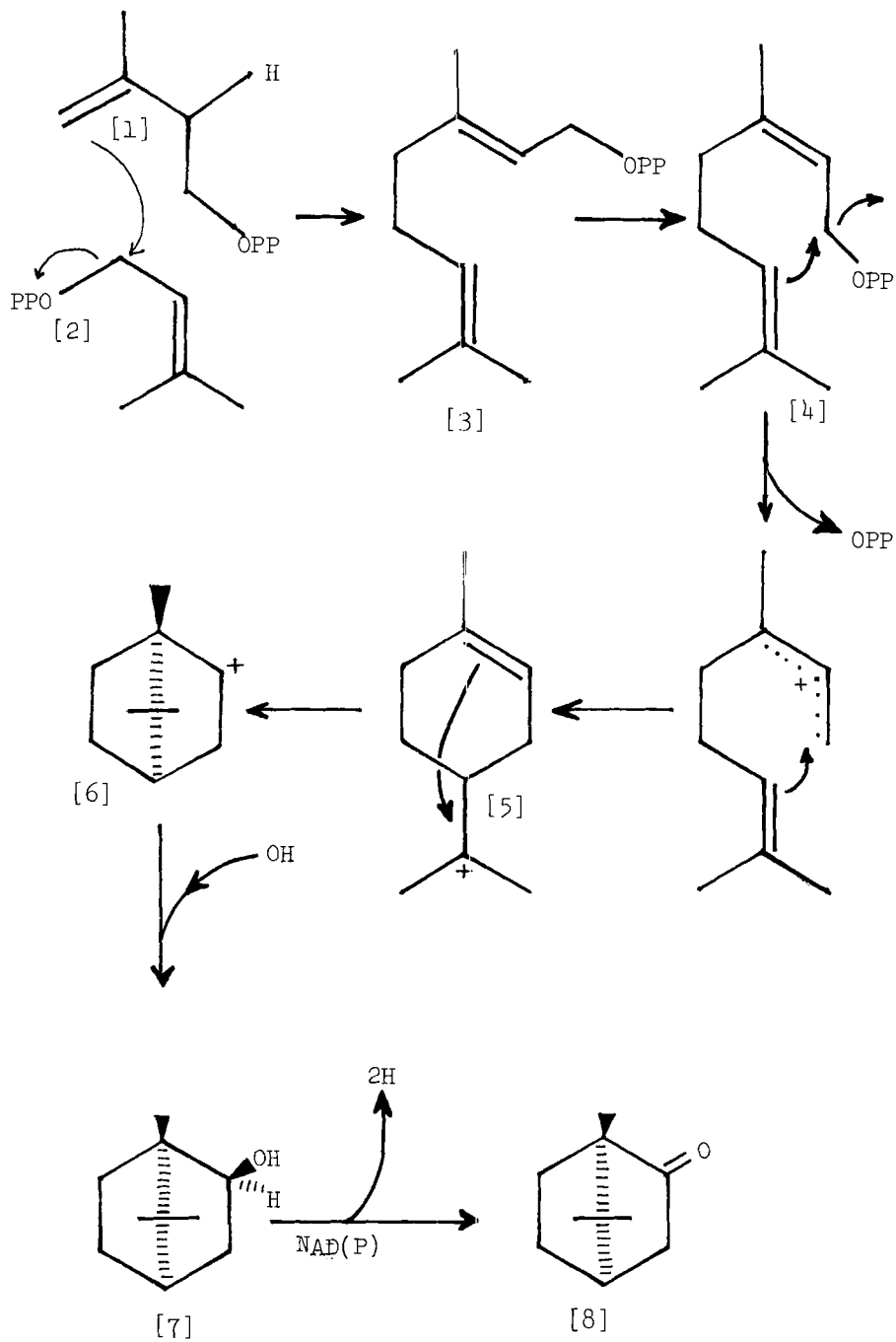
The biosynthesis of monoterpenoids and camphor has been described by several authors (108-114). Ruzicka (115,116) proposed a unified biogenetic scheme for terpenes. The biosynthetic building blocks for these terpenes are isoprene units. The biosynthetically active isoprene units are isopentenyl pyrophosphate [1] and dimethyl allyl pyrophosphate [2] the compounds that are derived from acetate via mevalonic acid (Scheme V). Geranyl pyrophosphate [3] is the C-10 precursor for the terpenes (117). Banthorpe and Baxendale (118) confirmed the biosynthetic pathway of camphor via acetate mevalonate by conducting degradation study of camphor, biosynthesized from  $^{14}\text{C}$  labelled mevalonic acid. The biosynthesis of camphor is summarised in Scheme VI.

The isopentenyl pyrophosphate [1] derived from mevalonic acid isomerized into dimethyl allyl pyrophosphate [2] and



Scheme V

Scheme VI



the subsequent condensation yield geranyl pyrophosphate [3]. The geranyl pyrophosphate again isomerized into neryl pyrophosphate [4]. The neryl pyrophosphate will be converted into a common enzyme bound cyclic intermediate,  $\alpha$ -terpineyl cation [5] which would undergo proton loss from C-5 and 1-2 hybriide shift with the expulsion of enzyme to yield borneyl cation [6]. This on further cyclization gives borneol [7] and then finally camphor [8].

## 6. METABOLISM OF CAMPHOR

(-) and (+) Camphor are metabolised by hydroxylation of a methylene group and subsequent glucuronide conjugation. Hydroxylation produced the 3-*endo* derivative [2] and the 5-*endo*-derivative [3], the latter predominating. These results are consistent with the more strained nature of the 5-versus the 3-position of camphor. The two hydroxyl derivatives canjugate with glucuronic acid to give the 3- and 5-glucuronides respectively and excreted as such. Also it is hydroxylated in the 2-position to give (+) borneol [1] (119,120).

Metabolism of Camphor is presented in Scheme VII.

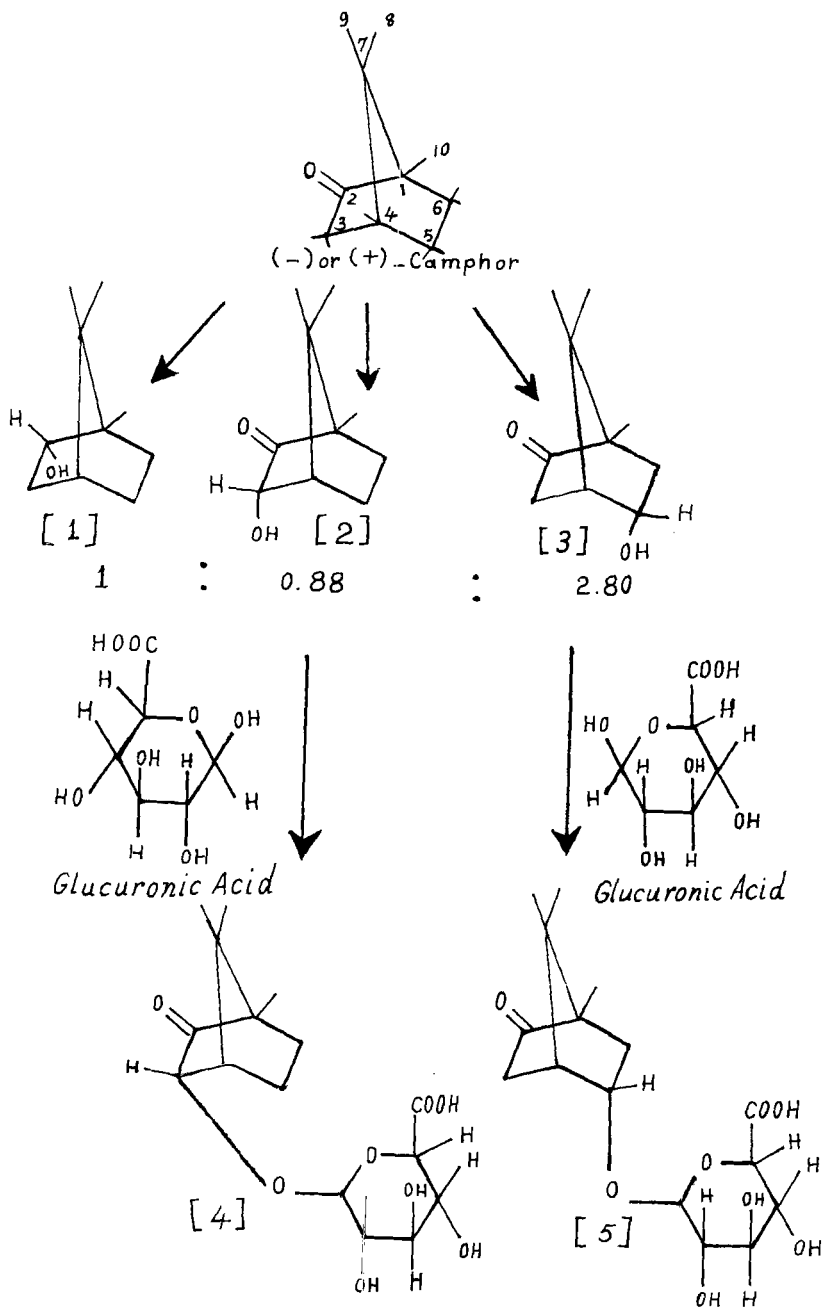
## 7. PHARMACOLOGY

### 7.1 Action and Use

Taken internally camphor is irritant and carminative. It has been used as a mild expectorant and relieves gripping. Applied externally, camphor acts as a rubefacient and mild analgesic and is employed in liniments as a counter-irritant in fibrositis, neuralgia and similar conditions. Camphor has been administered as a solution in oil by subcutaneous or intramuscular injection as a circulatory and respiratory stimulant, but there is little evidence of its value for this purpose (121-125).

### 7.2 Toxicity

Poisoning has occurred through the accidental administration of camphor liniment to young children by mistake for castor oil. The symptoms are nausea, vomiting, colic disturbed vision, delirium, mental confusion and epileptic convulsions. Recovery is the rule. But in rare cases death may occur from respiratory failure (126-128).



Scheme VII



### 7.3 Dosage

120 to 300 mg (2 to 5 grains); 60 to 200 mg (1-3 grains) by injections (12).

### 7.4 Preparations

The following are the preparations of camphor official in Pharmacopoeas:

- a) Camphor Liniment (4)
- b) Camphorated opium Tincture (126)
- c) Turpentine Liniment (4)
- d) Camphor Ointment (126)
- e) Camphor Spirit (126)
- f) Camphor Water (126)
- g) Camphor Injection (126)
- h) Camphorated Parachlorophenol (152).

## 8. METHODS OF ANALYSIS

### 8.1 Identification

#### 8.11 Pharmacopoeial Test

The following tests have been described in British Pharmacopoeia for the identification of Camphor (4):

- a) Burns readily with a bright smoky flame, and volatilises at ordinary temperature.
- b) The light absorption in the range 230 to 350 nm, of a 2-cm layer of a 0.25 percent w/v solution in alcohol (95%) exhibits a maximum only at 289 nm; extinction at 289 nm, about 1.06.

#### 8.12 Colour Tests

The following colour tests have been described for the identification of camphor:

- a) Bohrisch camphor reaction: When 0.5g of natural camphor is treated with 1 cc. of vanillin hydrochloride solution (1 part of vanillin dissolved in 100 parts of 25% hydrochloric acid) and heated on a water-bath to 75-100°, a blue colour passing to green is obtained. Synthetic camphor gives no colour (129-131).

Bohrisch (132) in reexamining the official tests for true camphor, substitutes the following: To 0.1 g. of powdered camphor in test tube is added 2 cc. of 1% vanillin-hydrochloride solution and the mixture put into a beaker with water which is warmed gradually. At 30° the colour is yellow, at 60° bluish green, at 75-80° indigo-blue. The latter colour persists for several hours, even after cooling. Synthetic camphor by the same treatment gives only a yellow colour.

- b) When 1 g of natural camphor is treated with 10 drops of Rosenthal's Reagent (equal volume of vanillin-hydrochloride and concentrated sulphuric acid) a dull green colour changing in 7-8 hours to indigo-blue is obtained (129).
- c) With barium peroxide-sulphuric acid reagent, camphor gives a yellow colour (133).
- d) When 2 ml of a 0.5% of alcoholic solutions of camphor is treated with 5 ml of 5% phosphomolybdic acid solution and 5 ml of sulphuric acid, a greenish-yellow precipitate is obtained which rapidly turns green and disappears on shaking (134).
- e) With furfural and sulphuric acid camphor gives reddish brown liquid which slowly turns purplish-violet for synthetic and purple for natural (134).
- f) Fluorescence test: When camphor is irradiated by x-rays it exhibits a bluish violet fluorescence in the visible region (135).

#### 8.13 Detection of Synthetic Camphor in Natural Camphor:

- a) About 1 g of camphor is thoroughly mixed with 2 g of lime, the mixture heated until the camphor is completely volatilized, the residue is extracted with water, and the solution filtered. The filtrate, acidified with nitric acid should give no precipitate with  $\text{AgNO}_3$  if synthetic camphor is absent from the original substance (136, 137).

- b) 5 g of the camphor to be tested is dissolved in 50 cc 90% alcohol, an aqueous solution of 5 g hydroxylamine hydrochloride and 8 g. sodium hydroxide are added, and then enough alcohol to produce a clear solution. The solution is heated for 90 min. on a water-bath, water is then added, and if turbidity appears, the presence of isoborneol or camphene is proved. If the turbid solution is now neutralized with hydrochloric acid, the precipitate which forms should be soluble in excess of hydrochloric acid and also in sodium hydroxide (136).

## 8.2 Gravimetric Methods

In order to determine the camphor content of essential oils (which contain no other carbonyl compounds) the gravimetric determination proposed by Aschan (138) may be employed:

Procedure: Introduce about 1 g. of the oil, accurately weighed, into a test tube and dissolve the oil in 2 g. of glacial acetic acid. Add 1 g. of semicarbazide hydrochloride and 1.5 g. of freshly fused anhydrous potassium acetate. Triturate thoroughly with a glass rod, stopper the tube with a plug of absorbent cotton and heat three hours in a water bath at 70°. Cool the mixture, add 10 to 15 cc. of water, stir thoroughly and transfer the precipitate quantitatively to a tared 4 to 5 cm. filter. Wash with water until all water soluble matter is removed, air dry, wash with petroleum ether and dry in air to constant weight. Determine the weight of semicarbazone from the increase in the weight of the filter. Calculate the content of camphor in the original oil by means of the following formula:

$$\text{Percentage of camphor} = \frac{72.7p}{s}$$

where: p = weight of semicarbazone in grams;  
s = weight of oil in grams.

Other methods for determination of camphor via semicarbazone, in pharmaceutical preparations were also reported (139, 140).

Estimation of camphor in camphor spirit (141):

The method is based on the ability of camphor to enter

into molecular combination with salol, to yield salolcamphor,  $C_{10}H_{16}O \cdot C_{13}H_{10}O_3$ .

Into a 100 cc. weighed Erlenmeyer flask introduce about 10g. (accurately weighed) camphor spirit and 70 cc of water. Add exactly 8 g. desiccated chemically pure salol, shake the flask, finally allowing the camphor and salol to collect on the bottom of the container. Pass the clear supernatant liquid through a weighed Gooch crucible (the bottom of which carries 2 small filters covered by a porcelain net). Wash the crucible with a few drops of concentrated salol spirit. Place the Erlenmeyer flask in a water bath at 50-60° whereby the entire content of the flask melts to a uniformly oily liquid consisting of salol-camphor and salol. Remove the container from the bath, set aside 1 hr. in a cold room in order that the salol excess may crystallize. Should this not take place, shake the flask, whereupon rapid crystallisation usually results. Transfer the entire content of the flask to the crucible separating the oily salolcamphor from the crystals salol by suction. Wash the latter by suction with 5 cc. concentrated salol spirit. Remove the combined portions of salol to a sheet of smooth paper. Wash the inside of the flask with a few cc of ether, receiving and evaporating the latter on a watch glass. Press the salol residue on the watch glass between 2 sheets of white paper until no further grease spot forms. Add this salol to the main portion, then dry at first in the air until the odor of camphor is no longer perceptible, then in a desiccator to constant weight. The percentage of camphor is then determined according to the following formula:

$$\% \text{ camphor} = (a \cdot 71.06) / b$$

a = is the amount of camphor combined with salol.

b = the amount of sample taken for analysis

and the value 71.06 = the molecular weight of salol into that of camphor multiplied by 100.

Good results are reported.

Another method utilizing hydroxylamine hydrochloride (142, 143) is as follows:

10 g. of spirit of camphor, 1 g. of hydroxylamine hydrochloride and 2 g. of sodium hydroxide in 20 cc distilled water are heated in a flask provided with a reflux condenser on a water bath for three hours. After cooling dilute sulfuric acid is added, phenolphthalein being used as indicator. The liquid is then extracted with ether three times. The ethereal solution of camphor oxime is dried with anhydrous sodium sulphate and poured into a weighed flask. After distilling the greater part of ether, the remaining ether is removed with air at room temperature, until a crystal mass is formed which is dried in a dessicator and weighed. The results obtained by this method are satisfactory.

Hampshire and Page (144) evolved a useful practical method for the determination of camphor in galenicals using 2,4-dinitrophenylhydrazine as a precipitant. The procedure used is as follows:

Dissolve about 0.2 g of camphor, accurately weighed by difference, in 25 ml aldehyde-free ethanol in a 300 ml conical flask and slowly add with constant shaking, 75 ml of the reagent (prepared by dissolving 1.5 g of 2,4-dinitrophenylhydrazine in a mixture of 10 ml of concentrated sulphuric acid and 10 ml of water, diluting with water to 100 ml and filtering; the reagent decomposes on standing and must be made up just before use). Then heat the mixture on a water-bath under a reflux condenser for four hours, allow it to cool, dilute with 2 per cent v/v sulphuric acid to 200 ml (weaker acid allows crystallisation of the excess of base from the solution) and allow to stand for twenty-four hours. Collect the precipitate on a weighed gooch crucible with paper mat, or on a sintered-glass filter, wash it with successive quantities of 10 ml of cold distilled water until the washings are no longer acid, and dry at 80°; 1 g of camphor 2,4-dinitrophenylhydrazine corresponds to 0.458 g of camphor.

A comparison of various methods for determination of camphor and other compounds utilizing 2,4-dinitrophenylhydrazine were also reported (145-149).

A vacuum oven method and a comparison with the U.S.P.X. method as well as a modification, utilizing anti-oxidants such as pyrogallol,  $\alpha$ -naphthol,

hydroquinone and paraphenylenediamine for the determination of camphor in camphor liniment were described by Poe (150, 151).

The U.S.P. XIX (152) described a method for the determination of camphor in camphorated para chlorophenol.

Procedure: Transfer about 300 mg of Camphorated Parachlorophenol, accurately weighed, to a 200 ml pressure bottle containing 50 ml of freshly prepared dinitrophenylhydrazine TS. Close the pressure bottle, immerse it in a water bath, and maintain it at about 75° for 4 hours. Cool to room temperature, then transfer the contents to a beaker with the aid of 100 ml of dilute sulfuric acid (1 in 12), and allow it to stand overnight. Collect the precipitate on a tared filter crucible, wash with 100 ml of dilute sulfuric acid (1 in 12) and then with 75 ml of cold water, in divided portions, to remove the acid. Dry at 80° for 2 hours, cool and weigh. The weight of the precipitate so obtained, multiplied by 0.4581, represents the weight of  $C_{10}H_{16}O$  in the sample taken.

Another official method described by the pharmacopeia of Japan IX (153) for the assay of camphor is as follows:

Transfer about 0.2 g of d-camphor, accurately weighed, to a 300-ml glass stoppered flask, and dissolve in 25 ml of aldehyde-free ethanol. Add slowly 75 ml of 2,4-dinitrophenylhydrazine TS while shaking, and heat for 4 hours on a water bath under a reflux condenser. Cool, add 100 ml of diluted sulfuric acid (1 → 50), and allow to stand for 24 hours. Collect the precipitate so obtained on a glass filter (G3), wash the residue on the filter with cold water until the washings become neutral, dry for 2 hours at 105° and weigh as the 2,4-dinitrophenylhydrazone of d-camphor ( $C_{16}H_{20}N_4O_4$ :332.36).

Amount (mg) of d-camphor ( $C_{10}H_{16}O$ ) = amount (mg) of  
2,4-dinitrophenylhydrazone of d-camphor  
( $C_{16}H_{20}N_4O_4$ ) X 0.4580.

The official method of NF XIV (154) for the assay of camphor in camphor spirit is given below:

Assay: Transfer 2.0 ml of camphor spirit to a suitable pressure bottle containing 50 ml of freshly prepared dinitrophenylhydrazine TS. Close the pressure bottle, immerse it in a water bath, and maintain at about 75° for 16 hours. Cool to room temperature, and transfer the contents to a beaker with the aid of 100 ml of dilute sulfuric acid (1 in 12). Allow to stand not less than 12 hours at room temperature, transfer the precipitate to a tared filter crucible, and wash with 100 ml of dilute sulfuric acid (1 in 12) followed by 75 ml of cold water in divided portions. Continue the suction until the excess water is removed, dry the crucible and precipitate at 80° for 2 hours, cool, and weigh. The weight of the precipitate so obtained, multiplied by 0.4581, represents the weight of  $C_{10}H_{16}O$  in the sample taken.

### 8.3 Titrimetric Methods

#### 8.31 Aqueous

Mital and Gaid (155) claim good recoveries for camphor in pharmaceutical preparations with a volumetric method in which hydroxylamine hydrochloride is used. For spirit, liniment and solution of camphor in turpentine, the following procedure is applicable:

Reflux the sample (containing 0.2 g of camphor) for four hours with 20 ml of aldehyde-free 95 per cent ethanol, 10 ml of a 4 percent solution of hydroxylamine hydrochloride in aldehyde-free 90 per cent ethanol and 0.3 g of sodium bicarbonate. Cool, rinse the condenser with 20 ml of light petroleum (b.p. 50° to 60°), collecting the rinsings in the flask and titrate the mixture first with 0.2N hydrochloric acid to dimethyl yellow and then with 0.2N potassium hydroxide to phenolphthalein. Carry out a control omitting the camphor and adding 5 ml of steam-distilled turpentine oil before refluxing for the spirit and solution and 1 g of arachis oil before refluxing for the liniment. Calculate the amount of camphor present from the difference in the volume of 0.2N potassium hydroxide used in the two titrations. 1 ml of 0.2N KoH = 0.0304 g camphor.

Other reported procedures with minor modifications and use of different indicators have been also reported (156-165). A method was also described for determination of camphor in dilute solutions by photometric titration using bromophenol blue as the indicator (166).

### 8.32 Vanadometry

An indirect vanadometric method of assay for camphor was developed. The method involves the formation of 2,4-dinitrophenylhydrazone of camphor. The nitro groups in the hydrazone are then reduced to amines by treatment with vanadium sulfate ( $VSO_4$ ) and the excess of the reagent is back titrated with sodium dichromate ( $Na_2Cr_2O_7$ ). The entire procedure is carried out in a modified separatory funnel into which standard vanadate,  $6N H_2SO_4$ , and zinc amalgam are introduced. A carbondioxide current is then passed and the mixture is shaken to obtain a blue colour after which the zinc amalgam is drawn off. Then sulfuric acid is added together with several drops of phenosafranine and excess vandate is titrated with standard sodium dichromate solution.

### 8.33 Polarographic

Schwabe and Berg (167) have studied the polarography of terpene derivatives. The solvents were 80% ethanol, 80% dioxane, 80% acetone or water and the supporting electrolytes were 0.1 M LiCl, LiOH,  $Et_4NI$ , or  $Et_4NOH$ . Camphor was found to depress the polarographic maxima and this could be used for its determination.

### 8.4 Refractometric Method

Rapaport and Solyanik (168) have developed a rapid method of refractometric determination of 23 mixtures containing anesthesin, barbamy, bromcamphor, bromisoval, camphor, antipyrine, amidopyrine, acetalsalicylic acid, barbital, codeine, salol, terpin hydrate, hexamethylenetetramine, and phenobarbital. The method described is suitable for analysis of pharmaceutical mixtures containing compounds which are insoluble in water and soluble in ethanol. A mixture of 2 pharmaceuticals (0.1 g) is dissolved in 1 ml. ethanol and  $n_D$  of this solution is determined. One component is



chemically determined in another 0.1 g of the sample. The amount of such component is calculated from formulas:

$$A = [(n - n_0) - (C_x F)] P / 100 F_1 P_1$$

$C = VT / 100$ ,  $B = VTP / P_1$  where A is wt. of the 1st component in grams, V is the volume of 0.1N solution used for titration of the 2nd component, T is the g. equivalent of the 1st compound, B is the wt. of the 2nd component (g.), n is the refractive index of the solution,  $n_0$  is that of ethanol, C is the amount of the 2nd component in 100 ml. solution (chemically determined), F and  $F_1$  are constants determined experimentally, P is total wt. of the mixture and  $P_1$  is wt. of sample (0.1 g.).

Other reported procedures for the determination of camphor, by using refractive index and other physical constants have also been reported (169,170).

### 8.5 Nephelometric Method

Nephelometric methods for determination of camphor vapors in air (171) and camphor in spirits were described (172). Optimum results were obtained with a mixture consisting of ether, 96%, ethanol and water in the proportion 0.1 : 0.9 : 3. The turbidity of the mixture is increased by the presence of camphor, in proportion to the concentration of camphor in solution. Approximately, 0.2 mg of camphor can be determined in 2 ml of the mixture (0.01 mg / L can be determined if 100 L of air and 10 ml of the solution are used).

### 8.6 Chromatographic Methods

#### 8.61 Thin Layer Chromatography

Miller and Kirchner (173) have developed an analytical method for volatile constituents including camphor. They used silicic acid coated-glass chromatostrips. Ethyl acetate hexane, chloroform, benzene, isopropyl ether and their mixtures were used as developers and the strips were examined with various reagents like bromine, fluorescence, O-anisidine, sulphuric acid - nitric acid, and concentrated sulphuric acid. The Rf values were calculated.

Table 7: Rf value of Camphor on Silicic acid using different solvents.

S. No.	Solvent System.	Colour of spot with H <sub>2</sub> SO <sub>4</sub> +Formaldehyde Reagent.	Rf Value.
1.	n-Hexane.	Light green.	0.00
2.	Carbon tetrachloride	,, ,,	0.31
3.	Cyclohexane	,, ,,	0.33
4.	Benzene	,, ,,	0.10
5.	Chloroform	,, ,,	0.61
6.	Ethyl acetate	,, ,,	Spot appears at the solvent front.
7.	Benzene : Carbon tetrachloride (50 : 50)	,, ,,	0.62
8.	Ethyl acetate : n-Hexane (10:90)	,, ,,	0.38
9.	Ethyl acetate : n-Hexane (15:85)	,, ,,	0.39
10.	Ethyl acetate : Carbon tetrachloride (15:85)	,, ,,	0.42
11.	Ethyl acetate : Cyclohexane (15:85)	,, ,,	0.42
12.	Ethyl acetate : Benzene (15:85)	,, ,,	0.37

Conditions

Glass plates 10.5 x 25 cm. coated with silicic acid (Mesh No.100).

Temperature 25°C.

Relative humidity 25 ± 5%.

Normal Chromatographic Chamber, saturated for 15 minutes before use.

Egon Stahl (174) has invented a special chromatographic device for the separation of volatile oils using uniform thickness of adsorbent on plate.

El-Deeb, et al (175) have reported the separation and evaluation of camphor by TLC technique on silicic acid plate. The behaviour of camphor on chromatoplates was studied using different solvents of increasing polarity and their mixtures as developers. The spots were located by spraying the plates first with sulphuric acid and then with 40% solution of Formaldehyde and heating the plate to 100°C for 3 to 5 minutes. The Rf values were calculated. It is given in Table 7.

### 8.62 Column Chromatography

The column technique has been used for the separation and determination of the purity of camphor.

Montes (176) analysed the essential oil constituents using the principle of chromatographic adsorption with the aid of columns of silicic acid and bentonite. The compounds were then crystalized and their melting points were determined.

Gustave Vavon and Bernard Gastambide (177) and Bernard Gastambide (178), studied the effects of steric hindrance of camphor and fenchone on chromatographic alumina column.

Eero Sjostrom (179) has described a method of the separation of camphor and other ketones using anion exchange resins column.

A successful attempt of separation and identification of camphor from mixtures was achieved by Karawya (180) and by Karawya and El-Deeb (181) using alumina column.

El-Deeb, et al (175) have reported the comparative study of differential adsorption of camphor on both aluminium oxide and silicic acid. The retention volumes were determined by using Petroleum-ether and ether as solvents. 100 mg of material was chromatographed on column of 1 cm diameter and 10 g of either Alumina or silicic acids (mesh No. 30). The results are presented in Table 8.

Table 8: Retention of Volume of Camphor on different columns.

S. No.	Adsorbent.	Retention volume	
		Petroleum Ether	Ether
1.	Silicic acid	More than 25 ml.	20 ml.
2.	Alumina	100 ml.	15 ml.

### 8.63 Gas Liquid Chromatography

A GLC method for the determination of camphor (synthetic and natural) has been carried out in our laboratory (182), using a Varian GC-3700 gas chromatograph equipped with Varian CDS 111 integrator.

Column conditions: 3% OV1 on chromosorb W-Hp (80-100 mesh size); glass column (2m x 2mm). The column run isothermally at 100° → 200°C for 2 minutes (the temperature is increased at the rate of 10° per minute), Carrier gas: Nitrogen, flow rate was adjusted to 40 ml/minute. Detector: FID, hydrogen and air, flow rates were adjusted to 30 ml/minute and 300 ml/minute respectively. Ethanol was used as solvent. The injection temperature was 150°C and the chart speed was adjusted to give 1 cm/minute. The retention time = 1.45 minutes. Camphor content of the sample is calculated from the chromatogram by using peak area ratio method. The GLC of camphor is presented in Fig. 11.

Camphor has been determined by Baines and Proctor (183) in a number of essential oils and pharmaceutical preparations using gas chromatographic technique. The apparatus employed has a thermal conductivity detector with 4 in. platinum wire 0.001 in. diameter of nominal resistance 250 Ohms. The wires in the channels being matched to within 0.1 Ohm. The bridge current was 200 mA and the output recorded on a 2.5 mV recorder. 2% ethylbenzene was added to the standards and samples as an internal standard. The operating conditions were as

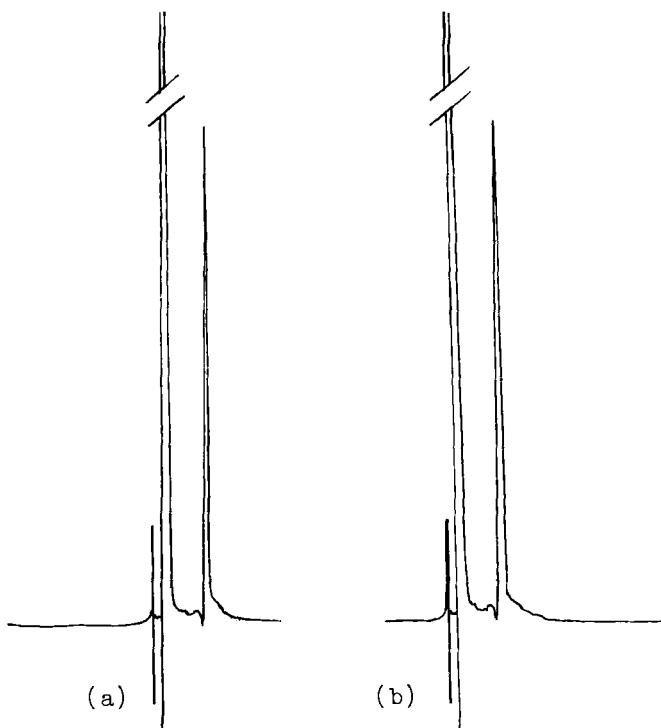


Fig. 11: GLC of Camphor.

- (a) : Natural Camphor.  
(b) : Synthetic Camphor.

follows: Column length 7 ft. Column diameter 4 to 5 mm. Column temperature 130°C. Stationary phase 20% of squalane on 100 to 120 mesh celite. Sample size 30  $\mu$ l, Carrier gas 4:1 hydrogen and nitrogen mixture at 100 ml/min. The retention volumes were camphor 1200 ml, ethylbenzene 270 m. camphor content of the sample is calculated from the chromatogram by using peak area ratio method.

Yoshio Hanada and Masayoshi Kitajima (184) have reported the gas chromatographic determination of camphor using naphthalene as the internal standard.

GLC procedure for the separation of camphor and other terpenes on polypropyleneglycol-impregnated

column was also described by Egon Stahl and Trennheuser (185). El-Deeb, et al (175) have reported GLC technique for determining the constituents of volatile oil using silicon oil DC 550 as a stationary phase.

#### 8.64 High-Performance Liquid Chromatography

Quantitative analysis of both camphor and parachlorophenol in camphorated parachlorophenol by high-pressure liquid chromatography is described (186).

The HPLC chromatograph (Waters Associate Model A.I.C. 202) was used with solvent system of 60% hexane and 40% chloroform. A UV detector at 254 nm was used at an attenuation of x8 for camphor and x 64 for parachlorophenol and phenol. The flow rate was 2.5 ml/minute at an inlet pressure of 0.131 Nm<sup>-2</sup>. The chart speed was 0.5 cm/min. The column (Hi-Eff Micropart) was 0.64 cm O.d X 15 cm long, packed with 5  $\mu$ m silica gel (Applied Science Laboratories). The injection volume was 7-15  $\mu$ l and the elution over was camphor (2.76 min.), parachlorophenol (10.64 min) and phenol (12.61 min).

The results indicated that camphor and parachlorophenol can be simultaneously assayed by simple dilutions of the sample and injection into a high pressure liquid chromatograph. The linearity curve indicated that the components have been analysed give linear response. Results are shown in Table 9.

Table 9: Assay Results on USP Camphorated Parachlorophenol

Assay	Camphor, %	Parachlorophenol, %
1	64.3	34.7
2	65.6	34.9
3	65.0	35.4
4	64.6	35.6
SD	0.56	0.42
CV	0.86	1.19
Average deviation.	0.43	0.35

## 8.7 Spectroscopic Methods

### 8.7.1 Colorimetric

Camphor can be determined colorimetrically with sulfuric acid and furfural (187). To 1cc of camphor solution, add 3 cc of ethanol (95%) and 10 drops of 1% solution of furfural in 95% ethanol. Stir the mixture, add two drops of concentrated sulfuric acid and heat in a water bath. Then cool and add 5cc. of ethanol and compare the red to violet colour produced with a standard. The amount present can be determined within 10%.

The colour reactions of camphor with aldehydes have been applied with p-dimethylaminobenzaldehyde in sulfuric acid (188). The yellow colour formed conforms to Beer's law and checks closely with the official NF method (154). The assay is carried out as follows:

Dissolve 0.125 g of p-dimethylaminobenzaldehyde in 65 ml of concentrated sulphuric acid and 35 ml of water. Add 7 ml of this reagent by burette to an aliquot of a dilution of the sample in chloroform to contain between 0.6 and 7.5 mg of camphor. Stopper, shake and allow to stand two hours for colour development and measure the extinction at 460 m $\mu$ .

Prepare a standard curve in a similar manner from aliquots of a prepared solution of camphor in chloroform in the range 0.6 to 7.5 mg of camphor.

A method has been described for determination of camphor in dilute solutions and in plants (189). The assay is based on the formation of a yellow colour with phenylhydrazine (Camphor phenylhydrazone). The colour produced after absorption on Whatman No.120 paper and the intensity of the yellow colour produced was compared with that produced from a sample of known concentration.

A microanalytical procedure was reported for determination of camphor (190). The method is based on the formation of camphor oxime. The oxime is subjected to acid hydrolysis and the resulting hydroxylamine reacted with formalin and

ferric ammonium. The resulting ferric formohydroxamate is treated with ammonium persulphate and the colour produced is then determined.

### 8.72 Nuclear Magnetic Resonance

Hassan et al (29) recently have described a proton magnetic resonance analytical procedure for the determination of camphor as bulk drug and the simultaneous assay of camphor and p-chlorophenol. The method is based on the integration of the nine protons of the three methyl groups of camphor centred at 0.88 ppm and the ten aromatic protons of benzophenone centred at 7.55 ppm employed as an internal reference standard. The method is specific and accurate with standard deviation of  $\pm 0.86$  and an average recovery of 100.28%. In case of camphor parachlorophenol assay, integration of the nine protons of the three methyls of camphor centred at 0.83 ppm, the four aromatic protons of parachlorophenol centred at 6.97 ppm, and the two olefinic protons singlet of maleic acid appearing at 6.37 ppm were carried out. The method was specific and accurate with standard deviations of  $\pm 1.13$ ,  $\pm 1.01$  for camphor and  $\pm 0.62$ ,  $\pm 0.89$  for parachlorophenol, in standard mixtures and in camphorated parachlorophenol respectively. The PMR spectrum in addition, provides a very specific means for identification of camphor and parachlorophenol.

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# CHLOROQUINE

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## 1. History

Chloroquine is one of a large series of 4-aminoquinolines investigated in connection with the extensive cooperative program of antimalarial research in the United States during World War II. The objective was to discover more effective and less toxic suppressive agents than quina-crine. Although the 4-aminoquinolines had previously been described as potential antimalarials by Russian investigators, serious attention was not paid to this chemical class until the French reported that 3-methyl-7-chloro-4-(4-diethylamino-1-methylbutylamino) quinoline (SN-6911; Sontichin, Sonotoquin) was well tolerated and had high activity in human malaras. Beginning in 1943, thousands of these compounds were synthesized and tested for activity in avian malaria and for toxicity in mammals; ten of the series were then examined in human volunteers with experimentally induced malaras. Of these, chloroquine proved most promising and was released for field trial. When hostilities ceased, it was discovered that the chemical had been synthesized and studied under the name of Resochin by the Germans as early as 1934. (1)

## 2. Description

### 2.1 Nomenclature

#### 2.1.1 Chemical Names

$N^4$ -(7-chloro-4-quinolinyl)- $N'$ ,  $N'$ -diethyl-1, 4-pentanediamine.  
7-Chloro-4-(4-diethylamino-1-methylbutylamino) quinoline.

The CAS Registry No. is [54-05-7]. (2 & 3)

#### 2.1.2 Generic Name

Chloroquine  
RP 3377, SN 7, 618. (2)

#### 2.1.3 Trade Names

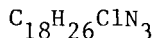
Base: Aralen, Nivaquine B, Sanquine, Artrichin, Bipiquine, Reumachlor, Bemaphate, Tanakan, Resoquine. (3)

Diphosphate: Malaquine, Resochin, Silbesan, Tresochin, Arechin, Avloclor, Imagon. (2, 3)

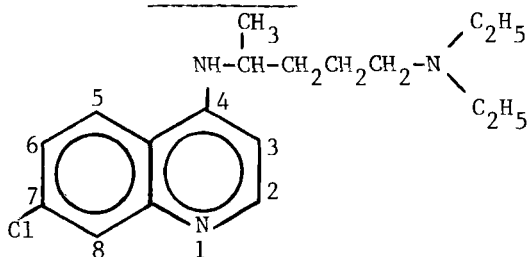
Sulphate: Nivaquine and Bemasulph. (2,3 & 8)

## 2.2 Formulae

### 2.2.1 Empirical



### 2.2.2 Structural



Chloroquine

### 2.2.3 Wiswesser Line Notation

T66 BNJ EM & 3  
N2 & 2 1G8QP  
QQO 2 DL (4)

## 2.3 Molecular Weight

319.89

## 2.4 Elemental Composition

C 67.59%, H 8.19%, Cl 11.08% N 13.14%. (2)

## 2.5 Appearance, Color, Odor and Taste

White or slightly yellow crystalline powder, odorless and bitter in taste. (5 & 6).

## 3. Physical Properties

### 3.1 Melting Point

Melts between 87° and 97°. (6)



### 3.2 Boiling Point

212<sup>o</sup> to 214<sup>o</sup> at 0.2 mm Hg. (7)

### 3.3 Solubility

Very slightly soluble in water; soluble in dilute acids, chloroform and ether. (7)

### 3.4 Acidity

Chloroquine phosphate has a pH of 3.5 to 4.5 of 10% solution. While the 10% solution of chloroquine sulphate has a pH of 4 to 5. (7)

### 3.5 Moisture Content and Hygroscopicity

Not more than 1.5% determined by drying at 105<sup>o</sup>. It absorbs insignificant amounts of moisture at temperatures up to 37<sup>o</sup> at relative humidities up to about 80%. (7)

### 3.6 Dissociation Constants

pKa Values are 8.4, 10.8 (20<sup>o</sup>). (7)

### 3.7 Stability

Solutions of pH 4 to 6 are stable when heated but sensitive to light. (7)

### 3.8 Storage

It should be protected from light. (7)

### 3.9 Sterilization

Solutions for injection are sterilized by heating in an autoclave or by filtration. (7).

### 3.10 Spectral Properties

#### 3.10.1 Ultraviolet Spectrum

The ultraviolet spectrum of chloroquine in neutral methanol solution in the region of 200 to 400 nm exhibits maxima at 218 nm, 253 nm and 328 nm and minima at 243 and

275 nm. The spectrum is shown in Fig. 1. The spectrum was obtained on Varian AG UV-Vis Spectrophotometer model DMS 90.

### 3.10.2 Infrared Spectrum

The infrared spectrum of chloroquine is presented in Fig. 2. The spectrum was obtained from KBr disc on a Perkin-Elmer Infrared Spectrophotometer model 580 B, in the range of 4000 - 400  $\text{cm}^{-1}$ . It shows the following:

<u>Frequency (<math>\text{cm}^{-1}</math>)</u>	<u>Assignments</u>
3260	NH group
1540	
1580	quinoline
1610	

In addition to other bending and stretching vibration bands for the aliphatic moiety of the molecule.

Clarke (8) reported that the principal peaks for chloroquine base in KBr disc are 1573, 1538, 1372 or 1448.

### 3.10.3 Mass Spectrum

The chemical ionisation (CI) mass spectrum Fig. 3, was recorded on a Finnigan 4000 Mass Spectrometer with ion source pressure 0.3 Torr, ion source temperature 150°C, emission current 300  $\mu\text{A}$ , electron energy 100 eV using methane as a reagent gas. The electron impact (EI) mass spectrum Fig. 4, was recorded on Varian MAT 311 Spectrometer, with an ion source pressure  $10^{-6}$  Torr, ion source temperature 180, emission current 300  $\mu\text{A}$  and electron energy of 70 eV.

Table 1 and the following figure illustrate the prominent fragments in the EI spectrum and their proposed assignments.

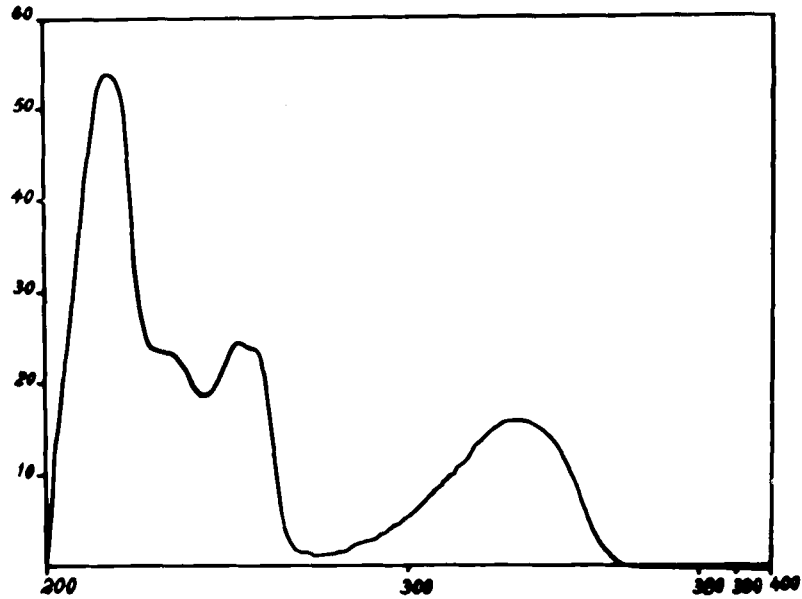


Fig. 1. Ultraviolet spectrum of chloroquine:  
neutral methanol.

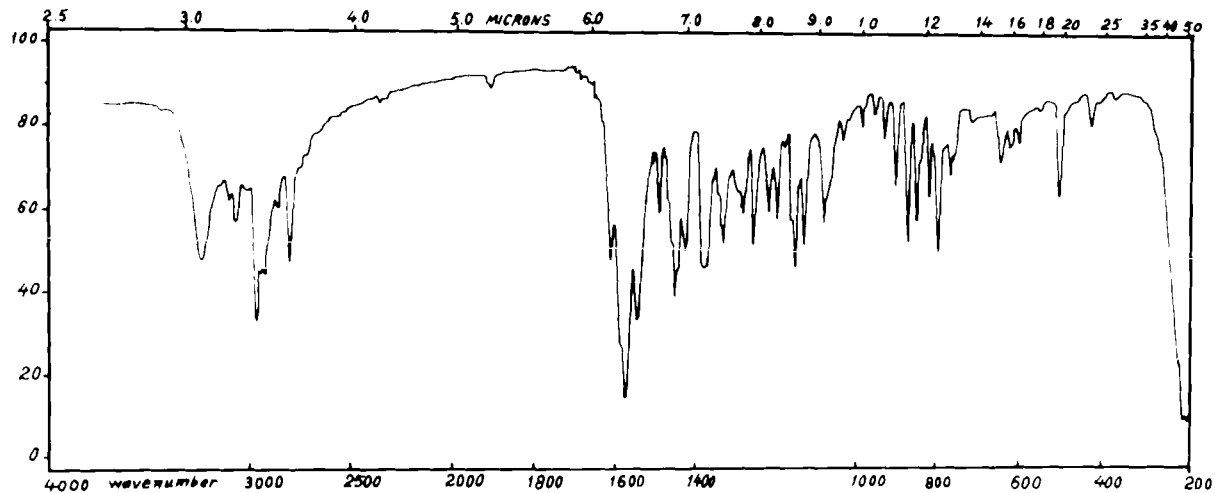


Fig. 2. Infrared spectrum of chloroquine in KBr disc.

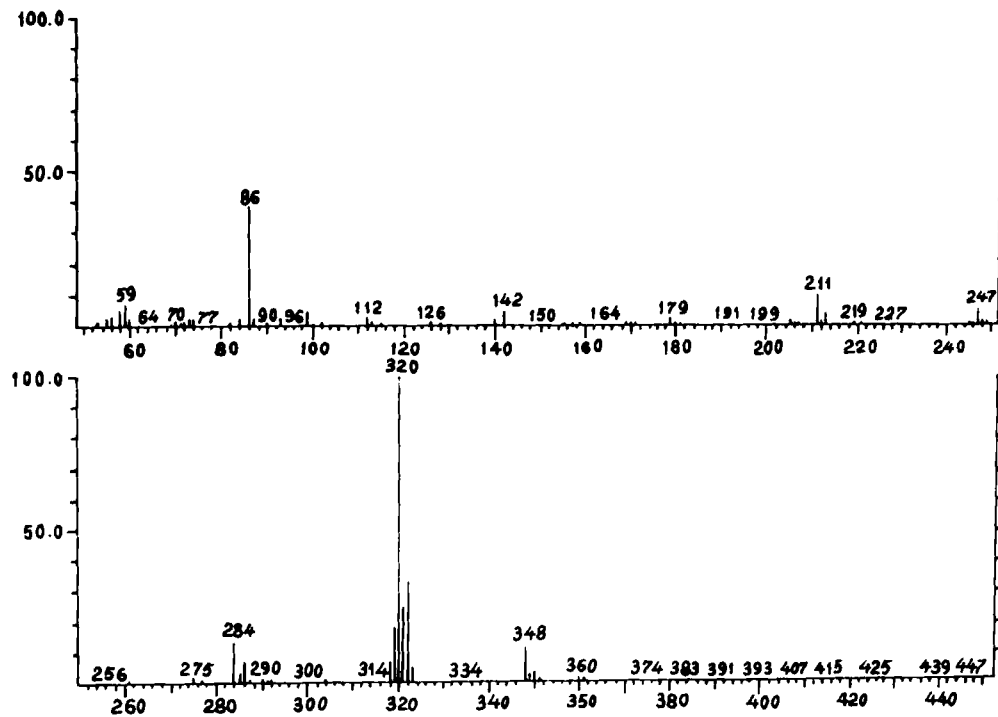


Fig. 3. Mass spectrum of chloroquine, CI.

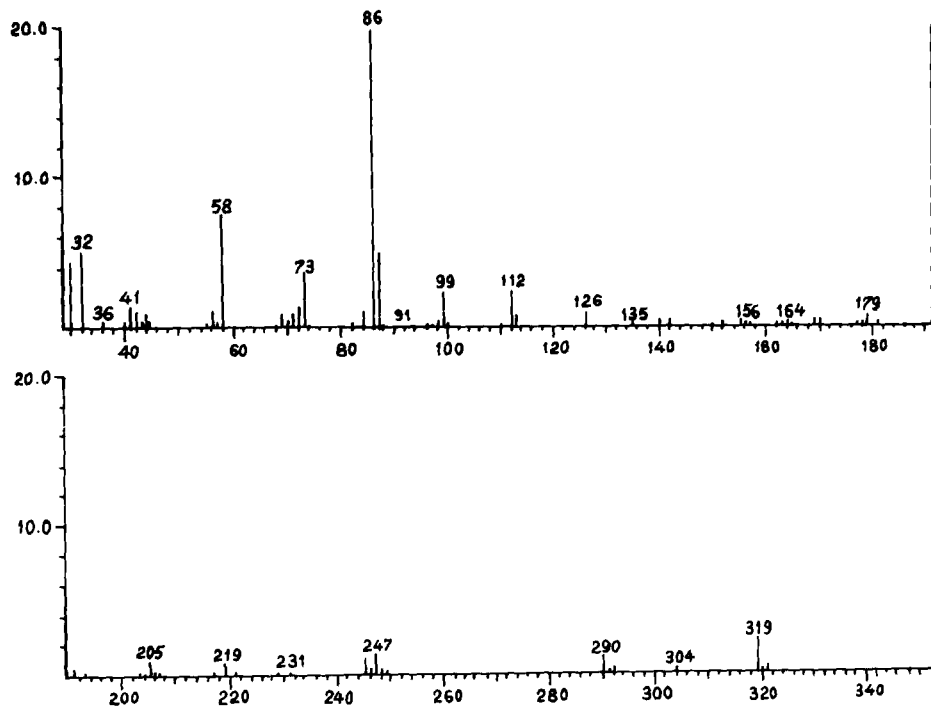


Fig. 4. Mass spectrum of chloroquine, EI.

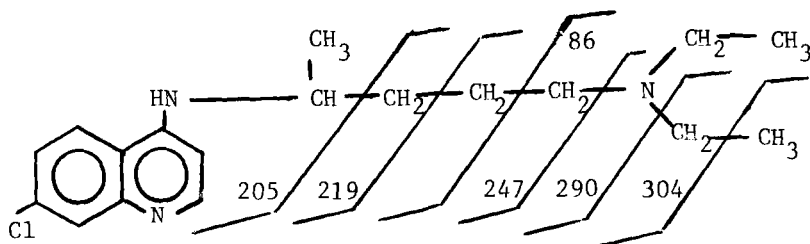


Table 1 EI Mass Spectrum Assignments of Chloroquine.

m/e	Relative Abundance	Ion or Fragment
319	13	M <sup>+</sup>
304	3	M-CH <sub>3</sub> <sup>+</sup>
290	6	M-C <sub>2</sub> H <sub>5</sub> <sup>+</sup>
247	7	M-NEt <sub>2</sub> <sup>+</sup>
231	2	M-CH <sub>2</sub> -NEt <sub>2</sub> <sup>+</sup>
219	5	M-(CH <sub>2</sub> ) <sub>2</sub> -NEt <sub>2</sub> <sup>+</sup>
205	5	M-(CH <sub>2</sub> ) <sub>3</sub> NEt <sub>2</sub> <sup>+</sup>
126	5	C <sub>4</sub> H <sub>6</sub> NEt <sub>2</sub> <sup>+</sup>
112	13	C <sub>3</sub> H <sub>4</sub> NEt <sub>2</sub> <sup>+</sup>
99	13	C <sub>2</sub> H <sub>3</sub> NEt <sub>2</sub> <sup>+</sup>
86	100	CH <sub>2</sub> NEt <sub>2</sub> <sup>+</sup>
58	40	$\begin{array}{c} \text{CH}_2 \\    \\ \text{H}-\text{N}-\text{Et} \end{array} \text{ } ^+$

3.10.4  $^1\text{H-NMR}$  Spectrum

The  $^1\text{H-NMR}$  spectra of chloroquine base and phosphate are shown in Fig. 5 and 6 and were obtained on Varian T6-A NMR Spectrometer with  $\text{CDCl}_3$  and  $\text{D}_2\text{O}$  respectively as solvents and tetramethylsilane as an internal reference. The signals are assigned in Table 2.

Table 2 .  $^1\text{H-NMR}$  Spectral Assignments of Chloroquine.

Proton	Chemical Shift (ppm) and Multiplicity [ ]	
	Base	Phosphate
$\text{CH}_3\text{CH}_2$ ; $\text{CH}_3\text{-CH}$	0.83 - 1.33 [m]	1.2 - 1.5 [m]
$\text{-CH-CH}_2\text{CH}_2\text{-CH}_2\text{-}$	1.66 [t]	1.82 [b s]
$\text{-CH}_2\text{-N}\begin{matrix} \text{CH}_2\text{-} \\ \text{CH}_2\text{-} \end{matrix}$	2.33 - 2.67 [m]	3.2 [q]
$\begin{matrix} \text{CH}_3 \\   \\ \text{-CH-} \end{matrix}$	3.66 [q]	4.00 [b s]
NH	5.64 [d]	Exchanged
Aromatic $\text{C}_2\text{H-}$	8.43 [d]	8.2 [d]
Aromatic $\text{C}_3\text{H-}$	6.4 [d]	6.65 [d]
Other Aromatic Protons	7.2 - 7.96 [m]	7.15 - 7.9 [m]

s = singlet    d = doublet    t = triplet    q = quartet

b s = broad singlet    b m = broad multiplet.



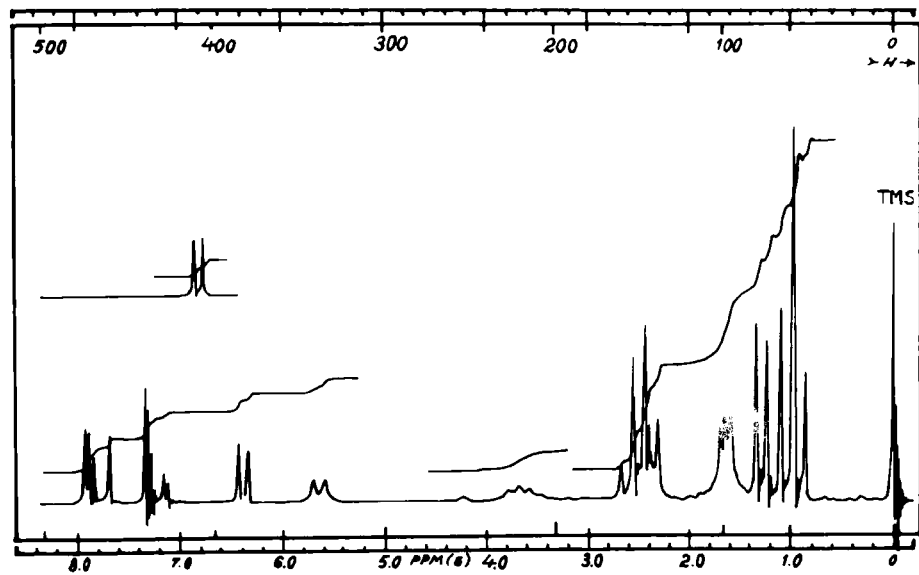


Fig. 5.  $^1\text{H-NMR}$  spectrum of chloroquine in  $\text{chloroform-d}_3$ .

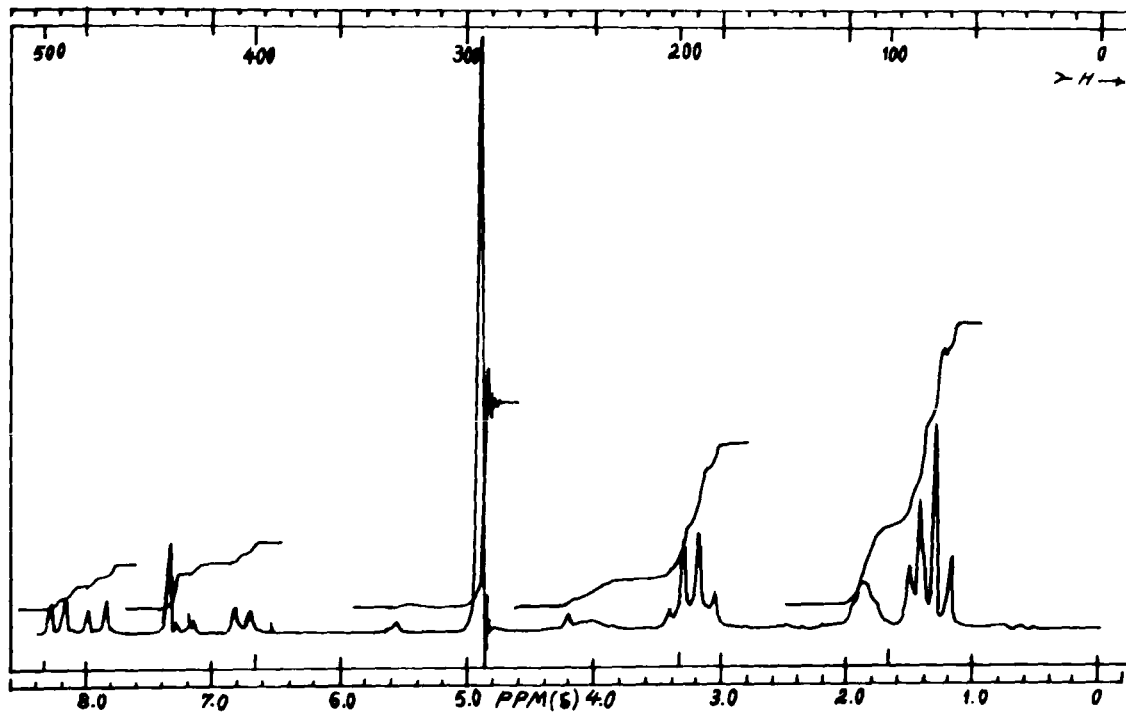


Fig. 6.  $^1\text{H}$ -NMR spectrum of chloroquine in  $\text{D}_2\text{O}$ .

### 3.10.5 C-13 NMR Spectrum

The noise-decoupled and off-resonance C-13 NMR spectra of chloroquine are presented in Figures 7 and 8 respectively. The spectra were obtained on Varian FT-80A Spectrometer. Spectral assignments are listed in Table 3.

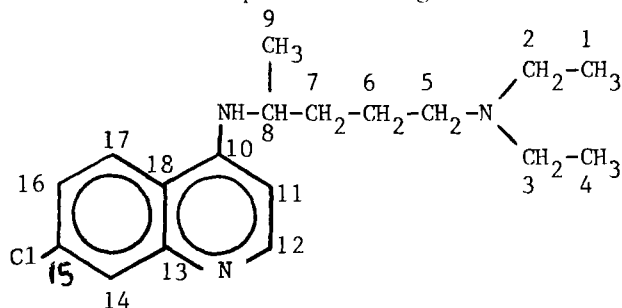


Table 3 . Carbon-13 NMR Spectral Assignment of Chloroquine.

Carbon No.	Chemical Shift (ppm) relative to TMS	Multiplicity
1, 4	11.7	quartet
2, 3	46.27	triplet
5	52.25	triplet
6	23.60	triplet
7	33.46	triplet
8	47.72	doublet
9	19.83	quartet
10	149.60	singlet
11	98.79	doublet
12	151.85	doublet
13	149.47	singlet
14	127.55	doublet
15	133.36	singlet
16	123.71	doublet
17	124.40	doublet
18	117.67	singlet

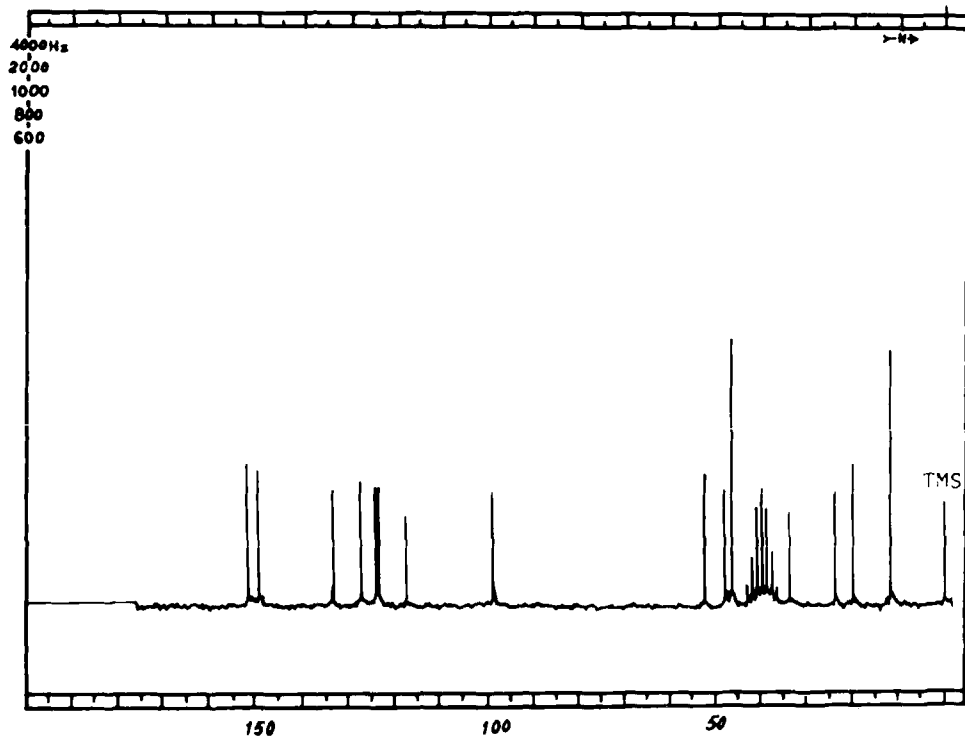


Fig. 7.  $^{13}\text{C}$ -NMR spectrum of chloroquine in  $\text{DMSO-D}_6$ , noise-decoupled.

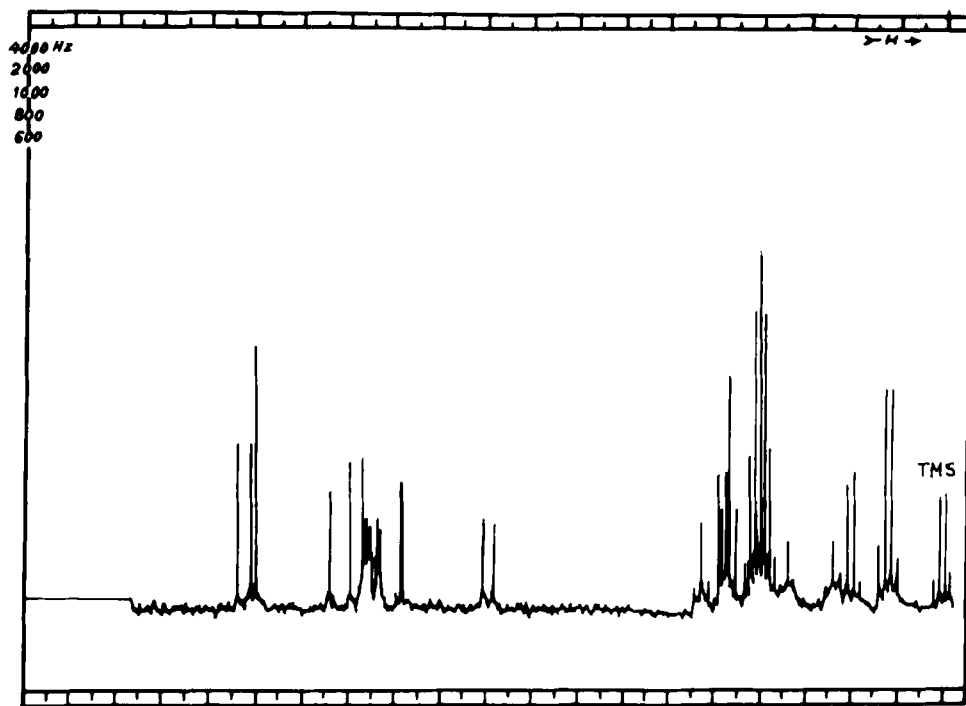
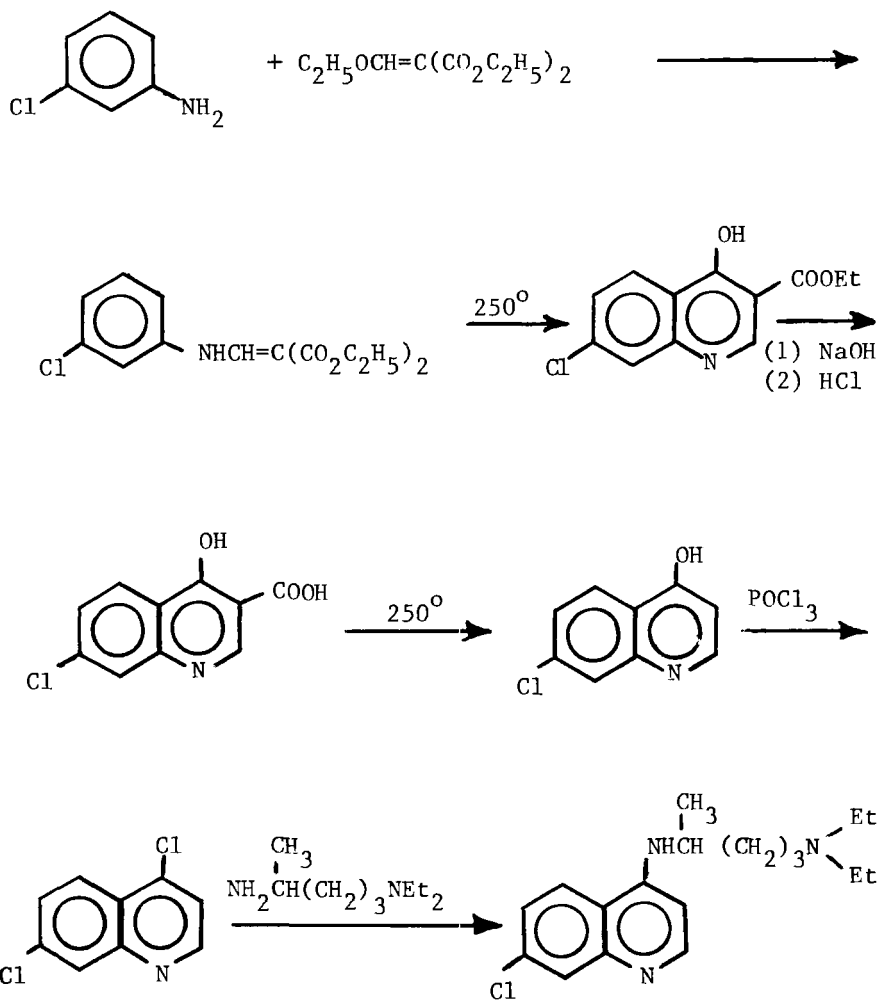


Fig. 8.  $^{13}\text{C}$ -NMR spectrum of chloroquine in  $\text{DMSO-D}_6$ , off-resonance.

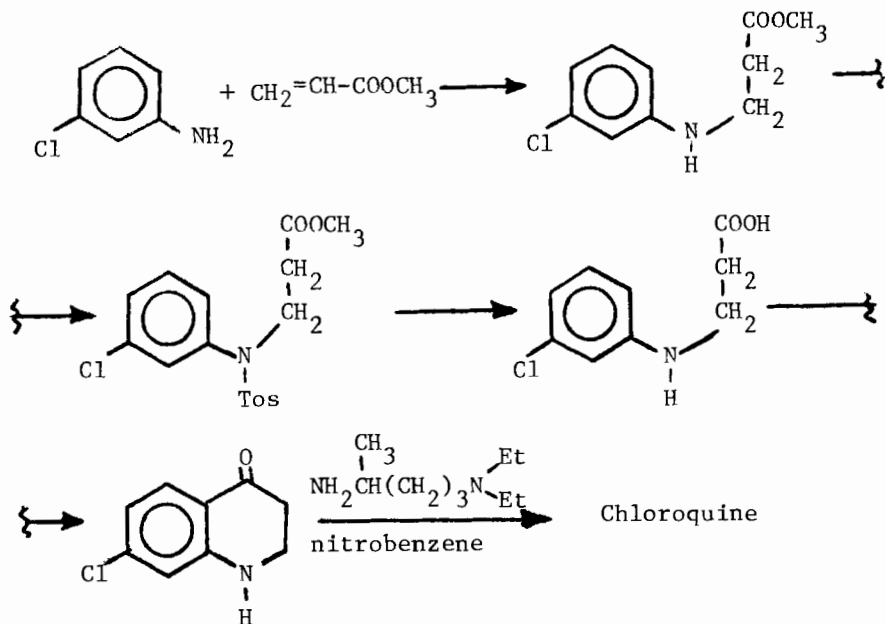
4. Synthesis

- a) One of the earliest and most effective antimalarials is "chloroquine", in whose synthesis 4,7-dichloroquinoline, prepared from 7-chloro-4-quinolone, is key intermediate. (9, 10).

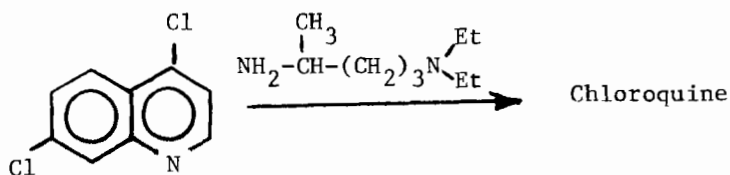


Chloroquine

- b) In another synthesis methyl acrylate is reacted with m-chloroaniline, the resulting methyl m-chloroanilino-propionate is tosylated and then hydrolyzed to the acid. The acid is converted to acid chloride and cyclized to give 4-keto-7-chloro-1,2,3,4-tetrahydroquinoline reaction of this compound with 4-diethyl-amino-1-methylbutylamine under the dehydrogenating influence of nitrobenzene gives chloroquine in about 25% overall yield (11 & 12).



- c) Chloroquine is obtained by coupling 4,7-dichloroquinoline with 5-diethylamino-2-aminopentane. (13)



## 5. Pharmacokinetics

### 5.1 Absorption, Distribution, Metabolism and Excretion

Chloroquine is readily absorbed from the gastrointestinal tract and about 50% in the circulation is bound to plasma protein. It delays gastric emptying. It accumulates in high concentrations in some tissues, such as the kidneys, liver lungs and spleen and is strongly bound in melanin-containing cells such as those in the eyes and the skin; it is also bound to double stranded DNA, present in red blood cells containing schizonts (1). At a dose of 310 mg, plasma concentrations reach a plateau at 10 days with a concentration of about 120 ng/ml; after a 500 mg dose, concentrations of about 700 ng/ml are attained in haemolysed blood in 4 hours and after single oral doses of 400 to 500 mg, peak plasma concentrations of 35 to 220 ng/ml are attained in 2 to 6 hours (6). About 70-85% of the total whole blood content of chloroquine and its metabolite desethylchloroquine were recovered in blood cells isolated from whole blood, indicating that these compounds have a cell/plasma concentration ratio (14). It has been reported that small amounts of chloroquine may be found in the blood and urine of patients for as long as 5 years after the last known administration, and the existence of large tissue reservoirs has been postulated (15 & 16). Other studies are published on unidentified metabolites present in plasma and urine of the treated subjects. The polar and non-polar metabolites of chloroquine are shown below:

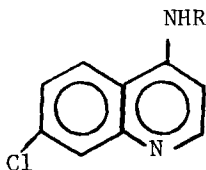


Fig. 9

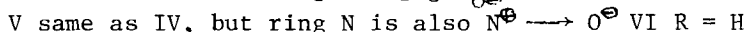
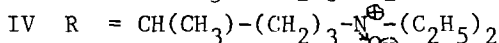
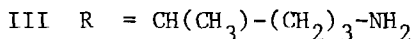
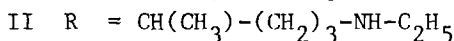
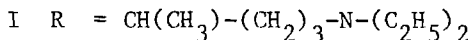




Fig. 9 Chloroquine (I), desethyl-(II) and didesethylchloroquine (III). Side-chain N-oxide (IV), di-N-oxide (V), and 7-chloro-4-aminoquinoline (VI). IV and V, polar metabolites; II and III, relatively non-polar metabolites. (17).

Chloroquine and its metabolite desethylchloroquine found in thrombocytes and granulocytes in vivo and in vitro. Chloroquine is characterized by its extensive tissue distribution which causes a slow elimination from the body. Plasma half-life is variable  $2\frac{1}{2}$ -7 days. Brohult et al (18) obtained a value for plasma half-life of chloroquine is 4 days, whilst Frisk-Holmberg et al (19) found half-lives of 3.1 hours to 13 days depending upon the dose. Metabolic reactions involve oxidative N-dealkylation and oxidative deamination and also conjugation, possibly with glucuronic acid, of the carboxylic acid metabolites derived from dealkylation and deamination; the metabolites include mono- and bisdesethylchloroquine, 4-(7-chloroquinol-4-ylamino) pentan-1-01, and 4-(7-chloroquinol-4-ylamino) pentanoic acid and its conjugate (7). Chloroquine is eliminated very slowly from the body and it may persist in tissue for a prolonged period. About 55% is excreted in the urine and about 10% in the faeces by 90 days following therapy with 310 mg for 14 days; the urinary excretion of the unchanged drug is dependent upon urinary pH and larger amounts are excreted in acid urine than in alkaline urine; of the material excreted in the urine, about 70% is unchanged, 23% is desethylchloroquine, 1 to 2% is bisdesethylchloroquine and an unidentified metabolite, and 1 to 2% is excreted as carboxylic acid metabolites in conjugated form. (21 & 22).

## 5.2 Distribution and Metabolism in Neonates

Chloroquine, its N-dealkylated metabolites, and chloroquine N-oxides were detected in the urine of pregnant women who were receiving chloroquine medication whereas chloroquine and its nonpolar metabolites, desethyl and didesethylchloroquine and 7-chloro-4-aminoquinoline have been found in the neonates' urine, blood and cord blood. That chloroquine and its relatively nonpolar metabolites (including one without the alkyl side-chain, 7-chloro-4-aminoquinoline) cross the placenta is demonstrated by the

presence of these compounds in the cord blood, neonatal systemic blood, and neonatal urine. The selective transfer of the compounds across the placenta has also been reported (17).

Both in vitro and in vivo metabolic studies have shown that some drug metabolism occurs or can be induced in placental tissues (20). Further degradation of parent chloroquine that reaches the placenta would result in the appearance of less chloroquine and more of the nonpolar metabolites in the fetal or neonatal circulation. (17).

## 6. Therapeutic Uses

It is highly effective in terminating acute vivax and falciparum malaria. It is also valuable in the treatment of intractable amebiasis and some cases of giardiasis (1). Chloroquine also produces symptomatic improvement in *Barbesia* infections. It can be used as an antirheumatic agent (23).

## 7. Toxicity

In therapeutic doses chloroquine may cause headache, visual disturbances, gastrointestinal upset and pruritis. In some cases it may cause discoloration of nailbeds and mucous membranes. Prolonged treatment may cause lichenoid skin eruption, visual blurring and bleaching of hair. Prolonged large doses (250 to 750 mg daily) may cause loss of central visual acuity, granular pigmentation of macula and retinal artery constriction. The visual loss appears to be irreversible (24). Ototoxicity has been reported in few cases. Hart and Naunton, (25) have implicated chloroquine in fetal abnormality. Blood dyscrasias have been reported rarely. They include reversible agranulocytosis, thrombocytopenia, and neutropenia (6).

## 8. Methods of Analysis

### 8.1 Identification

- a) Dissolve about 25 mg in 20 ml of water and add 8 ml of trinitrophenol solution; a precipitate is produced which, after washing successively with water, alcohol (95%), and ether, melts at about 207°. (8).

- b) Micro: Picric acid - rosettes of plates (sensitivity: 1 in 1000); styphnic acid - rosettes of plates (Sensitivity 1 in 1000). (8).

## 8.2 Microbiological Assay

A rapid semiautomated microdilution method for the microbiological assay of the chloroquine has been developed by Desgardins (26). Antimalarial activity of chloroquine may be studied against cultured Plasmodium falciparum, microplates are used to prepare serial dilution of the drug. Parasites obtained from continuous stock cultures are subcultured in the micro-plates for 42 h. Inhibition of uptake of a radio labeled nucleic acid precursor by parasites serves as the indicator of antimicrobial activity.

## 8.3 Spectrophotometric Methods

### 8.3.1 Ultraviolet Spectrophotometric Analysis

#### 8.3.1.1 $\Delta p_j$ Method

Chloroquine has been assayed spectrophotometrically (27) using the  $\Delta p_j$  method (28) that depends on differences in orthogonal function coefficients. The latter method is based on the fact that the peaks of certain compounds may split into subsidiary peaks without any appreciable change in intensity by changing the pH in a suitable interval. The behaviour of these compounds restricts the application of the  $\Delta A$  method (29 & 30) because  $\Delta A$  may not fulfil the requirements specified for its successful application. In these circumstances, the  $\Delta p_j$  method offers a solution for the determination of such compounds in the presence of irrelevant absorption. Thus, the contribution of a pH-insensitive irrelevant absorption may be cancelled by means of

$$\Delta P_{ji} = [\alpha_{jia} C_x + p_{ji}(Z)] - [\alpha_{jib} C_x + p_{ji}(Z)]$$

$$\text{and } C_x = \Delta p_{ji} / \Delta \alpha_{ji}$$

where  $p_j$  is the coefficient of the polynomial,  $p_j$  (4)  $\alpha_j$  is  $p_j$  (1%, 1 cm), the coefficient for A (1%, 1 cm) of the

pure compound,  $x$ ;  $C_x$  is the concentration;  $p_{ji}(Z)$  denotes the contribution from irrelevant absorption; the subscripts  $i$ ,  $a$ , and  $b$  denote the wavelength range and the two different solutions, respectively. Thus, by choosing  $P_j$  and also the set of wavelengths,  $i$ , so that  $p_{ji}$  is optimum in one solvent and negligibly small in the other solvent,  $\Delta p_{ji}$  can be used to evaluate compound  $x$ .

The assay parameters using the  $\Delta p_j$  method for chloroquine:

Solvents	:	0.1 N $H_2SO_4$ & 0.1 N NaOH.
Concentration	:	1.6 mg/100 ml solvent.
$P_j$	:	$P_4$ (the quartic polynomial).
Number of Points	:	12-point orthogonal polynomials.
Wavelength range	:	324-346 nm each 2 nm.
$\Delta p_4$	:	- 0.00170
$N_4^*$	:	8008
$(\Delta p_4 \cdot N_4^{1/2})^{**}$	:	0.160

---

\*  $N_4$  = normalizing factor for  $P_4$ .

\*\* should exceed 0.14 for the coefficient of variation to be less than 1%.

### 8.3.2 Fluorimetric Method

A highly sensitive, accurate and improved fluorimetric method has been developed for analysis of chloroquine in biological samples (31). The chloroquine is extracted with heptane or methylene dichloride from an alkali medium buffered with borate, pH 9.5. Chloroquine is reextracted into 0.1 N HCl which is then mixed with an equal volume of 0.2 M alcoholic NaOH and fluorescence is read at activation and emission wavelengths of 335 and 400 nm respectively.

## 8.4 Chromatographic Methods

### 8.4.1 Paper Chromatography

Several solvent systems have been described, which are used for the identification of chloroquine (8) as shown in the following table:

Solvent Systems	Visualizing Agents ( $R_f$ )
Citric acid: water: n-butanol 4.8 g: 103 ml: 870 ml.	Ultraviolet, iodoplatinate spray (strong reaction), bromocresol green spray (strong reaction) (0.19).
Acetate buffer (pH 4.58)	Ultraviolet, iodoplatinate spray (positive reaction). (0.70).
Phosphate buffer (pH 7.4)	Ultraviolet, iodoplatinate spray. (0.14).

### 8.4.2 Thin-Layer Chromatography

Clarke (8) described the following system for the identification of chloroquine.

Solvent System: Strong ammonia solution: methanol (1.5 : 100) should be changed after two runs.

Absorbent: Silica Gel G

Visualizing Agent: Acidified iodoplatinate spray (positive reaction).

$R_f$  : 0.31.

#### 8.4.3 Gas Chromatography

- a) Clarke (8) has described the following gas chromatographic method for the identification of chloroquine.

Column: 1% SE-30 on 100-120 mesh Arkrom ABS. 6 ft X 4 mm interval diameter borosilicate glass column.

Column Temperature : 250°.

Carrier Gas: Argon.

Gas Flow: 80 ml per min.

Detection and Retention Time: Argon ionisation detector or flame ionisation detector. Rt 1.44 relative to codeine.

- b) Churchill et al 1983 (23) has developed two gas chromatographic methods using Tandem fused-silica capillary for the determination of chloroquine and its major metabolite desethylchloroquine. The method I employed a single extraction step and internal standardization to permit rapid precise analysis for chloroquine in whole blood. Whereas method II employes derivatization with pentafluoropropionic anhydride before extraction and estimation of chloroquine and desethylchloroquine the detection limit for chloroquine in blood by both methods are 5 ng/ml and for desethylchloroquine is 15 ng/ml. Following specification column:

1.83 m X 2 mm ID glass column packed with OV-101 on 100-120 macsh gas chrome Q and a helium flow rate 15 ml/min.

Temperature : 250°C.

Electron impact ionisation performed at 70 eV.

#### 8.4.3.1 Liquid Chromatography

The liquid chromatographic system consisted of an M45 pump a U6K injector. The excitation wavelength was 335 nm and a 370 nm filter was used. The column (0.15 m X 4.6 mm I.D.) was slurry-packed with Lichrosorb Si 60, 5 µm and eluted. The flow rate of the eluent was 1 ml/min. After addition of water and extraction with diethyl ether, the organic phase was dried (sodium sulphate) and the solvent evaporated, the product could be used directly. The limit of detection of this method was 1 ng/ml of plasma for chloroquine and 0.5 ng/ml for desethylchloroquine. The precision of the method was 3.5% of (n=10) at 50 ng/ml plasma (urine) for chloroquine and 5% (n=10) at the 25 ng/ml for desethylchloroquine. (32).

#### 8.4.4 High-Pressure Liquid Chromatography

##### 8.4.4.1 Reversed-Phase HPLC Analysis

Bergqvist and Frisk-Holmberg, (33) reported a rapid and sensitive method for the analysis of chloroquine and its metabolite desethyl chloroquine in plasma, blood and urine using high-performance liquid chromatography. Chloroquine is extracted from the alkalized biological fluid with ethylene dichloride and re-extracted with dilute acid. It is then chromato-

graphed on a reversed-phase column. Ultraviolet absorption (254 or 340 nm) or fluorescence detectors are used. Chloroquine and desethylchloroquine concentrations in the range of 10 n mol/l (UV-detection) and of 0.5 n mol/l (fluorescence detection) could be accurately measured with a relative standard deviation of 12%.

#### 8.4.4.2 Straight-Phase HPLC Analysis

Bergqvist and Olin (34) have described a straight-phase HPLC method for the analysis of chloroquine as follows:

LiChrosorb Si 100 (7  $\mu\text{m}$ ) was used as support. The mobile and stationary phases were equilibrated before use. A tube (length 1.5 m and volume 4 ml) was inserted in front of the injector and placed in a thermostat in order to ensure good temperature control of the mobile phase. 25 ml of an aqueous perchlorate solution was present as a separate layer in the solvent reservoir in order to keep the mobile phase saturated. The column coating was achieved by the pumping technique. Columns were first tested in the adsorption mode with n-hexane + 1-butanol (199 + 1) as the mobile phase. The void volume  $V_m$ , was 2.86 ml. It was determined with toluene, which is not retained. The concentration of 1-pentanol in the mobile phase was controlled by gas chromatography on a Chromosorb 102 column with 1-butanol as internal standard.

#### 8.4.4.3 Ion-Pair HPLC Analysis

Recently a method for determination of chloroquine and its metabolites has been described by Brown (35).



This method is simple specific and sensitive for separation and quantification of chloroquine desethylchloroquine and bidesethylchloroquine in biological fluids using ion pair reverse phase HPLC procedure. It can detect amount as low as 5 ng and analysis time is 12 minutes. The following conditions have been used.

Column : 30 mm X 3.9 mm I.D.  
packed with 10  $\mu$ m  
U Bondspak C<sub>18</sub>.

Mobile Phase : 0.02ml 1-heptane  
sulphonic acid and  
acetonitrile.

pH : 3.4

Pumping ratio: 66:34 of PIC B-7 to  
acetonitrile.

Column Pres- : 62-76 bar.  
sure

### 8.5 Photolysis Analysis

Aron and Fidanza 1982 has described a photochemical method for determination and separation of chloroquine at nanogram level covering a range between 4 and 10<sup>4</sup> ng. They showed that reversible photo isomerization of chloroquine takes place and yields a fluorescent photoproduct. The chloroquine solution are spotted on silica gel plates and developed in alcohol ammonia mixture. The dried plates are irradiated with the image of mercury are focused on chloroquine spots. The fluorescence intensity was recorded. For photolysis studies chloroquine zone were scraped from the plates and mixed well with the 4 ml of water. The clear supernatant decanted and studied by absorption photometry and spectrofluorometry (36).

### Acknowledgements

The authors are thankful to Mr. Syed Rafatullah for his skillful assistance and Mr. Tanvir A. Butt for typing the manuscript.

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# CIMETIDINE

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## INTRODUCTION

Cimetidine is a histamine H<sub>2</sub>-receptor antagonist which inhibits the secretion of basal and gastric hydrochloric acid secretion and also reduces the output of pepsin. The drug has been widely used in the treatment of duodenal and gastric ulceration. It has also been used in the treatment of reflux esophagitis and for the reduction of gastric acid secretion and for the management of Zollinger-Ellison syndrome. The compound is given orally as a tablet, syrup, i.v., and as an injection fluid.

### 1. Description

#### 1.1 Nomenclature

##### 1.11 Chemical Names

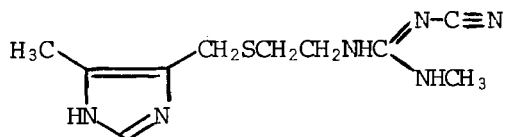
- (a) N"-cyano-N-methyl-N'-[2-[ (5-methyl-1H-imidazol-4-yl)methyl]thio]ethyl]guanidine (c.a. name)
- (b) N"-cyano-N-methyl-N'-[2-[ (5-methyl-imidazol-4-yl)methylthio]ethyl]guanidine

##### 1.12 Trade Name

Marketed by SmithKline Corporation under the trade name, Tagamet.

1.2 Formula, Molecular Weight Structure1.21 Empirical Formula, Molecular Weight

$C_{10}H_{16}N_6S$  252.352

1.22 Structure1.23 Hydrochloride Salt

$C_{10}H_{16}N_6S \cdot HCl$  288.816

1.3 Appearance, Color, Odor

The free base and hydrochloride salt are white crystalline solids with little or no odor. A slight sulfur-mercaptan odor may be present.

2. Physical Properties2.1 Spectral Properties2.11 Infrared Spectra

Figure 1 is the infrared spectrum of cimetidine free base. Figure 2 is the infrared spectrum of cimetidine hydrochloride. The spectra were obtained as mineral oil dispersions from 400-625  $cm^{-1}$  on a Perkin-Elmer Model 457A infrared spectrometer. The significant absorption bands in the spectra are:

Cimetidine

<u>Wavelength (<math>cm^{-1}</math>)</u>	<u>Assignment</u>
3220-3150	NH stretch
2180	-C≡N stretch
1620	>C=N-, cyano-guanidine
1590	C-C, C-N, aromatic



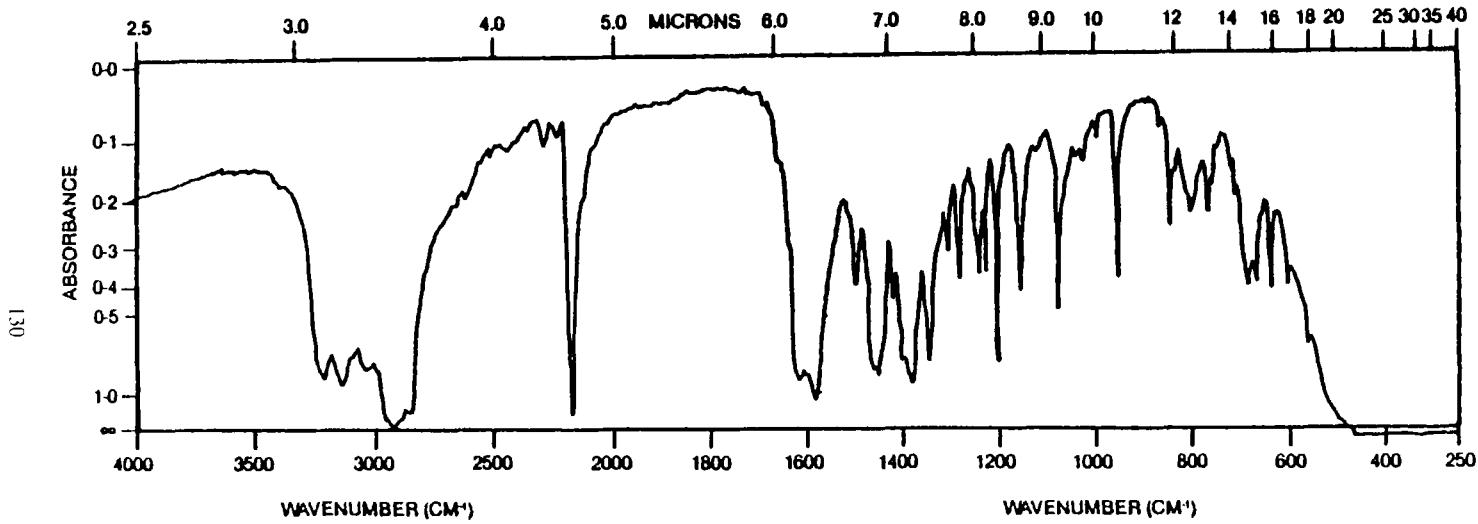


Fig. 1. Infrared spectrum of cimetidine.

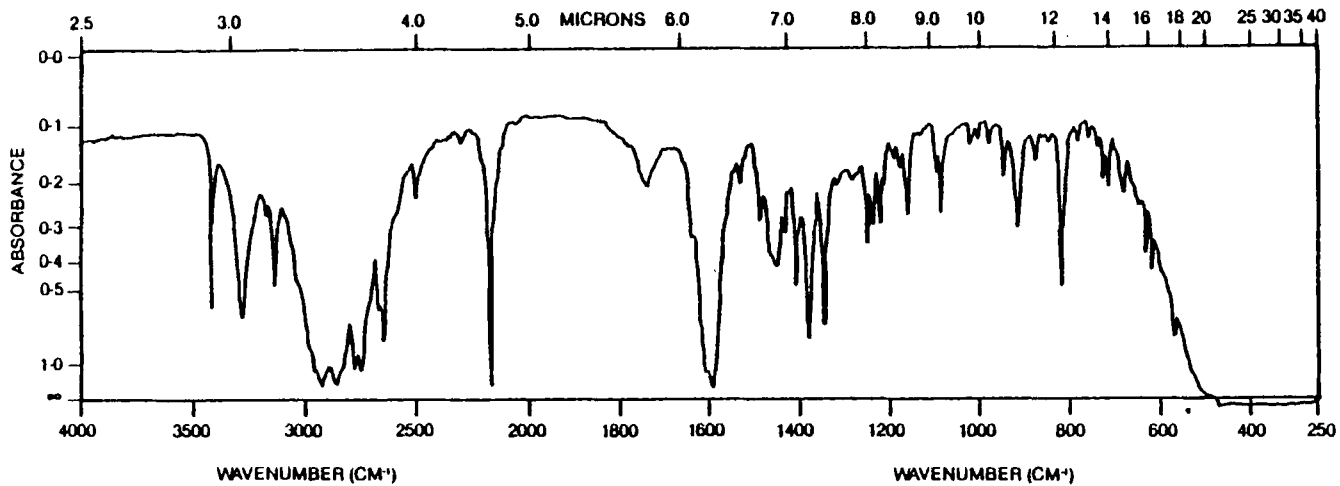


Fig. 2. Infrared spectrum of cimetidine hydrochloride.

Cimetidine Hydrochloride:

<u>Wavelength (cm<sup>-1</sup>)</u>	<u>Assignment</u>
3418	NH stretch
3280	NH stretch
3130	NH stretch
2780-2600	NH <sub>2</sub> <sup>+</sup>
2175	>N-C≡N stretch
1705-1595	>C-N stretch

An infrared study of the intramolecular hydrogen bonding in cimetidine and several related H<sub>2</sub>-receptor antagonists has been reported by R. C. Mitchell.<sup>1</sup> His study shows that cimetidine can form intramolecular bonds in solution and that it can adopt a ten-membered ring conformation in which the basic imidazole nitrogen atom is believed to be intramolecular hydrogen bonded to the NH group furthest from the imidazole ring.

2.12 Ultraviolet Spectrum

The ultraviolet absorption spectrum of cimetidine in 0.1N aqueous sulfuric acid is shown in Figure 3. A summary of the ultraviolet absorbance characteristics obtained in several solvents is shown in Table 1.

Table 1 Summary of Cimetidine Ultraviolet Absorption Data

<u>Solvent</u>	<u>λ max (nm)</u>	<u>ε max-ε<sub>260</sub></u>	<u>log ε</u>
0.1N aqueous H <sub>2</sub> SO <sub>4</sub>	218	20,530	4.31
95% ethanol	220	22,600	4.35
0.1N aqueous HCl	216	19,360	4.29

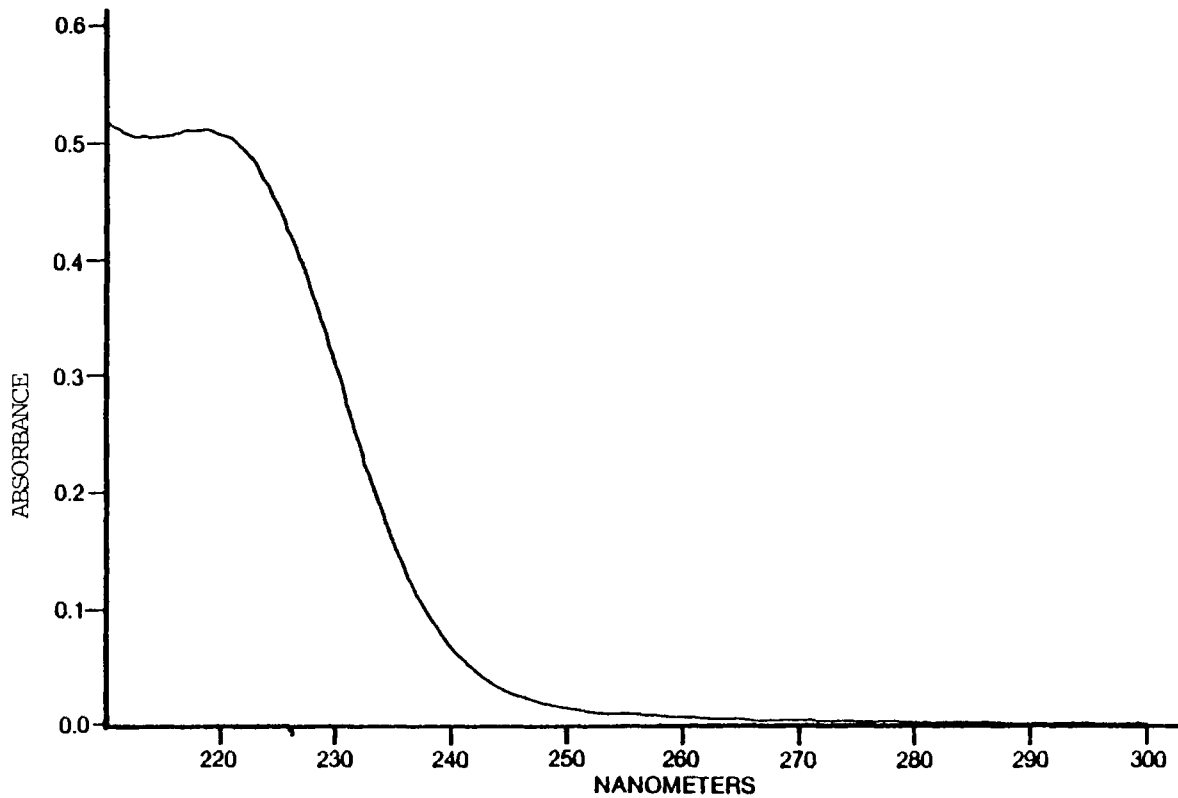


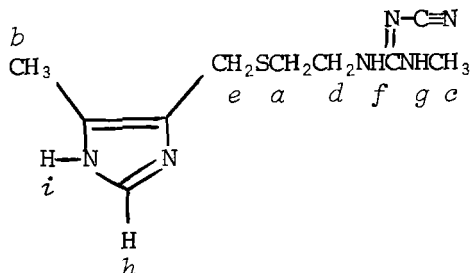
Fig. 3. Ultraviolet absorption spectrum of cimetidine in 0.1 N aqueous sulfuric acid.

The absorption maximum is due primarily to the  $\pi \rightarrow \pi^*$  transition of the cyanoimino ( $C=N-C\equiv N$ ) group with a partial contribution from the conjugated double bond system of the imidazole ring. The pH absorption dependency of cimetidine from pH 1.36 to 10.35 has been described by Kajfez, et al.<sup>2</sup>

## 2.13 Nuclear Magnetic Resonance Spectra

### 2.131 Proton Magnetic Resonance of Spectrum Cimetidine

The proton magnetic resonance spectrum (Figure 4) of cimetidine was recorded for a deuterated dimethyl sulfoxide solution containing approximately 100 mg/ml of the compound with tetramethylsilane as the internal standard. The spectrum illustrated was obtained using a Perkin-Elmer R32 proton magnetic resonance (CW) spectrometer. The resonances are:



<u>Protons</u>	<u>Chemical Shift</u>	<u>Multiplicity</u>	<u>Number</u>	<u>Integral</u>
<u>At</u>	<u>(ppm)</u>		<u>of Protons</u>	
$C_a$	2.50-2.58	multiplet	2	2
$C_b$	2.60	singlet	3	3
$C_c$	2.65	doublet	3	3
$C_d$	3.09-3.44	multiplet	2	2
$C_e$	3.60	singlet	2	2
$N_f, N_g$	6.45-7.34*	multiplet	2	2
$C_h$	7.43	singlet	1	1
$C_i$	11.7	singlet (broad)	1	1

\*quartet and triplet overlapped

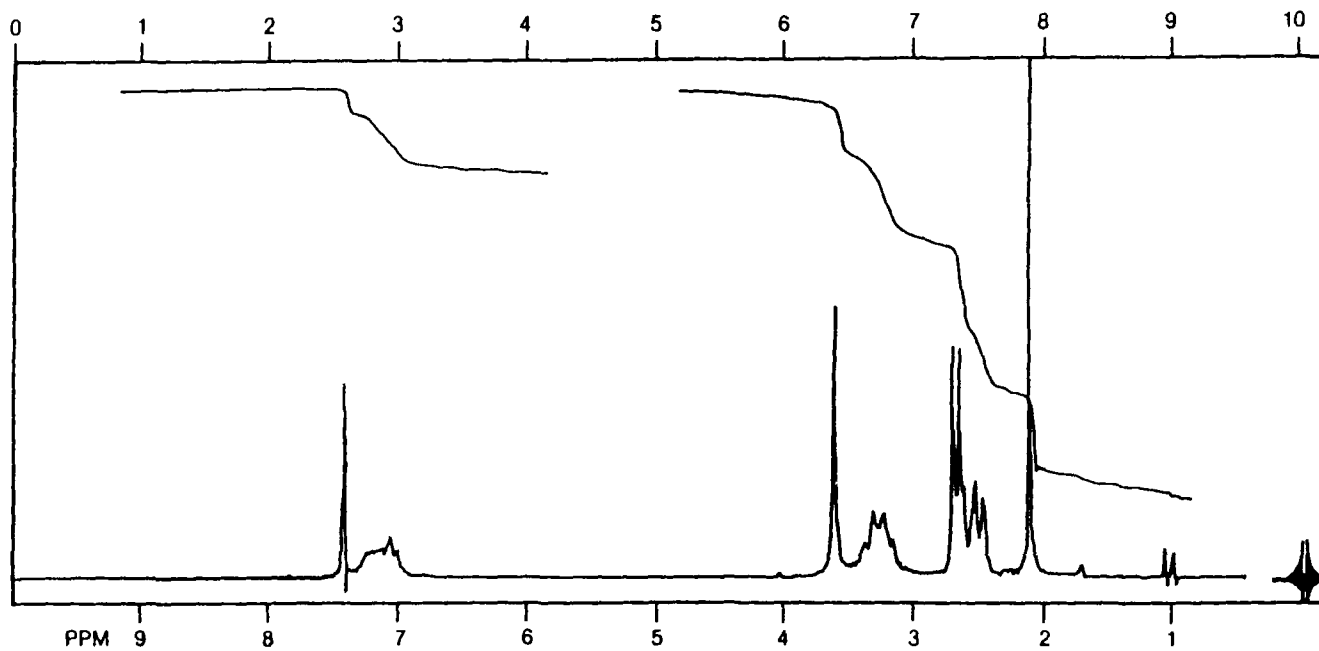
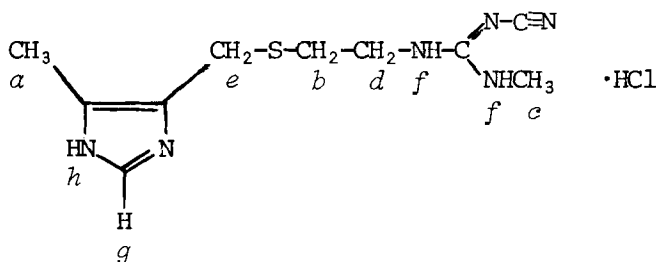


Fig. 4. Proton magnetic resonance spectrum of cimetidine in deuterodimethylsulfoxide.

2.132 Proton Spectrum—CimetidineHydrochloride <sup>3,4</sup>

The proton magnetic resonance spectrum of cimetidine hydrochloride was obtained by preparing a solution of approximately 100 mg/ml of the chemical in deuterated dimethyl sulfoxide containing tetramethylsilane as the internal standard. The chemical shift values of the hydrochloride salt differ from those of the free base and are:



<u>Proton Position</u>	<u>Structure</u>	<u>Chem. Shift (ppm)</u>	<u>Multiplicity</u>	<u>No. of Protons</u>
<i>a</i>	ring-CH <sub>3</sub>	2.29	singlet	3
<i>b</i>	S-CH <sub>2</sub>	2.60	triplet overlapped by DMSO multiplet at 2.5 ppm & a doublet at 2.7 ppm	2
<i>c</i>	-NHCH <sub>3</sub>	2.70	doublet overlapped by -SCH <sub>2</sub> triplet	3
<i>d</i>	-CH <sub>2</sub> CH <sub>2</sub> NH	3.32	multiplet	2
<i>e</i>	ring-CH <sub>2</sub> S	3.87	singlet	2
<i>f</i>	NH-CH <sub>3</sub> CH <sub>2</sub> NH-C(=N-CN) N-	7.18	multiplet	2
<i>g</i>	N-CH=N	8.99	singlet	1
<i>h</i>	-NH	9.0-10.0	broad singlet	1

The chemical shifts at positions *d* and *f* were verified by E. S. Pepper <sup>4</sup> using spin decoupling and spin-decoupling

with D<sub>2</sub>O exchange, respectively. These shifts were later confirmed by Kajfez, et al.<sup>2</sup>

2.133 Double Resonance Spin Decoupling and Deuterium Exchange Studies of Cimetidine

Spin Decoupled at: 7.12 ppm

Results: 2.69 ppm doublet collapses to a singlet. Shows that a coupling exists between NH-CH<sub>3</sub>.

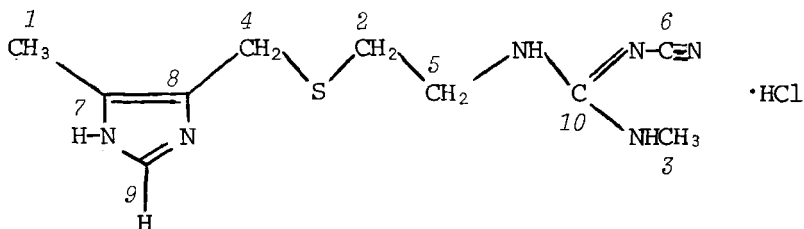
Spin Decoupled at: 7.1 ppm

Results: 2.09-3.44 multiplet (2H) is reduced to a less complex pattern. This is assigned to -CH<sub>2</sub>NH-C(=NCN) protons.

Deuterium (D<sub>2</sub>O) exchange causes the chemical shifts at 7.18 and 9.0-10.0 to disappear. These resonances are assigned to three exchangeable hydrogens attached to nitrogen on the imidazole ring and in the side-chain.

2.134 Carbon-13 NMR Spectrum

The broad band decoupled carbon-13 NMR spectrum of cimetidine hydrochloride (Figure 3) was obtained by using a solution of approximately 100 mg/ml in deuterated dimethylsulfoxide. The deuterium signal of dimethylsulfoxide was used as the internal reference and the spectrum was obtained on a Varian Associates Model FT-80 fourier transform NMR spectrometer. The chemical shift assignments are:





<u>Position of Carbon</u>	<u>Chemical Shift, ppm</u>
1	9.68
2	26.01
3	28.17
4	30.05
5	40.81
6	118.18
7	124.91
8	130.00
9	133.34
10	159.96

#### 2.14 Mass Spectrum

##### 2.141 Electron-Ionization Impact Spectrum <sup>3,4</sup>

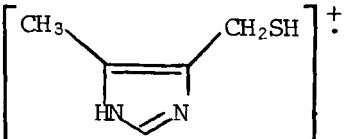
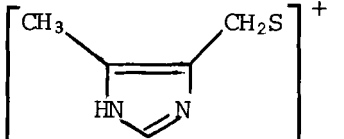
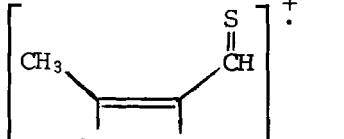
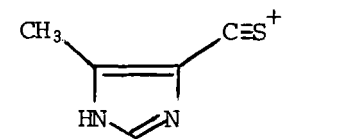
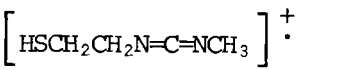
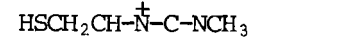
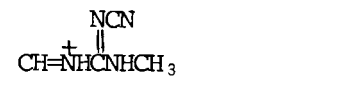
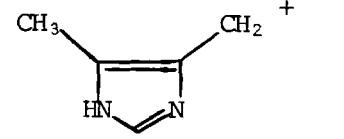

The mass spectrum of cimetidine using a Hitachi Perkin-Elmer RMU-7E medium resolution mass spectrometer and a direct insertion probe of the sample is shown in Figure 6. The mass spectrum was recorded on magnetic tape and relative abundances were calculated with a PDP-8/I digital computer coupled to the instrument. The results are presented in tabulated form in Table 2.

Table 2 Cimetidine Mass Spectral Fragment Ions By Electron-Ionization Impact <sup>5</sup>

<u>m</u>	<u>ion</u>
252	M <sup>+</sup>
158	$\left[ \begin{array}{c} \text{HSCH}_2\text{CH}_2\text{NHC} \\ \text{N-CN} \\ \text{NHCH}_3 \end{array} \right]^+$
157	$\left[ \begin{array}{c} \text{HSCH}_2\text{CH}=\text{NHC} \\ \text{N-CN} \\ \text{NHCH}_3 \end{array} \right]^+$

(continued)

Table 2 Continued

<u>m</u>	<u>ion</u>
128	
127	
126	
125	
116	
115	
111	
95	
82	

(continued)

Table 2 Continued

<u>m</u>	<u>ion</u>
69	$\text{CH}_2=\overset{+}{\text{N}}-\text{C}-\text{NCH}_3$
30	$\text{CH}_2-\overset{+}{\text{N}}\text{H}_2$

The fragmentation of cimetidine base appears to follow the three major pathways. Shown in Schemes 1, 2, and 3. The molecular ion is observed at  $m/e = 252$ .

#### 2.142 Mass Spectrum-Field Desorption

The field desorption mass spectrum of cimetidine base and a table of the fragmentation peaks are presented in Figure 7 and tabulated in Table 3. The spectrum was obtained using a Varian MAT CH-5 DF mass spectrometer. The emitter was loaded by the dipping techniques from an acetone solution. An emitter current of 20 milliamperes was used to obtain the spectrum. The spectrum shows a molecular ion,  $M^+$ , at  $m/e = 252$  and a  $MH^+$  at  $m/e = 253$ . The peak at  $m/e = 258$  is due to acetone used for instrument tuning purposes. Several low intensity fragment ions are found and are related to the electron-ionization fragmentation mechanism described above.

#### 2.143 Mass Spectrum-Chemical Ionization (CI)

The chemical ionization mass spectrum of cimetidine and the major fragmentation ions are presented in Figure 8 and Table 4. The spectrum was obtained using a Finnigan Model 3200 quadrupole mass spectrometer fitted with a chemical ionization source. The sample, applied to the probe from an acetone solution, was introduced via the direct inlet system. Methane was used as the reactant gas.

The spectrum shows an  $(M+1)^+$  peak for the molecular ion and characteristic

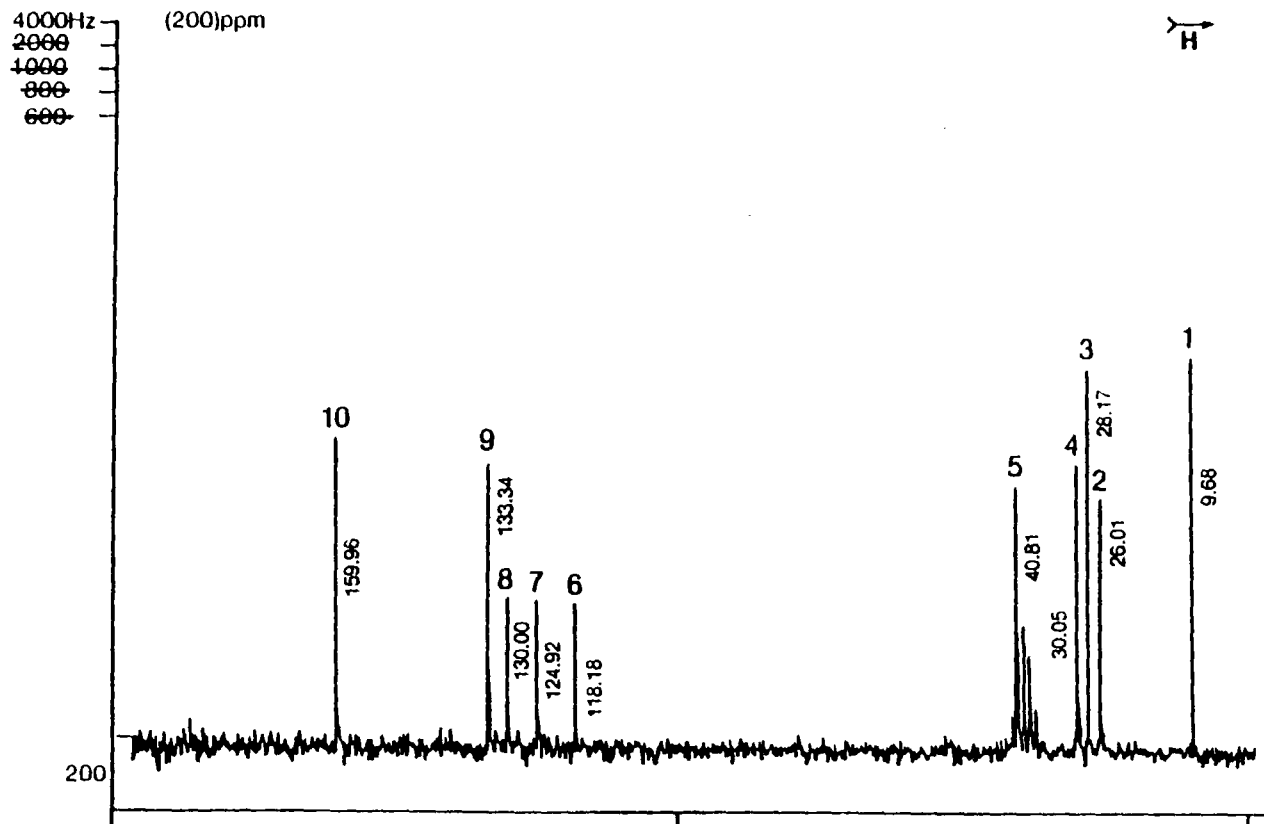


Fig. 5. Carbon-13 NMR spectrum of cimetidine hydrochloride.

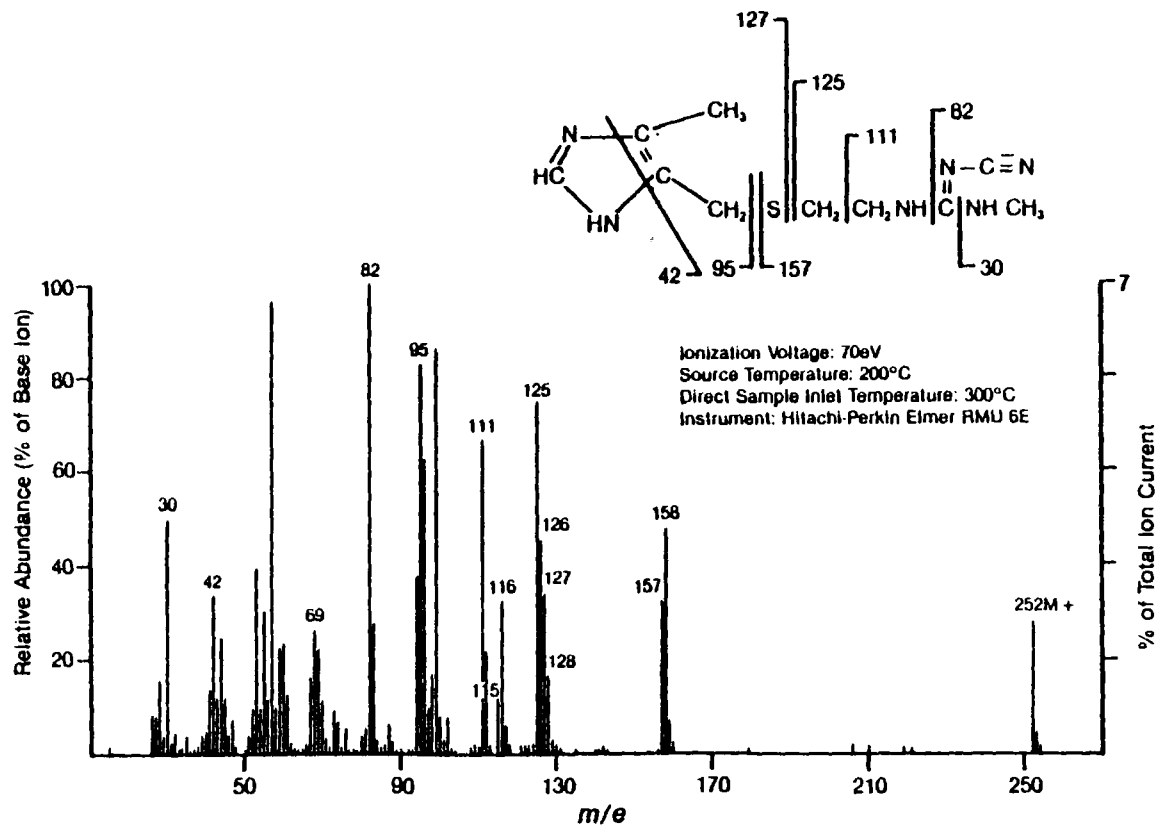


Fig. 6. Electron-ionization mass spectrum of cimetidine.

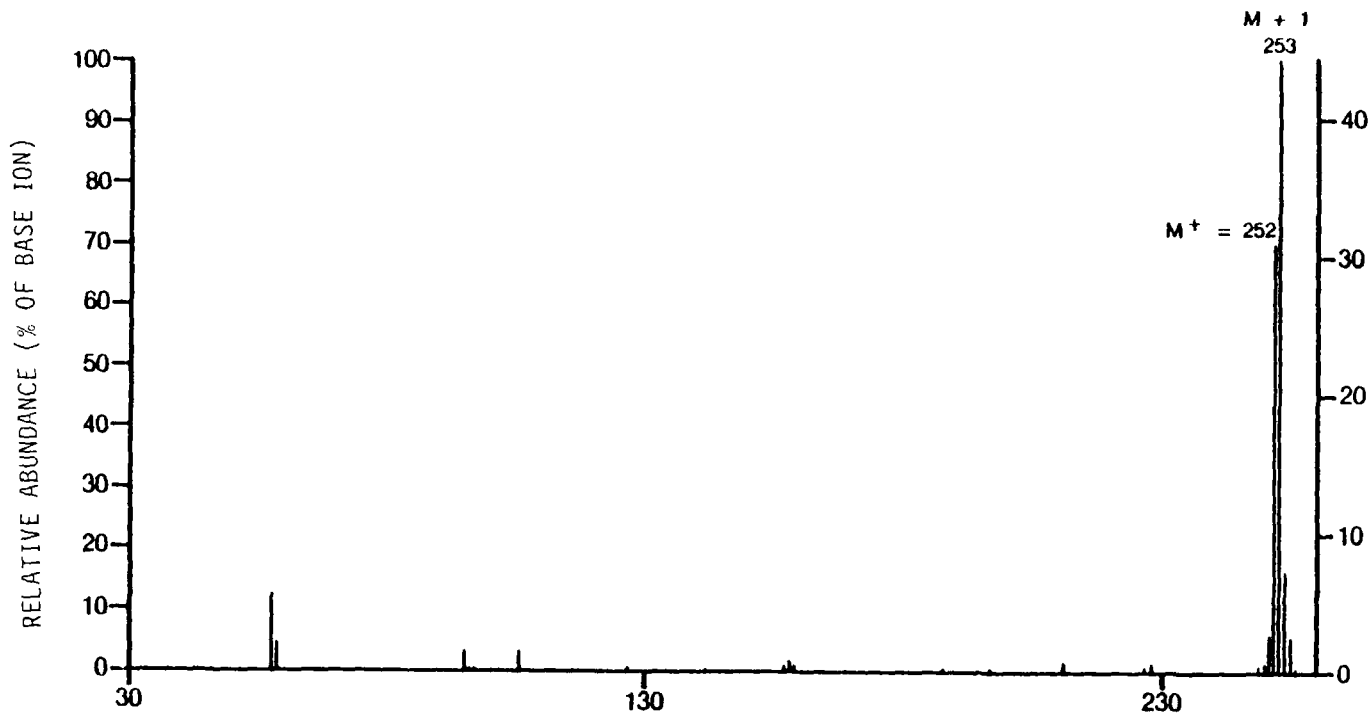


Fig. 7. Field desorption mass spectrum of cimetidine.

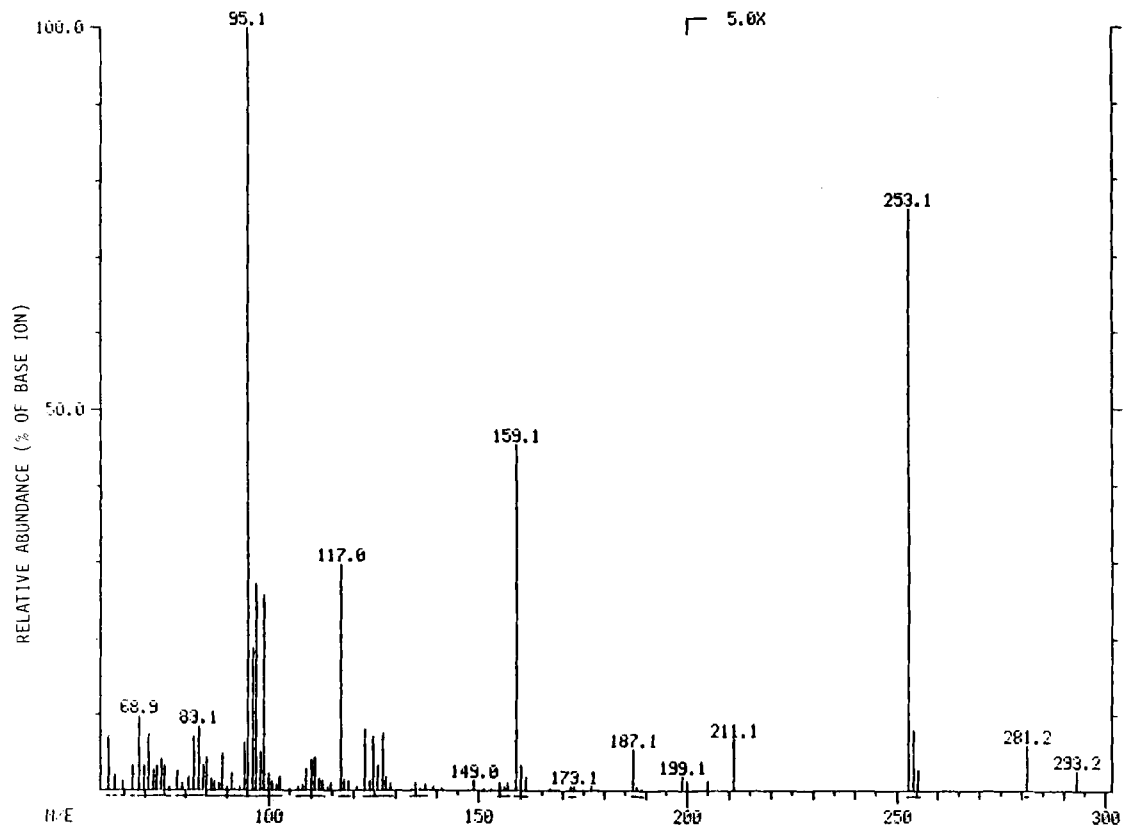


Fig. 8. Mass spectrum cimetidine- chemical ionization- methane reactant gas.

Table 3 Field Desorption Mass Spectrum of  
Cimetidine. Peak Intensity Data.

---

	04/16/81/SPEC#	185/KM/FD 20 MA
	BASE	SUM
	11306	27545
	<hr/>	
PEAK	I/BASE	MASS
<hr/>		
1	0.30%	43.8
2	12.48%	58.0
3	4.49%	59.0
4	0.26%	77.9
5	0.09%	81.0
6	3.02%	95.1
7	0.44%	96.1
8	0.14%	97.0
9	2.98%	105.9
10	0.09%	106.3
11	0.12%	117.0
12	0.62%	125.9
13	0.64%	127.0
14	0.38%	141.9
15	0.92%	157.0
16	1.78%	158.0
17	1.27%	158.9
18	0.37%	188.0
19	0.35%	188.1
20	0.13%	197.0
21	1.71%	211.1
22	0.78%	226.9
23	1.40%	228.0
24	0.84%	249.0
25	1.63%	250.0
26	6.09%	251.0
27	69.95%	252.1
28	100.00%	253.1
29	16.45%	254.0
30	5.98%	255.0
31	0.51%	256.1

---



peaks at  $(M+29)^+$  and  $(M+41)^+$  when using methane as the reactant gas at 1  $\mu$  torr pressure. The major fragmentation ions are given in Table 4. An INCOS computer system coupled with the mass spectrometer was used to obtain, normalize, and plot the spectral data.

Table 4 Chemical Ionization Mass Spectral Fragment Ions of Cimetidine ( $\text{CH}_4$ -Reaction Gas)

m	ion
293.2 (m + 41)	$(M + \text{C}_3\text{H}_5)^+$
281.2 (m + 29)	$(M + \text{C}_2\text{H}_5)^+$
253.1	$(M + \text{H})^+$
159.1	$\left[ \begin{array}{c} \text{N-CN} \\    \\ \text{HSCH}_2-\text{CH}-\text{CNHCH}_3 \\   \\ \text{+H} \end{array} \right]^+$
117.0	$\left[ \begin{array}{c} \text{HSCH}_2-\text{CH}_2-\text{N}=\text{C}=\text{NCH}_3 \\   \\ \text{+H} \end{array} \right]^+$
95.1	
68.9	$\text{CH}_2=\overset{+}{\text{N}}-\text{C}-\text{NCH}_3$

#### 2.144 Negative Ion Mass Spectrum

The negative ion mass spectrum of cimetidine was obtained using a Finnigan Model 3200 quadrupole mass spectrometer equipped with an INCOS data system. The mass spectrum is presented in Figure 9 and the major fragments are tabulated in Table 5. A comparison of the molecular ion peak intensity obtained in the negative ion mode with that obtained in the chemical ionization mode shows the former to be 11.4 times more intense. As the fragmentation in the negative ion mode is less, identification of smaller size samples of cimetidine can be made.

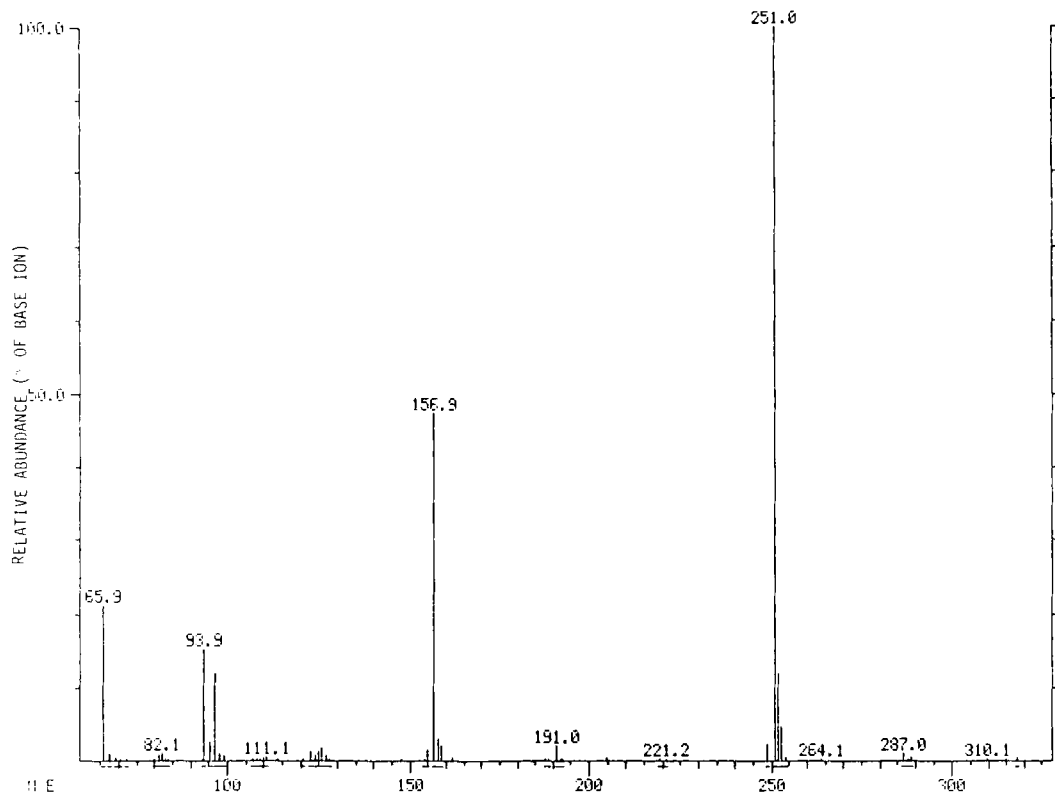
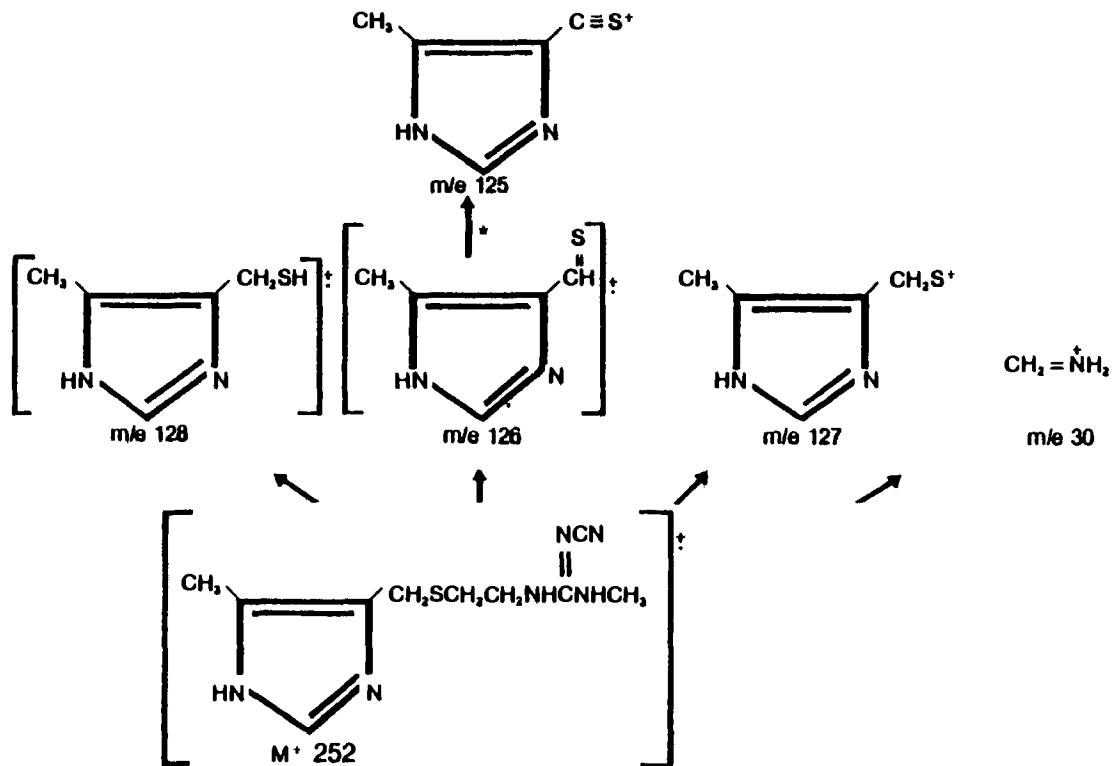
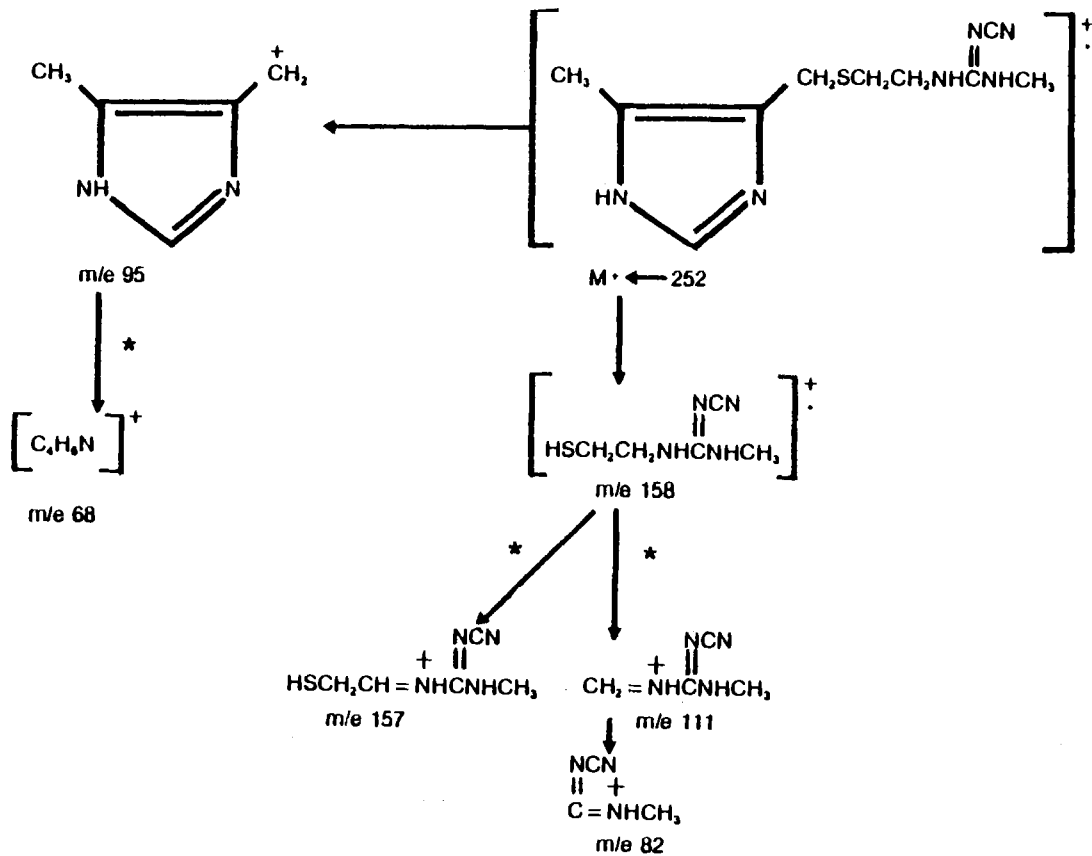


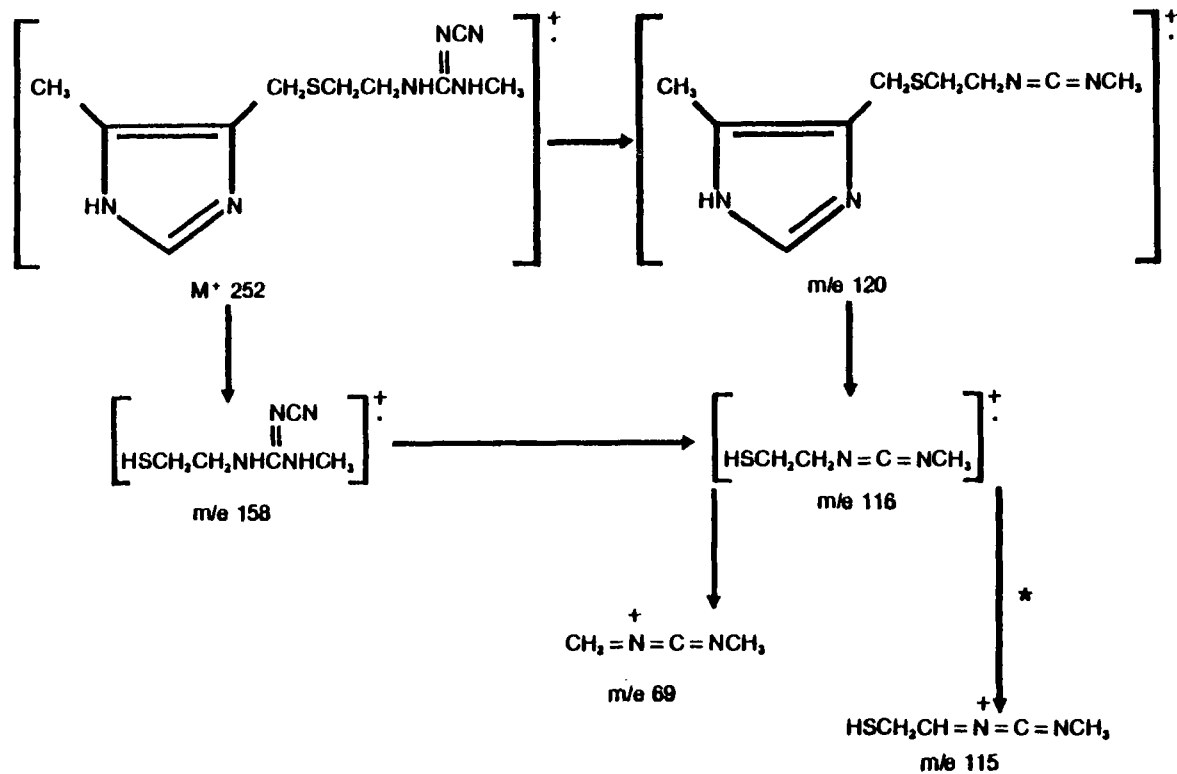
Fig. 9. Negative ion-mass spectrum of cimetidine.



Scheme 1. Fragmentation of cimetidine.

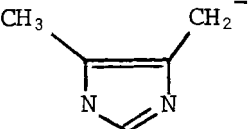


Scheme 2. Fragmentation of cimetidine.



Scheme 3. Fragmentation of cimetidine.

Table 5 Negative Ion Mass Spectral Fragment Ions of Cimetidine

m	ion
251.0	$[M-H]^-$
156.9	$-S-CH_2CH_2-NH-C(=N)CN$
93.9	

### 2.15 Photoelectron Spectrum of Cimetidine

The photoelectron spectrum of cimetidine has been reported<sup>2</sup>. The following bands were found and their assignments have been described:

<u>Band</u>	<u>Assignment</u>
8.41	Ionization potential of both imidazole $\pi$ electrons and the lone electron pair of imidazole N (3) atom.
9.03	Ionization potential of unshared sulfur electron pair.
9.4	$\pi_2$ electrons of imidazole ring and $\pi$ electron pairs of three nitrogen atoms in guanidinium unit.
11.57, 12.08	$\pi$ electrons $-C\equiv N$
13.03, 13.36, 14.20, 14.95, 15.88	} Not assigned

The photoelectron spectrum was obtained<sup>4</sup> with a Vacuum Generator UV 63 instrument at low resolution (35 eV) using He excitation at  $140^\circ$  and xenon internal calibration.

### 2.16 X-Ray Diffraction

Single crystal x-ray diffraction studies of

cimetidine were carried out by Hadicke, Frickel, and Franke.<sup>6</sup> "Cimetidine was found to form monoclinic crystals (space group  $P2_1/C$ ). The molecule is internally hydrogen bonded by the N...N-N bond between the imidazole and guanidine residue to form a ten-membered ring system."<sup>2</sup> The X-ray powder diffraction spectrum of cimetidine is shown in Figure 10. The spectrum was obtained using a General Electric XRD-5 powder diffractometer using  $\text{CuK}\alpha$  irradiation. Table 6 lists the d-spacings (interplanar distances), the diffraction angle,  $2\theta$ , and the relative peak intensities. Crystals were found to be prismatic and monoclinic. Prodic-Kojic, et al.<sup>7</sup> have reported the cell constants to be  $a = 6.82$  (1),  $b = 18.813$  (3), and  $c = 10.374$  (2) Å,  $\beta = 106.42$  (1)°.

## 2.2 Physical Properties of the Solid

### 2.21 Identity Tests

#### 2.211 Thin Layer Chromatography/Color Tests

This test may be used with the chemical, tablet, or liquid dosage form containing cimetidine or cimetidine hydrochloride. A methanol extract of the sample is prepared and chromatographed on a Silica Gel GF chromatoplate with ethyl acetate, methanol, ammonium hydroxide (100, 10, 10, v/v) mobile phase. The  $R_f$  comparison to a reference similarly chromatographed, the response to diazotized p-nitroaniline (imidazole nucleus detection), U.V. (254 nm), and  $\text{I}_2$  vapors serves to identify cimetidine.

#### 2.212 Infrared Identification

The infrared spectrum of a mineral oil dispersion of the sample, previously dried at 105°C for four hours, corresponds to the reference sample. Refer to Section 2.11.

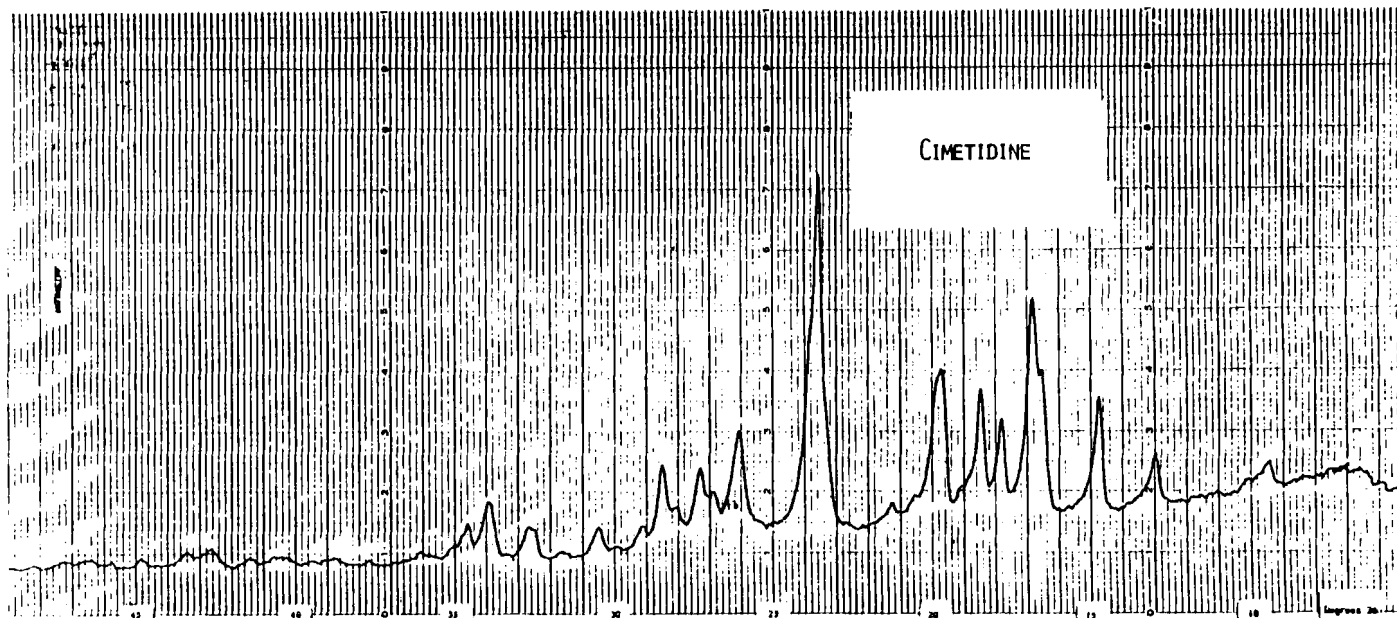


Fig. 10. X-ray powder diffraction pattern of cimetidine.



Table 6 X-Ray Diffraction Pattern of Cimetidine

$2\theta$	$d, \text{\AA}^*$	Relative Intensity
7.20	12.27	5.7
9.40	9.40	7.4
10.10	8.75	2.8
13.00	6.80	14.5
14.75	6.00	31.9
16.58	5.34	38.3
16.80	5.27	58.2
17.80	4.98	24.1
18.46	4.80	32.3
19.10	4.64	4.6
19.72	4.50	39.0
20.58	4.31	4.3
21.26	4.18	4.3
23.58	3.77	100.0
26.06	3.42	24.1
26.90	3.31	6.4
27.30	3.26	14.2
28.10	3.17	5.7
28.50	3.13	19.1
29.10	3.07	4.3
29.96	2.98	0.7
30.50	2.93	7.1
31.62	2.83	1.4
32.60	2.74	7.8
33.92	2.64	14.9
34.60	2.59	9.2
35.00	2.56	2.8
36.06	2.49	2.1
37.64	2.39	1.4
38.80	2.32	2.1
40.50	2.22	2.1
41.40	2.18	2.1
42.64	2.12	5.3
43.40	2.08	4.3
44.80	2.02	2.1
46.40	1.96	2.1
47.30	1.92	1.4

$$* d = \text{interplanar distance} = \frac{n\lambda}{2 \sin \theta}$$

### 2.213 Ultraviolet Absorption Identification

The ultraviolet absorption spectrum of a solution of cimetidine in 0.1N sulfuric acid or 0.1N HCl exhibits a maximum between 217-219 and a minimum between 210 nm and 212 nm, (Figure 4). Refer to Section 2.12.

### 2.214 Color Identification Test I

To 0.1 ml of solution of the sample prepared by dissolving 1 mg of cimetidine in 1 ml of ethanol add 5 ml of a solution of 1 g citric acid in acetic anhydride to make 50 ml, (freshly prepared). Heat the mixture on a waterbath for ten to fifteen minutes. A red violet color develops.

### 2.215 Color Identification Test II

To 0.1 g of sample add 5 ml of 1N hydrochloric acid, and heat gently. Add 5 ml of sodium hydroxide solution (3 g in 10 ml H<sub>2</sub>O) to the solution and heat additionally. The reaction mixture evolves an ammonia odor. When a piece of moistened red litmus paper is exposed to the evolved gas, it turns blue.

## 2.22 Thermal Properties

### 2.221 Melting Range

Cimetidine melts between 140 to 143.5°C using the USP XX procedure for class 1 substances.

### 2.222 Differential Scanning Calorimetry (DSC)

The DSC thermogram for cimetidine is shown in Figure 11. A single endotherm is found at 142°C. As it does not recrystallize from its melt after prolonged cooling at room temperature, seeding, scratching, or annealing, it is considered an amorphous glass.

The hydrochloride salt of cimetidine shows a single endotherm at 193° C (Figure 12).

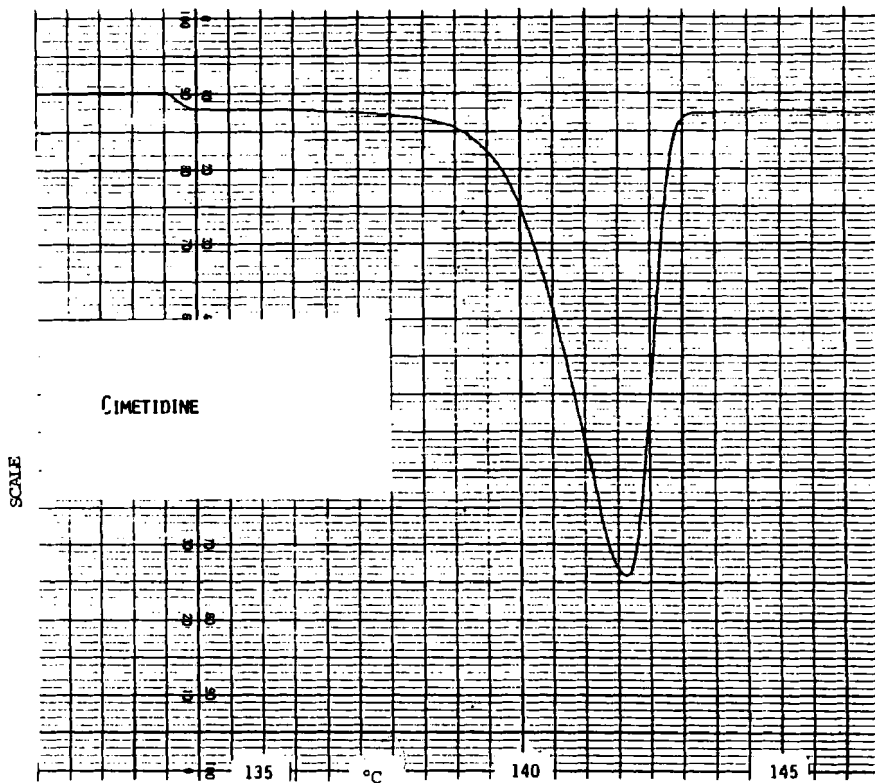


Fig. 11. Differential scanning calorimetric thermogram of cimetidine.

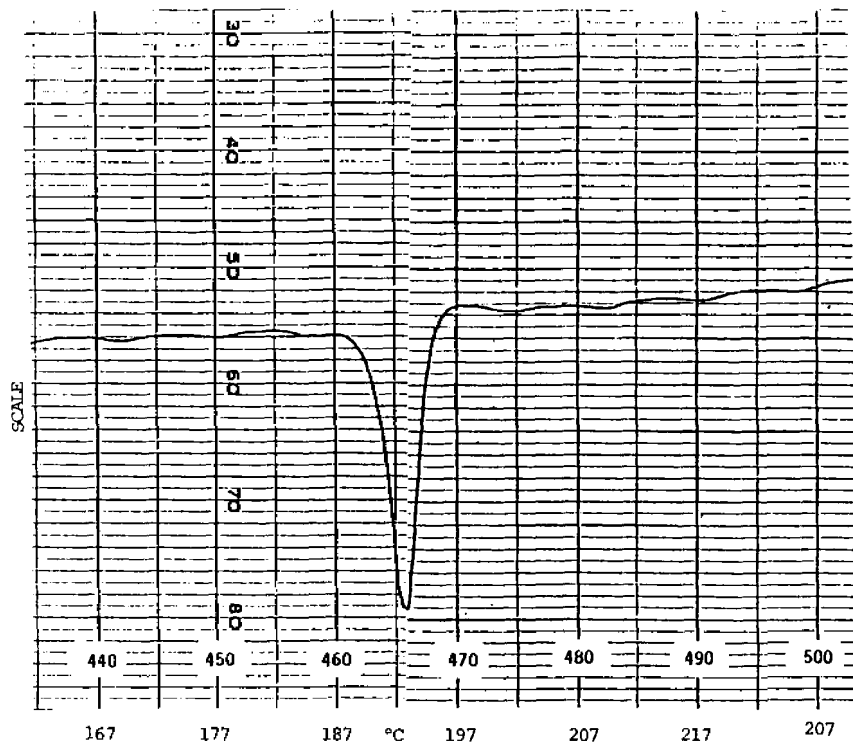


Fig. 12. Differential scanning calorimetric thermogram of cimetidine hydrochloride.

### 2.23 Density/Specific Surface Area<sup>8</sup>

Density measurements were carried out by the use of an air comparison pycnometer and the specific surface area was determined on milled and unmilled sample using a Stroklein areameter. The data are summarized in Table 7.

Table 7

<u>Property</u>	<u>Units</u>	<u>Unmilled</u>	<u>Milled</u>
Mean Particle Size	Microns	19.0	7.8 12.1
Specific Surface Area	m <sup>2</sup> /g	0.666	1.304
Absolute Density	g/cc	1.2815	-
Tapped Density	g/cc	0.40	0.51

### 2.24 Thermogravimetric Analysis (TGA)

The thermogravimetric curves for cimetidine and cimetidine hydrochloride are shown in Figure 13 and 14, respectively. The curves show that the compounds are stable up to a temperature of approximately 175-185°C and then decompose. The thermograms were obtained at a heating rate of 10°C/minute.

## 2.3 Solubility of Cimetidine

### 2.3.1 Solubility in Various Solvents

The solubility of cimetidine<sup>9,10</sup> was determined in a variety of solvents using essentially the method of Schefter and Higuchi.<sup>11</sup>

<u>Solvent</u>	<u>Solubility (mg/ml)</u>				
	<u>20°C</u>	<u>24°C</u>	<u>25°C</u>	<u>37.0°C</u>	<u>37.5°C</u>
Acetonitrile		2.7			
Chloroform	1.0				
Cyclohexane	<0.01				
Ethanol (USP)	58.0				64.5
Isopropanol	13.2				
Diethyl Ether	0.01				
Methanol	122	144			
Polyethylene Glycol 400	51				
Water	5		6.15	11.4	
0.1N HCl				>250	

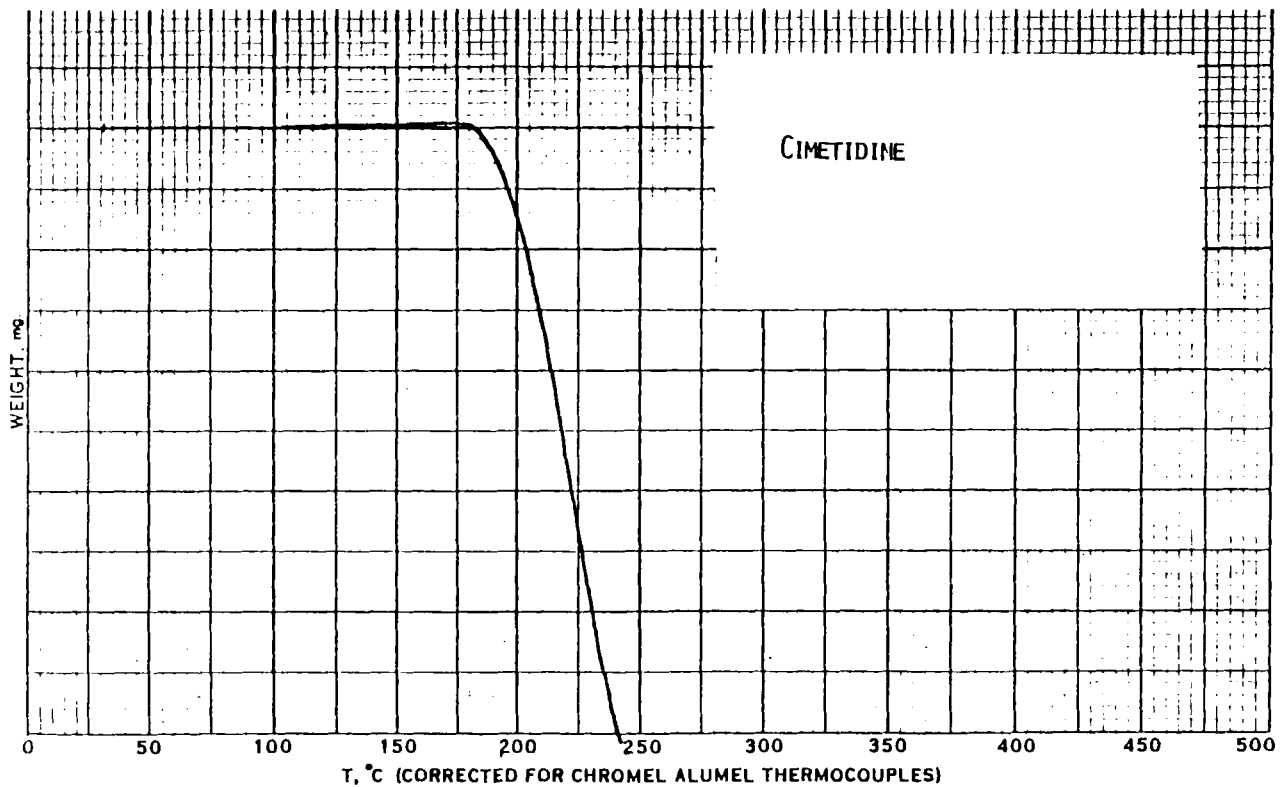


Fig. 13. Thermogram (TGA) for cimetidine.

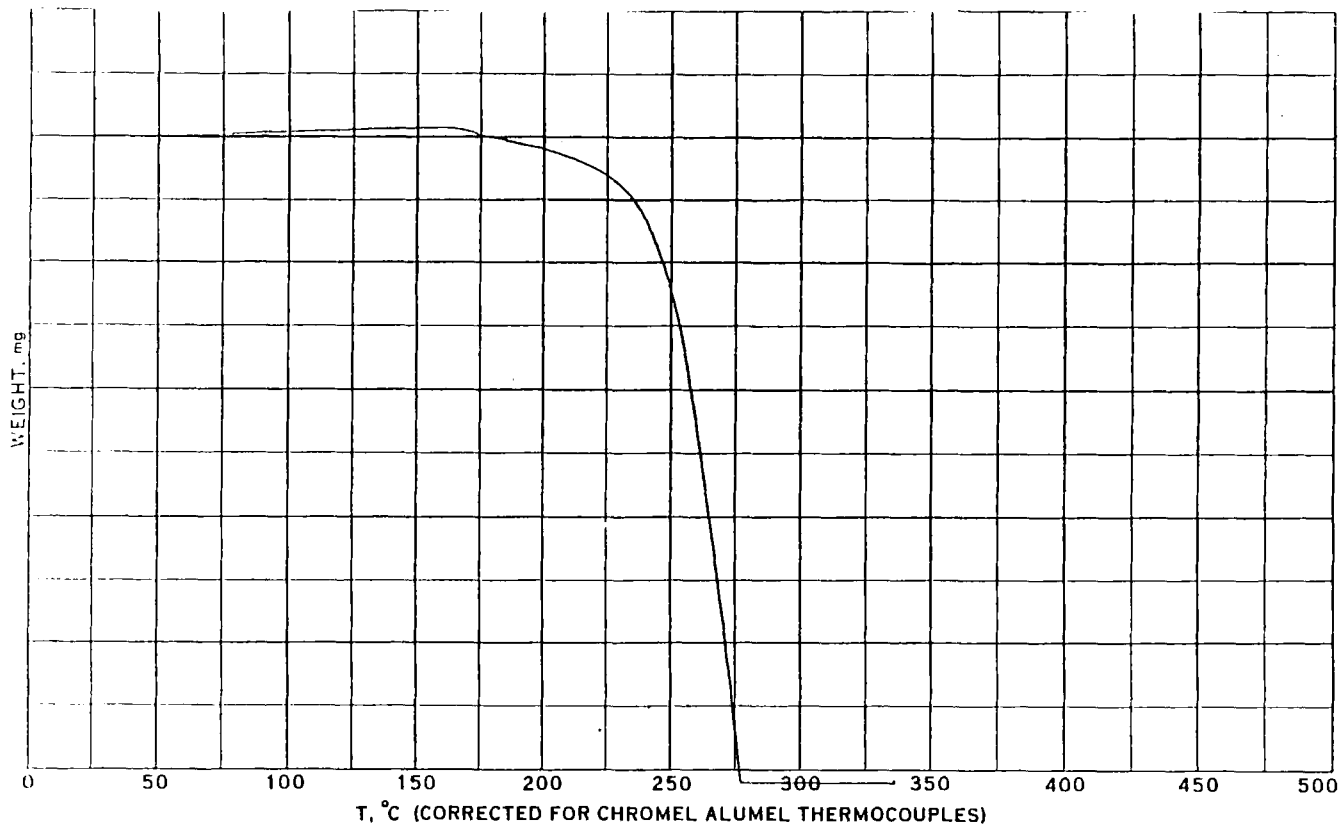


Fig. 14. Thermogram (TGA) of cimetidine hydrochloride.

A. Prodic-Kojec, et al<sup>7,9</sup> reported a high dependence of equilibrium solubilities of cimetidine which ranged from 0.75% at pH 8.92 to 2.72% at pH = 2.26 when determined at  $35.0 \pm 0.1^\circ\text{C}$ . Similar data was obtained by Mitchell.<sup>1</sup>

#### 2.311 Aqueous Solubility of Cimetidine Hydrochloride

In a study of apparent equilibrium solubility cimetidine hydrochloride is approximately 1.0 g/ml at  $25^\circ\text{C}$ . The equilibrium solubility is approximately 160 mg/ml.

### 2.4 Physical Properties of Cimetidine Solutions<sup>14</sup>

Aqueous solutions of cimetidine and cimetidine hydrochloride are usually colorless to a very pale straw color. A saturated solution of the free base has a pH of approximately 9.0 and a 15% solution of the hydrochloride salt, a pH of approximately 3.7.

#### 2.41 Dissociation Constants

The apparent pKa of cimetidine is  $7.11 \pm 0.04$  at  $25^\circ\text{C}$  measured potentiometrically in 0.1N aqueous KCl<sup>12</sup>. In water, the pKa of the imidazole ring nitrogen (-NH-) is 6.80.<sup>12</sup> At pH 7.4, 20.7% of cimetidine is present as cations. The nitrogen atom on the side chain to which the cyano (-C≡N) group is attached is essentially neutral and has a pKa =  $-0.4$ .<sup>13</sup> It is essentially non-ionized in a broad pH range (2-12).

#### 2.42 Partition Coefficient

The "Apparent Partition Coefficient" for cimetidine in an octanol/pH 7.4 phosphate buffer system at  $25^\circ\text{C}$  and in an octanol/water system at  $37^\circ\text{C}$  were found to be 2.0 and 2.5 respectively.<sup>9,13</sup>

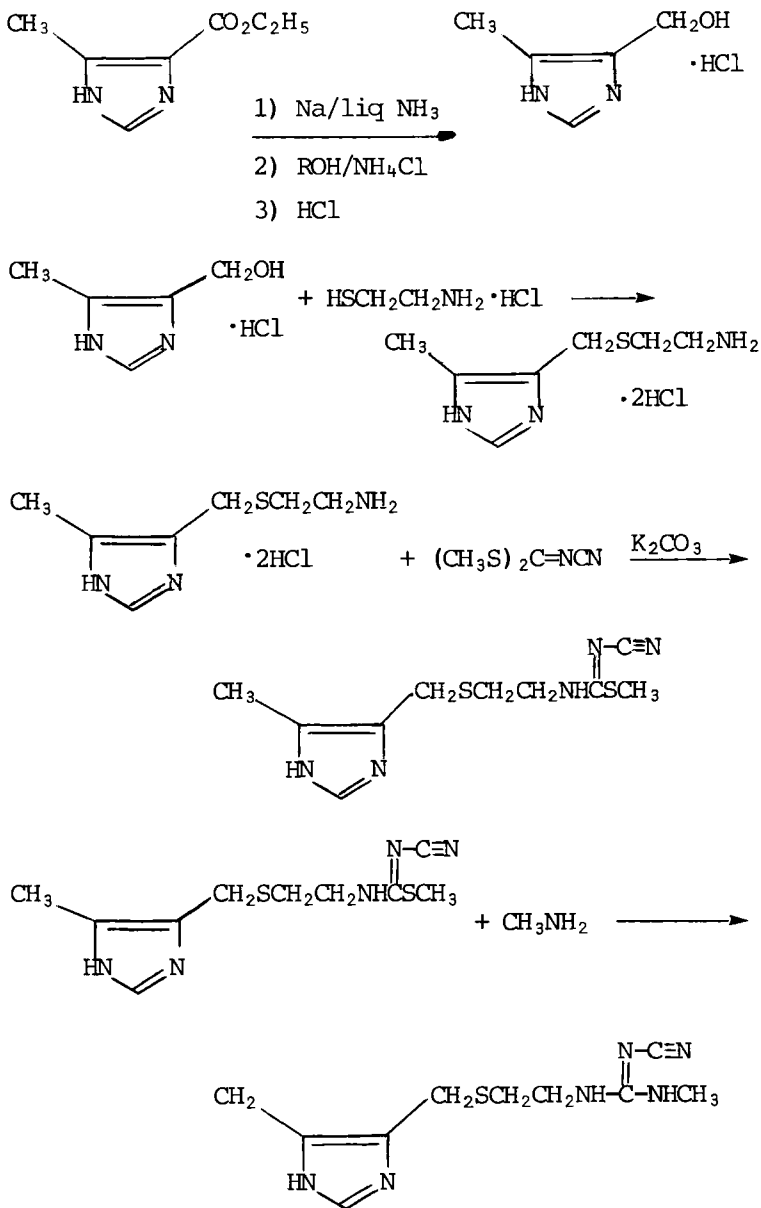
## 3. Synthesis

### 3.1 Synthesis of Cimetidine

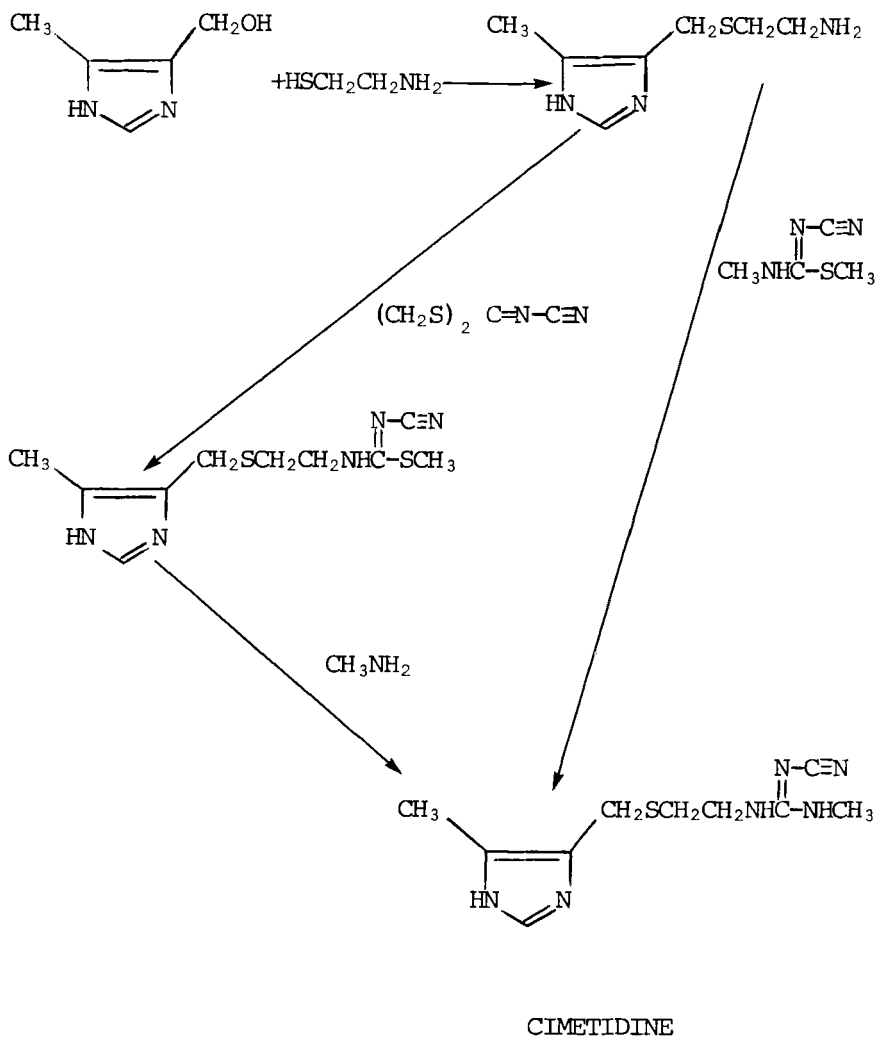
Synthesis of cimetidine has been described by Durant, G. J., et al.<sup>13,14</sup>



## Route 1



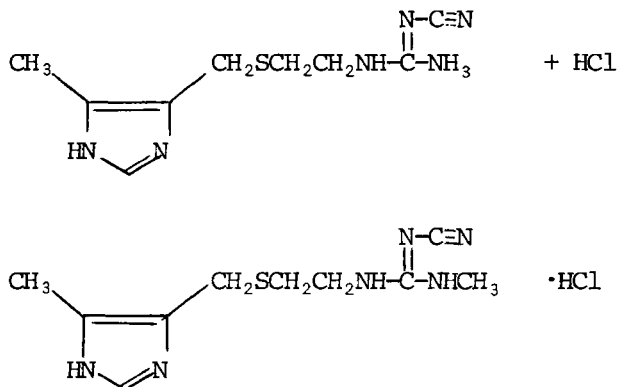
Route 2



### 3.2 Synthesis of Cimetidine Hydrochloride

N"-cyano-N-methyl-N'-[2-[(5-methyl-1H-imidazol-4-yl)methyl]thio]ethyl]guanidine, hydrochloride

To an ethanolic suspension of cimetidine, concentrated hydrochloric acid and ethyl acetate are added, the product is collected, washed with ethyl acetate, and dried. The schematic is illustrated below:



## 4. Stability

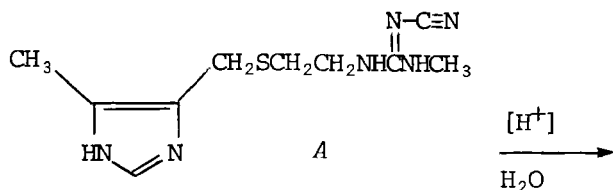
### 4.1 Cimetidine

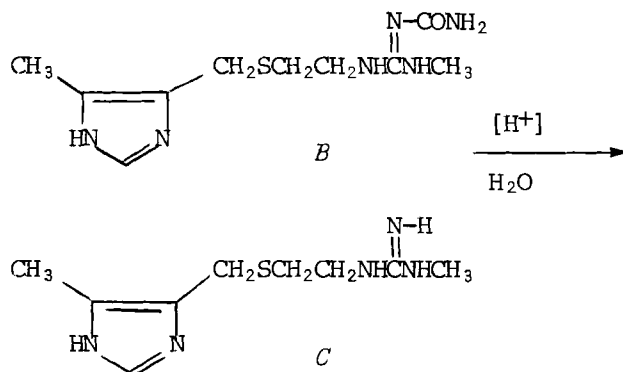
#### 4.1.1 Dry State

Cimetidine in the dry state, stored in a closed container at room temperature showed no decomposition after five years when examined by high pressure liquid chromatography, thin layer chromatography, infrared spectrophotometry, and mass spectrometry. It is stable for at least 48 hours at 100°C.

#### 4.1.2 Acid Hydrolysis

Acid hydrolysis of cimetidine<sup>13</sup> follows the following scheme:





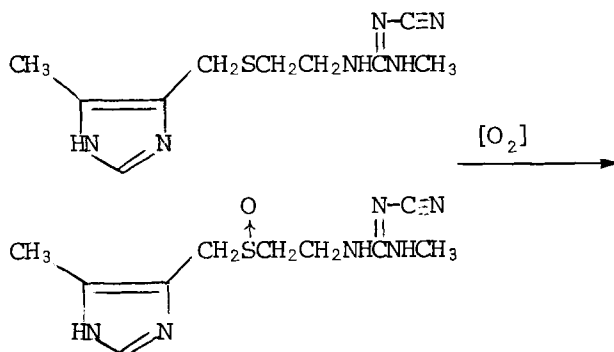
The formation of the amide is pH and temperature dependent. Solutions of cimetidine in hydrochloric acid at pH  $\sim 5.4$  showed no decomposition at  $50^\circ$  over a period of thirty days. Hydrolysis to the guanylurea (B) occurred when the compound was heated at  $45^\circ$  for 36 hours with an excess of 1N hydrochloric acid at pH  $< 1$ . The guanidine was formed by heating cimetidine for two hours at  $100^\circ\text{C}$  with concentrated hydrochloric acid.

#### 4.13 Effect of Ultraviolet Light

No decomposition of cimetidine was noted 15 after several months exposure to U.V. light.

#### 4.14 Effect of Hydrogen Peroxide and Oxygen

When cimetidine is treated with 3%  $\text{H}_2\text{O}_2$  at room temperature or exposed to oxygen at  $50^\circ\text{C}$  for short time periods, formation of sulfoxide is observed. Refer to the following:



## 4.2 Cimetidine Hydrochloride

The stability of cimetidine hydrochloride was reported by Rosenberg, et al.<sup>16</sup>

### 4.21 Aqueous Solutions

Aqueous solutions (vials for injection) showed a shelf life of at least two years when examined by high pressure liquid and examined by high pressure liquid and thin layer chromatography.<sup>16</sup>

### 4.22 Infusion Fluids

Solutions in common infusion fluids at concentrations of 120 or 500 mg/100 ml were found to be stable for at least one week at room temperature when examined by high pressure liquid and thin layer chromatography.<sup>16</sup>

## 5. Analytical Chemistry

### 5.1 Identity Tests

#### 5.11 Identification Tests for Cimetidine

A. Dissolve about 20 mg of sample in 2 ml of distilled water with gentle warming. Add 1 ml of 5% aqueous sodium nitrite and allow to stand for approximately three minutes. Add 1 ml of sulfanilic acid test solution (250 mg of sulfanilic acid dissolved in 25 ml of 10% v/v aqueous HCl) and allow to stand for about five minutes. The formation of a yellow-orange to reddish brown color indicates the presence of cimetidine.<sup>17</sup>

B. Place about 20 mg of sample in a small test tube. Add one drop of concentrated hydrochloric acid, 5 ml of glacial acetic acid, stir to dissolve, and then add 1 ml of mercuric acetate test solution (USP). The formation of a white precipitate indicates the presence of cimetidine.

C. A solution of the sample of the hydrochloride salt responds positively to the test for chloride.

#### 5.12 Identity Tests by IR, UV, TLC and HPLC

A. Cimetidine and cimetidine hydrochloride have characteristic spectra (Figures 1 and 2).

B. The ultraviolet absorption spectrum of a solution of cimetidine or cimetidine hydrochloride in 0.1N H<sub>2</sub>SO<sub>4</sub> exhibits a maximum between 217 and 219 nm and a minimum at 210-212 nm (Figure 3).

C. Characteristic TLC and HPLC retention times are described for cimetidine in the text and may be used as confirmatory tests for cimetidine (Section 5.4, 5.5).

D. Single crystals or trace amounts of cimetidine respond to color tests and will give a positive mass spectral peak for the molecular ion. These have been described above.

## 5.2 Non Aqueous Titration

### 5.21 Chemical

Dissolve about 240 mg of sample, accurately weighed, in 75 ml of glacial acetic acid. Titrate with standardized 0.1 N perchloric acid in acetic acid to a potentiometric endpoint using glass-calomel electrodes. Each ml of 0.1N perchloric acid is equivalent to 25.234 mg of cimetidine.

### 5.22 Tablet

Determine the average tablet weight of twenty tablets. Powder and weigh accurately a sample equivalent to about one gram of cimetidine into a 200 ml volumetric flask. Add about 150 ml of methanol, and shake mechanically for thirty minutes. Dilute a volume with methanol, mix and filter through a Whatman #1 filter paper, discarding the first 15 ml of the filtrate. To 50 ml add 25 ml of glacial acetic acid and titrate with 0.1N acetous perchloric acid to a potentiometric endpoint using glass-calomel electrodes.

#### Calculation:

mg Cimetidine tablet =

$$\frac{\text{ml} \times \text{N} \times 252.34 \times 200 \times \text{Average Tablet Weight}}{\text{Sample Weight (mg)} \times 50}$$

### 5.3 Spectrophometric Procedure

#### 5.31 Chemical

Transfer about 65 mg of sample, accurately weighed, to a 200 ml volumetric flask. Dissolve in and dilute to volume with 0.1N sulfuric acid. Transfer 5.0 ml of this solution to a 200 ml volumetric flask, and dilute to volume with the same solvent. Record the ultraviolet absorption spectrum of this solution in 1 cm cells on a suitable spectrophotometer from 320 to 210 nm using 0.1N sulfuric acid as a blank. Measure the absorbance difference ( $\Delta A$ ) between the absorbance at 218 nm (maximum) and that at 260 nm.

#### Calculation:

% Cimetidine =

$$\frac{\Delta A \times 0.01212^* \times \text{Dilution Factor} \times 100}{\text{Weight of Sample (mg)}}$$

\*0.01212 = mg cimetidine/ml/unit  $\Delta A$

This absorptivity factor was experimentally determined using accurately weighed samples of cimetidine reference standard carried through the same procedure.

#### 5.32 Tablet

Weigh and finely powder twenty tablets. Weigh accurately a portion of the powder, equivalent to about 100 mg of cimetidine and transfer to a 200 ml volumetric flask. Add 75 ml of 0.1N sulfuric acid, and shake mechanically for thirty minutes. Dilute to volume with the same solvent, mix, and filter through Whatman #1 filter paper, discarding the first 15 ml of filtrate. Pipet 5.0 ml of filtrate into a 200 volumetric flask, add 0.1N sulfuric acid to volume, and mix. Record the ultraviolet absorption spectrum of this solution from 300 to 215 nm in a 1 cm cell versus 0.1N sulfuric acid on a suitable spectrophotometer. Subtract the absorbance at 260 nm from the absorbance of the maximum at about 218 nm ( $\Delta A$ ).

Calculation:

mg cimetidine/tablet =

$$\frac{\Delta A \times 0.01212^* \times \text{Average Tablet Weight (mg)} \times \text{Dilution Factor}}{\text{Sample Weight (mg)}}$$

#### 5.4 Thin Layer Chromatography

Adsorbent: Silica Gel GF, 250 microns

Eluting Solvent: ethyl acetate, methanol,  
concentrated ammonium hydroxide  
(10:1:1, v/v)

Ethyl acetate must be freshly  
distilled.

Equilibration: 15 minutes in a closed paper lined  
tank

Concentration: 50 mg/ml methanol

Detection: iodine, UV (254 nm)

Spotting: 100  $\mu$ g

The solvent is allowed to rise to the 15 cm line, the plate is removed from the chromatographic chamber and dried in a current of air until no solvent odor is detected. The plate is examined under UV light at 254 nm. To obtain maximum sensitivity the plate is placed in a chamber containing iodine crystals for about thirty to forty minutes or until maximum contrast of the spots is obtained. The Rf of cimetidine is approximately 0.35.

Several additional thin layer chromatography systems have been reported by Kajfez, et al.<sup>14</sup> These systems separate the sulfoxide of cimetidine from the free base. All work was carried out using Merck-Kieselgel 60F<sub>254</sub> plates. Visualization of the spots UV 254 and iodine absorption.



	ELUTING SOLVENT	COMPOSITION	<u>Rf</u>	
			CIMETIDINE	SULFOXIDE
a)	EtOAc:iso-PrOH:conc. NH <sub>4</sub> OH (25%)	(9:7:4)	0.76	0.56
b)	CHCl <sub>3</sub> :CCl <sub>4</sub> :MeOH	(2:1:1)	0.48	0.27
c)	Me <sub>2</sub> CO:CHCl <sub>3</sub> :n-BuOH:NH <sub>4</sub> OH (25%)	(3:5:3.4:1)	0.64	0.32
d)	EtOAc:iso-PrOH:DMF	(9:7:4)	0.62	0.33
e)	EtOAc:MeOH:DMF	(10:7:1)	0.62	0.40
f)	MeOH:n-BuOH	(3:2)	0.60	0.40
g)	MeCN:iso-PrOH:NH <sub>4</sub> OH (25%)	(9:3:2)	0.73	0.50

## 5.5 High Pressure Liquid Chromatographic Procedure<sup>20</sup>

Adsorption and reverse phase systems have been used to evaluate cimetidine. The procedures are rapid, have high specificity and are amenable to the analysis of cimetidine and cimetidine formulations. Table 9 contains the parameters of several cited investigations.

### 5.51 Analysis of Cimetidine

#### Mobile Solvent:

Mix 975 ml of acetonitrile, 20 ml of distilled water, and 5 ml of concentrated ammonium hydroxide.

#### Assay Preparation:

Accurately weigh about 50 mg of sample into a 50 ml volumetric flask. Dissolve in and dilute to volume with mobile solvent.

#### Standard Preparation:

Accurately weigh about 50 mg of cimetidine reference standard into a 50 ml volumetric flask. Add 25 ml of mobile solvent, shake for one-half hour, dilute to volume with mobile solvent, and mix.

#### Procedure:

Inject 20  $\mu$ l of Assay and Standard Preparations into a liquid chromatograph adjusted to the following operating conditions:

Instrument: Chromatronix 3100

Column: Zorbax-Sil (DuPont)

Column Diameter: 2.1 mm i.d. and 0.25 inch o.d.

Column Length: 25 cm

Column Temperature: Ambient

Column Pressure: 500 psig

Attenuation: 16

Flow Rate: 0.2 ml/minute

Detector: UV (254 nm)

NOTE: Under the above operating conditions, cimetidine has a retention time of about

10 minutes.

Calculation:

$$\% \text{Cimetidine} = \frac{C \times A_u \times \text{dilution factor} \times 100}{A_s \times \text{Sample Weight (mg)}}$$

Where:  $A_u$  = area under the peak relating to the Assay Preparation

$A_s$  = area under the peak relating to the Standard Preparation

$C$  = concentration of cimetidine reference standard in mg/ml

#### 5.52 Analysis of Tablets:

##### Mobile Solvent:

Mix 975 ml of acetonitrile, 20 ml of distilled water, and 5 ml of concentrated ammonium hydroxide.

##### Assay Preparation:

Weigh and finely powder twenty tablets. Weigh accurately a portion of powder (equivalent to 80 mg of cimetidine) into a 50 ml volumetric flask. Add 25 ml of mobile solvent, shake for one-half hour, dilute to volume with mobile solvent, and mix.

##### Standard Preparation:

Transfer about 80 mg of cimetidine reference standard accurately weighed, into a 50 ml volumetric flask. Add 25 ml of mobile solvent, shake for one-half hour, and dilute to volume with mobile solvent, and mix.

##### Procedure:

Inject 20  $\mu$ l of Assay and Standard Preparations into a liquid chromatograph adjusted to the following operating conditions:

Instrument: Chromatronix Liquid Chromatograph or equivalent

Column: Zorbax-Sil (DuPont), or equivalent

Column Diameter: 2.1 mm i.d. and 0.25 inch o.d.

Column Length: 25 cm

Column Temperature: Ambient

Column Pressure: 1200 psi

Attenuation: x 16

Flow Rate: 0.54 ml/minute

Detector: UV (254 nm)

NOTE: Under the operating conditions, cimetidine has a retention time of about 10 minutes.

Calculation:

mg cimetidine/tablet =

$$\frac{C \times A_u \times \text{dilution factor} \times A_v \text{ Tablet Wt (mg)}}{A_s \times \text{Sample Weight (mg)}}$$

Where:  $A_u$  = area under the peak relating to Assay Preparation

$A_s$  = area under the peak relating to the Standard Preparation

$C$  = concentration of cimetidine reference standard in mg/ml

### 5.53 Analysis of Capsules

Mobile Phase (0.05M borax):

Dissolve 39.1 g of  $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10 \text{H}_2\text{O}$  in distilled water and dilute to 2000 ml. Adjust the pH to 7.5 with formic acid.

Internal Standard Solution:

Dissolve about 120 mg of benzoic acid (ACS grade) in 100 ml of mobile phase.

Assay Preparation:

Transfer as completely as possible the contents of not less than twenty capsules to a tared container, determine the average content weight per capsule, and mix the combined contents thoroughly. Transfer an accurately weighed portion of the powder, equivalent to about 50 mg of cimetidine, to a 25 ml volumetric flask. Add about 8 ml of methanol, and shake mechanically for 10 minutes. Add 15.0 ml of Internal Standard Solution, dilute to volume with methanol and mix.

Table 9 HPLC Parameters For Cimetidine

Column	Mobile Phase	Flow Rate (mL/min.)	UV Detector (nm)	~RT (min.)	Reference
Lichrosorb Si 60	CH <sub>3</sub> CN, H <sub>2</sub> O, CH <sub>3</sub> OH, conc. NH <sub>4</sub> OH (1000, 20,60,5)	0.8	228	10.0	40
Lichrosorb Si 60	0.1% NH <sub>4</sub> OH/CH <sub>3</sub> CN	0.8	228	40.0	40
Zorbax Sil	CH <sub>3</sub> CN, CH <sub>3</sub> OH, H <sub>2</sub> O, conc. NH <sub>4</sub> OH (1000, 500,20,2)	3.0	228	3.4	38
Zorbax Sil	CH <sub>3</sub> CN, H <sub>2</sub> O, conc. NH <sub>4</sub> OH (975,20,5)	0.5	228	10.0	37
μ Bondapak C18	CH <sub>3</sub> CN, 10mM potas- sium phosphat e buffer (pH 3.0) (90,910)	2.0	220	4.0	39
μ Bondapak C18	0.3% (NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub> , CH <sub>3</sub> OH (450,550)	1.0	254	16.5	41
Partisil SCX	0.9% Aq (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , CH <sub>3</sub> OH (200,800)	1.0	228	3.5	40
Partisil SAX	0.1M Borax Buffer (pH 8.5)	0.75	254	2.8	42
Partisil 10-ODS	CH <sub>3</sub> CN, H <sub>2</sub> O, NH <sub>4</sub> OH (1000,50,1)	2.5	228	-	43

Standard Preparation:

Transfer ~50 mg of cimetidine reference standard, accurately weighed, into a 25 ml volumetric flask. Dissolve in 8 ml of methanol, add 15.0 ml of Internal Standard Solution, dilute to volume with methanol, and mix.

Procedure:

Using a loop injector, inject 20  $\mu$ l of Sample and Standard Preparation.

Instrumental Conditions:

The following instrumental conditions are typically employed when a Chromatronix Liquid Chromatograph is employed:

Instrument: Chromatronix, Model 3100

Column Packing: Strong Anion Exchange Resin

Column Diameter: 2.1 mm (i.d.)

Column Length: 1 meter

Column Temperature: Ambient

Column Pressure: ~500 psi

Flow Rate: ~0.40 ml/minute

Attenuation: x 64

Chart Speed: 4 minutes/cm

Detector: UV (254 nm)

Using the above conditions, the peaks are recorded in the order: cimetidine (approximately 4 minutes) and benzoic acid (approximately 8 minutes).

Calculation:

Calculate the ration 'R' for each chromatogram where:

$$R = \frac{\text{Cimetidine Peak Height}}{\text{Benzoic Acid Peak Height}}$$

mg cimetidine/capsule =

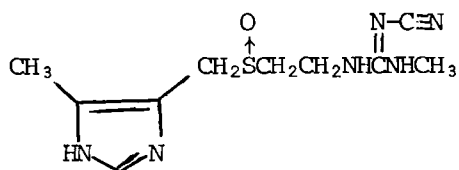
$$\frac{R_A \times \text{Wt of Std (g)} \times 1000 \times \text{Av Net Contents (g)}}{R_S \times \text{Wt of Sample (g)}}$$

Where:  $R_A$  = Ratio of Assay Preparation

$R_S$  = Ratio of Standard Preparation

### 5.6 Determination of Cimetidine Sulfoxide Content

White<sup>21</sup> used the previously cited procedure (Section 5.5) to separate and estimate the amount of the sulfoxide of cimetidine in the new drug substance. With sample concentration of 1 mg/ml and an injection of 20  $\mu$ l, as little as 1 ppm of the 'sulfoxide' could be detected and estimated at the RT of about 13 minutes.



'Sulfoxide'

White<sup>40</sup> also determined the presence of the 'sulfoxide' using a Partisil SCX column (25 cm x 4.6 mm i.d.); Mobile Phase: 80% CH<sub>3</sub>OH and 20% H<sub>2</sub>O containing 1.8 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>/l, a flow rate of about 1.5 ml/minute; Detection: UV at 228 nm and an injection of 25  $\mu$ l of a 25 mg/ml solution of sample.

### 6. Metabolism and Pharmacokinetics

The metabolism and pharmacokinetics of cimetidine has been studied and reviewed by several investigators.<sup>22</sup> The compound is absorbed rapidly whether given orally or i.v.. About 15-20% of cimetidine appears to be plasma protein bound and this is reported to be of no pharmacological significance.<sup>23</sup> In rats and dogs, cimetidine is readily absorbed and has a plasma half-life of about one-half to two hours. In human studies, cimetidine was shown to have a half-life of 123  $\pm$  12 minutes in the blood.<sup>23</sup> Cimetidine has been found from radioactive trace studies with 2-<sup>14</sup>C-cimetidine in healthy humans as well as dogs and rats to be mainly excreted in the urine. It is widely distributed throughout all the tissues and is rapidly eliminated with the exception of the liver, kidney and adrenal cortex.<sup>24, 25</sup> TLC, HPLC, and radioautographic studies indicated 56-85% of cimetidine (I) was unchanged, up to 30% was excreted as the sulfoxide (II), 5-8% as the hydroxymethyl (III) compound, approximately 2% as the

guanylurea (IV) and 7-17% as unidentified material.<sup>26-28</sup>

The reported metabolism products are illustrated in Figure 15.

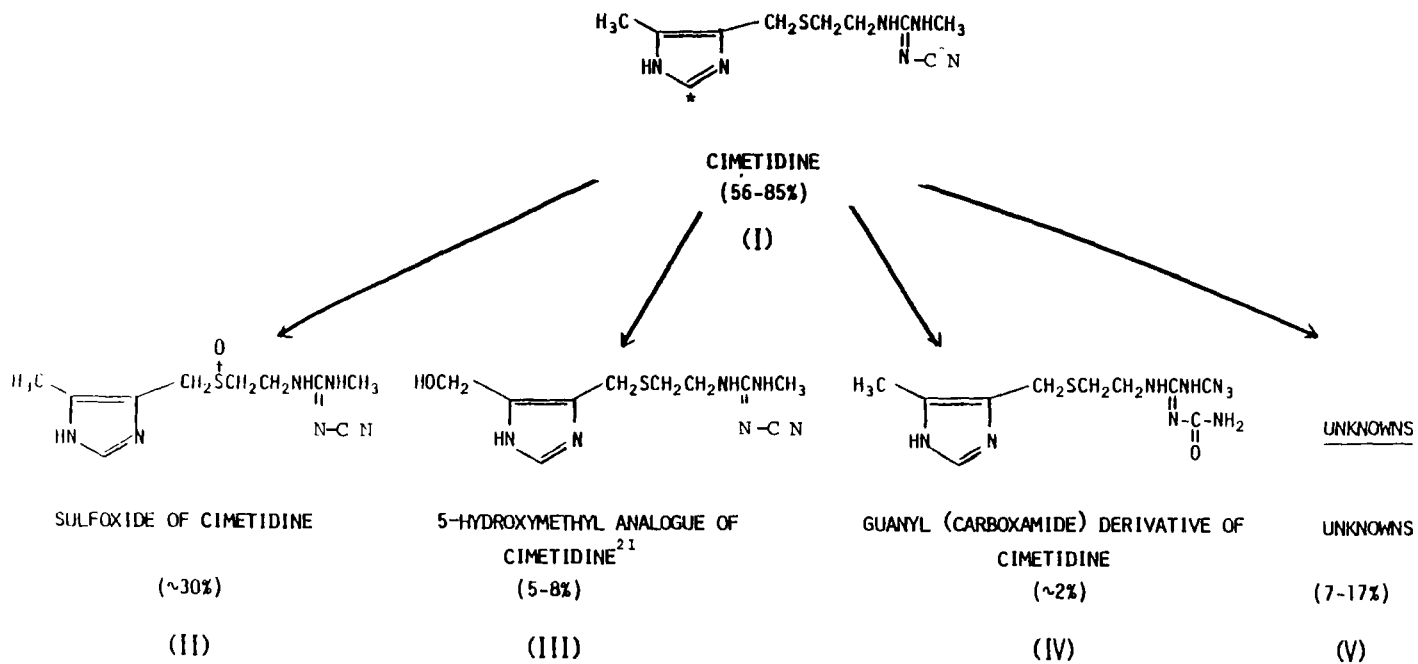
### 6.1 Determination of Cimetidine and its Metabolites in Biological Fluids

Cimetidine may be assayed in blood, urine, plasma,<sup>29, 30, 31, 32, 33, 38</sup> serum, bile, pancreatic fluid, gastric fluid, pleural fluid, cerebrospinal fluid, ascitic fluid and body tissues by HPLC procedures.<sup>32, 34</sup> The major identified metabolite, cimetidine sulfoxide<sup>22</sup> which is eliminated from the body mainly by renal excretion has been determined in blood and urine by Lee and Osborne using a high pressure liquid chromatographic procedure.<sup>32</sup> Their procedure allows the operator to cleanly separate creatinine, which is frequently present in clinical samples of patients suffering from renal failure and to determine both the cimetidine as well as the cimetidine sulfoxide.

The separation of cimetidine and its metabolites is usually carried out by extraction of the biological medium with 1-octanol from an aqueous alkaline pH 9 solution followed by mixing, addition of an internal standard and centrifugation. The extraction with octanol is repeated and the combined extracts are re-extracted with dilute hydrochloric acid. The aqueous acid solution is then separated, ethanol is added and mixed. This is then followed by saturating the mixture with a large amount of potassium or sodium carbonate to "salt out" the ethanol layer which contains the cimetidine and its metabolite, the sulfoxide. Several different internal standards have been used: Metiamide, 1-methyl-3-[2-[[5-methyl-imidazole-4-yl)-methyl]thio]ethyl]-2-thiourea,<sup>19, 31, 34</sup> (N-cyano-N'-methyl-N''-(3-(4-imidazolyl)-propyl)guanidine<sup>32</sup>, and  $\beta$ -hydroxy-theophylline.<sup>30</sup> After extraction the samples are either evaporated to dryness and reconstituted with a known amount of ethanol, injected directly or dissolved in the mobile phase for the HPLC analysis.

The columns used for HPLC were silica<sup>29, 31, 32, 34</sup> and a reverse phase C18  $\beta$  Bondapak<sup>®</sup><sup>30</sup>. The separation of cimetidine sulfoxide<sup>11</sup> and the guanidine derivative of cimetidine, N-methyl-N'-[2-((4-methyl-5-imidazolyl)methyl)thio]ethyl]guanidine, (VI) plus the polar decomposition and metabolic products of





\*POSITION OF <sup>14</sup>C LABEL

Fig. 15. Metabolites of cimetine.

cimetidine may be carried out using a Partisil SCX<sup>®</sup> (Whatman Inc.) strong ion exchange column using the following set of conditions<sup>3,3</sup>:

Column: Partisil SCX<sup>®</sup> 10  $\mu$  (25 cm x 4.6 mm i.d.)  
Whatman Inc.

Mobile Phase: Weigh 1.8 grams of ammonium sulfate into a one liter volumetric flask. Add 200 ml of distilled water to dissolve, then make up to volume with methanol.

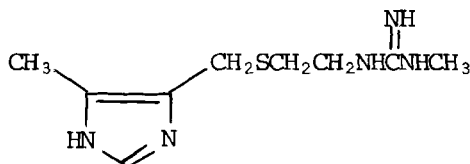
Flow Rate:  $\sim$ 1 ml/minute

Detector: UV (228 nm)

Sensitivity: Variable

Sample Diluent: Mobile Phase

<u>Retention Times</u> :	<u>Minutes</u>
Guanol (carboxamide) Derivative (IV) SK&F 92422	$\sim$ 10
Guanidine Derivative (VI) SK&F 92408	$\sim$ 15



'Guanidine' Derivative of Cimetidine

VI

In the recent method of Ziemniak, et al<sup>3,3</sup>, a liquid chromatographic procedure has been developed which separates and permits quantitative analysis of cimetidine (I), cimetidine sulfoxide (II), its hydroxymethyl (III) and guanol urea derivatives (IV). Metiamide, SK&F 92058, is used as the internal standard. Their procedure involves the precipitation of protein with acetonitrile, addition of anhydrous  $K_2HPO_4$ , extraction of the separated aqueous phase with methylene chloride and  $KH_2PO_4$  to saturate and salt out the solution. The methylene chloride is evaporated to dryness, the sample is reconstituted with mobile phase ( $CH_3CN:CH_3OH:H_2O:NH_4OH$ ,

1000:50:50:2). Detection is by UV at 228 nm and a Zorbax Sil Column, 4.6 mm x 25 cm (DuPont Instruments, Wilmington, DE) equipped with a Whatman HC Pellosil precolumn (Whatman Inc., Clifton, NJ) is used. An apparent pH of 10.5 has been used, and according to the authors column performance was satisfactory for more than a five months period in spite of the high alkalinity.

Identification of decomposition products and metabolic products is most conveniently done by their isolation using thin layer chromatography and identification by use of field desorption mass spectrometry.

#### Acknowledgements

The authors would like to acknowledge and thank members of SmithKline Corporation's Analytical & Physical Chemistry staff and other scientific staff at Philadelphia, Guayama, Welwyn, Tonbridge, Ireland, and France for their assistance, advice, recommendations and their personal efforts. The authors would further like to thank SmithKline Corporation for supporting them to carry out this work.

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# DISOPYRAMIDE PHOSPHATE

Alan Wickman and Patricia Finnegan

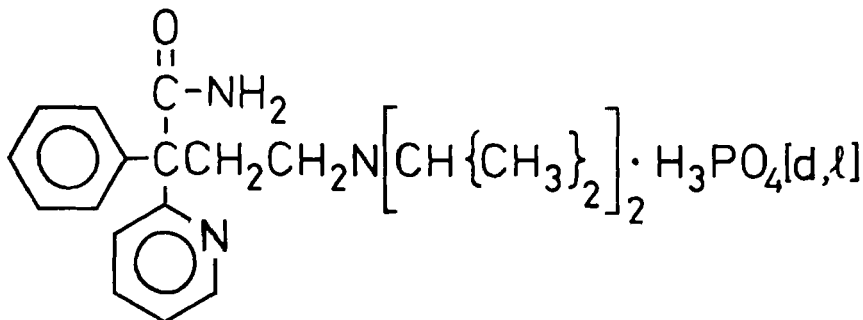
*G. D. Searle and Co.  
Skokie, Illinois*

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## 1. Description

1.1. Disopyramide Phosphate  $C_{21}H_{29}N_3O^+H_3PO_4$

Mol. Wt. 437.5



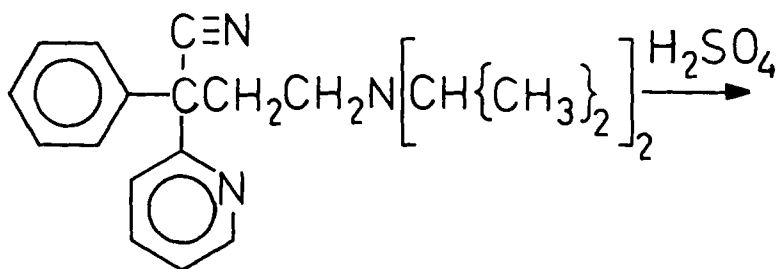
$\alpha$ -[2-(Diisopropylamino)ethyl]- $\alpha$ -phenyl-2-pyridineacetamide phosphate (1:1)

## 1.2. Color, Odor, Appearance

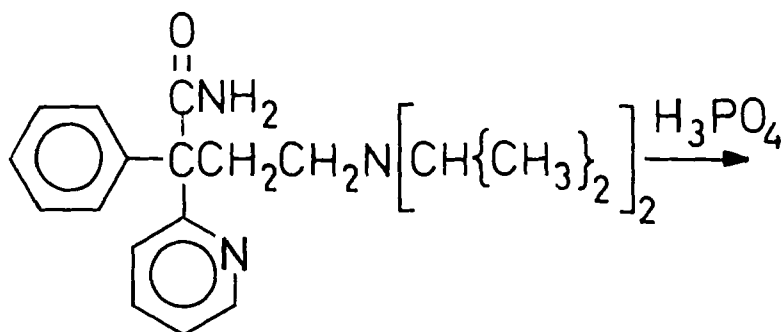
Disopyramide phosphate is an odorless white, or slightly off-white free flowing powder.

## 2. Synthesis

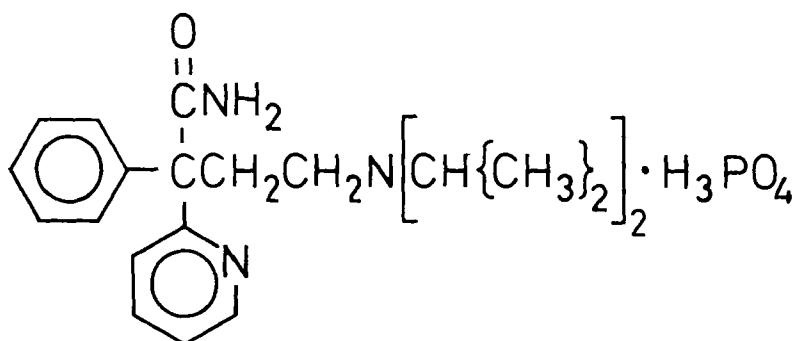
Nitrilopyramine is heated with concentrated sulfuric acid for 4 hours on a steam bath, the mixture is poured into ice after which it is made alkaline with 10 normal sodium hydroxide. The pH of the solution is then adjusted to 6 with acetic acid and the solution is washed with toluene. The mixture is again made alkaline with 10 normal sodium hydroxide and extracted with toluene. The toluene is evaporated and the residue dissolved in ethanol and treated with activated carbon. The ethanol is then evaporated and the residue recrystallized from hexane to give disopyramide. The phosphoric acid salt of disopyramide is prepared by reacting disopyramide with a phosphoric acid solution.<sup>1</sup> The synthesis pathway is illustrated in Figure 1.



Nitrilopyramine



Disopyramide



Disopyramide Phosphate

Figure 1 - Synthesis of Disopyramide Phosphate



### 3. Physical Characteristics

#### 3.1. Infrared Spectrum

The infrared spectrum of disopyramide phosphate in a Nujol and Fluorolube split mull is shown in Figure 2.<sup>2</sup> Band assignments are summarized below.

<u>Band</u> (cm <sup>-1</sup> )	<u>Assignment</u>
3480, 3290	amide N-H stretch
2300, broad	N <sup>+</sup> -H stretch
1678, 1640	amide C=O stretch and NH <sub>2</sub> deformation
1590, 1560 1480, 1460	benzene and pyridine ring vibrations
1395	CH <sub>2</sub> -N <sup>+</sup> methylene deformation
1263	C-N <sup>+</sup> stretch
1065, 940	H <sub>2</sub> PO <sub>4</sub> <sup>-</sup> stretches
760	4 adjacent H wag (α - substituted pyridine)
740, 695	5 adjacent H wag (mono-substituted benzene)

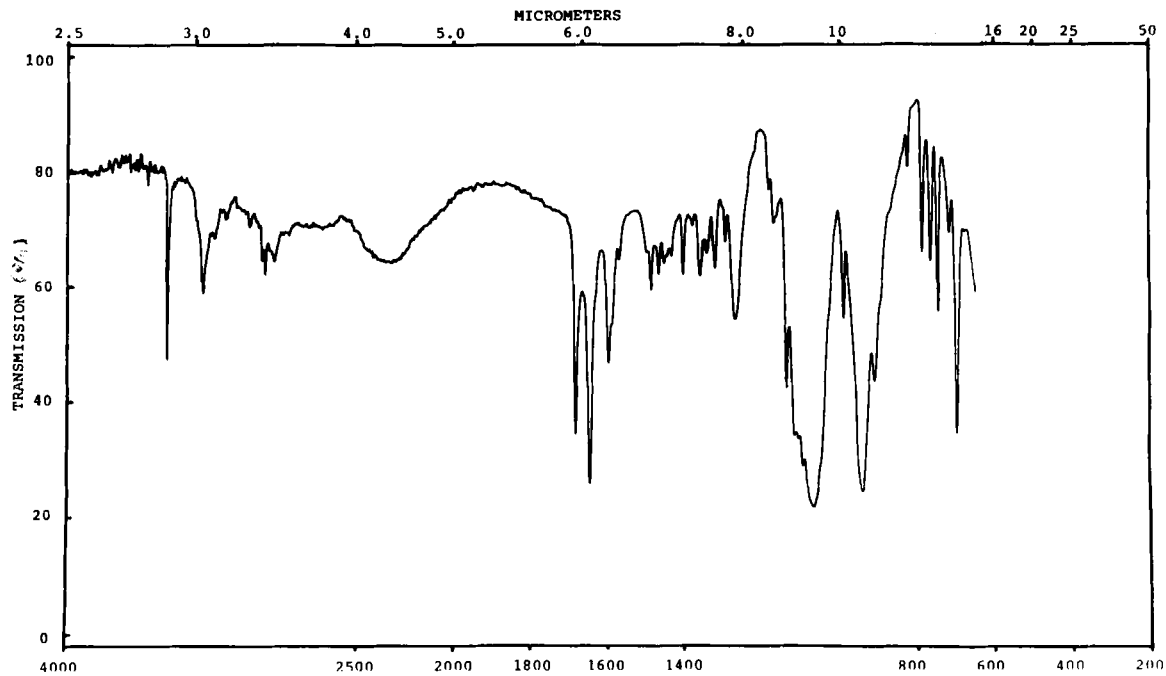
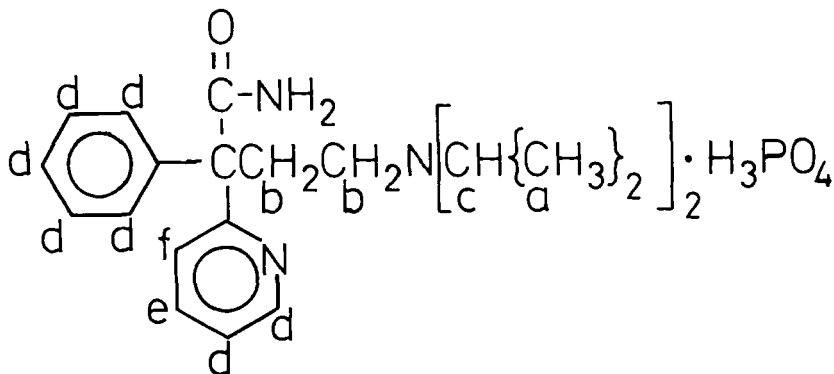


Figure 2  
Infrared Spectrum of Disopyramide Phosphate

### 3.2. Proton Magnetic Resonance Spectrum

The PMR spectrum of disopyramide phosphate in deuterated water is shown in Figure 3.<sup>2</sup> Shifts are reported in ppm downfield from the methyl signal of 3-trimethylsilyl propanoic acid-d<sub>4</sub> sodium salt which was used as an internal reference. Band assignments are summarized below.



<u>Band (ppm)</u>	<u>Assignment</u>
1.24 doublet	a
2.93 singlet	b
3.67 septet	c
7.10 - 7.55 multiplet	d
7.88 triplet of doublets	e
8.57 doublet of doublets	f

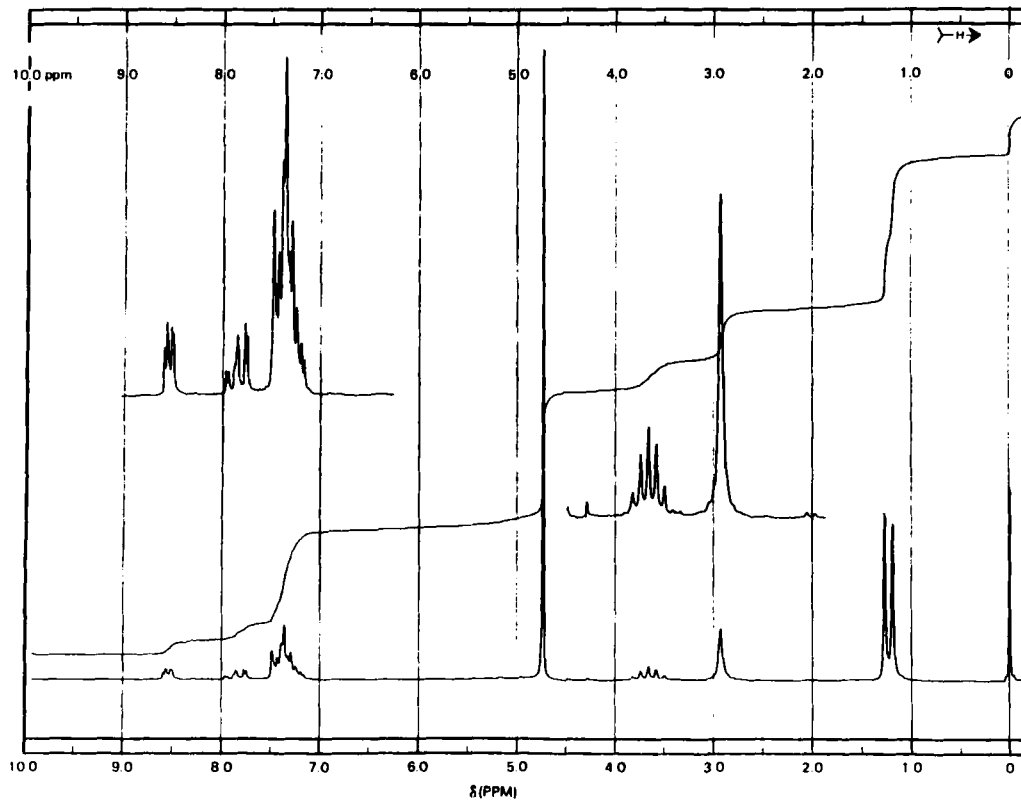
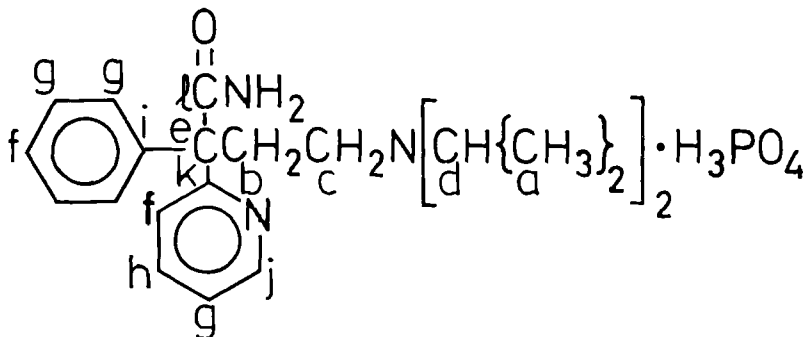


Figure 3 - Proton Magnetic Resonance Spectrum of Disopyramide Phosphate

### 3.3. Carbon Magnetic Resonance Spectrum

Noise and single frequency off-resonance decoupled CMR spectra of disopyramide phosphate in deuterated water solution are shown in Figures 4 and 5.<sup>2</sup> Shifts are reported in ppm downfield from the shift of tetramethyl silane as referenced to a shift of 67.4 ppm for dioxane which was used as an internal reference.

Band assignments are given below.



<u>Band (ppm)</u>	<u>Assignment</u>
18.7, 17.1	a
35.4	b
45.2	c
55.8	d
62.7	e
125.4, 124.5	f
130.0, 129.2	g
139.4	h
141.8	i
149.6	j
160.6	k
178.2	l

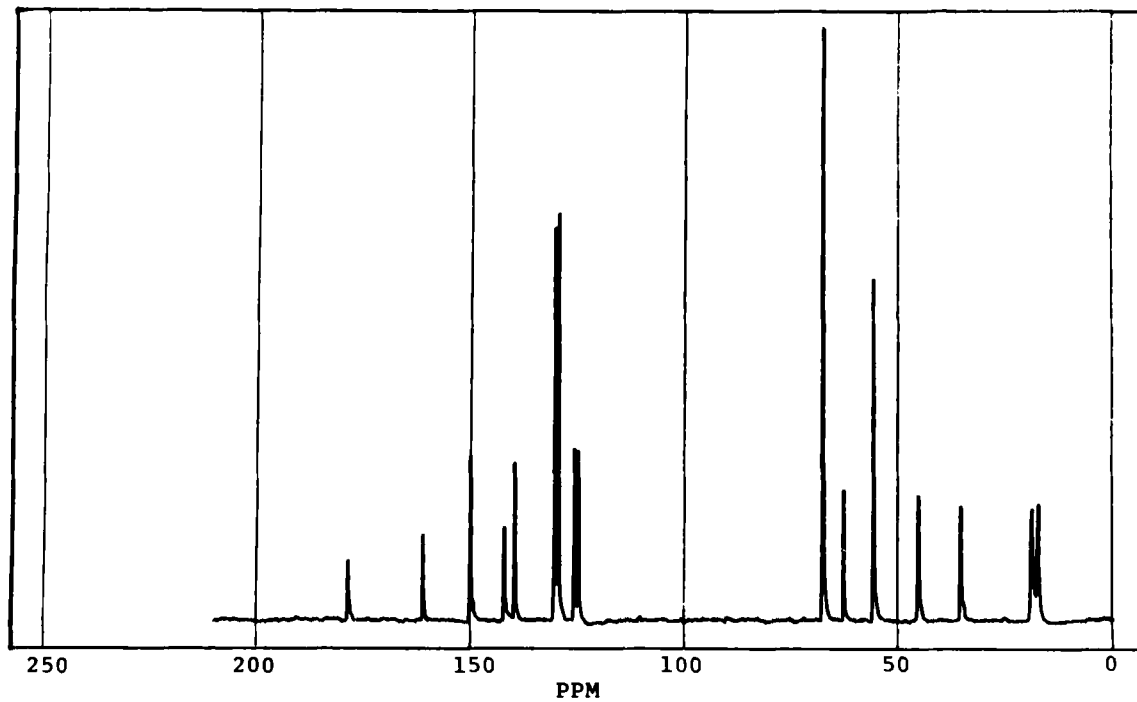


Figure 4 - Noise decoupled carbon magnetic resonance spectrum of disopyramide phosphate.

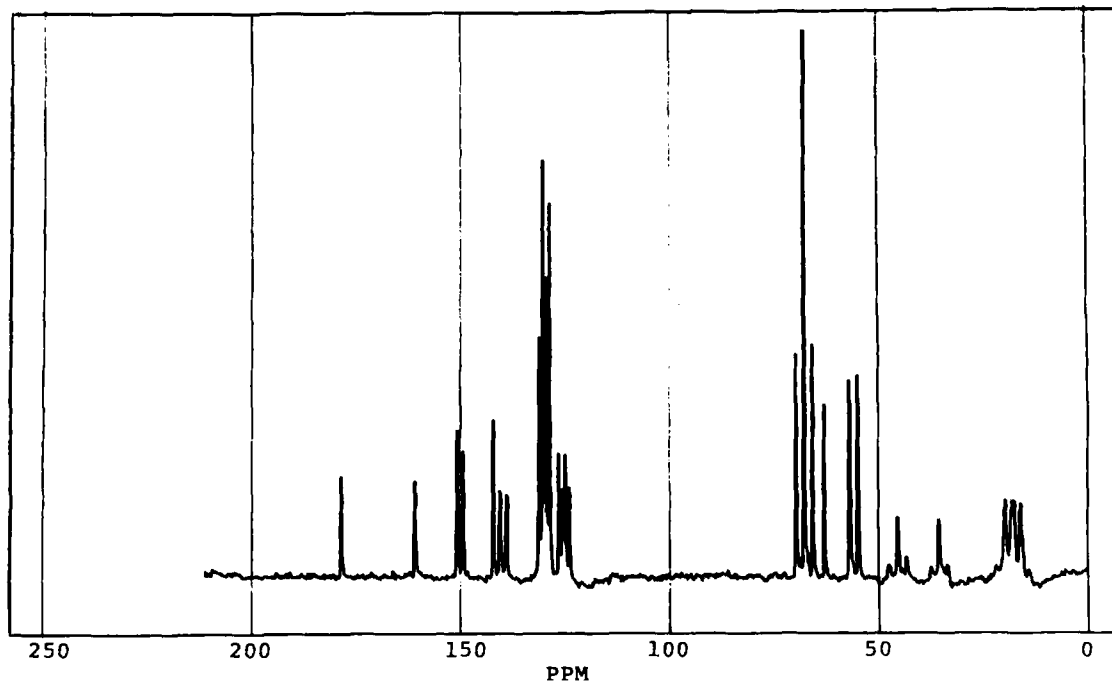


Figure 5 - Single Frequency Off-Resonance Proton Decoupled Carbon Magnetic Resonance Spectrum of Disopyramide Phosphate

## 3.4 Ultraviolet Spectrum

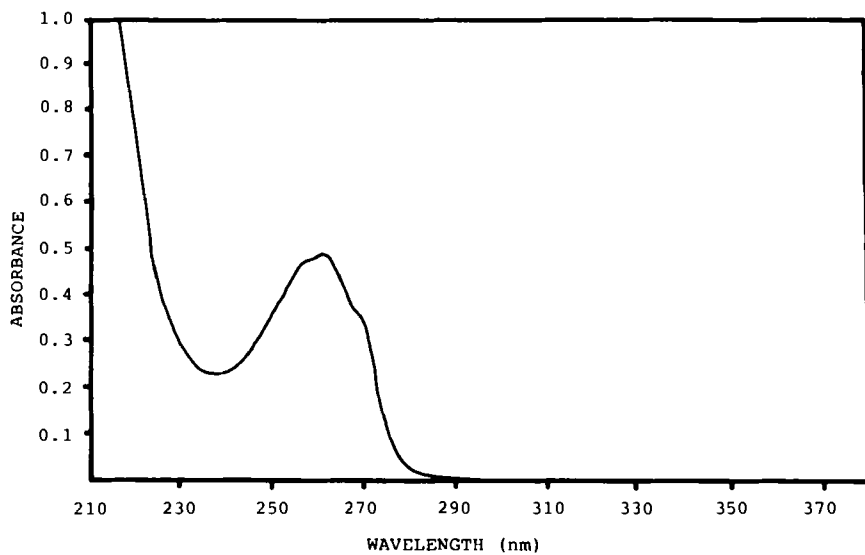


Fig. 6. Ultraviolet spectrum of disopyramide phosphate.



### 3.5. Thermal Analysis

The DSC thermogram of disopyramide phosphate obtained using a heating rate of 10°C/minute is shown in Figure 7.<sup>2</sup> The endothermic change of 138 J/g at about 213°C is due to melting and, as can be seen by the TG in Figure 8,<sup>2</sup> is accompanied by weight loss.

The TG was obtained using a heating rate of 20°C/minute. The maximum rate of weight loss is at 236°C. The total weight loss is 82.4% of the sample weight.

### 3.6. Mass Spectrum

A chemical ionization mass spectrum of disopyramide phosphate contains a base peak at  $M/z = 340$  which is due to the protonated free base of disopyramide phosphate. No peaks greater than 10% of the base peak are observed below  $M/z = 340$ . No peak due to the salt is present.<sup>2</sup>

### 3.7. Solubility

The solubility of disopyramide phosphate in various organic solvents is summarized in Table 1 below.<sup>3</sup>

Table 1

Solubility in Organic Solvents\*

<u>Solvent</u>	<u>Solubility (g/L)</u>
Ethanol	1.089
Isopropanol	0.103
Chloroform	$1.413 \times 10^{-2}$
Cyclohexane	$6.524 \times 10^{-3}$

\* Solubility determined at 24 - 26°C

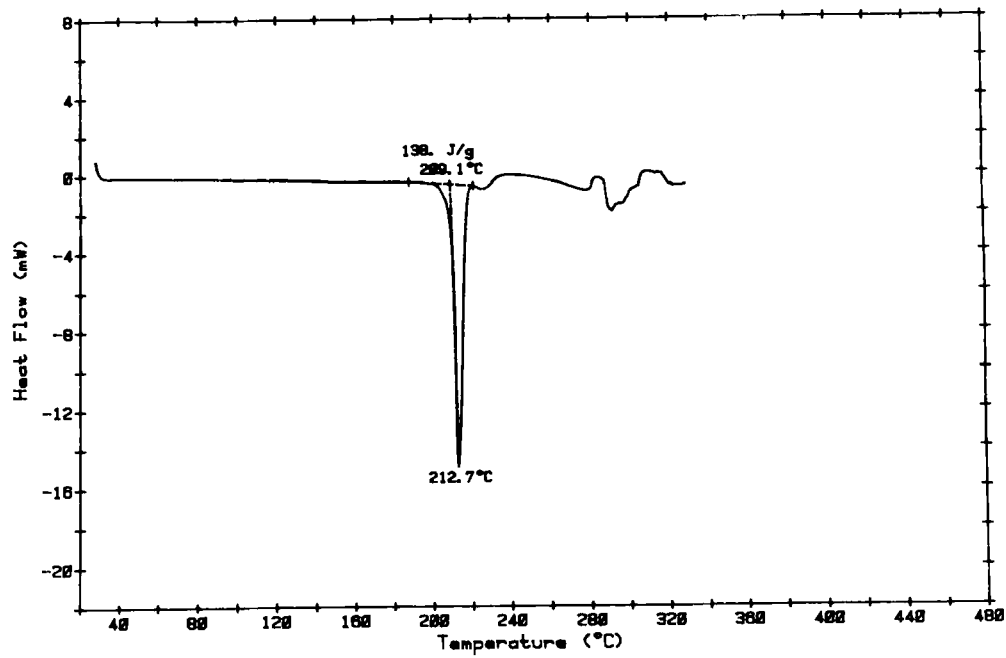


Figure 7 - DSC Thermogram of Disopyramdie Phosphate

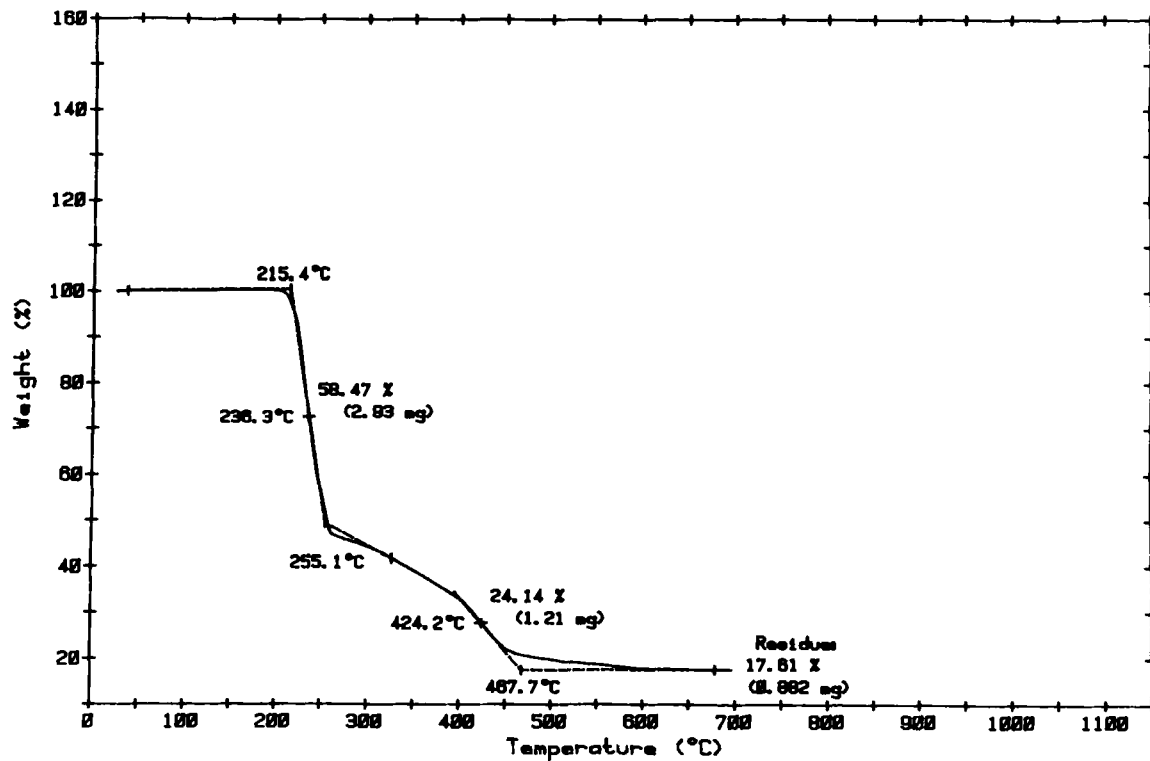


Figure 8 - TG Thermogram of Disopyramide Phosphate

The solubility of disopyramide phosphate free base in various aqueous media is summarized in Table 2 below.<sup>3</sup>

Table 2

Solubility in Various Aqueous Media**			
<u>Aqueous Media</u>	<u>Molarity (M)</u>	<u>pH***</u>	<u>Solubility (mg/ml)</u>
Acetate	0.1	3.93	62.0
Phosphate	0.1	5.85	32.0
Phosphate	0.16	7.45	8.71
Phosphate	0.1	7.98	4.32
Glycerine	0.1	9.86	3.78
0.1 N NaOH	0.1	12.7	1.29

\*\* Solubility at 27°C

\*\*\* Represents pH of Aqueous Media prior to Addition of Disopyramide

#### 4. Metabolism and Pharmacokinetics

After oral administration of 100 mg of the drug to healthy men and women<sup>4,5</sup>, peak plasma levels of 2.4 ug/ml were obtained 2 hours after administration. The drug had an absorption rate of 1.35 hours<sup>-1</sup>. The drug was eliminated with a biological half life of 7 hours. Twenty four hours after administration, plasma levels were low but urinary excretion was still appreciable. An average of 60% of the oral dose was recovered unchanged in the urine within 48 hours, with 50% being recovered within 24 hours of administration.

After oral administration of the drug to dogs and rats, the biological half life, after peak plasma levels were obtained, is 1.5 hours and 1.4 hours respectively. Urinary excretion after 24 hours was much lower for dogs (20%) and rats (35%) than for man. It is assumed that the rapid disappearance of disopyramide from the blood of both dog and rat is due to both urinary excretion and formation of metabolites which undergo biliary excretion.

Karim et al<sup>6</sup> determined that the main pathway of disopyramide metabolism involved N-dealkylation of the isopropyl group and arylhydroxylation, with a marked species difference in biotransformation between dog, rat, and man.

Oral administration of <sup>14</sup>C labeled disopyramide to dogs yielded 79% of radioactive compound in the urine after 72 hours. Of this 17% was the unchanged compound, 12% the mono-N-dealkylated species 2, 29% the pyrrolidone species 4, and 18% a water soluble conjugate that gave the pyrrolidone upon acid hydrolysis. Figure 9 is the proposed metabolic pathway for disopyramide in dogs. (See Figure 9)

Intraperitoneal administration of <sup>14</sup>C labeled disopyramide to rats yielded 44% of the radioactive compound in the urine after 72 hours. Of this, 80% was the unchanged disopyramide 1. The minor metabolic products recovered were two phenolic compounds 5 and 6 arising from arylhydroxylation. (See Figure 10.)

Oral administration of disopyramide 1 to man yielded 56% of disopyramide and 4% of the mono-N-dealkylated species 2 in the urine after 24 hours (refer to Figure 9).

## 5. Methods of Chemical and Dosage Form Analysis

### 5.1. Titrimetric Analysis

Accurately weigh a portion of the disopyramide phosphate and add it to 50 mL of glacial acetic acid in a 250 mL Erlenmeyer flask. Titrate with 0.1 N perchloric acid to a potentiometric endpoint. A blank determination is performed and any necessary corrections made. Each mL of 0.1 N perchloric acid is equivalent to 21.87 mg of disopyramide phosphate.<sup>7</sup>

### 5.2. Spectrophotometric Analysis

The analysis of disopyramide phosphate may be performed by UV spectrophotometric analysis employing 0.1 N sulfuric acid in absolute methanol as solvent. The ultraviolet absorption maximum is at about 268 nm.<sup>8</sup>

### 5.3. Chromatographic Analyses

#### 5.3.1. Thin Layer Chromatography

Several TLC systems are available for the analysis of disopyramide phosphate.<sup>9</sup> Solvent, adsorbent, and detection parameters are summarized in Table 3.

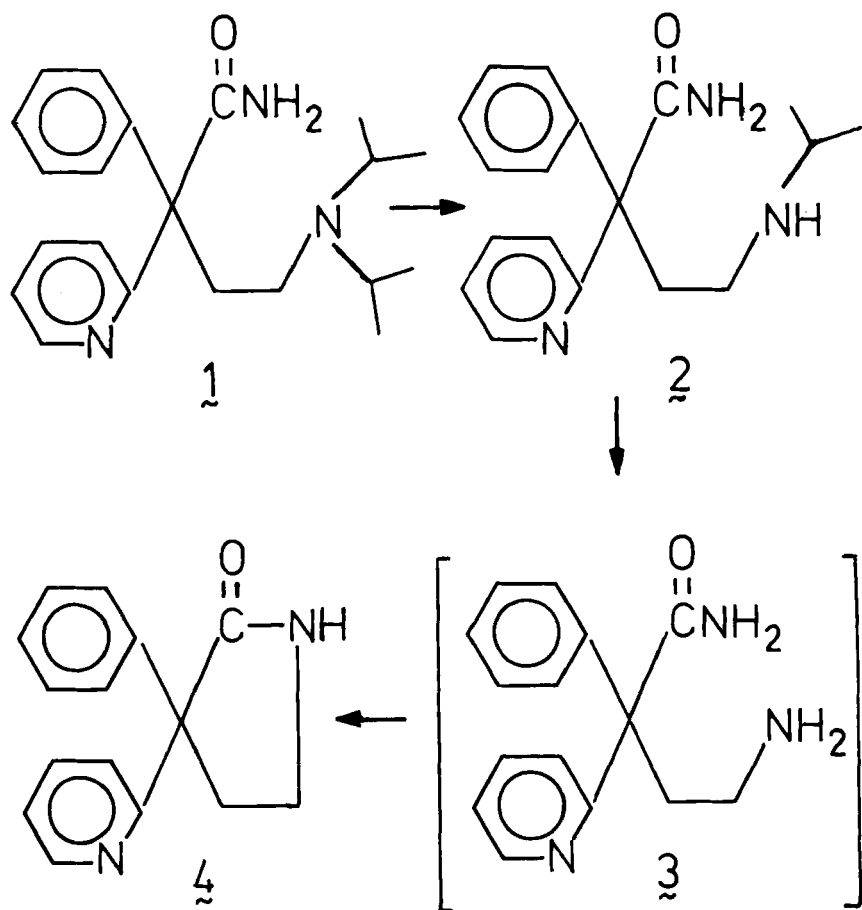


Figure 9 - Proposed Metabolic Pathway of Disopyramide in Dogs

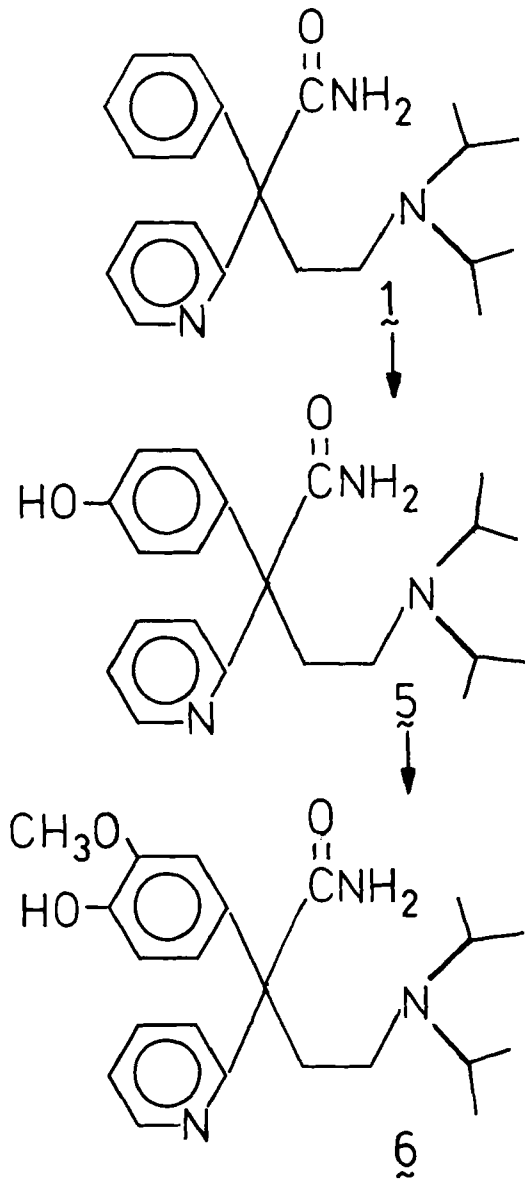


Figure 10 - Proposed Metabolic Pathway of Disopyramide in Rats



Table 3

Solvent System	Adsorbent	Detection	R <sub>f</sub>
Benzene:ethanol 2B:ammonium hydroxide 170: 28:2 (v/v/v)	Silica Gel 0.25 mm	1,2	0.39
Toluene:ethanol 2B:ammonium hydroxide 170: 28:2 (v/v/v)	Silica Gel 0.25 mm	1	.34
Methylene chlo- ride:ethanol 2B:ammonium hydroxide 70: 28:2 (v/v/v)	Silica Gel 0.25 mm	2	0.65

#### Detection Systems

1. Spray with Dragendorff Reagent
2. Color test the chromatographic plate by exposure to excess t-butyl hypochlorite. Evaporate the excess reagent (20 minutes in fume hood) and then spray the plate with 0.5% (w/v) potassium iodide and 0.5% (w/v) starch in water.

#### 5.3.2. High Performance Liquid Chromatography

Disopyramide phosphate may be chromatographed under the following HPLC conditions.<sup>10</sup>

##### System 1

Column: u-Bondapak C18 (30 cm x 4.0 mm i.d.)

Mobile Phase: 70% triethyl ammonium phosphate buffer/30% methanol (v/v)

Flow Rate: 2 mL/min

Detection: 254 nm  
Temperature: Ambient  
Retention Time: Approximately 6  
minutes

#### System 2

Column: DuPont Zorbax C8 (15  
cm x 4.6 mm i.d.)  
Mobile Phase: 65% TEAP Buffer pH  
3.7/35% MeOH  
Flow Rate: 2 mL/min  
Temperature: Ambient  
Detection: 254 nm  
Retention Time: Approximately 5  
minutes

#### 5.3.3. Gas Liquid Chromatography

Disopyramide phosphate can be chromatographed as the free base. The free base is obtained by dissolving disopyramide phosphate chemical or capsule in an aqueous base medium and extracting into an organic solvent such as chloroform, ethyl ether, methylene chloride, etc.. Disopyramide is chromatographed under the following conditions<sup>11</sup>.

#### System 1

Column: 3% OV-1 on Gas Chrom Q,  
80/100 mesh (1.8 m x 2  
mm i.d., glass column)  
Oven Temp.: 210°C  
Carrier: Helium  
Flow: 50 mL/min

Detection: Flame Ionization

### System 2

Column: 3% Silar 10C on Gas Chrom Q 100/120 (1.8 m x 2 mm i.d., glass column)

Oven Temp.: 250°C

Carrier: Helium

Flow Rate: 50 mL/min

Detection: Flame Ionization

## 6. Methods of Analyses in Biological Fluids

### 6.1. Spectrophotometric Analysis

A portion of the plasma/serum is added to 0.5 M phosphate buffer pH 7.5 and extracted with methylene chloride. The organic portion is washed with potassium hydroxide and then extracted with 0.1 N sulfuric acid. The ultraviolet absorption of the aqueous extract is measured at about 260 nm to quantitate the disopyramide<sup>1,2</sup>.

### 6.2 Spectrofluorometric Analysis

A portion of the sample fluid is added to sodium hydroxide solution and extracted with methylene chloride. The methylene chloride solution is then extracted with 50% sulfuric acid.

To quantitate disopyramide, the fluorescence of the sulfuric acid extract is determined at  $\lambda$  emission = 410 nm,  $\lambda$  excitation = 275 nm<sup>5</sup>.

### 6.3. Chromatographic Analyses

#### 6.3.1. Gas Liquid Chromatography

Many investigators have used gas chromatography to analyze disopyramide <sup>13</sup> - <sup>20</sup>, and to a lesser extent disopyramide and its mono-N-dealkylated metabolite <sup>21</sup> - <sup>23</sup>, in biological fluids.

The majority of the methods involve basifying the sample fluid and extracting the free base into an organic medium. This organic medium may then be evaporated and the sample reconstituted in ethanol or another solvent, or the sample can be injected directly into the chromatograph. Some of the methods described use a double extraction where the organic medium containing disopyramide is extracted with acid solution, which is then made basic and again extracted with organic solvent. Many of the methods also employ an internal standard for quantitation. Internal standards commonly used are p-chlorodisopyramide, and aminopentamide. Table 4 gives a summary of the gas chromatographic conditions employed.

Table 4

Column	Column Temp.	Detector	Carrier Flow	Ref.
1.5% SE30 on Gas Chrom Q (1.8 m x 2 mm, glass)	230°C	FID	30 mL/min N <sub>2</sub>	13
3% OV-17 on Gas Chrom Q 100/200 mesh (1 m x 2 mm, glass)	255°C	Nitrogen	20 mL/min N <sub>2</sub>	14
3% OV-101 on Gas Chrom Q 80/100 mesh (1.8 m x 6 mm, glass)	230°C	FID	30 mL/min N <sub>2</sub>	15
3% OV-1 on Supelcoport 80/100 mesh (1.5 m x 4 mm, silanized glass)	240°C	FID	40 mL/min N <sub>2</sub>	16
2% SP2250, 2% OV-101 on Chromosorb W-HP 100/120 mesh (1.2 m x 2 mm, glass)	230°C	Nitrogen	30 mL/min N <sub>2</sub>	17
3% OCW 98 on Gas Chrom Q 100/120 mesh (1.7 m x 2.6 mm, glass)	210°C	FID	100 mL/min N <sub>2</sub>	18
3% OV-1 Gas Chrom Q 100/100 mesh (2 m x 2 mm, glass)	200°C	Nitrogen	40 mL/min N <sub>2</sub>	19
3% OV-17 on Gas Chrom Q 100/120 mesh (60 cm x 2 mm, glass)	260°C	FID	30 mL/min N <sub>2</sub>	20
3% OV-17 on Chromosorb W 100/120 mesh (1 m x 2 mm, glass)	250°C	FID	28 mL/min N <sub>2</sub>	21
3% OV-17 on Gas Chrom Q 100/120 mesh (60 cm x 2 mm, glass)	245°C	Nitrogen	25 mL/min	22
2.6% OV-17 on Chromosorb W-HP 80/100 mesh (.6 m x 2 mm, glass)	210°C/ 230°C	FID	60 mL/min He	23

### 6.3.2. High Performance Liquid Chromatography

Several HPLC methods have been reported that can be used to analyze disopyramide phosphate alone<sup>24 - 28</sup> or in combination with its mono-N-dealkylated metabolite<sup>29 - 32</sup>. Sample preparation is similar to that for gas chromatography. For most methods the sam-

Table 5

Column	Mobile Phase	Detection (nm)	Flow Rate (mL/min)	Ref.
5 micron ODS-18	Acetonitrile:potassium phosphate (monobasic) buffer	254	---	24
10 $\mu$ ODS-18 (25 cm x 3 mm i.d.)	1% Acetic acid:methanol: triethylamine (54.5:45: .5)	254	1	25
$\mu$ Bondapak C18 (30 cm x 4 mm i.d.)	Methanol:hexane:sulfonic acid (pH 4.5) (60:40)	254	1	26
Lichrosorb SI60 (15 cm x 4 mm i.d.)	Dichloroethane:methanol: perchloric acid (95.7:4: .3)	265	1	27
$\mu$ Bondapak C18 (30 cm x 4 mm i.d.)	.05 M Potassium phosphate (debasic):acetonitrile (65:35)	258	2	28
$\mu$ Bondapak CN (30 cm x 4 mm i.d.)	Acetonitrile:.01 N sodium acetate (pH 4) (50:50)	254	1.2	29
$\mu$ Bondapak CN (25 cm x 5 mm i.d.)	.06 M Sodium acetate, 4.7% acetic acid (pH 3.5); methanol (85:15)	254	1.9	30
ODS C18 (25 cm x 5 mm i.d.)	Methanol:water with .005 M heptanesulfonic acid (53:47)	254	1	31
Lichrosorb RP-8 10 micron (25 cm x 4.6 mm i.d.)	0.5 M Sodium phosphate: acetonitrile (73:27)	254	1.8	32

ple fluid is basified and extracted into an organic medium. The medium is then evaporated and the sample reconstituted in mobile phase solution or other organic medium. Table 5 gives a summary of different HPLC Systems.

#### Acknowledgments

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# INDOMETHACIN

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## 1. Introduction

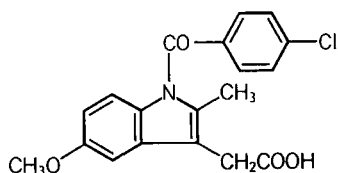
### 1.1 Historical

Indomethacin is a non-steroidal, anti-inflammatory agent with anti-pyretic and analgesic properties discovered and developed by the Merck Sharp and Dohme Research Laboratories(1).

Indomethacin has been used effectively in the management of patients with moderate to severe rheumatoid arthritis, ankylosing spondylitis, osteoarthritis, acute painful shoulder (bursitis and/or tendinitis) and acute gouty arthritis(2-6). Recently, indomethacin has been found effective in the treatment of neonates with patent ductus arteriosus and in patients with acute cystoid macular edema following cataract surgery(7-9). Worldwide, indomethacin has been formulated into many dosage forms, including formulations designed for long duration of activity. The discovery of this compound continues to provide new insights into medical treatment of disabling diseases(10).

### 1.2 Name, Formula, Molecular Weight

Indomethacin (I) is 1-(p-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid. Other names for the compound include (1-p-chlorobenzoyl-5-methoxy-2-methylindol-3-yl) acetic acid(10). Proprietary names for indomethacin include AMUNO INDACID, INDACIN, INDOCIN, INDOCIN-SR, INDOCIN-IV, INDOMEET, INDOPTIC, METINDOL, and MEZOLIN. Additional chemical and proprietary names are listed in the monograph for indomethacin in the Merck Index(11).



Official monographs for indomethacin are given in U.S.P. XX B.P. 1980 , European Ph.1980 ,and the Nord. Ph.1969 , (12-15)

### 1.3 Definition

Indomethacin is defined as the Form I crystalline, non-solvated free acid moiety of the compound unless otherwise noted. Indomethacin as described below can exist as several crystalline forms but has been used in pharmaceutical preparations principally as Form I and less frequently as the crystalline sodium trihydrate.

### 1.4 Appearance, Color, Odor

Pale yellow to yellow-tan, crystalline powder that is odorless or almost odorless.

## 2. Physical Properties

### 2.1 Ultraviolet Absorbance

Indomethacin was first characterized by Shen *et al.* as having ultraviolet absorbance maxima at 319 and 230 nm with an inflection at 260 nm in ethanol. The corresponding values reported were 6290, 20800 and 16200, respectively(1).

U.S.P.XX lists a UV absorbance maximum at 318 nm in methanolic 0.1N hydrochloric acid. Figure 1 shows an ultraviolet absorption spectrum of indomethacin (Merck Standard 6375-66-1) in methanolic 0.1N HCl at a concentration of 1.429mg/100 ml. Absorbance maximum is at 318 with  $E_{1\%}^{1cm}$  value of 182 ( $\epsilon=6510$ ).

### 2.2 Mass Spectrum

The mass spectrum of indomethacin obtained on an LKB-5000 at 70 eV ionization energy is found in Figure 2. Important Features of the spectrum include:

- (1) Molecular ion peak at 357 (m/e).
- (2) Peak at m/e 312 (M.W.-45) attributed to loss of CO<sub>2</sub>H.
- (3) The most intense peak of the spectrum occurs at m/e 139 which corresponds to p-chlorobenzoyl(16).

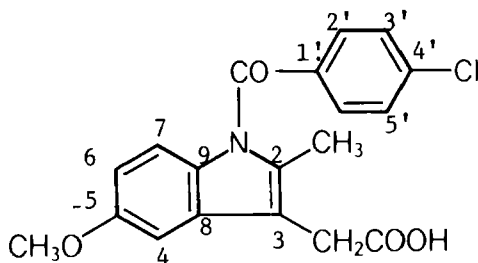
## 2.3 Nuclear Magnetic Resonance

2.3.1 The proton NMR spectrum of indomethacin in deuterated dimethylsulfoxide ( $d_6$  DMSO) at a concentration of 20% (w/v) is reproduced in Figure 3. A tabulation of the assignments and chemical shifts is found in Table I(17).

The carboxylic proton is not visible in the above spectrum due to exchange broadening involving the water in the solvent.

### 2.3.2 Carbon 13 Spectrum

The  $^{13}\text{C}$  magnetic resonance spectrum is given in Figure 4 and was obtained using a Varian CFT-20 (FT mode) spectrometer with  $d_6$  DMSO as the solvent at a concentration of 10% w/v. The spectrum is consistent with the structure and the assignments are in Table II (17).



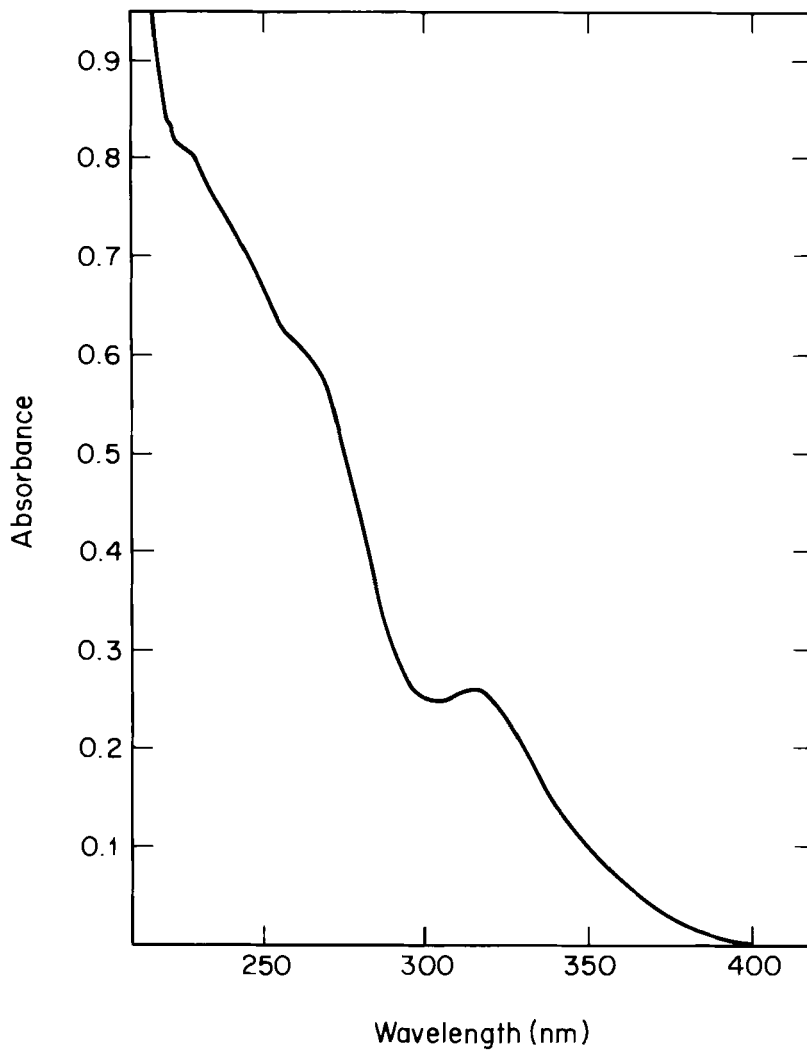


Figure 1. Ultraviolet Absorption Spectrum of Indomethacin in Methanolic Hydrochloric Acid at 0.00143% Concentration; max @ 318 nm;  $E_{1\%}^{1cm} = 182$ . Merck Standard 6375-66-1

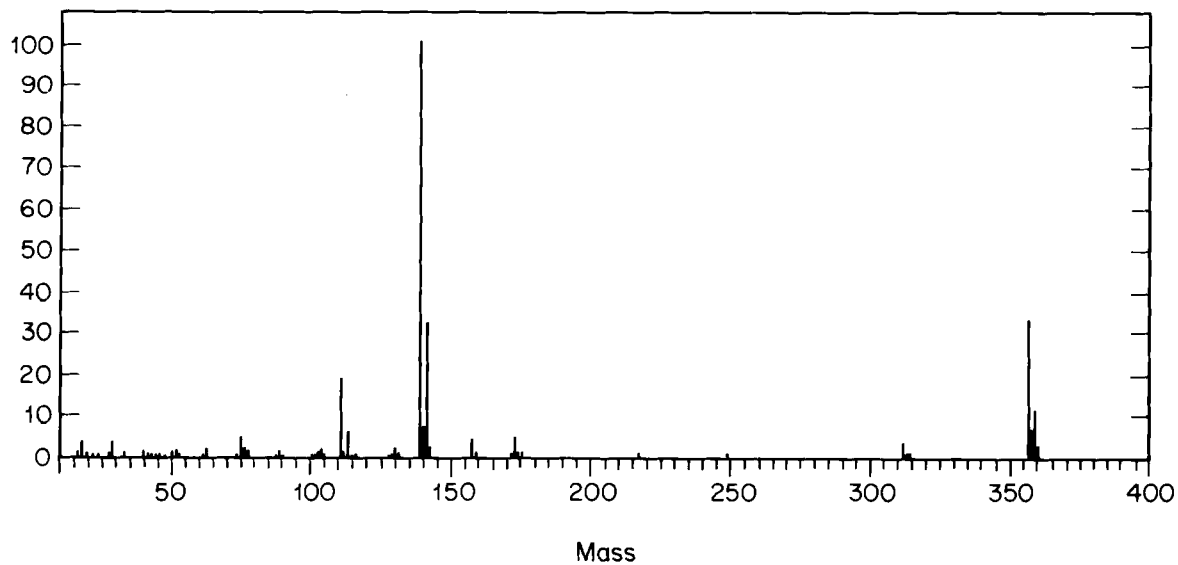
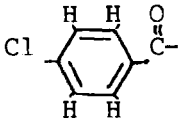


Figure 2. Mass Spectrum of Indomethacin(LKB 5000-70 eV ionization energy).  
Merck Standard 6375-66-1

Table I. Proton NMR Spectrum of Indomethacin  
Merck Standard 6375-66-1

Solvent:  $d_6$  DMSO; Conc: 20% w/v; Inst.: JEOL C-60 Hz

Chemical Shift (ppm) <sup>a</sup>	Integral (mm)	Rel. No. Proton	Assignment
2.22 (s)	43	[2.8 <sub>7</sub> ]	2-CH <sub>3</sub>
3.67 (s)	75 1/2	[5.0 <sub>4</sub> ]	3-CH <sub>2</sub> CO <sub>2</sub> H
3.74 (s)			OCH <sub>3</sub>
6.55-7.10 (m)	44 1/2	[2.9 <sub>7</sub> ]	H <sub>4</sub> , H <sub>6</sub> and H <sub>7</sub>
7.45-7.80 (m)	61 1/2	[4.1 <sub>1</sub> ]	

a - TMS used as internal reference.

Table II. Carbon 13 Chemical Shifts for Indomethacin

Shift $\delta^{13}\text{C}$ (ppm)	Assignment*
172.0	CO <sub>2</sub> H
167.8	>N-C=O
155.5	C <sub>5</sub>
137.6	C <sub>4</sub> '
135.1	C <sub>8</sub>
134.1	C <sub>1</sub> '
131.1	C <sub>2</sub> ' , C <sub>6</sub> '
130.7	C <sub>2</sub>
130.2	C <sub>9</sub>
129.0	C <sub>3</sub> ' , C <sub>5</sub> '
114.5	C <sub>7</sub>
113.4	C <sub>3</sub>
111.2	C <sub>6</sub>
101.7	C <sub>4</sub>
55.3	OCH <sub>3</sub>
29.5	3-CH <sub>2</sub>
13.1	2-CH <sub>3</sub>



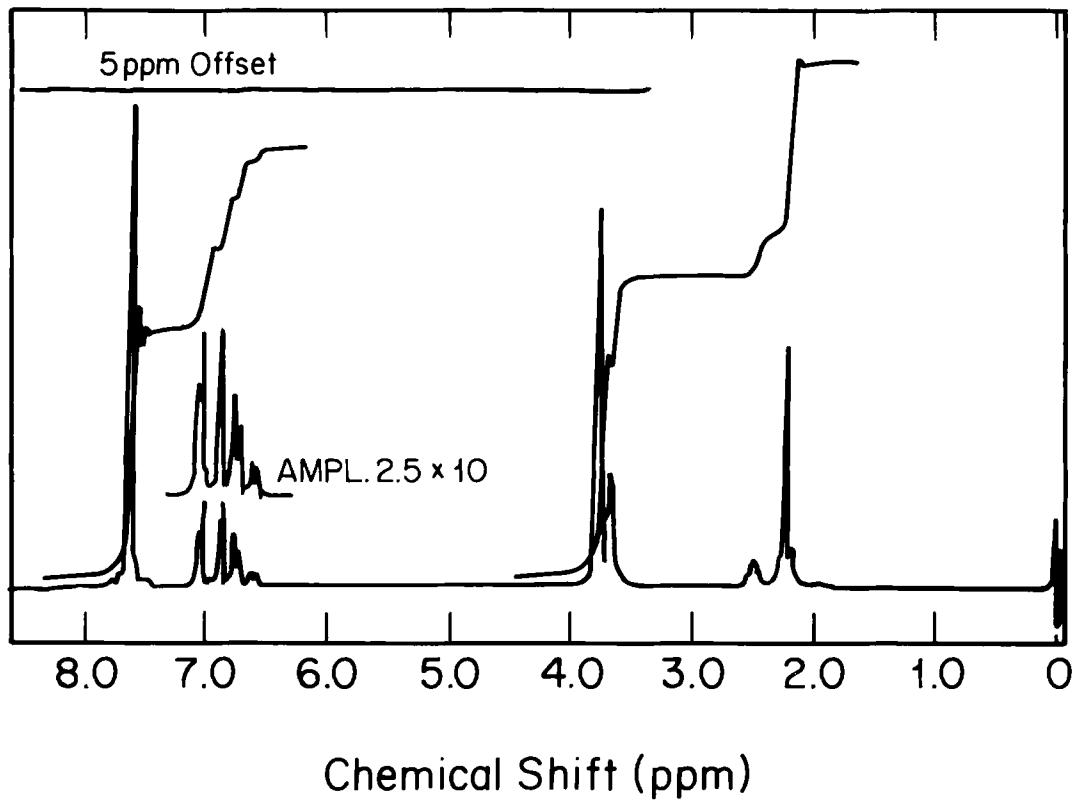


Figure 3. Proton NMR Spectrum of Indomethacin in  $d_6$ -DMSO.  
Merck Standard 6375-66-1

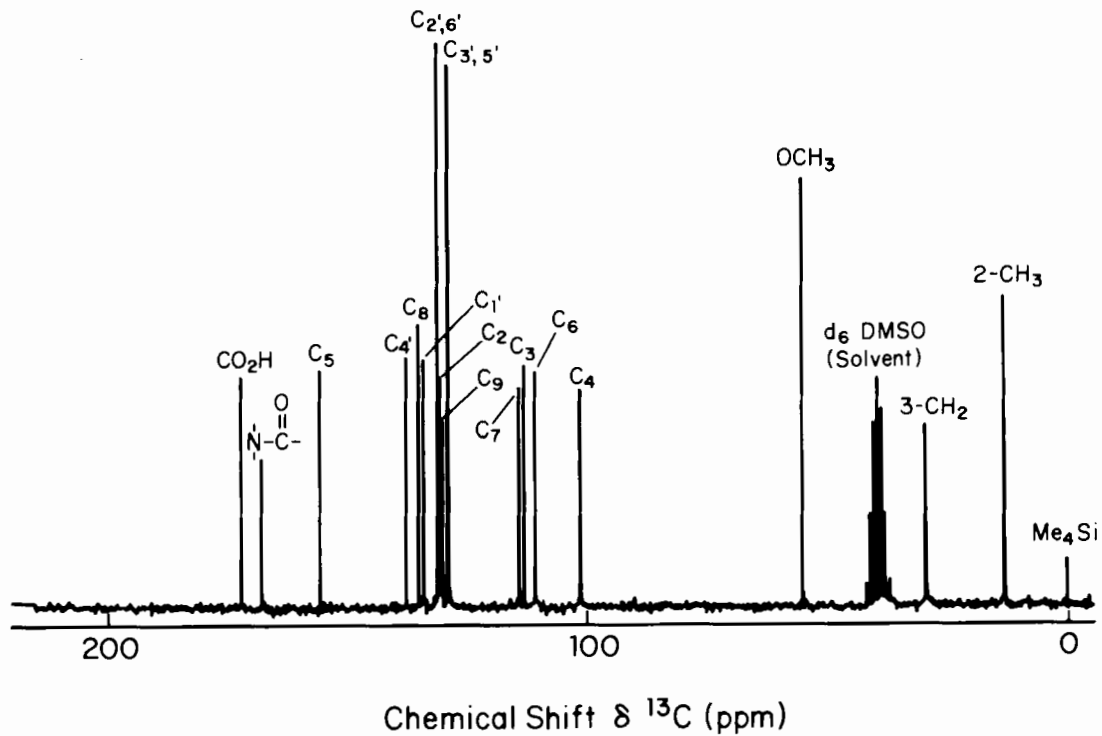


Figure 4.  $^{13}\text{C}$  NMR Spectrum of Indomethacin. Merck Standard 6375-66-1.

## 2.4 Crystal Properties

Indomethacin is known to exist in more than one nonsolvated crystalline modification. Yamamoto described three polymorphs  $\alpha$ ,  $\beta$  and  $\gamma$  with melting point, IR and x-ray powder diffraction data(18). Monkhouse and Lach reported two modifications characterized by melting point and IR(19). Borka found four and reported hot-stage microscopic melting point, IR and solubility data (solubility on only three)(20). Others have also observed four crystalline polymorphs(21). While there is disagreement as to the existence of some of the polymorphs, all agree on two of the crystalline modifications. There are consistent melting point, IR and x-ray powder diffraction data for these two modifications. Most authors refer to these polymorphs as Form I and Form II. Yamamoto, however, uses  $\gamma$ -type and  $\alpha$ -type, respectively. Form I is the highest melting (see 2.6 below) and lowest solubility (see 2.7 below) polymorph and is, therefore, the thermodynamically stable crystalline modification of indomethacin. However, from a practical view, both Form I and II are equally biologically available and active(22).

The x-ray powder diffraction data for Forms I and II of indomethacin are found in Table II. The d-spacings and relative intensities for Form I are consistent with those cited in the National Formulary XIV (1975) (13).

Kistenmacher and Marsh determined the crystal and molecular structure of indomethacin by single crystal x-ray diffraction methods(23). Although not explicitly stated by Kistenmacher and Marsh, the crystals grown from anhydrous acetonitrile are most likely Form I. They reported that the crystals are triclinic, space group P1, with cell constants  $a = 9.295(2)\text{\AA}$ ,  $b = 10.979(1)\text{\AA}$ ,  $c = 9.742(1)\text{\AA}$ ,  $\alpha = 69.38(1)^\circ$ ,  $\beta = 110.79(1)^\circ$ ,  $\gamma = 92.78(1)^\circ$  and  $Z = 2$ . The calculated density is  $1.37\text{ gm/cm}^3$ ; the observed density is  $1.38(1)\text{ gm/cm}^3$ .

In addition to the polymorphism described above, indomethacin is known to form solvates with benzene and t-butyl alcohol(21). Borka has also observed solvates with a number of different solvents(20).

Table III

X-Ray Powder Diffraction of Indomethacin Forms I and II  
 Sample Merck Standard  
 Cu K $\alpha$  Radiation

Form I			Form II		
2 ( $^{\circ}$ )	d( $\text{\AA}$ )	Rel. Intensity	2 ( $^{\circ}$ )	d( $\text{\AA}$ )	Rel. Intensity
10.18	8.69	22	4.85	18.22	14
11.60	7.63	100	6.89	12.83	43
12.75	6.94	15	8.80	10.05	100
16.65	5.32	54	10.20	8.67	36
17.00	5.21	89	11.35	7.80	32
17.30	5.13	25	11.85	7.46	77
18.55	4.78	19	13.85	6.39	34
19.30	4.60	36	14.19	6.24	80
19.60	4.52	60	14.45	6.13	79
20.30	4.37	10	14.70	6.03	30
20.90	4.25	15	14.90	5.95	30
21.80	4.08	89	16.05	5.52	31
22.90	3.88	17	17.50	5.07	45
23.15	3.84	15	18.00	4.93	50
24.00	3.71	22	18.90	4.70	55
25.50	3.49	13	19.00	4.67	16
26.62	3.35	42	19.70	4.51	54
27.50	3.24	15	20.15	4.41	39
28.3	3.15	11	20.60	4.31	46
28.9	3.09	23	21.10	4.21	29
29.35	3.04	31	22.02	4.04	77
30.40	2.94	14	22.60	3.93	68
33.60	2.67	10	23.35	3.81	52
34.15	2.63	11	24.01	3.71	39
34.75	2.58	8	24.50	3.63	57
37.50	2.40	13	24.90	3.58	23
			25.28	3.52	38
			26.25	3.40	36
			27.20	3.28	14
			28.35	3.15	39
			31.0	2.89	25

## 2.5 Infrared Absorbance

The infrared absorption spectrum for Form I of indomethacin in KBr is given by Hayden *et al.* and Sadtler(24,25). Monkhouse and Lach, and Borka, in addition to IR spectrum for Form I, give IR spectra for the other crystalline modifications of indomethacin that they observed(19,20).

The IR spectrum which is consistent with the literature for Form I of indomethacin is found in Figure 5. Some band assignments for the solid state infrared absorption spectrum are given below(26).

<u>Wavelength (cm<sup>-1</sup>)</u>	
~ 3400-2500	Aromatic C-H stretch Carboxylic acid O-H stretch
1715, 1695	C=O stretch
1600	Aromatic C=C stretch
1450	O-CH <sub>3</sub> deformation
1230	? (C-O) stretch plus O-H deformation
925	Carboxylic O-H out of plane deformation
900-600	Various C-H out of plane deformation for substituted aromatic
750	C-Cl ?

## 2.6 Thermal Behavior

Shen *et al.* initially reported a melting point of 153-154° for indomethacin(1). The N.F. XIV states that the melting point of indomethacin is 162°C while N.F. XIII gives 155° and 162° for the melting points of two polymorphs of indomethacin. The British Pharmacopoeia 1980 specifies a melting point of 158° to 162°C(14).

Tabulated in Table IV are the melting points observed for the various polymorphs previously reported in the literature.

Table IV

Melting Points of Indomethacin Polymorphs	
Form	Melting Point (°C)
Form I (type $\gamma$ )	160-161.5(18)
	160(20,18)
	158(19)
Form II (type $\alpha$ )	154.5-155.5(28)
	154(20,28)
	152(19)
Form III	148(21)
Form IV	134(21)
Type $\beta$	158-160.5(18)

A DTA curve for indomethacin (Form I - Merck Standard) obtained on a du Pont 990 thermal analyzer is given in Figure 5. Observed DTA peak temperature is 162°C.

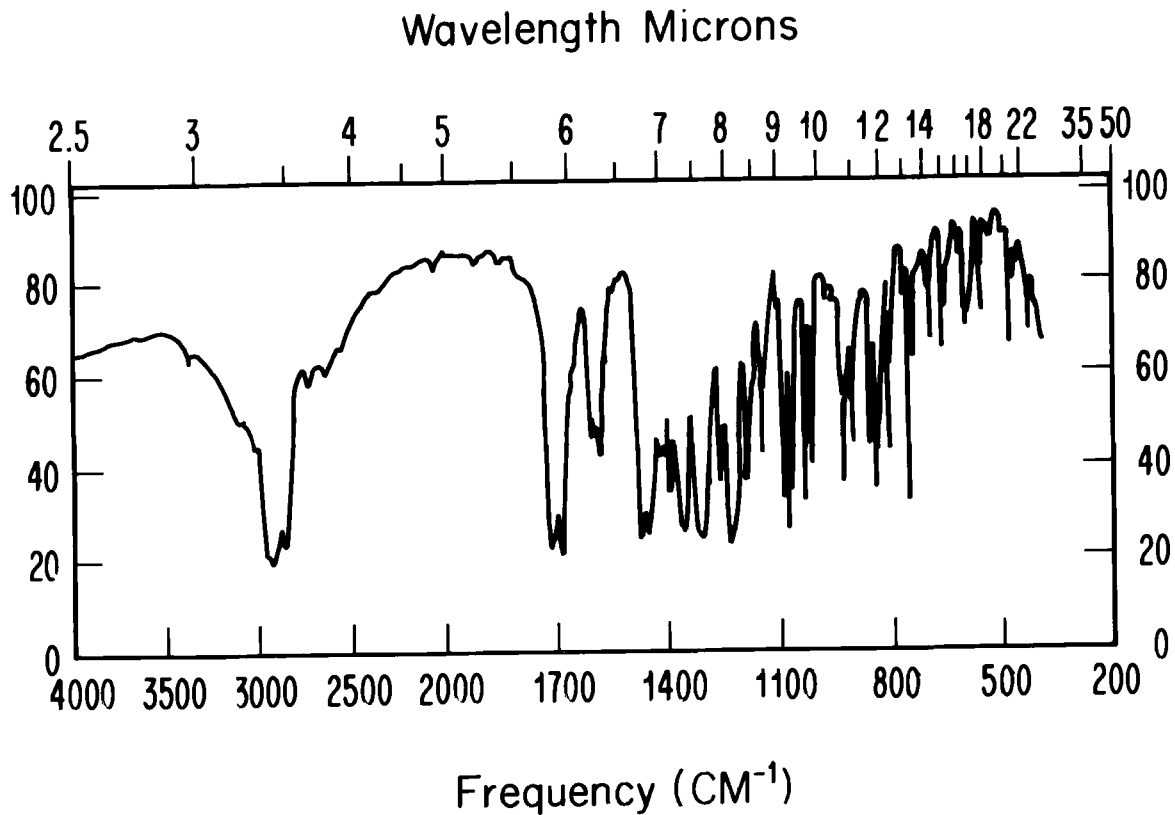


Figure 5. Infrared Spectrum of Indomethacin (Form I) in Nujol Mull  
Merck Standard 6375-66-1]]

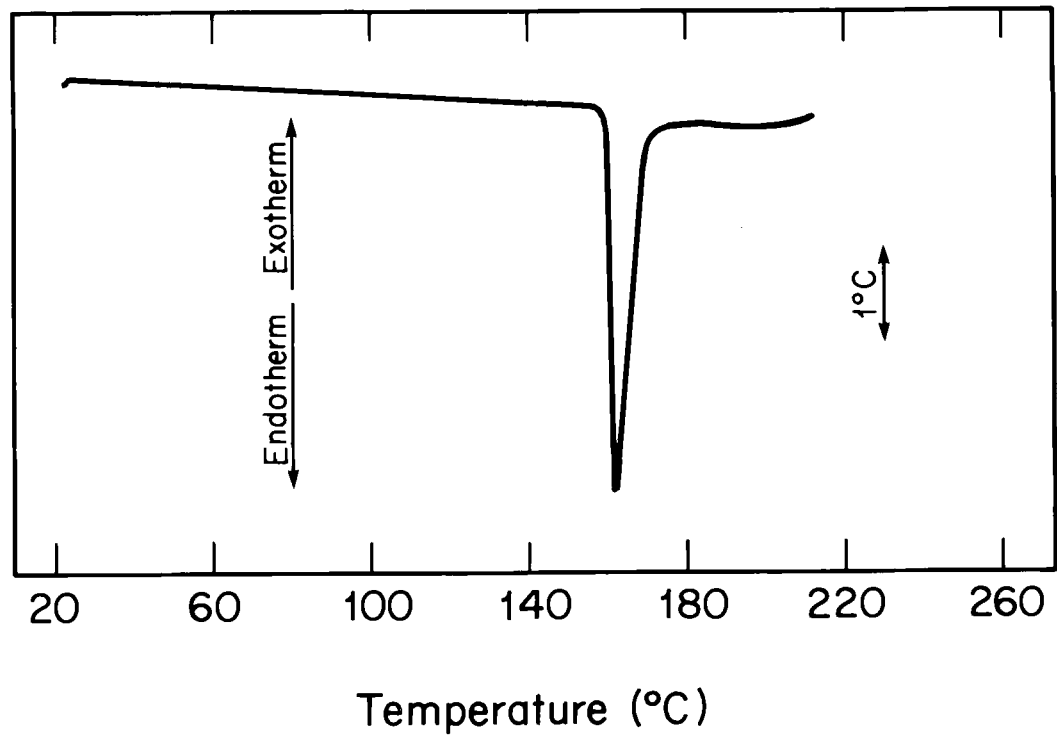


Figure 6. D.T.A. Curve of Indomethacin (Form I). Merck Standard 6375-66-1.



2.7 Solubility


---

The following solubility data have been reported:

Solvent	Temp(°C)	Solubility	Reference
Water	25	0.40 mg/100 ml <sup>a</sup>	20
Water	25	0.52 mg/100 ml <sup>b</sup>	20
Water	25	0.88 mg/100 ml <sup>c</sup>	20
Water	RT	Practically Insoluble	13
Phosphate Buffer pH 5.6	25	3 mg/100 ml <sup>a</sup>	28
Phosphate Buffer pH 5.6	25	5 mg/100 ml <sup>b</sup>	28
Phosphate Buffer pH 6.2	25	11 mg/100 ml <sup>a</sup>	28
Phosphate Buffer pH 6.2	25	16 mg/100 ml <sup>b</sup>	28
Phosphate Buffer pH 7.0	25	54 mg/100 ml <sup>a</sup>	28
Phosphate Buffer pH 7.0	25	80 mg/100 ml <sup>b</sup>	28
Ethyl alcohol (95%)	RT	1 in 50	13
Chloroform	RT	1 in 30	13
Ether	RT	1 in 45	13
Methanol	25	32 mg/gm	29
Benzene	25	4 mg/gm	29
n-butanol	25	19 mg/gm	29
sec-butanol	25	27 mg/gm	29

---

<sup>a</sup>Form I,    <sup>b</sup>Form II,    <sup>c</sup>Form III

## 2.8 Dissociation Constant

A pKa of 4.5 for the carboxyl group of indomethacin was calculated from aqueous solubility data(30). Potentiometric titration data for indomethacin in 50% methanol-water yielded pKa of 4.5 using a correction factor for the solvent(31).

## 2.9 Partition Behavior

Apparent distribution coefficients for indomethacin are tabulated below(31). Additional information is given in the references.

Solvent Pairs	K*
Methylene Chloride/pH 7.1 Phosphate Buffer	16.3
Ether/pH 7.1 Phosphate Buffer	8.2

$$K^* = \frac{\text{conc. in organic phase}}{\text{conc. in aqueous phase}}$$

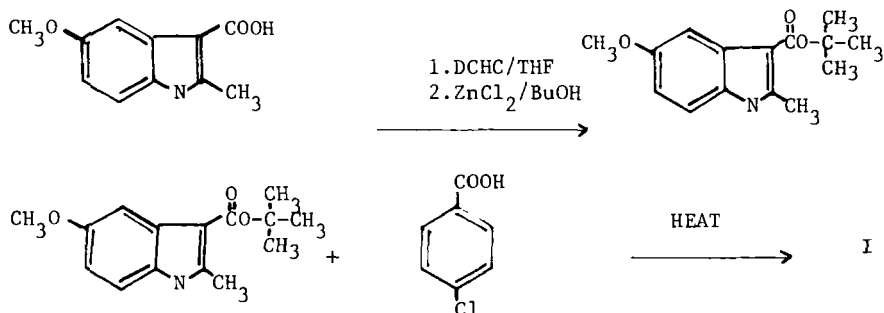
## 3. Synthesis

### 3.1

Indomethacin(I) was originally prepared as shown in the scheme below by conversion of 5-methoxy-2-methylindole-3-acetic acid to its anhydride using dicyclohexylcarbodiimide in THF(1). Treatment of the anhydride in the presence of zinc chloride and butanol produced the butyl ester, which was then acylated with p-chlorobenzoic acid to yield indomethacin butyl ester. The ester was then pyrolyzed and purified to produce indomethacin.

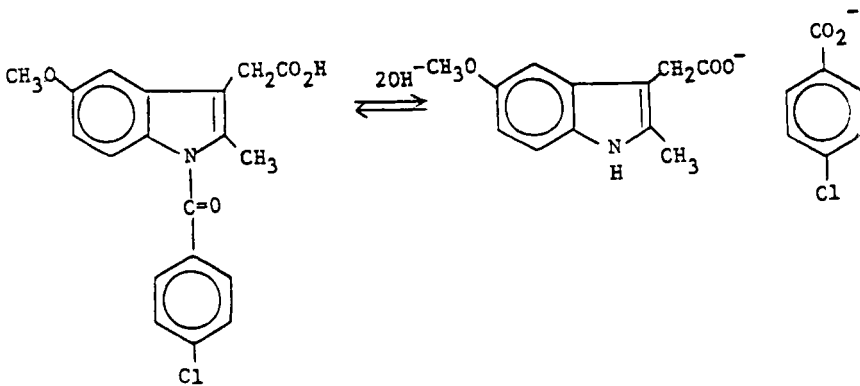
Indomethacin ethyl ester is synthesized by the reaction of N-p-chlorobenzoyl-N-p-methoxyphenylhydrazine with l-hydroxy-2-propanone and 2-bromoethylacetate after the method of Yamamoto et.al. (32)

Indomethacin has also been synthesized from sodium p-methoxyphenylhydrazine sulfonate and from saffrole(33,34).



#### 4. Stability

In general, the integrity of indomethacin powder and formulated products exists for at least five years at room temperature(35,36). Exposure to strong direct sunlight induces an increase in the color of indomethacin(37);however,degradation is slight, but the precaution of employing light resistant containers should be taken to minimize discoloration of solid. Indomethacin undergoes alkaline hydrolysis to p-chlorobenzoate and 2-methyl-5-methoxy-indole-3-acetate. These transformation products are also primary metabolic products(section on metabolism and pharmacokinetics).



The half-life at room temperature is about 200 hours in pH 8.0 buffer and about 90 minutes in pH 10.0 solutions(36). In a patent specification, Sumitomo Chemical Co. Ltd. reports stability conditions for seven different injectable formulations of indomethacin(38). Several of these formulations were stable after four months storage at 50°C.

Formulations of indomethacin as the free-acid and as the sodium salt are available in various parts of the world. They include:

Capsules 25mg and 50mg  
 Time Release Capsules 75mg  
 Injection 1.0mg/ml  
 Ophthalmic Suspension 10mg/ml  
 Oral Suspension 10mg/ml  
 Suppositories 50mg and 100mg

## 5. Methods of Analysis

### 5.1 Elemental Analysis

Analysis of Merck Sharp & Dohme reference lot 590,226-0A38 for C, H, N and O was reported as follows:

	<u>Theory</u>	<u>Observed</u>
C	63.78	63.87
H	4.51	4.44
N	3.92	4.09
O	9.91	10.01

Reference material is obtainable from both the U.S. Pharmacopeia and the British Pharmacopeia (13-14).

### 5.2 Spectrophotometric Analysis

Analysis of capsules containing indomethacin is described in the USP/NF and the British Pharmacopeia (13,14). After an extraction into methylene chloride from a methanolic pH 7.2 phosphate buffer, the ultraviolet absorbance is measured near 318 nm. This analysis measures intact indomethacin in the presence of its hydrolytic degradation products. If esterification has occurred, an ether wash prior to extraction from phosphate buffer has been used to prevent interference caused by esters(39). This procedure is also applicable to injections, suppositories, suspensions and tablets.

Allesandro and co-workers have also described analysis of indomethacin by formation of a nitroso derivative which has an ultraviolet absorption maximum at 317 nm(40).

### 5.3 Fluorescence Analysis

The indomethacin hydrolysis product 2-methyl-5-methoxy indole acetic acid fluoresces at 385 nm after excitation at 300 nm in 0.1N NaOH,(41) and at 387 nm after excitation at 312 nm in pH 11.6 buffer(42). The latter procedure claimed a three-fold increase in detectability. Neither method distinguishes indomethacin from salicylates. Clinical studies employing subjects administered aspirin must use a separation prior to fluorescence analysis. Without adequate separation the indole metabolites as well as salicylate, produce a positive assay bias.

## 5.4 Polarographic Analysis

In aqueous methanol, indomethacin exhibits a half-wave potential ( $E_{1/2}$ ) at the dropping mercury electrode which is dependent upon pH. In 0.1M methanolic lithium chloride, indomethacin has two waves between -1.4v and -1.6v (vs. S.C.E.). The first step height is diffusion controlled and corresponds to a two electron reduction of the amide carbonyl. The second wave is believed to be a kinetic wave. The method as described is specific for nonhydrolyzed indomethacin and is suitable for analysis of capsules, suppositories and suspensions with precision of  $\pm 1.2\%$ ,  $\pm 0.7\%$  and  $\pm 1.2\%$  for the respective formulations(43).

## 5.5 Mass-transport Techniques

### 5.5.1 Liquid-liquid Extraction

Distribution ratios of indomethacin and its metabolites, N-deschlorobenzoylindomethacin and O-desmethylindomethacin, have been described for heptane and aqueous solutions of buffers between pH 5.0 and 7.0(44). In addition, a comparison was made on samples extracted from sera of man, dog and rat in the above pH range.

### 5.5.2 Paper Chromatography

Harman and co-workers described a series of solvent systems for ascending chromatography on Whatman 3MM paper(45). The following table compares the  $R_f$  values of indomethacin (I), N-deschlorobenzoylindomethacin (II) and O-desmethylindomethacin (III).

<u>System</u>	<u><math>R_f</math></u>		
	<u>I</u>	<u>II</u>	<u>III</u>
A	0.75	0.40	0.66
B	0.95	0.88	0.92
C	0.95	0.95	0.95

System A - isopropyl alcohol - 15N ammonium hydroxide-water (8:1:1)

B - methanol-water-n-butyl alcohol-benzene (2:1:1:1)

C - acetic acid - isopropyl alcohol (5:95)

Other workers achieved separation of I, II, and III using as a developing system Xylene-toluene-dioxane-isopropanol-20% morpholine (1:1:3:3:2) (46).

### 5.5.3 Thin-Layer Chromatography

Sondergaard and Steines measured indomethacin in plasma and urine by direct quantitative TLC using chloroform-methanol(30:6) on silica gel(47). Reliable quantitation was achieved at the 30 ng/ml level(+12%).

In addition to the systems described above, Harman and co-workers developed TLC systems on silica gel(45).

<u>System</u>	<u>R<sub>f</sub></u>		
	<u>I</u>	<u>II</u>	<u>III</u>
D	0.50	0.55	0.65
E	0.57	0.37	0.15
D	ethyl acetate - isopropyl alcohol-10% ammonium hydroxide (5:4:3)		
E	acetic acid - chloroform (5:95)		

Allessandro and co-workers using silica gel G were able to separate indomethacin from drugs of similar pharmacological properties(40). Likewise Thompson and Johnson reported R<sub>f</sub> values for a variety of analgesics, antipyretics and anti-inflammatory drugs which were separable from indomethacin(48). Polyamide gels were also used by Hsiu *et al.* to resolve a series of antipyretics including indomethacin(49).

Curran, *et al.* , used TLC to resolve and identify impurities in formulations(50). The systems employed had detection limits on the order of 0.1 to 0.2 ug.

<u>System</u>	<u>R<sub>f</sub></u>				
	<u>I</u>	<u>II</u>	<u>PCB</u>	<u>IV</u>	<u>V</u>
A	0.80	0.75	0.90	0.24	0.35
B	0.40	0.38	0.50	0.33	0.28

PCB = parachlorobenzoic acid

IV = alpha monoglyceride of I from suppositories

V = alpha monoglyceride of PCB from suppositories

#### 5.5.4 Gas-Liquid Chromatography

GC-MS characterization of indomethacin has been reported using trimethylsilyl esters separated on SE-52 liquid phase(51). The same paper also reports precision and accuracy using an electron capture detector, which for plasma was  $92 \pm 19\%$  (5ng/ml) and  $96 \pm 1.5\%$  (1000ng/ml). For aqueous humors, the values were  $97 \pm 5.6\%$  and  $99 \pm 2.2\%$ , respectively.

Indomethacin has been determined without any preparatory work by Saito and Hara on 2% (w/w) OV-17 supported on Diasolid L and on 1.5% (w/w) SE-52 supported on high purity Chromosorb W(52).

Indomethacin has also been determined as its ethyl ester on a 1m x 2.5 mm column packed with 100-120 mesh Chromosorb W (AW-DMCS) coated with 2% (w/w) OV-1(53). Under optimum conditions utilizing an electron capture detector, a recovery from spiked serum of  $96 \pm 3\%$  was attained. Formation of the ethyl ester prevents assay inclusion of O-desmethylin domethacin, a metabolite which after methylation would be identical with the methyl ester of indomethacin.

Measurement with an electron capture detector has been made using the pentafluoropropyl derivative in the plasma of neonates with comparable precision and linearity over the range 10 to 1000 ng(54).

#### 5.5.5 Liquid-Solid Chromatography

Separation of salicylic acid and indomethacin was accomplished by packing 6 gms. of kieselguhr ground with citrate buffer on top of 2 gms. of kieselguhr mixed with pH 7.0 phosphate buffer. Material was eluted with heptane(42).

#### 5.5.6 High Performance Liquid Chromatography

Table V contains references for chromatographic systems used to measure indomethacin in various matrices.

Table V SEPARATION SYSTEMS FOR INDOMETHACIN BY HPLC

<u>Sample</u>	<u>Column</u>	<u>Mobile Phase</u>	<u>Detection</u>	<u>REF</u>
Biological	Hypersil ODS	76% methanol in 0.025M pH4 Phosphate Buffer	Fluorometric exc @ 295nm emm @ 340nm 1.5ng/ml	55
Dosage Form	Ultrasphere ODS	Methanol-water-acetonitrile- acetic acid(55:35:10:1)	Ultraviolet@254	56
Biological	Zorbax ODS	Mobile phase optimization	Ultraviolet@235	57
Dosage Form	Silica,10um	Gradient: A:8% acetic acid in heptane B:8% acetic acid and 20% ethanol in heptane	Ultraviolet@254	58
Dosage Form	C18uBondapak	27%acetonitrile in 1% aqueous formic acid	Ultraviolet@254	59
Dosage Form	C18uBondapak	60% methanol in 2.5% phos- phoric acid	Ultraviolet@240	60
Bulk Drug	C18uBondapak	Gradient: A:250ml acetonitrile in 750 phosphate buffer ( pH 7, 0.02M) B:700ml acetonitrile in 300	Ultraviolet@254	61



## 5.6 Titrimetric Analysis

Assessment of indomethacin powder had been specified in the U.S.P XIX and the British Pharmacopeia, 1980 as a back titration with hydrochloric acid after alkaline hydrolysis(13,14). This method can attain a precision of  $\pm 0.8\%$ (62). A direct titration for tablets and capsules is described using sodium hydroxide. If performed rapidly with phenolphthalein indicator, a precision of  $\pm 0.3\%$  is attainable.<sup>58</sup> The latter procedure serves to differentiate ester formation as well as hydrolysis products from intact material. The presence of parachlorobenzoic acid and 5-methoxy-2-methylindole-3-acetic acid are cause for positive error.

## 6. Metabolism and Pharmacokinetics

The only known routes of indomethacin elimination are renal, biliary and metabolic(46,63). Unchanged indomethacin (I), O-desmethyl-indomethacin (DMI), N-deschlorobenzoylindomethacin (DBI) and O-desmethyl-N-deschlorobenzoylindomethacin (DMBI) and their respective acyl glucuronides account for all drug-related moieties in plasma urine and feces. I, DMI and DBI are the major components in plasma and urine; they appear predominantly as conjugates in urine but totally unconjugated in plasma. DMBI is the major chemical species in the feces.

In all animal species studied, all four chemical moieties undergo enterohepatic circulation to varying degrees. The same can be inferred from the time courses of each in man. Quantitatively, it has been estimated that approximately 50% of an intravenous dose undergoes enterohepatic circulation as I.

Because of enterohepatic circulation the amount of I available to the systemic circulation can exceed the administered dose. On the average, the bioavailability of orally and rectally administered I is 100 and 80% relative to an intravenous reference dose.

Within the therapeutic range, there is no evidence that the disposition of I is route- or dose-dependent. The mean plasma, renal, biliary and metabolic clearances of I are 107, 28, 60, 68 ml/min., respectively. Because of the sporadic and variable nature of gall bladder discharge and consequently the ensuing reabsorption attempts to determine plasma half-life directly tend to be futile. Thus, values ranging from 90 minutes to 16 hours have been reported in the literature. An effective half-life of about 4.5 hours has been estimated from the time course of I accumulation during repeated drug administration and is the mean value which must have prevailed for the observed accumulation to obtain.

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# KETOTIFEN

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## 1. Foreword, History, Therapeutic Category

Ketotifen is a nonspecific, oral, mast cell stabilizer introduced in 1972 (1). The prominent biochemical-pharmacological activities of ketotifen are  $H_1$ -receptor antagonism, phosphodiesterase inhibition, inhibition of the formation SRS-A, and inhibition of calcium flux in smooth muscle preparations: all these actions are suited to prevent a development of asthmatic conditions. Promising results were obtained in early clinical trials: ketotifen was equipotent with disodium cromoglycate in prevention of asthma induced by spontaneous exercise or by antigens. Also, ketotifen is more specific than clemastine as a  $H_1$ -receptor antagonist. However, more recent, controlled trials failed to substantiate the early therapeutic optimism. The beneficial effect of ketotifen in the treatment of asthma is only small; it was noted that this effect is associated with pronounced sedation (2).

## 2. Description

### 2.1. Nomenclature

#### 2.1.1. Chemical Name

4-(1-Methyl-piperidylidene)-4H-benzo-[4,5]-  
-cyclohepta-[1,2-b]-thiophene-10(9H)-one hydrogen-  
fumarate

#### 2.1.2. Generic Name

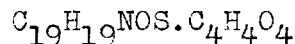
Ketotifen

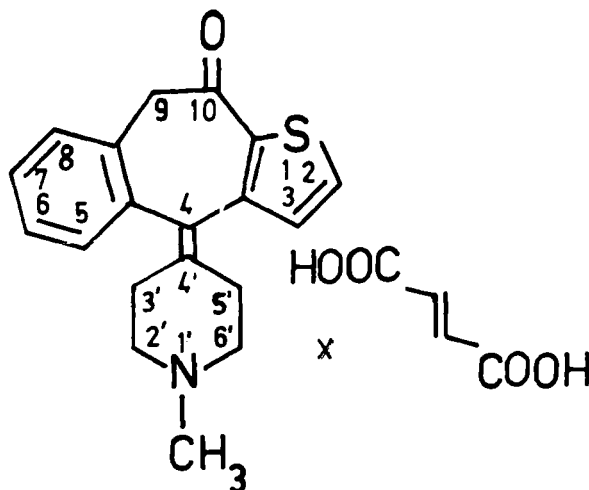
#### 2.1.3. Trade Name

Zaditen

## 2.2. Formula

### 2.2.1. Empirical



2.2.2. Structural2.3. Molecular Weight

425.504

2.4. Appearance, Color, Odor

The drug is marketed as the salt, either containing 2.5 molecule of water, or anhydrous. In either form it is a white, odorless crystalline powder.

Ketotifen base: a yellow, odorless crystalline powder.

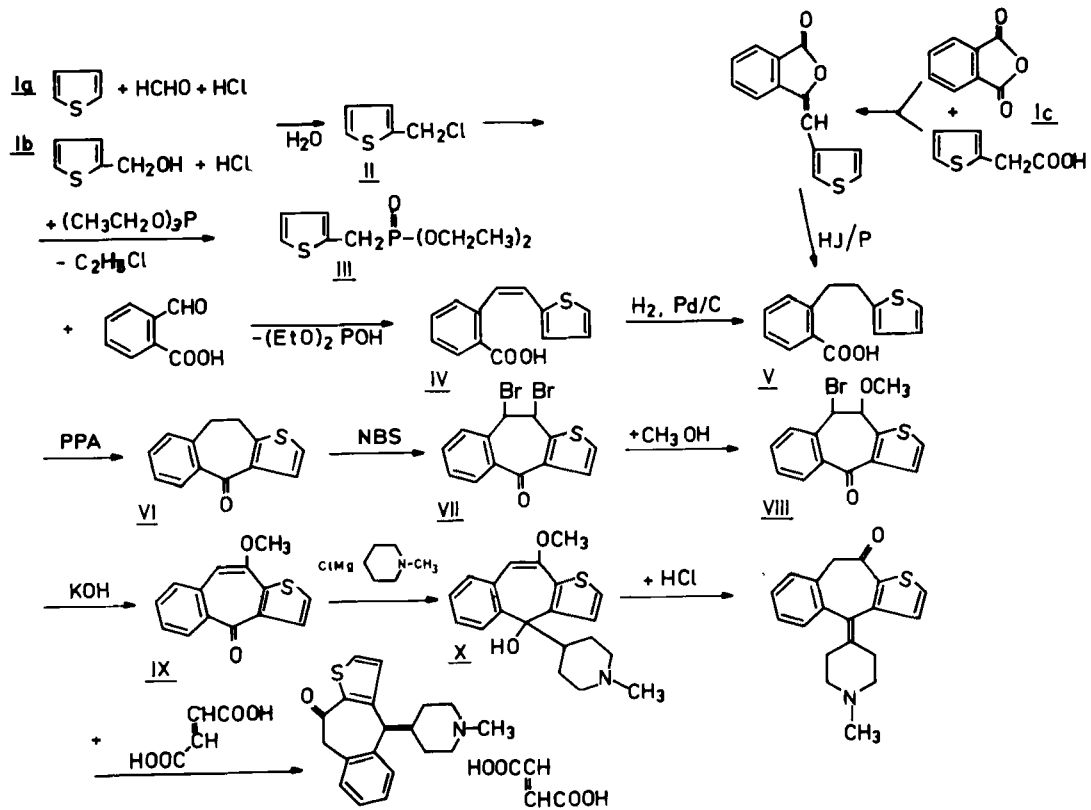
3. Synthesis

The reaction sequence leading to ketotifen hydrogen fumarate is shown in scheme I.

2-Thienylchloride (II), prepared by chloromethylation of thiophene (Ia) or 2-hydroxymethylthiophene (Ib), was subjected to Arbuzov's rearrangement to give 2-thienyl-diethylphosphonate (III). This product was reacted with 2-carboxybenzaldehyde whereupon 2-/2-(thienyl)-vinyl/-benzoic acid (IV) was obtained; IV was hydrogenated to 2-/2-(thienyl)-ethyl/-benzoic acid (V). Cyclisation, under the influence of polyphosphoric acid, (PPA), resulted in 9,10-dihydro-4H-benzo[4,5]-cyclohepta[1,2-b]-thiophene-4-one (VI).

Alternatively (V) may be prepared by reduction of 2-thienylidene-phthalide obtained from 2-thi-





Scheme I.

enyl acetic acid (Ic) and sodium ethylate (3).

Bromination of (VI) gives the dibromo derivative which, on refluxing in methanol, followed by dehydrobromination with KOH in refluxing methanol, gave lo-methoxy-4H-benzo-[4,5]-cyclohepta-[1,2-b]-thiophene-4-one (IX). This compound was condensed with the magnesium derivative of 4-chloro-1-methylpiperidine in tetrahydrofuran, and the resulting lo-methoxy-4-(1-methyl-4-piperidyl)-4H-benzo[4,5]-cyclohepta-[1,2-b]-thiophene-4-one (X) was finally dehydrated and demethylated with HCl at 95-100°C, to give the base of ketotifen (4).

#### 4. Physical Properties

##### 4.1. Spectra

##### 4.1.1. Infrared

The IR absorption spectrum of ketotifen base, and that of ketotifen hydrogen fumarate with 2.5 molecules of water, are shown in Fig. 1. These spectra were recorded with KBr-pelleted samples using a Pye Unicam SP-200 Infrared Spectrophotometer (5). Some of the major frequencies and band assignments are given in Tables 1. and 2.

Table 1. Characteristic IR bands of ketotifen base

frequency (cm <sup>-1</sup> )	assignment
3100	thiophene C-H stretching
3000-2840	aromatic and CH <sub>3</sub> stretchings
1640	C=O stretching
1620	C=C (ring), stretching
1400 and 1280	C-O-H, interaction
1450	C-H, bending
1120	C-C(=O)-C, stretching and bending
1060	C-H, in-plane bending
935-700	thiophene

##### 4.1.2. Ultraviolet

Fig. 2. shows the UV spectra (Pye-Unicam SP-8-100 Spectrophotometer) of ketotifen base (1) and ketotifen hydrogen fumarate with 2.5 molecule of crystal water (2), both compounds being in methanolic solution (5).

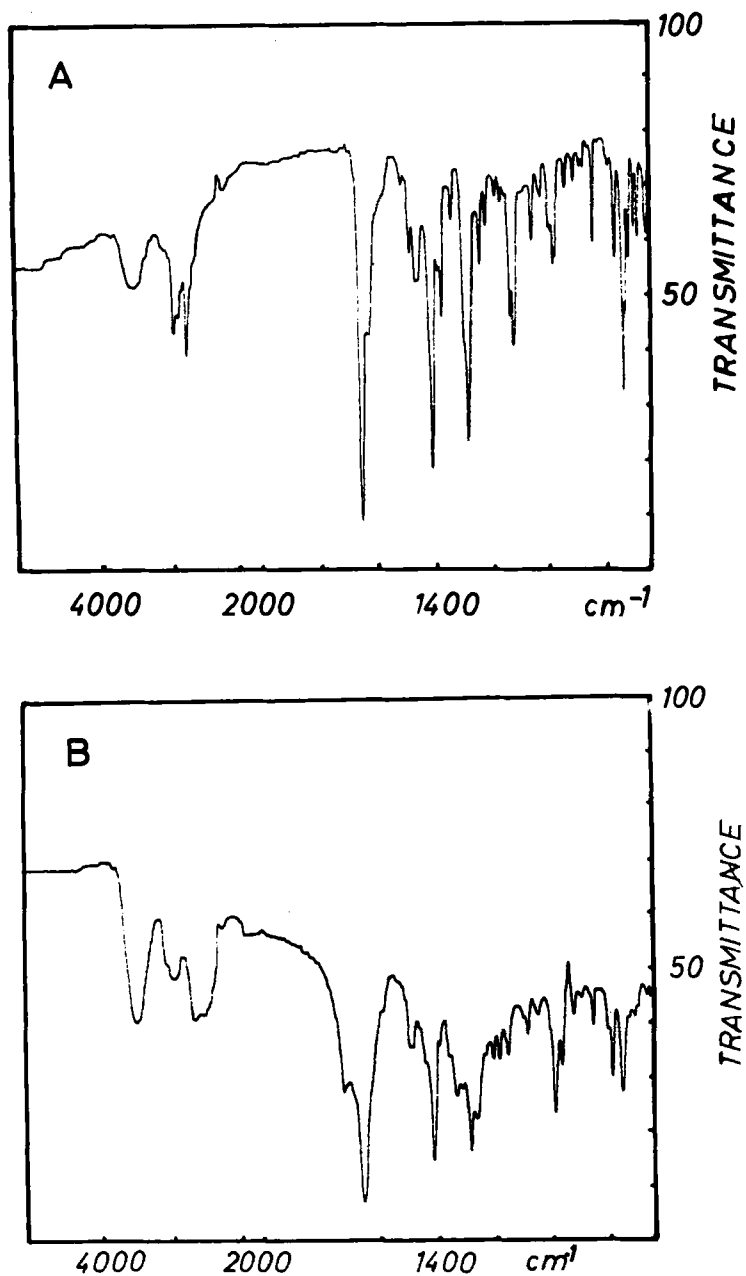


Fig. 1. Infrared absorption spectra of ketotifen base (A) and ketotifen hydrogen fumarate with 2.5 H<sub>2</sub>O (B). Instrument: Pye-Unicam SP-200.

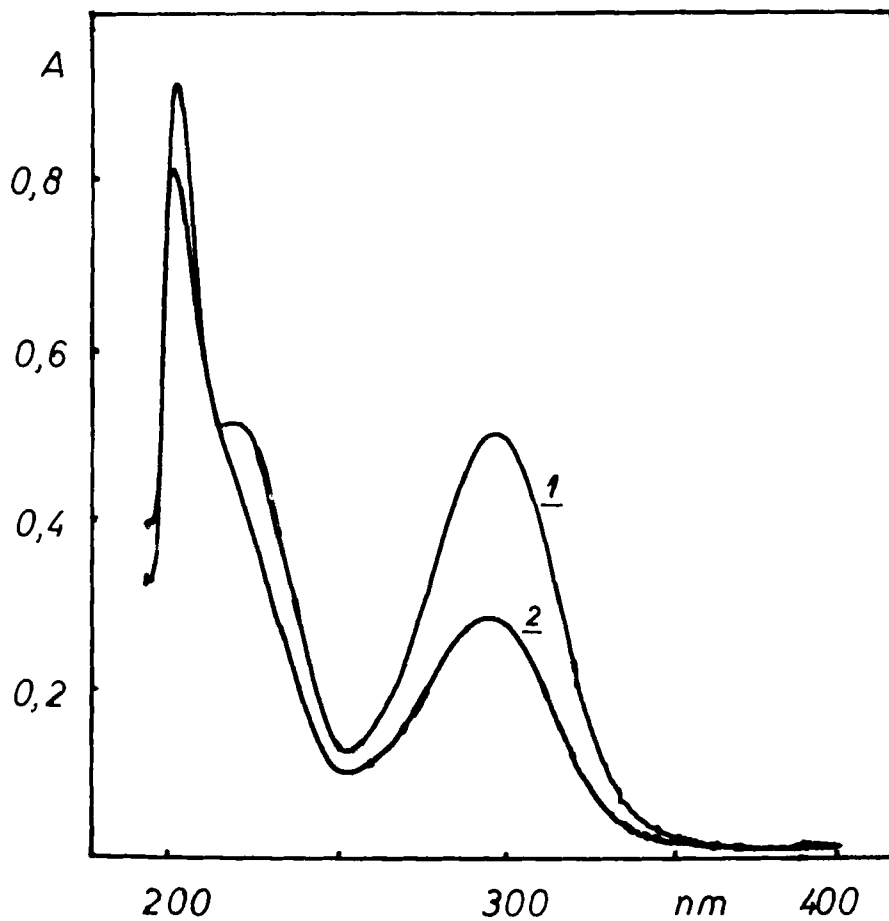


Fig. 2. Ultraviolet spectra of ketotifen base (1), and ketotifen hydrogen fumarate with 2.5 crystal H<sub>2</sub>O (2). Instrument: Pye-Unicam SP-8-100.

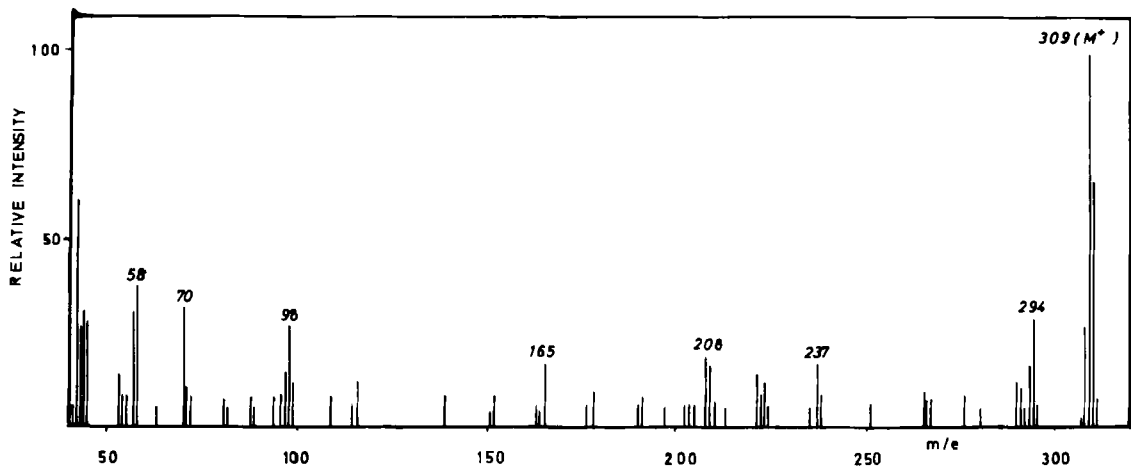


Fig. 3. Mass spectrum of ketotifen hydrogen fumarate. The electron energy was 70 eV and the temperature of the ion source was 250°C. Instrument: mass spectrometer CEC-21-110 B.

Table 2. Characteristic IR bands of ketotifen hydrogen fumarate containing 2.5 molecules of crystal water

frequency ( $\text{cm}^{-1}$ )	assignment
3550-3450	OH, broad band
3080-3030	aromatic CH, stretching
3095-3075	=CH <sub>2</sub> stretching
3100	thiophene CH, stretching
2800-2200	COOH and NH
1710	COOH (fumaric acid)
1640	C=C (ring), stretching
1390	C-N, stretching
1280	-C-O-H, interaction

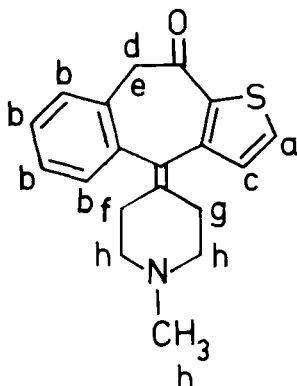
#### 4.1.3. Mass

The mass spectrum (CEC-21-110 B Mass spectrometer) of ketotifen hydrogen fumarate is shown in Fig. 3. The strongest peak corresponds to the molecular ion of ketotifen hydrogen fumarate ( $m/e$  309). Fragmentation apparently starts by a separation of the methyl group ( $m/e$  15) from the piperidylidene moiety, and the remainder of the original molecule ( $m/e$  294) does not fragment further, at least not markedly. The heterocyclic parts of the molecule, thiophene and piperidine, should give the fragments  $\begin{array}{c} \diagdown \\ \text{S} \\ \diagup \end{array}$  ( $m/e$  58) and  $\text{CH}_2\text{-N-CH}_3$  ( $m/e$  57), respectively (5).

#### 4.1.4. Proton Magnetic Resonance

The proton MR spectra (Jeol FX-100 Spectrometer) were recorded in different solvents: that of ketotifen base in deuterated chloroform, and that of ketotifen hydrogen fumarate in deuterated dimethyl sulphoxide (in either case against TMS as internal standard) (5). These spectra are presented in Figs. 4. and 5. Characteristic features of the spectra are given separately in Tables 3. and 4. The proton MR spectrum of ketotifen base was partly described by Waldvogel et al. (6).

When ketotifen hydrogen fumarate was shaken with  $\text{D}_2\text{O}$ , the carboxylic group protons of fumaric acid and the  $\text{N}^+\text{H}$  protons were exchanged for deuterons from  $\text{D}_2\text{O}$ , as shown by the absence of the broad peak at 9.14 ppm (see Table 4.) (5).

Table 3.  $^1\text{H}$ -NMR spectrum of ketotifen base; characteristics and assignments

chem. shift $\delta$ (ppm)	intens.	multipli- city	coupling const. J (Hz)	assign- ment
7.50	1 H	doublet	$J_{a,c} = 4.9$	$\text{H}_a$
7.36-7.10	4 H	multiplet		$\text{H}_b$
7.03	1 H	doublet	$J_{c,a} = 4.9$	$\text{H}_c$
4.24	1 H	doublet	$J_{d,e} = 13.18$	$\text{H}_d$
3.74	1 H	doublet	$J_{e,d} = 13.18$	$\text{H}_e$
2.94-2.58	2 H	multiplet		$\text{H}_f$
2.49-2.39	2 H	multiplet		$\text{H}_g$
2.28-1.99	7 H	multiplet		$\text{H}_h, \text{N-CH}_3$

#### 4.1.5. $^{13}\text{C}$ -Nuclear Magnetic Resonance

$^{13}\text{C}$ -NMR spectra were recorded with a Jeol FX-100 spectrometer at 25.05 MHz. The sample of ketotifen base was dissolved in both  $\text{CDCl}_3$  and  $\text{DMSO-d}_6$ , and hydrogen fumarate only in  $\text{DMSO-d}_6$  (5). The samples were measured in 5 mm tubes with TMS as internal standard and using internal deuterium lock.

The broad band  $^{13}\text{C}$ -NMR spectra of  $\text{DMSO-d}_6$  solutions of base and the hydrogen fumarate are shown in Figs. 6. and 7. In both cases all the signals were clearly resolved. The assignments of saturated carbon atoms can be made on the basis of their well

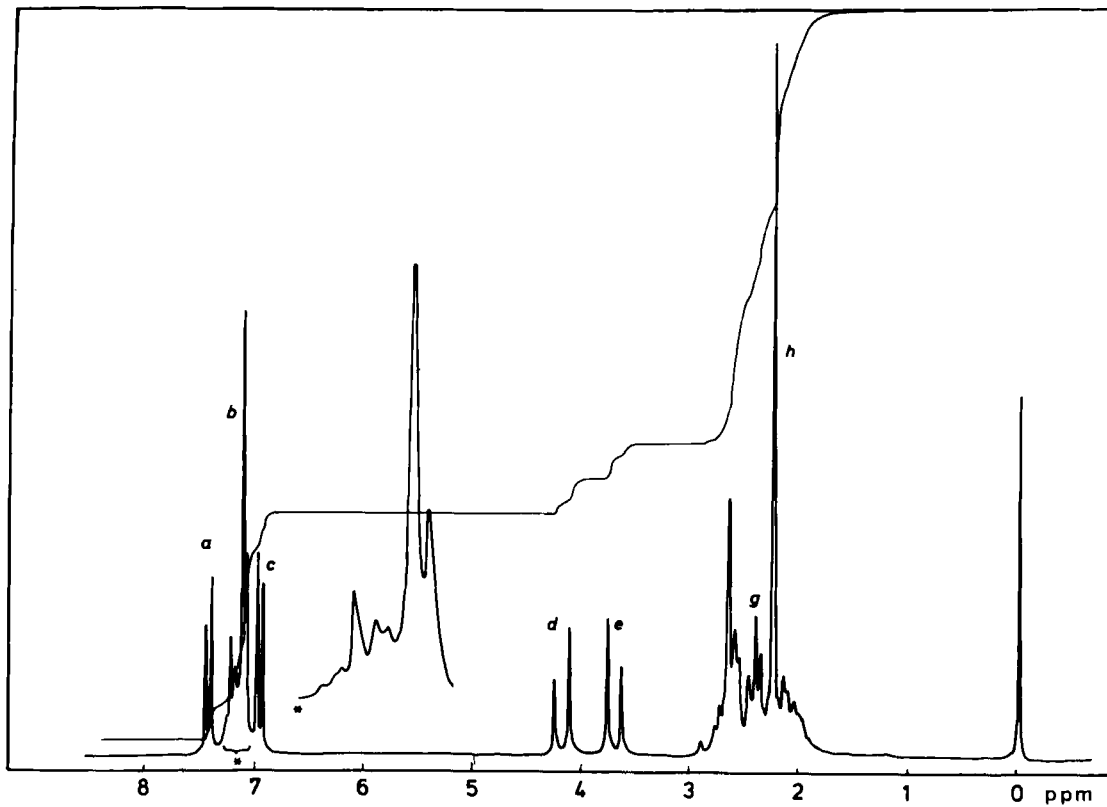


Fig. 4.  $^1\text{H}$ -Nuclear magnetic resonance spectrum of ketotifen base. Instrument: Jeol FX-100 at 100 MHz.



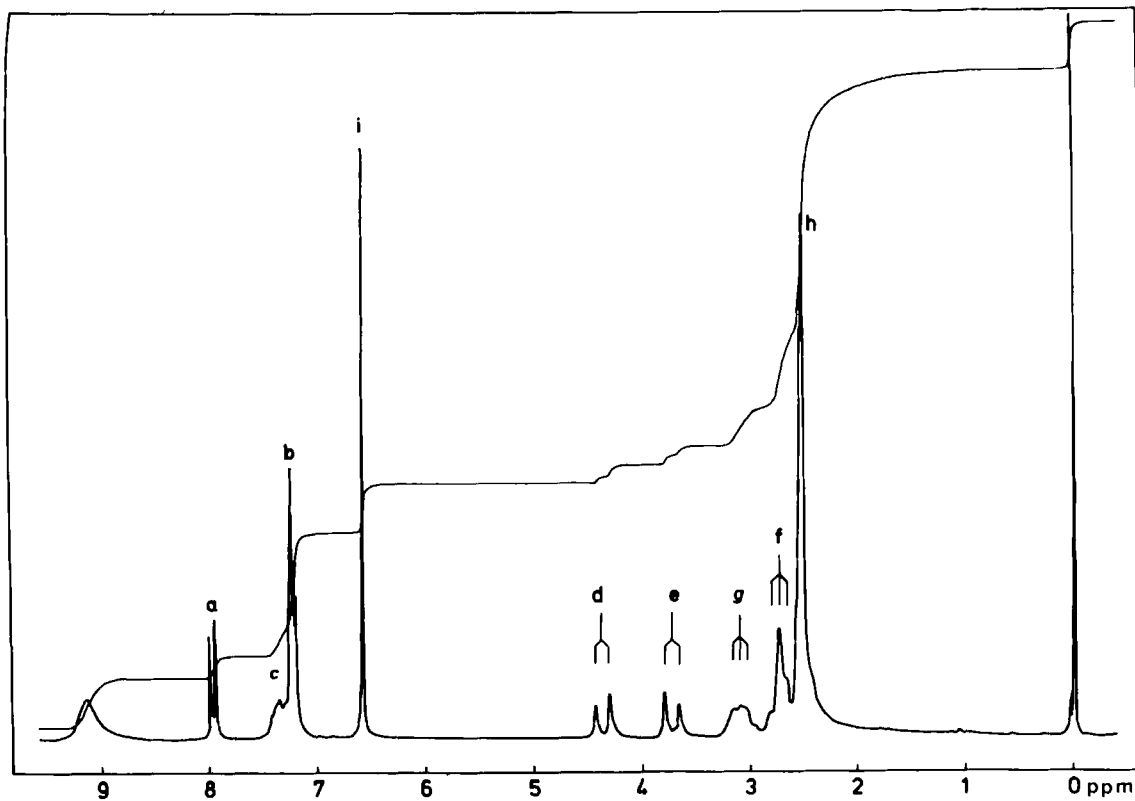
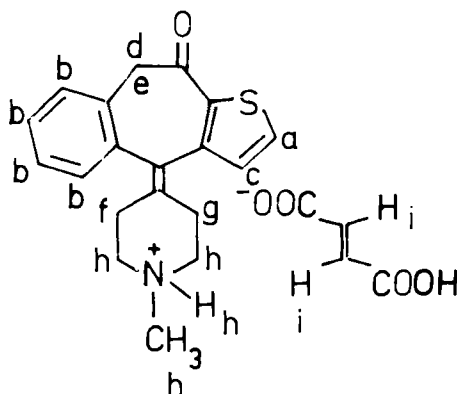


Fig. 5.  $^1\text{H}$ -Nuclear magnetic resonance spectrum of ketotifen hydrogen fumarate with 2.5  $\text{H}_2\text{O}$ . Instrument: Jeol FX-100 at 100 MHz.

Table 4.  $^1\text{H-NMR}$  spectrum of ketotifen hydrogen fumarate

chem. shift $\delta$ (ppm)	intens.	multipli- city	coupling const. J (Hz)	assign- ment
9.14	2 H	broad singlet		COOH
7.98	1 H	doublet	$J_{a,c} = 4.9$	$H_a$
7.43-7.26	1 H	multiplet		$H_c$
7.26-7.21	4 H	singlet		$H_b$
6.60	2 H	doublet	$J_{d,e} = 13.17$	$H_i$ (fumaric ac.)
4.37	1 H	doublet	$J_{e,d} = 13.17$	$H_d$
3.72	1 H	multiplet		$H_e$
3.17-3.03	2 H	multiplet		$H_g$
2.80-2.66	2 H	multiplet		$H_f$
2.54-2.51	8 H	multiplet		$H_h$

defined absorption regions (7), but due to very closely spaced chemical shifts of numerous aromatic and two quaternary olefinic carbons, the assignment was possible only by means of gated decoupled (NOE) spectra. Because of the solute-solvent interaction in  $\text{DMSO-d}_6$  solutions these spectra were not quite suitable for an unambiguous interpretation. Therefore we have recorded the NOE spectrum of the base in  $\text{CDCl}_3$ .

From the multiplets of the aliphatic part of the completely coupled spectrum, it was possible to assign the signals belonging to C-2', C-3' and

Table 5.  $^{13}\text{C}$  Chemical shifts<sup>a</sup> and  $^{13}\text{C}$ - $^1\text{H}$  coupling constants<sup>b</sup> for ketotifen base dissolved in  $\text{CDCl}_3$

C-atom	$\delta$ (ppm)	$^1J_{\text{CH}}$	$^2J_{\text{CH}}$	$^3J_{\text{CH}}$	$^4J_{\text{CH}}$
C-2	131.80 (d)	185.1	6.3	-	-
C-3	130.28 (d)	169.9	3.9	-	-
C-4	137.73 (s)	-	-	7.8	-
C-5	126.52 (d)	161.1	$\approx 1.0$	7.5	-
C-6	128.58 (d)	159.0	$< 1.0$	6.8	-
C-7	129.34 (d)	157.6	$\approx 2.0$	4.0	-
C-8	130.28 (d)	158.0	$\approx 1.0$	5.0	-
C-9	49.67 (dd)	134.9	-	3.9	-
		125.9	-	3.9	-
C-10	189.05 (s)	-	8.0	-	-
C-11	147.95 (s)	-	-	9.3	4.6
C-12	132.39 (s)	-	overlaped with C-7 multiplet		-
C-13	141.02 (s) <sup>c</sup>	-	-	-	-
C-14	138.67 (s) <sup>c</sup>	-	-	-	-
C-2'	57.07 (t)	-	-	-	-
C-3'	31.47 (t)	129.2	3.4	-	-
C-4'	128.93 (s)	-	-	-	-
$\text{CH}_3$	45.91 (q)	133.3	-	$< 1.0$	-

<sup>a</sup>  $\delta$  in ppm downfield from internal TMS; precision  $\pm 0.05$  ppm; off-resonance multiplets are given in parentheses in the second column.

<sup>b</sup> J in Hz; the precision is  $\pm 0.5$  Hz.

<sup>c</sup> The assignment can be interchanged. The long-range couplings give complicated multiplets.

methyl group of the N-methyl-piperidine part of the molecule, as well as the methylene carbon (C-9) of the cyclic ketone part. The latter carbon atom displays a doublet of doublets indicating a pronounced departure of the  $\text{CH}_2$  group from the planarity. Two different first order C-H coupling constants were determined (Table 5.), which is in agreement with the  $^1\text{H}$ -NMR spectrum (Fig. 4, Table 3.) which shows the typical pattern for geminal protons.

The assignment of the aromatic-olefinic part of the spectrum was not possible using only known substitution rules (8) due to simultaneous interaction of few different moieties. However, the NOE spectrum enabled a sound interpretation based on the long-range carbon-hydrogen couplings. The com-

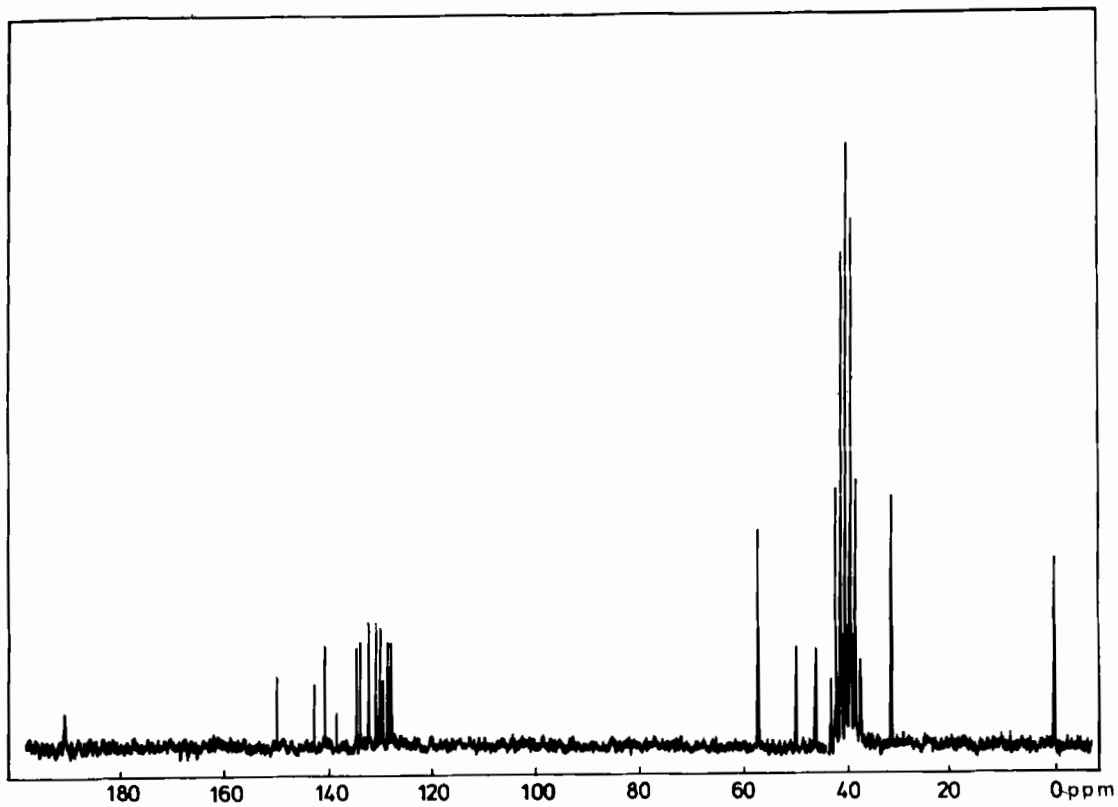


Fig. 6.  $^{13}\text{C}$  Broad band decoupled NMR spectrum of ketotifen base in  $\text{DMSO-d}_6$ . Instrument: Jeol FX-100 at 25.05 MHz.

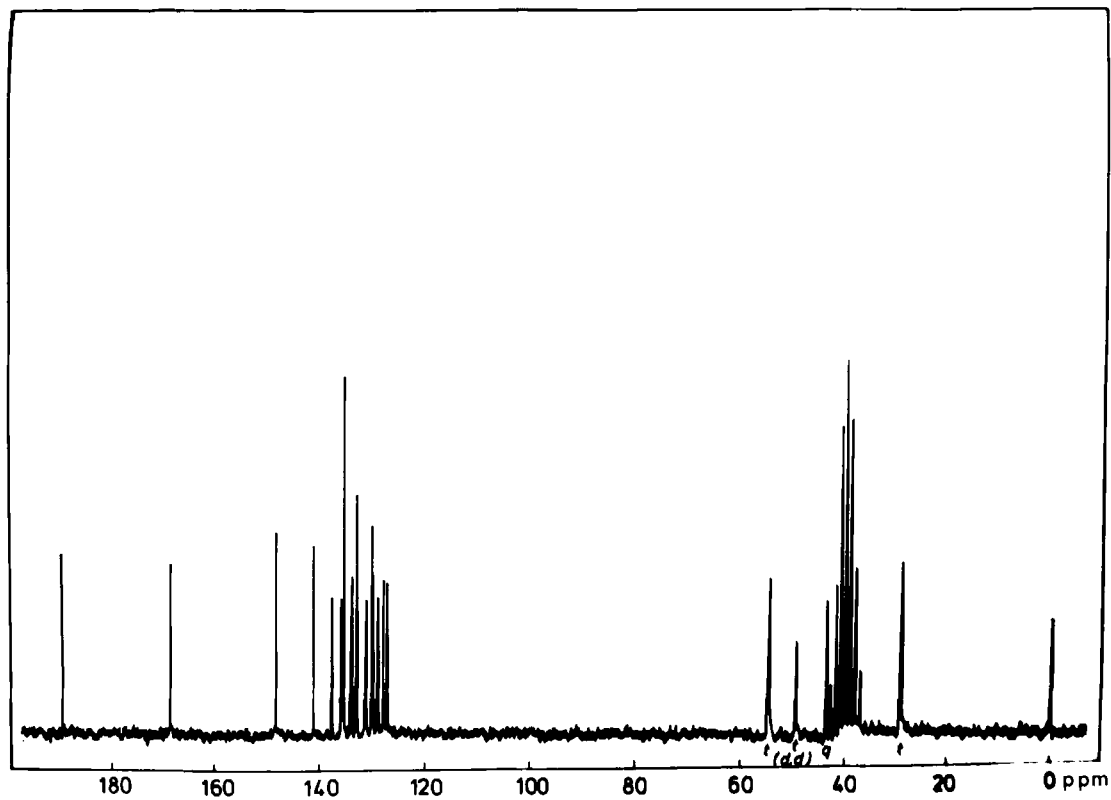


Fig. 7.  $^{13}\text{C}$  Broad-band decoupled NMR spectrum of ketotifen hydrogen fumarate in  $\text{DMSO-d}_6$ . Instrument: Jeol FX-100 at 25.05 MHz.

Table 6.  $^{13}\text{C}$  Chemical shifts<sup>a</sup> for ketotifen base and hydrogen fumarate in  $\text{DMSO-d}_6$  solution

C-atom	base	fumarate	$\Delta\delta^b$
C-2	132.26 (d)	132.26 (d)	0
C-3	130.00 (d)	130.54 (d)	+0.54
C-4	136.10 (s)	135.24 (s)	-0.84
C-5	126.53 (d)	126.66 (d)	+0.07
C-6	127.91 (d)	128.32 (d)	+0.41
C-7	129.24 (d)	129.47 (d)	+0.23
C-8	127.09 (d)	127.32 (d)	+0.23
C-9	48.96 (dd)	48.98 (dd)	+0.02
C-10	188.41 (s)	188.57 (s)	+0.16
C-11	148.05 (s)	147.43 (s)	-0.62
C-12	133.02 (s)	133.23 (s)	+0.21
C-13 <sup>c</sup>	140.89 (s)	140.43 (s)	-0.46
C-14 <sup>c</sup>	138.94 (s)	136.98 (s)	-1.96
C-2'	56.21 (t)	54.14 (t)	-2.07
C-3'	30.92 (t)	28.82 (t)	-2.10
C-4'	128.47 (s)	129.37 (s)	+0.9
$\text{CH}_3$	45.28 (q)	43.13 (q)	-2.15
C-1'', (C=C)	-	134.73 (d)	
C-2'', (C=O)	-	167.64 (s)	

<sup>a</sup>  $\delta$  in ppm downfield from internal TMS; precision  $\pm 0.05$  ppm; off resonance multiplets are given in parentheses.

<sup>b</sup> Changes in chemical shifts are going from base to fumarate; + denotes a downfield, and - an upfield shift.

<sup>c</sup> The assignments can be interchanged.

plete assignment is given in Table 5. including a number of long-range coupling constants up to the fourth order.

Figs. 6. and 7. show the broad-band decoupled spectra of ketotifen base and its fumarate in  $\text{DMSO-d}_6$ , and Table 6. lists the  $^{13}\text{C}$  chemical shifts. The assignment is made on the basis of a comparison with the values from Table 5. The most remarkable changes (an upfield shift of about 2.1 ppm) are observed for the saturated carbons of the heterocyclic moiety. The third column in Table 6. indicates the changes in chemical shifts for all carbon atoms common to both species.

It should be also mentioned that the addition

of  $D_2O$  to the  $CDCl_3$  solution causes not only the vanishing of the  $OH$  peak in the  $^1H$ -NMR spectrum, but the diminishing of the signal at 49.67 ppm belonging to C-9 as well. This indicates the exchange of protons by deuterons causing the splitting of the signal into a multiplet.

## 4.2. Solid Properties

### 4.2.1. Melting Characteristics

The melting range of ketotifen base is 156-158 °C, that of ketotifen hydrogen fumarate with 2.5  $H_2O$  is 124-130 °C, whereas the anhydrous hydrogen fumarate decomposes between 184 and 200 °C.

### 4.2.2. X-Ray Diffraction

X-Ray diffraction patterns were determined with a Mod. XRD-6 Spectrogoniometer (General Electric, Schenectady) (5). Pertinent data are presented in Table 7.

Table 7. X-Ray diffraction data of ketotifen hydrogen fumarate

$\Theta$ (°)	$d^a$ (Å) interplanar distance	$I/I_0^b$ relative intensity
4.94	8.95	3
5.37	8.24	6
6.08	7.28	14
6.42	6.89	9
7.05	6.28	12
7.76	5.71	23
8.79	5.04	17
9.00	4.93	20
9.61	4.62	67
9.90	4.48	93
10.79	4.12	26
11.46	3.88	14
12.02	3.70	48
12.42	3.58	100
12.99	3.43	16
14.32	3.12	12
15.65	2.86	10
17.45	2.57	9
17.76	2.53	11

<sup>a</sup>  $d = n\lambda/2 \sin \Theta$ ; <sup>b</sup> based on the highest relative intensity ( $I_0 = 100$ )

### 4.3. Solution Properties

#### 4.3.1. Solubility

Solubilities of ketotifen base and ketotifen hydrogen fumarate with 2.5 H<sub>2</sub>O in various solvents, at room temperature (18-20°C), are summarized in Tables 8. and 9. (5)

Table 8. Solubilities of ketotifen base

Solvent	Solubility
water	insoluble
methanol	soluble
ethanol	soluble
isopropanol	sparingly soluble
acetone	sparingly soluble
1,4-dioxane	sparingly soluble
diethyl ether	sparingly soluble
ethyl acetate	sparingly soluble
chloroform	freely soluble
dichloromethane	freely soluble
carbon tetrachloride	slightly soluble
toluene	sparingly soluble
n-hexane	insoluble

Table 9. Solubilities of ketotifen hydrogen fumarate hydrate (2.5 H<sub>2</sub>O)

Solvent	Solubility
water	slightly soluble
methanol	freely soluble
ethanol	freely soluble
isopropanol	sparingly soluble
acetone	sparingly soluble
1,4-dioxane	sparingly soluble
diethyl ether	sparingly soluble
ethyl acetate	very slightly soluble
chloroform	very slightly soluble
dichloromethane	very slightly soluble
n-hexane	insoluble

#### 4.3.1. Dipole Moment

The dipole moment of ketotifen base was determined with a Dipolmeter DM 01 (Wiss. Techn. Werkstätten, Weilheim), using 1,4-dioxane as the



solvent (5). The value obtained at  $20.0 \pm 0.1^\circ\text{C}$  was  $3.55 \pm 0.12$  D.

## 5. Methods of Analysis

### 5.1. Elemental Analysis

Elemental analysis of ketotifen hydrogen fumarate:  $\text{C}_{23}\text{H}_{23}\text{NO}_5\text{S}$ , calc.: C 64.90% (theor.)  
 H 5.45%  
 N 3.29%  
 O 18.81%  
 S 7.54%

## 5.2. Chromatographic Methods

### 5.2.1. Thin-Layer (TLC)

Thin layer chromatographic systems are given in Table 10. Spots were visualized under UV irradiation (5).

Table 10. Solvent systems for thin-layer chromatography of ketotifen base on silica gel plates

Solvent system	$R_f$
methanol	0.2
ethylacetate	0.3
methanol:ammonia (19:1)	0.9
ethylacetate:ammonia:methanol (1:0.1:0.9)	0.8
ethylacetate:ammonia:methanol (8:0.1:0.9)	0.5
ethanol:chloroform (1:5)	0.8

### 5.2.2. Gas (GC)

Ketotifen hydrogen fumarate was chromatographed on a glass capillary column 15 m x 0.32 mm I.D. with SE-30. The carrier gas was  $\text{H}_2$ , inlet pressure 0.45 bar. The temperature program used in analysis was  $120\text{--}240^\circ\text{C}$  at  $6^\circ/\text{min}$ . rate. The concentration of ketotifen hydrogen fumarate (solvent:ethanol) in the sample that gave the record shown in Fig. 8. was 1 mg/ml and the retention time was 12.3 min (5).

### 5.2.3. High Performance Liquid (HPLC)

Table 11. gives the HPLC conditions used for the analysis of ketotifen hydrogen fumarate (5). The column was Supelcosil LC-8 (15 cm x 4 mm I.D.), detection UV-230 nm, 0.16 A.U.F.S. (5).

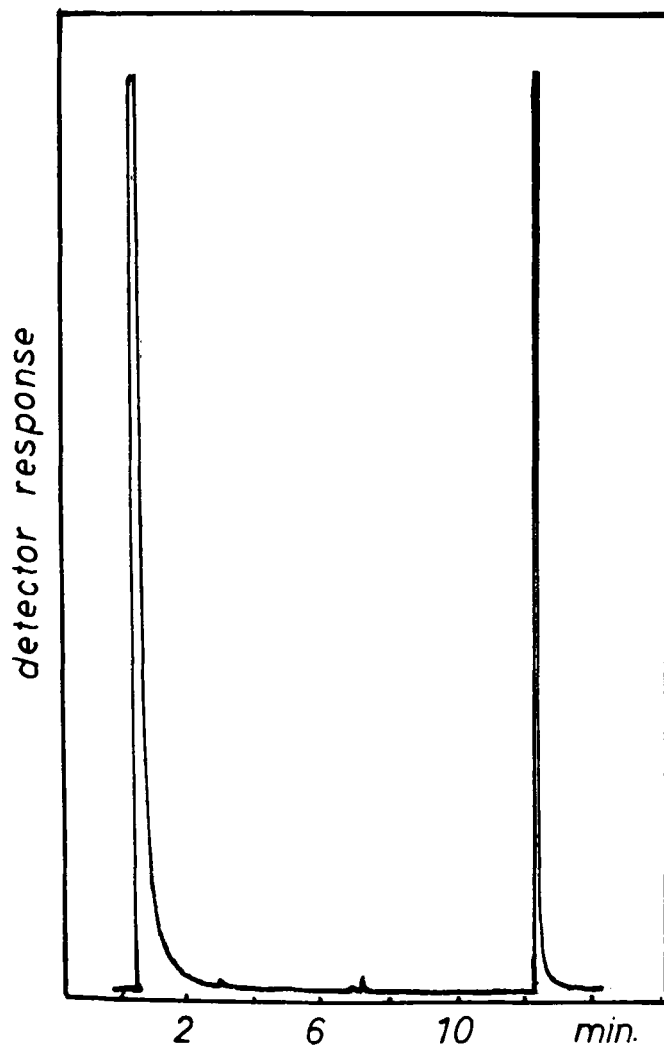


Fig. 8. The gas chromatogram of ketotifen base.  
Instrument: Pye-Unicam Mod. 204.

Table 11. HPLC conditions for ketotifen hydrogen fumarate

Mobile phase	Flow rate (ml/min)	Pressure (bar)	Retention time (min)
acetonitrile-dist. water- -acetic acid 90:10:0.5 (pH 3.5)	15	60	22.6
acetonitrile-dist. water- -methanol 120:20:30 (pH 7.5)	0.8	25-30	12.3

### 5.3. Titration

Fifty mg of ketotifen base was weighed ( $\pm 0.1$  mg) into a titration vessel and dissolved in 20 ml of glacial acetic acid. The resulting solution was titrated potentiometrically using a glass S.C.E. pair: 1.0 ml of 0.1 M  $\text{HClO}_4$  is equivalent with 30.94 mg of ketotifen base<sup>(5)</sup>. The content of ketotifen base in the sample was calculated by substituting numerical values into equation (1):

$$\text{content of ketotifen base (\%)} = \frac{V \times f \times 30.94}{W} \times 100 \quad (1)$$

where  $V$  = volume of 0.1 M  $\text{HClO}_4$  consumed (ml)

$f$  = normality factor of 0.1 M  $\text{HClO}_4$

$W$  = mass of the sample (mg).

The limits allowed are 98-102%.

Potentiometric titration of fumaric acid: fifty mg of ketotifen hydrogen fumarate with 2.5  $\text{H}_2\text{O}$  was weighed ( $\pm 0.1$  mg) into a titration vessel and dissolved in 10 ml of ethanol and 10 ml of water (5). The resulting solution was titrated potentiometrically with 0.1 M NaOH is equivalent with 5.8 mg of fumaric acid. The content of fumaric acid in the sample is calculated by using equation (2):

$$\% \text{ fumaric acid} = \frac{V \times f \times 5.8}{W} \times 100 \quad (2)$$

where  $V$  = volume of 0.1 M NaOH consumed (ml)

$f$  = its normality factor

$W$  = weight of the sample (mg).

The limits allowed are 24-25.2%.

### 6. Stability and Degradation

Tablets and the pure substance of ketotifen hydrogen fumarate with 2.5  $\text{H}_2\text{O}$  were tested in the

following way: one series of samples was kept at elevated temperatures, up to the 60°C and the relative moisture of 50% for seven days, and the other was exposed to daylight. Individual samples were withdrawn at 24-hr intervals, and subjected to HPLC analysis for degradation products. No changes were observed in the tablets; the pure substance assumed a slight yellow coloration at the end of the testing period.

### 7. Drug Metabolism, Pharmacokinetics, Bioavailability

Ketotifen appears to be a potent drug acting in the skin, nose and airways. There is little evidence from acute experiments to suggest that this drug has substantially different properties from a typical H<sub>1</sub> antagonist, e.g. clemastine (9). Administered to allergic patients, ketotifen decreased the required allergen challenge and showed antihistaminic activity (10). It was ineffective in reducing exercise-induced bronchoconstriction in 23 asthmatic children (11), so a higher dose of ketotifen is necessary to obtain a beneficial effect (12).

To study the bioavailability of ketotifen from sustained-release oral formulations, deuterated ketotifen (N-CD<sub>3</sub>) was used. The kinetic profiles of ketotifen in the plasma and in the urine shows that any isotopic effect due to the presence of deuterium is lacking (13).

### 8. Identification and Determination in Body Fluids and Tissues

Selective and sensitive gas-chromatographic methods have been developed for quantitative determination of ketotifen and its desmethyl metabolite in biological fluids. The ketotifen is detected by a nitrogen detector, or by mass fragmentometry, which allows determination at concentration down to 1 ng/ml and 0.05 ng/ml, respectively (14). The N-glucuronide of ketotifen is measured as ketotifen, after enzymatic cleavage. The nor-ketotifen is quantitated, after derivatisation with heptafluorobutyric anhydride, by gas chromatography with electron capture detection. On chromatograms obtained from urines of subjects who had taken ketotifen, peaks occurred. The structures of metabolites sepa-

rated by chromatography were ascertained by high and low-resolution mass spectrometry (14). The technique described was applied to establish the metabolic spectrum of ketotifen in monkeys (rhesus, baboon, gibbon, chimpanzee) and men (14).

### 9. Determination in Pharmaceuticals

The tablets were crushed in a mortar. A mass of powder equivalent with the average weight of one tablet was transferred into a 100 ml volumetric flask and 50 ml of methanol was added. The flask was then shaken automatically for 20 min, the resulting solution made up to the mark with methanol, and mixed thoroughly. A 20 ml aliquot was centrifugated at 4000 r.p.m. The absorbance of the clear supernatant was measured at 298 nm against methanol. A reference solution of ketotifen hydrogen fumarate 2.5-hydrate was prepared by dissolving 15.2(±0.1) mg of the salt in methanol (10-ml volumetric flask; concentration of ketotifen base, about 1 mg/ml). One ml of the standard solution was pipetted into a 100-ml volumetric flask, the solution made up to the mark with methanol, and the absorbance measured at 298 nm against methanol.(5). The ketotifen content in one tablet was calculated by using equation (3):

$$\text{mg ketotifen base in one tablet} = \frac{A_s \times C_r \times A.W.}{A_r \times W} \quad (3)$$

where  $A_s$  = absorbance of the sample solution

$A_r$  = absorbance of the standard solution

$C_r$  = concentration of ketotifen base in the standard solution

A.W. = average mass of one tablet (mg)

W = mass of powdered sample (mg)

The limits allowed are 90-110%.

### Acknowledgments

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# MELPHALAN

L. Valentin Feyns

*The United States Pharmacopeia  
Rockville, Maryland*

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## 1. Foreword, History, Therapeutic Category.

Melphalan is an antineoplastic drug, listed also as a Class I immunosuppressive agent (effective only when given prior to the immune stimulus) [1]. It is used for the treatment of multiple myeloma, ovarian carcinoma, tumors of the testes, chronic granulocytic leukemia, chronic lymphocytic leukemia, seminoma, Ewing's sarcoma, reticulum cell sarcoma, and thymoma [1,2]. Its use as an adjuvant to surgery in the management of primary breast cancer was one of the first illustrations of the therapeutic potential of combined modalities of treatment [3].

Chemically, it is a bis(2-chloroethyl)amine (a nitrogen mustard), and its biological activity is related to the ability to function as an alkylating agent in physiological conditions.

Attempts to use sulfur mustard, the chemical weapon of World War I, against experimental tumors or in the clinic were abandoned because of the high toxicity of the compound [4]. Extensive studies of the biological and chemical actions of aliphatic nitrogen mustards, directed mainly to their potential military use, were conducted before and during World War II. Noting the marked cytotoxic action of nitrogen mustards on lymphoid tissues and rapidly dividing cells, Goodman and Gilman initiated studies on experimental tumors and, in 1942, the first clinical trials on patients in the terminal stages of various neoplasms [2,5]. The publication of the results had to wait for the declassification of the military research at the end of the war. The series of papers published in 1946 in Science and in the Journal of the American Medical Association marked the emergence of chemotherapy as an alternative to surgery and irradiation in cancer treatment [5--8]. This emergence was followed by the synthesis and evaluation of thousands of nitrogen mustards, in a search for a selective, less toxic tumor inhibitor.

The synthesis of Melphalan, in 1954, was the result of two premises: (1) aromatic nitrogen mustards should be less reactive, thus less toxic, than the aliphatic analogs [9]; (2) attaching the nitrogen mustard moiety to a "carrier" selected from normal cellular constituents might confer greater selectivity to the molecule. Bergel and Stock at the Chester Beatty Research Institute in London described the synthesis of the nitrogen mustards of DL-, D-, and L-phenylalanine [10]; the latter is Melphalan. Shortly after that, a Russian team reported the synthesis and evaluation

of the hydrochloride of the racemic derivative, which they called Sarcolysin [Larionov, 11].

The success of the studies on experimental tumors and of the clinical trials prompted the synthesis of a wide range of Melphalan-related compounds such as the ortho isomer [12--15], the meta isomer [16--20],  $\beta$ -alanine analogs [19], esters, N-acyl derivatives, and peptides. Despite controversial reports of greater activity or better therapeutical index, none of these analogs replaced Melphalan in clinical use.

The higher inhibitory effect of the L-isomer on most of the experimental tumors represented one of the first examples of selectivity of action through optical isomerism in cancer chemotherapy [10, 21--23].

## 2. Description

### 2.1 Nomenclature

#### Chemical Abstracts Names

Current:

L-Phenylalanine, 4-[bis(2-chloroethyl)amino]-

Pre-1972:

Alanine, L-3-[p-[Bis(2-chloroethyl)amino]-phenyl]-

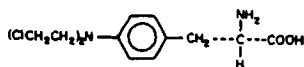
Other Names: Alkeran®, L-Sarcolysine, L-PAM, L-Phenylalanine Mustard, Compound CB 3025, Compound NSC-8806.

D-isomer: Medphalan

Racemic: Merphalan, Sarcolysin, Sarcochlorin

#### Chemical Abstracts Registry Numbers

Melphalan (L-form, free base)	:	148-82-3
L-hydrochloride	:	3223-07-2
D-free base	:	13045-94-8
D-hydrochloride	:	4213-32-5
DL-free base	:	531-76-0
DL-hydrochloride	:	1465-26-5

2.2 Formula, Molecular Weight

$\text{C}_{13}\text{H}_{18}\text{Cl}_2\text{N}_2\text{O}_2$       Molecular weight    305.20

2.3 Appearance, Odor, Color

In the original publication [10] and in the Merck Index [24] the monosolvated product obtained by crystallization from methanol is described as small colorless needles.

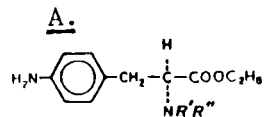
The compendial article is described as an ". . .off-white to buff powder, having a faint odor. . ." [25,26] or as ". . .a white or almost white powder; odourless. . ." [27].

3. Synthesis

Melphalan and the racemic analog have been prepared by two general routes (Scheme I). In Approach (A) the amino acid function is protected, and the nitrogen mustard moiety is prepared by conventional methods from aromatic nitro-derivatives. Thus, the ethyl ester of N-phthaloyl-phenylalanine was nitrated and reduced catalytically to amine I. Compound I was reacted with ethylene oxide to form the corresponding bis(2-hydroxyethyl)amino derivative II, which was then treated with phosphorus oxychloride or thionyl chloride. The blocking groups were removed by acidic hydrolysis. Melphalan was precipitated by addition of sodium acetate and was recrystallized from methanol. No racemization was detected [10,28--30]. The hydrochloride was obtained in pure form from the final hydrolysis mixture by partial neutralization to pH 0.5 [31]. Variants of this approach, used for the preparation of the racemic compound, followed the same route via the  $\alpha$ -acylamino- $\alpha$ -p-aminobenzyl malonic ester III [10,28--30,32,33] or the hydantoin IV [12].

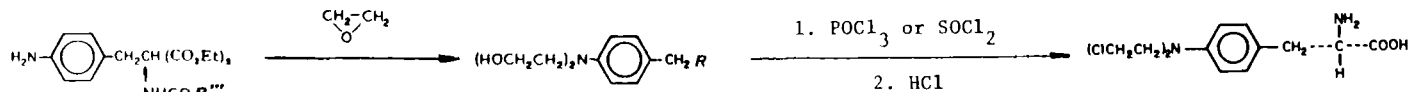
In Approach (B), aniline is converted into the corresponding nitrogen mustard, which is formylated to the benzaldehyde nitrogen mustard V. The alanine moiety is constructed via the Erlenmeyer reaction with hippuric acid, reduction, and hydrolysis [23,34--38].

High yields of optically pure products are claimed for the resolution of N-formyl-DL-melphalan through the brucine salt, followed by HCl hydrolysis [39].



I

or

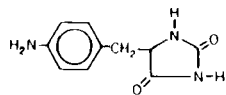


III

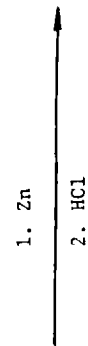
II

Melphalan

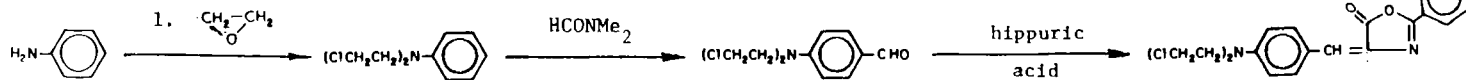
or



IV



B.



V

Scheme 1. Synthesis of melphalan.

Synthesis of Melphalan labelled at the nitrogen mustard function [40--42] or at the benzylic methylenic group [43] were reported. Deuteration at the positions ortho to the N-mustard substituent was reported when melphalan was refluxed in deuterium chloride in D<sub>2</sub>O (20% w/v) [44].

#### 4. Physical Properties

##### 4.1 Spectra

##### 4.1.1 Ultraviolet

The ultraviolet spectrum of a 1-in-100,000 solution of melphalan in alcohol exhibits an absorbance maximum at about 260 nm (absorptivity about 72) and a minimum at about 226 nm [45]. The spectrum of one compendial article in methanol (0.0005 percent w/v solution) has been defined as showing a maximum at 260 nm and a less well-defined maximum at 301 nm [27]; the wavelength of the second maximum is sometimes reported as 310 nm [25,46]. The 301 nm value appears to be the correct one (Figure 1).

##### 4.1.2 Infrared

Spectra of Melphalan and Melphalan Hydrochloride in KBr dispersions are shown in Figures 2 and 3.

The following prominent bands are present in the spectra:

(a) broad bands in the 2900--3100 cm<sup>-1</sup> region, assigned to CH and NH (in NH<sub>3</sub><sup>+</sup>) stretching vibrations; (b) a continuous series of bands and shoulders in the 2400-2900 cm<sup>-1</sup> region, characteristic for aminoacids and their hydrochlorides; (c) the asymmetrical and symmetrical vibrations of the COO<sup>-</sup> group at about 1590 and 1400 cm<sup>-1</sup> in the spectrum of Melphalan (In the spectrum of the hydrochloride, in which the ionization of the carboxylic group is suppressed, these bands are replaced by a strong carboxylic absorption at about 1740 cm<sup>-1</sup>.); (d) a strong band at about 1620 cm<sup>-1</sup> that can be assigned to an aromatic ring stretching vibration with some contribution from the deformation amino acid band 1; and (e) the medium band near 730 cm<sup>-1</sup> arising from the C-Cl (trans) stretching.

The assignments are in agreement with the general IR literature and with data reported for the meta isomer of Melphalan [17,20,49].

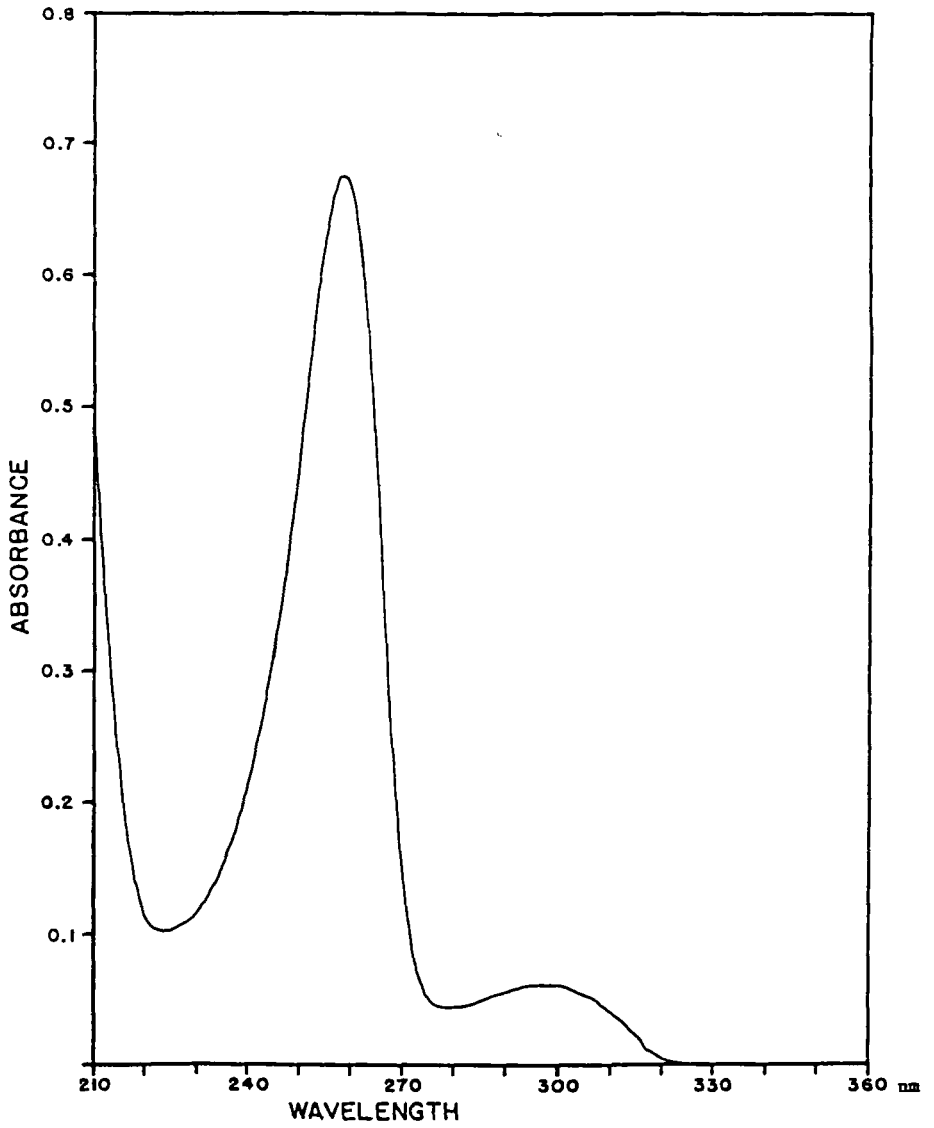


Figure 1. Ultraviolet Spectrum of Melphalan in Methanol (~10 ug/ml).

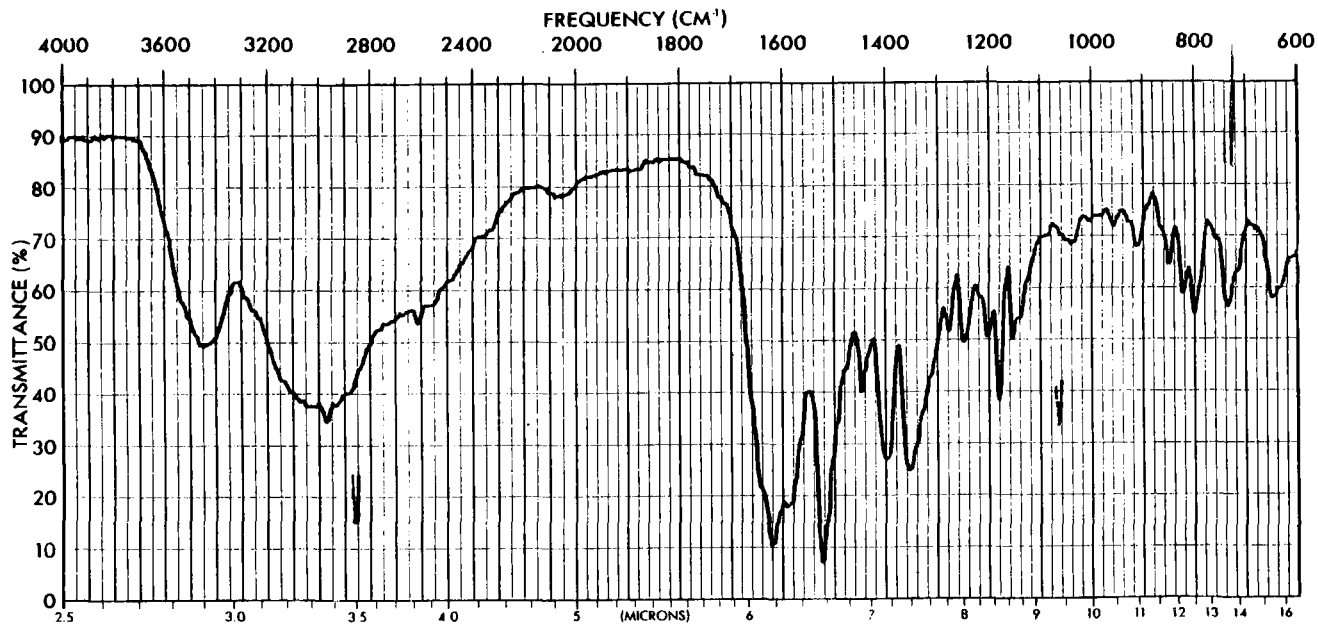


Figure 2. Infrared Spectrum of Melphalan (undried) KBr pellet.  
Instrument--Perkin Elmer 727.

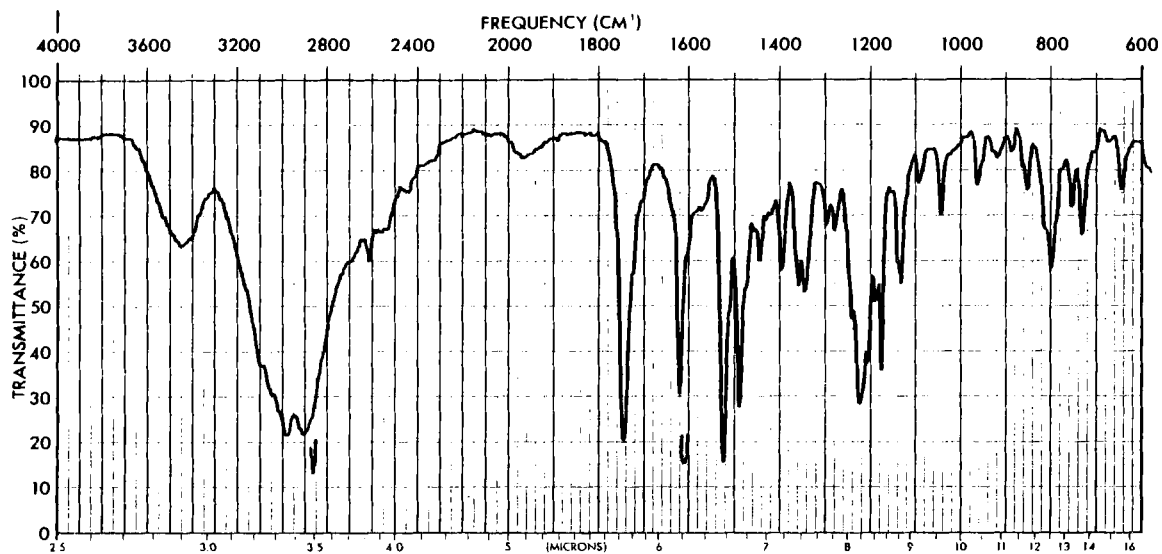


Figure 3. Infrared Spectrum of Melphalan Hydrochloride (undried).  
KBr pellet. Instrument--Perkin Elmer 727.



#### 4.1.3 Nuclear Magnetic Resonance

Proton magnetic resonance spectra of melphalan in dimethylsulfoxide (DMSO)-d<sub>6</sub> and D<sub>2</sub>O--DCl solutions and of melphalan hydrochloride in DMSO-d<sub>6</sub> are shown in Figure 4. The spectra were run on a Varian FT-80A spectrometer at 80 MHz. The assignments of the chemical shifts are tabulated in Table I.

They are within reasonable agreement with the literature data reported for phenylalanine [50] and melphalan [51], with one exception. Hsieh [33] reported for melphalan in D<sub>2</sub>O--DCl the following assignments:  $\text{CH}_2\text{Cl}$ , 4.10 ppm;  $\text{CH}_2\text{N}$ , 3.60 ppm. Based on a comparison of the spectra in DMSO-d<sub>6</sub> and D<sub>2</sub>O--DCl, we reversed the assignments; our assignment was confirmed by a two-dimensional proton--carbon correlation experiment run by Dr. James Shooley of Varian, Palo Alto, California.

Fully decoupled <sup>13</sup>C Spectra of Melphalan, recorded on a Varian FT-80A spectrometer are shown in Figure 5. The chemical shift assignments are presented in Table II.

Integration of the signals in the proton NMR spectra and multiplicity in the coupled <sup>13</sup>C NMR spectra support the assignments in Tables I and II.

In both types of spectra, the marked influence of the ionization form of the molecule on the chemical shifts is apparent.

#### 4.1.4 Mass spectra

A mixture of melphalan and its hydrolytic decomposition products was silylated and separated by gas chromatography; the peaks were identified by mass spectrometric analysis [52]. GC-MS was also used for the identification of the HPLC melphalan peak separated from plasma or hydrolysis mixtures [40,53,54].

A mass spectrum of the methyl ester of N-trifluoroacetylmelphalan was reported, without analysis of the fragmentation pattern [41]. The mass spectrum of melphalan-d<sub>2</sub> (direct insertion mode) showed a molecular ion at m/e 306 (3% relative intensity) and a base peak at m/e 232 (M-CH(NH<sub>2</sub>)COOH)<sup>+</sup>. A linear relationship was demonstrated between the composition of melphalan/melphalan-d<sub>2</sub> mixtures and the peak intensities at m/e 230 and m/e 232 [44].

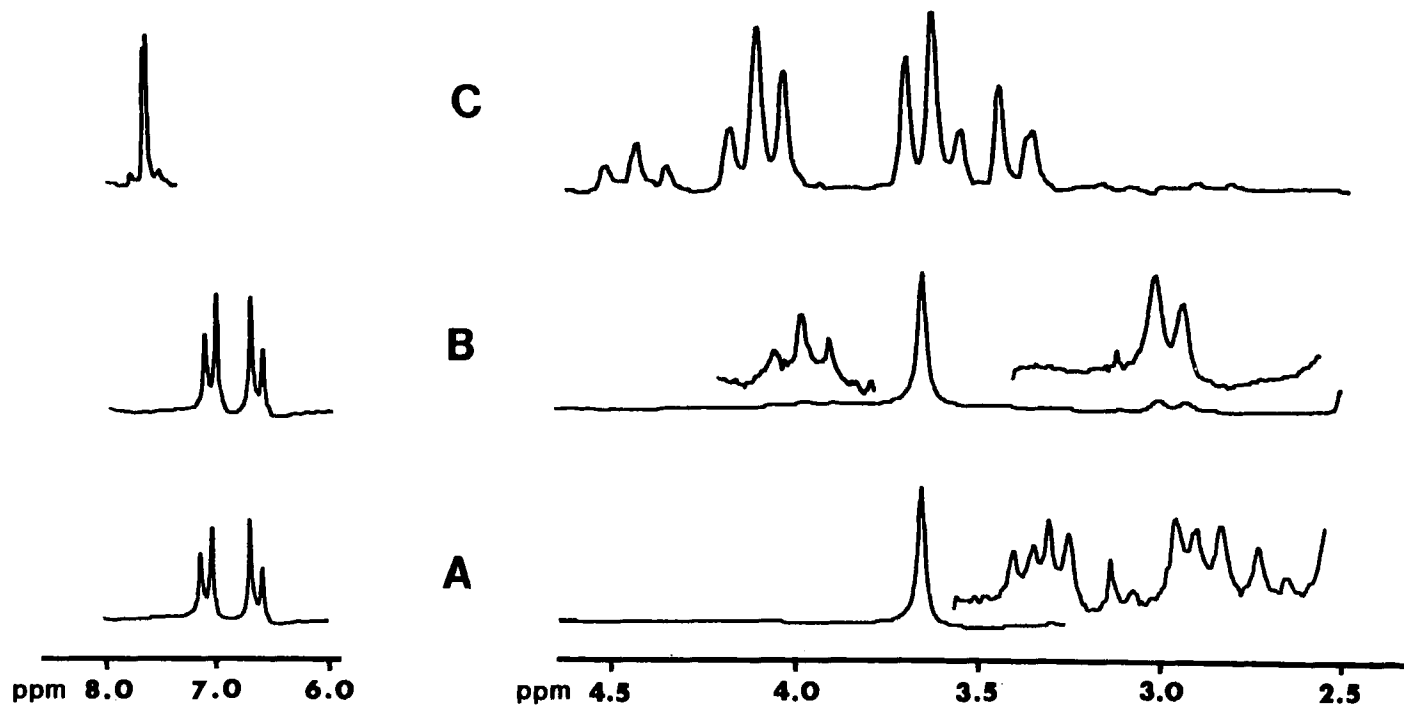
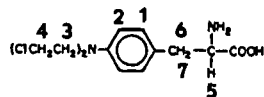


Figure 4. Proton NMR Spectra of Melphalan.

(A) Melphalan in  $\text{DMSO-d}_6$ . (B) Melphalan Hydrochloride in  $\text{DMSO-d}_6$ . (C) Melphalan in  $\text{DCI-D}_2\text{O}$ .

Table I. Chemical Shift Assignments in the Proton NMR Spectra of Melphalan



Proton #	Melphalan <sup>a</sup> (DMSO-d <sub>6</sub> )	Melphalan Hydrochloride <sup>a</sup> (DMSO-d <sub>6</sub> )	Melphalan <sup>b</sup> (conc. DCl-D <sub>2</sub> O 1:4, v/v)
1	7.12 (d, J <sub>1.2</sub> = 8.7 Hz)	7.12 (d, J <sub>1.2</sub> = 8.7 Hz)	7.69 (d, J <sub>1.2</sub> = 9.2 Hz)
2	6.67 (d)	6.70 (d)	7.61 (d)
3	3.70 (s)	3.71 (s)	4.16 (t, J <sub>3.4</sub> = 6.0 Hz)
4	3.70 (s)	3.71 (s)	3.67 (t)
5	3.36 (J <sub>5.6</sub> = 4.25 Hz) <sup>c</sup>	4.04 (t, J <sub>5,6</sub> = 6.2 Hz) <sup>d</sup>	4.45 (t, J <sub>5,6</sub> = 6.5 Hz)
6	3.03 (J <sub>6,7</sub> = 14 Hz) <sup>c</sup>	3.02 (d) <sup>d</sup>	3.42 (d) <sup>e</sup>
7	2.77 (J <sub>5,7</sub> = 7.75 Hz) <sup>c</sup>	3.02 (d, J <sub>5,7</sub> = 6.2 Hz) <sup>d</sup>	3.41 (d, J <sub>5,7</sub> = 7.0 Hz)

<sup>a</sup> in ppm, downfield from TMS; <sup>b</sup> in ppm, downfield from DSS

<sup>c</sup> ABC system, assignments according to [50b]; <sup>d</sup> probably unresolved ABC system

<sup>e</sup> For phenylalanine in 2N DCl in D<sub>2</sub>O, J<sub>5,6</sub> = 5.5 Hz, J<sub>5,7</sub> = 7.7 Hz, J<sub>6,7</sub> = -14.6 Hz [50a]

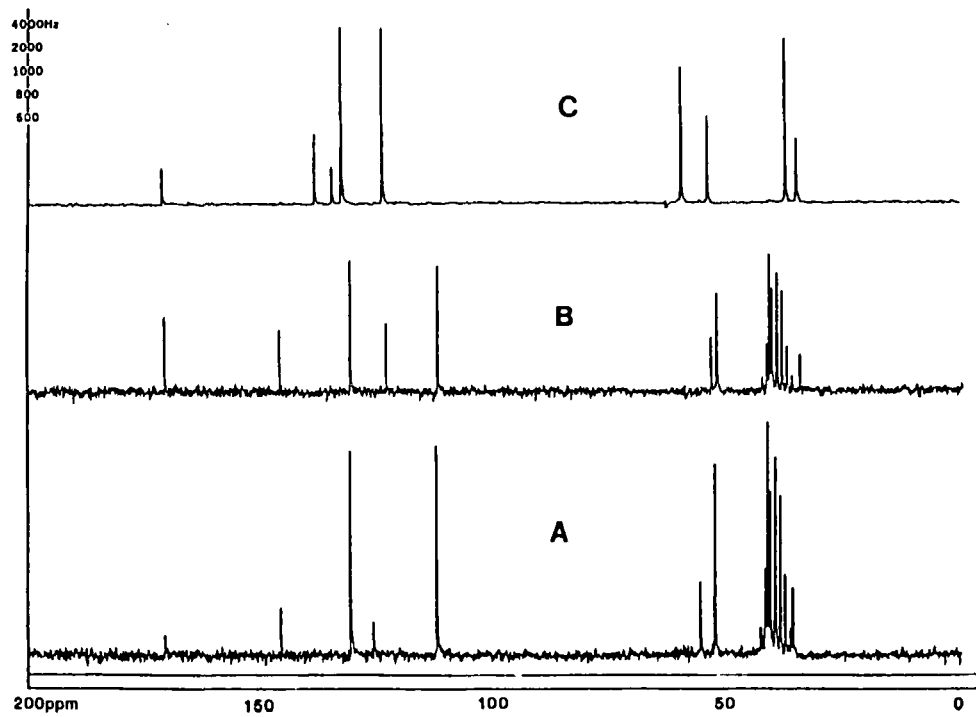
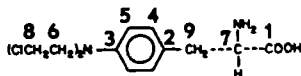


Figure 5. Carbon NMR Spectra of Melphalan.

(A) Melphalan in DMSO-d<sub>6</sub>. (B) Melphalan Hydrochloride in DMSO-d<sub>6</sub>. (C) Melphalan in D<sub>2</sub>O-DCI

Table II

Chemical Shift Assignments in the  $^{13}\text{C}$  NMR Spectra of Melphalan



Carbon atom #	Melphalan <sup>a</sup> D <sub>2</sub> O--DCI	Melphalan Hydrochloride <sup>b</sup> DMSO-d <sub>6</sub>	Melphalan <sup>b</sup> DMSO-d <sub>6</sub>
1	172.5	170.2	170.0
2	139.9 <sup>c</sup>	122.8	125.4
3	136.2 <sup>c</sup>	145.5	145.1
4	134.2	130.5	130.4
5	125.3	112.0	112.1
6	61.4	52.2	52.2
7	55.8	53.4	55.5
8	39.5	41.1	41.2
9	37.1	34.6	35.9

- a) in ppm, downfield from DSS, assignments from [50b]  
 b) in ppm, downfield from TMS  
 c) interchangeable assignment

Using chemical ionization with isobutane and selected ion monitoring of the M + H peak, the detection limit of the methyl ester of N-trifluoroacetyl-melphalan was less than 500 pg [41].

The electron impact ionization spectrum of melphalan is shown in Figure 6. It was obtained by direct-probe introduction of the sample on a Hewlett-Packard 5995A mass spectrometer (70 eV). The relative intensities of the most prominent fragments are given in Table III; the abundance cut-off was 1%.

#### 4.2 Optical Rotation

The following data were reported for the monosolvated crystals obtained by crystallization from methanolic solution [10]:

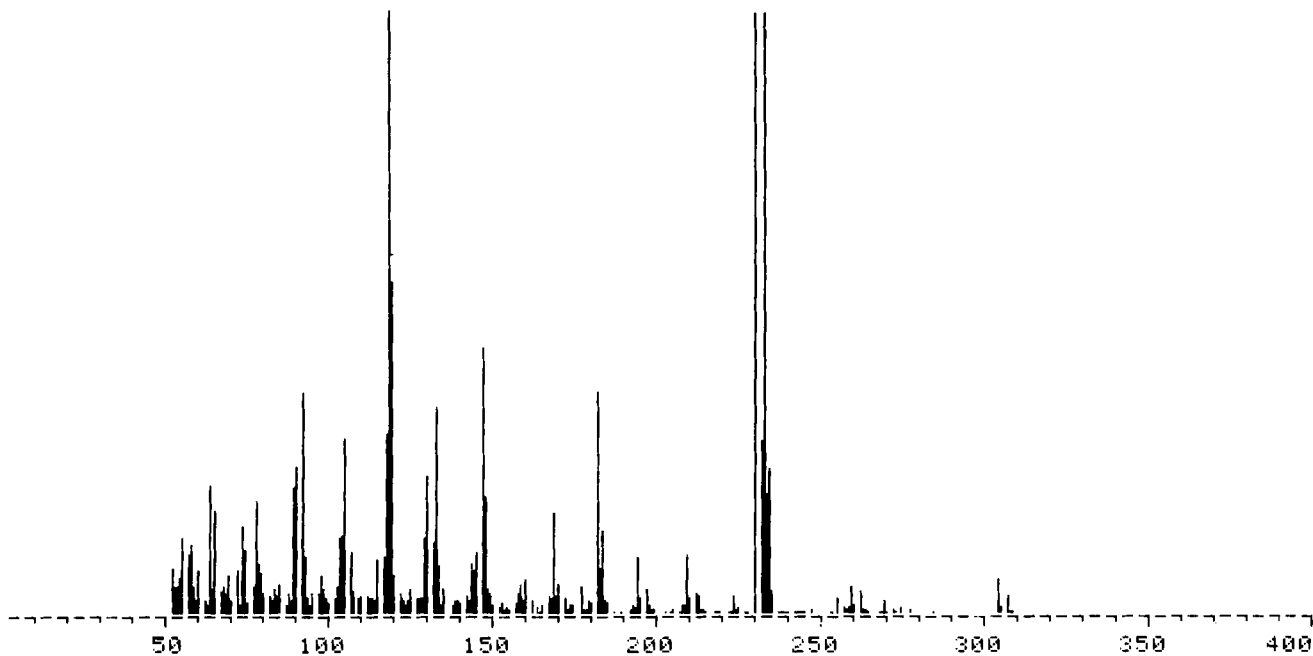


Figure 6. Mass spectrum of Melphalan.

(The peaks at m/e 118,230 and 232 are off-scale.)

Table III

Fragmentation pattern of melphalan			
Ion	Probable Structure	m/e	Relative Abundance, %
M <sup>+</sup>		304	2.6
		306	1.4
I <sub>1</sub> <sup>+</sup>	M <sup>+</sup> -COOH	259	2.3
		261	1.8
I <sub>2</sub> <sup>+</sup>	M <sup>+</sup> -CH <sub>2</sub> Cl	255	1.2
I <sub>3</sub> <sup>+</sup>	M <sup>+</sup> -CH(NH <sub>2</sub> )COOH	230	100
		232	64.8
		234	10.6
I <sub>4</sub> <sup>+</sup>	I <sub>3</sub> <sup>+</sup> -CH <sub>2</sub> Cl	181	15.9
		183	6.1
I <sub>5</sub> <sup>+</sup>	I <sub>3</sub> <sup>+</sup> -CH <sub>2</sub> =CHCl	168	7.4
		170	2.2
I <sub>6</sub> <sup>+</sup>	I <sub>5</sub> <sup>+</sup> -CH <sub>2</sub> Cl	119	23.9
I <sub>7</sub> <sup>+</sup>	I <sub>4</sub> <sup>+</sup> -CH <sub>2</sub> CH <sub>2</sub> Cl	118	70.7

<u>Solvent</u>	<u>Concentration,</u> <u>g/100 ml</u>	$[\alpha]_D^{22}$
1 N HCl	1.33	+7.5° ± 0.5°
Methanol	0.67	-31.5° ± 0.5°

The D-isomer in 6 N HCl had  $[\alpha]_D^{22} - 4.5^\circ \pm 0.25^\circ$ .

The USP XX monograph requires the specific rotation to be between  $-30^\circ$  and  $-36^\circ$ , calculated on the dried basis and determined at 7 mg/ml in methanol solution [25]. In the British Pharmacopeia the levorotatory optical activity is used as an identification test [27].

The following specific rotations of melphalan were determined at  $25^\circ$  in methanolic solution at a concentration of 7 mg/ml [47]:

<u><math>\lambda</math>, nm</u>	<u><math>[\alpha]</math>, (<math>^\circ</math>)</u>
589	-32.1 ± 0.6
578	-33.5 ± 0.8
546	-38.3 ± 0.7
436	-66.7 ± 1.0
365	-102.4 ± 1.6

#### 4.3 Melting range

All the melting ranges indicated below are uncorrected and are reported to occur with decomposition:

Melphalan.MeOH	182--183° [10] (from methanol)
D-Melphalan.MeOH	181.5--182° [10] (from methanol)
DL-Melphalan	180--181° (from methanol) [10,24]; 172--174° (from methanol) [29]; 167--170° (from aqueous alcohol [11])
DL-Melphalan.HCl	182--185° (from 95% ethanol) [11].

The official compendia require melting points of about  $177^\circ$  [27] or  $180^\circ$  [26].



#### 4.4 Solubility, Partition, Dissociation Constants

Approximate solubilities:

(a) Practically insoluble (1 part in more than 10,000 parts of solvent) in water [25,27,55], in chloroform [25,27], and in ether [25,27]; (b) slightly soluble (1 part in 100 to 1000 parts of solvent) in alcohol [25]; soluble in 150 parts of methanol [27]; (c) soluble (1 part in 10 to 30 parts of solvent) in dilute mineral acids [25,27]; and (d) soluble in propylene glycol [24].

The logarithm of the partition coefficient between benzene and pH 7.4 phosphate buffer is -1.70 [56]. A partition coefficient of 0.1 was reported for the heptane-water distribution [57].

The following pKa values were reported: approximately 2.5 in water; 5.9 in acetone--water, 9:1 (v/v) [58].

### 5. Methods of Analysis

#### 5.1 Elemental Analysis

The empirical formula  $C_{13}H_{18}Cl_2N_2O_2$  implies the following percentage compositions: C, 51.16; H, 5.94; N, 9.18; Cl, 23.3; and O, 14.48%.

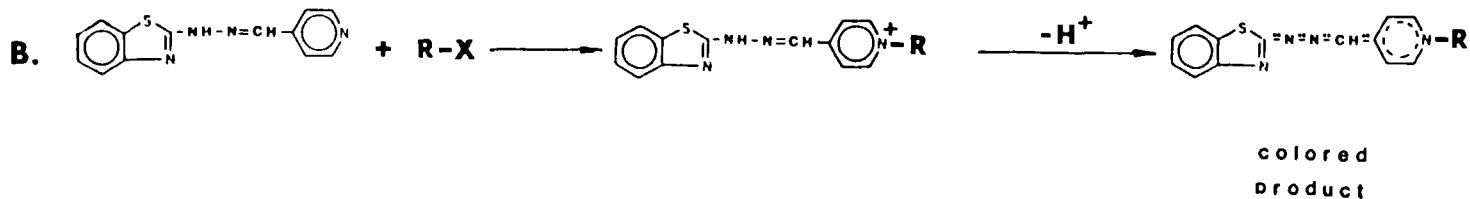
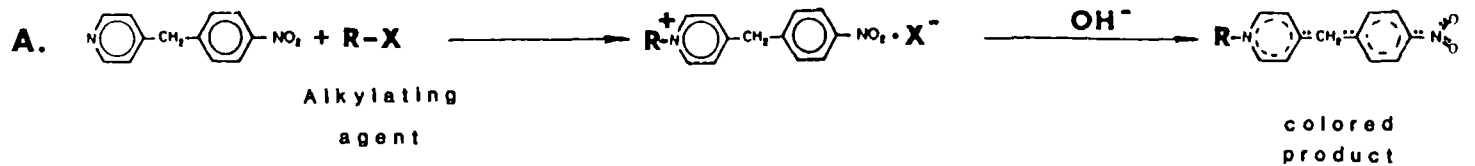
The following percentages were reported for the racemic compound: C, 51.1; H, 6.0; N, 8.9; and Cl, 23.2% [10]. Melphalan has been isolated from methanol as monosolvated crystals exhibiting N (8.3%) and Cl (21.1%) [10];  $C_{13}H_{18}Cl_2N_2O_2 \cdot CH_4O$  implies N (8.3%) and Cl (21.0%).

The USP XX limits for the nitrogen content of melphalan are 8.90--9.45% [25]. Several compendial assays are based on the determination of the chlorine liberated by alkaline hydrolysis (see 5.4).

#### 5.2 Spectrophotometric Methods

##### 5.2.1 Colorimetry

The use of 4-(p-nitrobenzyl)pyridine (NBP) as an analytical reagent for the determination of alkylating agents [59,60] is based on the series of reactions presented in scheme IIA. Melphalan is treated at 80--100° with a large excess of the chromogenic reagent to



Scheme II. Color reactions.

prevent competing reactions from traces of water or other nucleophilic reactants. Addition of base produces a purple color, the absorbance of which is read immediately at 565 nm [60]. The intensity of the color produced on alkalization is affected by the pH and/or the nature of the buffer used in the first step. The improvements of the original procedure [60] were aimed at a better reproducibility and increased sensitivity ( $\mu\text{g}$  level) [51,61]. The procedure has been used to follow the kinetics of hydrolysis [62].

A similar method was described for the quantitative determination of Melphalan on TLC plates [63b]. The plate is sprayed with an acidic solution of 4-pyridinecarboxaldehyde 2-benzothiazolyldiazide and heated over an acetophenone bath at 200°. After cooling, the plate is sprayed with triethylamine, and the red spots are measured at 530 nm with a densitometer. The detection limit is 0.2  $\mu\text{g}$ . The reactions are shown in scheme II B.

Sarcolysin fused with 2-nitroindan-1,3-dione and dissolved in acetic acid or 95% ethanol gives an orange--red color. Sarcolysin heated for 1--2 min with acetic anhydride gives a yellow color [64].

### 5.2.2 Ultraviolet

Melphalan has been determined spectrophotometrically at 262 nm in methanol solution [65]. The assay is linear in the 3 to 15  $\mu\text{g}/\text{ml}$  range, with precision of  $\pm 2\%$ . In contrast with the above mentioned colorimetric methods, the procedure is not stability-indicating; the major contaminant and degradation product, the bis(2-hydroxyethyl)derivative, has a UV spectrum very similar to that of Melphalan [48]. Ultraviolet spectrophotometric determinations in aqueous solution were also reported [66].

### 5.2.3 Fluorescence

In freshly prepared aqueous solutions the fluorescence intensity of Melphalan was found to be proportional to the concentration in the range of 0.05 to 5  $\mu\text{g}/\text{ml}$ , but the method does not discriminate between the parent compound and some of its hydrolytic products [48].

### 5.3 Chromatographic Methods

#### 5.3.1 Paper Chromatography

The solvent systems used for the analysis of Melphalan are listed below. The composition of the mixtures is given in volume to volume ratios. In all cases Whatman paper No. 1 was used.

<u>Developing solvent (v/v)</u>	<u>R<sub>f</sub></u>	<u>Reference</u>
n-Butanol--conc'd HCl--water 2:1:1		67
sec-Butanol--AcOH--water 5:1:4	>0.9	68
Phenol--water 4:1	>0.9	68
n-Butanol--(water-saturated)	0.43	43
n-Butanol--AcOH--water 5:2:3	0.87	43
n-Butanol--AcOH--water 12:3:5	0.80	48
i-Propanol--ammonia--water 20:1:2	0.53	48

Visualization of the spots was done by examination under UV-light or spraying with ninhydrin. The color-forming reaction with nitrobenzylpyridine (NBP) (see 5.2.1) has been used by dipping the chromatograms in solutions of NBP followed by heating at 90° and treating with triethylamine [68].

#### 5.3.2 Thin-layer Chromatography

The TLC systems reported in the literature for the analysis of melphalan are reported in Table IV.

The spots were located by examination under UV light or spraying with one of following solutions (detection limits in µg): fluorecamine (0.5), ninhydrin (1), NBP followed by heating and treatment with a base (5) [51,63a], and 0.5% iodine in chloroform. Visualization using 4-pyridine-carboxaldehyde 2-benzothiazolyl hydrazone has been described under 5.2.1.

Quantitative determinations were reported by densitometry [63,72] or, in the case of <sup>14</sup>C-labelled compounds, by scraping or cutting the chromatograms into strips and measuring the radioactivity in a liquid scintillation spectrometer [44,70].

Table IV

## Thin-layer Chromatography of Melphalan

Plate	Solvent (v/v)	Reference
Silica gel	<u>n</u> -Butanol--acetic acid--water (7:2:1) or (4:1:1) or (13:3:5) or (4:1:5, the upper phase)	47,51,69 70,71
Silica gel	<u>n</u> -Butanol--pyridine--acetic acid-- water (8:1:2:9, the upper phase)	51
Silica gel	<u>sec</u> -Butanol--3% ammonia (100:44)	51
Silica gel	Chloroform--methanol (9:1)	51
Silica gel	<u>n</u> -Butanol--0.1 N ammonium hydroxide (1:1)	47
Silica gel	<u>n</u> -Butanol--Acetic acid (2:1)	47
Silica gel	Methanol	47
Silica gel	Chloroform--methanol--acetic acid (75:20:5)	63a
Silica gel	Ethyl acetate--acetone--tetrahydro- furan--water (4:2:2:2, upper phase)	63a
Silica gel	<u>n</u> -Butanol water saturated	63a
Silica gel	<u>i</u> -Propanol--water (1:1)	63a
Silica gel	Benzene--methanol--acetone--acetic acid (7:2:6:5)	63a
Kieselguhr	<u>n</u> -Butanol--water 86:14	54
HPTLC Silica gel	Chloroform--methanol--acetic acid (35:17:3)	72
Cellulose	<u>n</u> -Butanol--Ethanol--Propionic acid--water (10:5:2:5)	73

### 5.3.3 Gas Chromatography

Melphalan has been converted to its trimethylsilyl derivative with bis(trimethylsilyl)acetamide and has been analyzed by GC on a 1.8 m x 3 mm column packed with 2.5% (w/w) SE-54 on acid-washed, silanized Chromosorb W (80-100 mesh) at 210° (injector temperature, 250°; flame ionization detector temperature, 215°) using nitrogen as the carrier at 30 ml/min. The order of elution from a partly hydrolyzed mixture was: melphalan, mono-hydroxy-derivative VI and di-hydroxy-derivative VII (Scheme III). The same elution order was obtained on a SE-30 column; it was reversed on a more polar liquid phase (OV-17). Identification of the peaks was done by mass spectrometry [52].

In another procedure, melphalan was derivatized by successive acylation with trifluoroacetic acid and eserification with diazomethane. The samples were analyzed on a 3% OV-101 Supelcoport (80--100 mesh) 5 foot x 2 mm glass column with temperature programming from 210° to 280° at 8°/min with a helium flow of 20 ml/min. Analysis of samples extracted from plasma was performed by mass spectrometry using chemical ionization with isobutane gas and melphalan-d<sub>8</sub> as internal standard. Selected-ion monitoring at the M + H peak of derivatized melphalan gave linear calibration curves over a range of 2 to 200 ng of drug per ml of plasma. The detection limit was less than 500 pg [41].

### 5.3.4 High-Pressure Liquid Chromatography

The HPLC procedures reported in the literature (see Table V) were developed mainly for biological and stability studies, and they do separate Melphalan from its hydrolytic degradation products. Detection was performed by UV absorbance measurements at 254--263 nm [40,53,74,75,76] or by fluorescence with excitation at 256 or 260 nm and emission at 350 or 360 nm [77,78]. For the UV measurements a linear standard curve was reported from 10 to 500 ng per injection [75]. With the fluorescence monitoring the detection limit was about 500 pg of drug injected [78].

Table V

HPLC Methods		
Column	Mobile phase (v/v)	Reference
10- $\mu$ m $\mu$ -Bondapak C <sub>18</sub>	2-methoxyethanol--0.1% AcOH gradient from 33.7:66.3 to 51.3:48.7	75
10- $\mu$ m Spherisorb ODS	Methanol--0.01 M NaH <sub>2</sub> PO <sub>4</sub> (pH 3) (1:1)	53
10- $\mu$ m HCODS/SIL X-C <sub>18</sub>	Acetonitrile--0.0175 M AcOH concave gradient from 12:88 to 80:20	76
10- $\mu$ m $\mu$ -Bondapak C <sub>18</sub>	Water--methanol (1:1), containing 1% acetic acid	40,74,79
10- $\mu$ m $\mu$ -Bondapak C <sub>18</sub>	2% acetic acid--acetonitrile (78:22)	54
5- $\mu$ m Spherisorb ODS	Methanol--water (40:60)	78
5- $\mu$ m Spherisorb ODS	Methanol--0.01 M NaH <sub>2</sub> PO <sub>4</sub> (pH 3)(7:3)	77

#### 5.4 Titration

Titration with silver nitrate is used both to confirm the lack of chloride ions in the sample and, after alkaline hydrolysis, as an assay for the drug. The compendial assays are performed potentiometrically using silver and calomel electrodes, the latter modified to contain saturated potassium sulfate solution [25--27]. Visual titration was also reported, the excess of silver being titrated with potassium thiocyanate in the presence of ferric ammonium sulfate indicator [51]. Mercuric nitrate titrations were also reported [62].

Potentiometric and thymol blue indicator titrations of sarcolysin in dimethylformamide solutions were performed using a 0.1 N sodium methoxide solution [80].

#### 5.5 Identification and Purity Tests in Official Compendia

Several of the following tests are included in melphalan monographs: (a) the UV spectrum of a methanolic solution should have maxima at 260 and 301 nm [25,27] or be in agreement with the spectrum of a reference standard [26]; (b) the IR spectrum in KBr dispersion should exhibit maxima only at the same wavelengths as a similar preparation of a

reference standard [26,79]; (c) a positive color reaction with 4-(p-nitrobenzyl)pyridine [25--27,79]; (d) a 0.5% (w/v) solution in methanol should be levorotatory; (e) a melting point of about 177° [27] or about 180° [26] with decomposition; (f) the loss on drying in vacuum at 105° to constant weight should not be more than 7.0%, and the residue on ignition should not be more than 0.3% [25] or 0.1% [26]; (g) a limit test for "ionisable chlorine," performed by direct potentiometric titration of the sample [25,27]; and (h) a positive test for chlorides after alkaline hydrolysis of the sample [25--27,79].

## 6. Stability, Degradation, Mechanism of Action

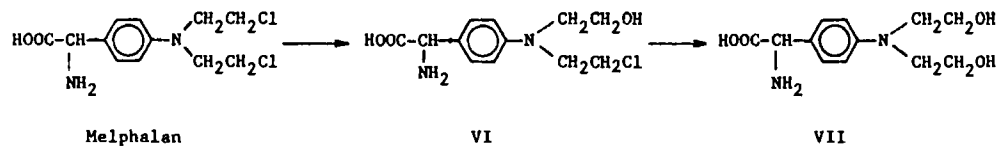
Indicative of the lack of selectivity of nitrogen mustards, their degradation and biological action proceed through a common mechanism, an outline of which is presented in Scheme III.

According to the nature of X, Scheme III illustrates a wide range of reactions, such as the hydrolytic degradation of the compound or biologically significant alkylations of reactive groups in proteins (carboxyl, sulfhydryl) and in nucleic acids (phosphoryl, heteroaromatic amino, heteroaromatic hydroxyl). The overall kinetics of the alkylation depend on the relative rates of the reactions involved. For the aliphatic nitrogen mustards, the fast formation of the cyclic immonium ion VIII and the overall second-order kinetics for the reaction have been supported by several analytical techniques including NMR [81]. There is less agreement on the reaction mechanism for the aromatic nitrogen mustards [4]. The reduced basicity of the aromatic nitrogen mustards retards the ionization step and makes it rate-determining. It was initially postulated that this ionization involves the formation of the carbocation IX [9,82,83], but this view has been challenged by authors who claim that the cyclic immonium ion is common to all nitrogen mustards [40,62,81].

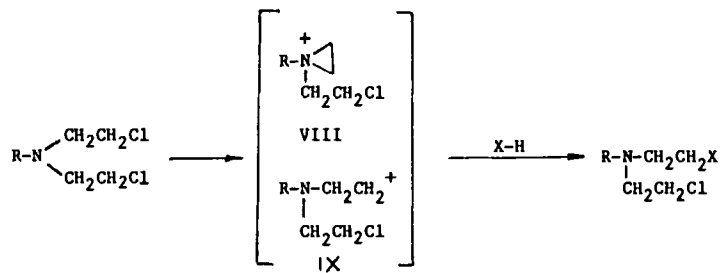
The hydrolysis of melphalan (and of most aromatic nitrogen mustards) represents the most important degradation mechanism and has been extensively studied. The first reports showed a 22% hydrolysis under the standard conditions designed by Ross for the assessment of aromatic nitrogen mustard reactivity (30 min reflux in acetone--water 1:1, v/v) [10,84]. Rate constants for the disappearance of melphalan in various media are listed in Table VI.



Scheme III



A. Hydrolysis of melphalan.



B. Possible mechanism of alkylation.

Table VI

## Rate Constants For the Disappearance of Melphalan in Various Media

Solvent	Temperature, °C	k, h <sup>-1</sup>	Reference
Water	0	0.04	40
Water	23	0.13	40
Water	37	0.83	40,90
0.01 M Citrate buffer (pH 3)	25	0.132	53
0.01 M Phosphate buffer (pH 5)	25	0.140	53
0.01 M Phosphate buffer (pH 7)	25	0.146	53
0.01 M Phosphate buffer (pH 7)	41	0.994	53
0.01 M tris(hydroxymethyl)- aminomethane (pH 9)	25	0.176	53
pH 7 Buffer + 0.019 M NaCl	25	0.103	53
pH 7 Buffer + 0.15 M NaCl	25	0.043	53
0.013 M HCl (pH 2.4)	37	0.44	87
0.013 M H <sub>3</sub> PO <sub>4</sub> (pH 2.6)	37	0.78	87
0.156 M NaCl (pH 5.4)	37	0.28	87
Burroughs-Wellcome injectable kit <sup>a</sup>	25	0.0057	53
Human plasma	37	0.31--0.45	40

<sup>a</sup> Three component kit. Melphalan (100 mg) is dissolved in 1 ml of 92% ethanol containing 2% (w/v) HCl. This solution is diluted with 9 ml of a solution consisting of 1.2% (w/v) K<sub>2</sub>HPO<sub>4</sub> and 60% (w/v) propylene glycol in water. The final solution has a pH of about 7.

The disappearance of melphalan from aqueous solutions has been shown to follow first-order kinetics [62,82,85] and the concentration profile during hydrolysis is consistent with a mechanism consisting of two consecutive pseudo first-order reactions (Scheme III A) [53].

Of the systems listed in Table VI, melphalan is most stable in the Burroughs-Wellcome injectable kit. The instructions recommend the use of the solution within 15--30 minutes. According to the manufacturer, 8.5% hydrolysis takes place in 24 hours after mixing [86]. The stability of melphalan increases in acidic solutions and higher temperatures accelerate the hydrolysis. The type and concentration of anionic species present in the solution alter the hydrolysis rate. The formation of the intermediate ionized species is retarded and/or reversed by the presence of chloride ions [9,82,87], resulting in a significantly increased stability [53,87,88]. The stability of melphalan is increased in the presence of bovine serum albumin, human plasma, bile acids, and bile, probably by hydrophobic interactions that make the chloroethyl moiety less accessible to nucleophilic attack [40,62,74,87,89--91].

Solutions in methanol were found to retain full integrity for more than 12 hours at room temperature and for at least 4 weeks at  $-20^{\circ}$  [78]. Melphalan is stable in solutions of dimethylsulfoxide at  $-20^{\circ}$  for over a month even with frequent thawing [51].

Melphalan is light-sensitive and the packaging and storing instructions call for tight, light-resistant containers.

## 7. Toxicity, Distribution, Metabolism, Excretion

### 7.1 Toxicity

Detailed data on the toxicology and pharmacology of melphalan (and other antitumor agents) were reported in 1965 by Schmidt and collaborators [21].

The  $LD_{10}$  and  $LD_{50}$  values for mice and rats show considerable variations among strains; intravenous and intraperitoneal administrations were twice as toxic as the oral dosing. The  $LD_{10}$  values ranged from 4 to 15 mg/kg for mice and from 1.3 to 3 mg/kg for rats [3,21].  $LD_{50}$  values of 2 to 16 mg/kg were reported for rats [21,77,92]. It was also reported that the  $LD_{50}$  values in mice varied with the time of dosing [93].

The clinical toxicology is mostly hematological [2].

### 7.2 Carcinogenicity

Melphalan induced cancer at the site of injection (peritoneal cavity) in mice and rats given three injections per week at the maximally tolerated dose [94]. It was also reported to increase the risk for development of leukemias [3].

### 7.3 Distribution

Following oral administration to human subjects, peak blood levels for melphalan were seen at 2 hour [44], much slower than in animals [3,91,95]. The drug is well absorbed when given to animals by the oral route [3,91], but considerable variability in absorption by humans was reported [41,96,97].

After intravenous administration, melphalan disappears from the plasma with half-lives of 67 min and 160 hour [44]. In a patient administered a 5-minute intravenous bolus of 140 mg of melphalan per m<sup>2</sup>, the alpha and beta half-lives in plasma were found to be 6.2 and 53.5 minutes, respectively [54]. Binding of the drug to plasma proteins is extensive [44], confirming the in-vitro studies [40,48,74,89,98]; this might have a profound effect on the in-vivo stability of the drug [97]. The dihydroxy derivative VII is detectable in plasma more than three weeks after administration [76]. It was suggested that the prolonged decay phase [54] is due to hydrolysis products and metabolites rather than the parent compound [41].

The absorption from the peritoneal cavity one hour after administration was 25% [57].

The kidney, liver, and spleen had the highest specific activity after administration of labeled melphalan to rats and mice [68,98], while in dogs and monkeys the highest levels of drug were in the bile [3,75,99]. No significant concentrations in the tumor were noted, and the inhibition of the tumor growth could not be attributed to interactions with proteins, DNA, or RNA within the tumor cell [68]. Cellular transport of melphalan by amino acid carrier systems has been reported [100--105].

### 7.4 Metabolism

Only the mono- and dihydroxy-derivatives VI and

VII were identified in plasma and urine [85,99], and it is difficult to distinguish between metabolic changes and chemical degradation. Up to ten  $^{14}\text{C}$ -labelled products of metabolism or hydrolysis of melphalan were detected without identification in the serum, urine, and bile of experimental animals. Metabolic pathways similar to that of phenylalanine were suggested [3].

### 7.5 Excretion

In man, urinary excretion accounts for most drug elimination after intravenous administration (50% within 24 hr, 75% over 7 days), and only small amounts are recoverable from the stool [44,86]. Following oral administration of labelled melphalan, 30% of the label was recovered in urine over 9 days and 20--50% in feces over 6 days [44].

In animals also, urinary excretion appears to be the major means of elimination of the drug from the body [3,68,91,99].

### 8. Determination in Body Fluids and Tissues

The separation and measurement of melphalan in biological samples are hampered by its instability and by the amphoteric nature of the molecule. Some of the earlier publications did not make corrections for reversible protein binding and decomposition during the work-up of the sample. They also lacked sensitivity and specificity.

The following techniques were reported: administration of labelled melphalan, followed by radioactivity measurements [44,91,98]; formation of a colored complex by oxidation with  $\text{FeCl}_3$  and  $\text{H}_2\text{O}_2$  [106]; colorimetric assay with 4-(p-nitrobenzyl)pyridine (detection limit--5  $\mu\text{g}/\text{ml}$  of plasma) [60,61,107,108]; fluorescence measurements (interference by tissue constituents) [48]; thin-layer chromatography and densitometry (detection limit--20  $\text{ng}/\text{ml}$  of plasma) [72]; thin-layer chromatography followed by radioactivity measurements or derivatization and mass spectrometry [44]; high-pressure liquid chromatography (detection limit with fluorescence detection--less than 5  $\text{ng}/\text{ml}$  of plasma) [3,40,54,74,76--78,91,99]; and derivatization--gas chromatography--chemical ionization mass spectrometry (detection limit--less than 2  $\text{ng}/\text{ml}$  of plasma) [41].

## 9. Identification and Determination in Pharmaceutical Preparations

### 9.1 Tablets

#### Identification

The Identification Tests in the official compendia are similar to those used for the drug substance: (a) extraction of the drug with methanol, treatment with 4-(p-nitrobenzyl)pyridine at 80° and alkalization with potassium hydroxide or ammonia after cooling, producing a violet to red--violet color [25,46,79]; (b) the UV spectrum of a methanolic extract exhibits a maximum at 260 nm and a less well defined maximum at 301 nm [25,46]; and (c) the retention time of the major peak in the HPLC chromatogram of the Assay Preparation is the same as that of a Standard Preparation of Melphalan Reference Standard [79].

#### Assay

The following procedures were reported for the assay of Melphalan Tablets: (a) hydrolysis with aqueous potassium hydroxide under reflux and potentiometric titration of the liberated chloride ions with silver nitrate in the presence of nitric acid. (Corrections for "ionisable chlorine" are made by titrating under the same conditions a sample that is not subjected to hydrolysis [25,46].); (b) HPLC Assay of an ethyl alcohol-acetic acid extract against a Reference Standard material (chromatographic conditions: reversed-phase mode; C<sub>18</sub> column; methanol--water--acetic acid, 50:49:1 (v/v), mobile phase; detection at 254 nm [79]); and (c) extraction of tablets with methanol or ethanol and UV measurements at 260 or 262 nm. Linearity in the 3--15 µg/ml and standard errors of less than 2% were reported [65,109,110]. A similar procedure is used for the determination of the Content Uniformity of the tablets [79].

The concentration profile during the dissolution testing of melphalan tablets in water and simulated gastric fluid was determined by HPLC. A method of data analysis was developed to take into account the spontaneous drug degradation [111].

### 9.2 Melphalan Injection [46]

Melphalan Injection is defined as a sterile solution of melphalan hydrochloride. It is available commercially as a kit comprising melphalan in a sealed

container, a dissolving solvent, and a diluting solvent (see footnote in Table VI).

The identification requirements include examination of the UV spectrum of a methanolic solution, positive reaction with 4-(p-nitrobenzyl)pyridine, positive reaction for chlorides after alkaline hydrolysis, levorotatory optical activity for a solution in methanol, and a melting point of about 177° with decomposition.

The assay is performed by potentiometric titration of the chloride ions liberated by alkaline hydrolysis, as described under 9.1 Assay (a).

Other purity requirements include a clarity and acidity of solution test, and limits for ionization chlorine, loss on drying, and sulphated ash.

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# MOXALACTAM DISODIUM

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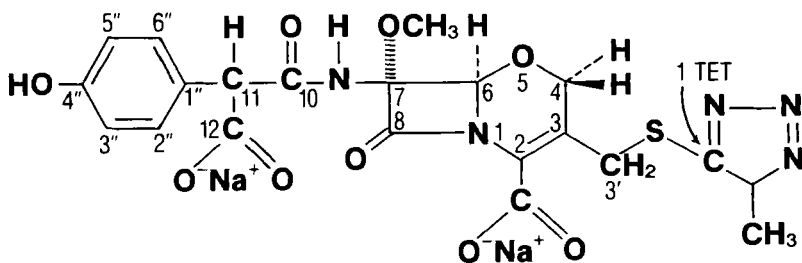


## 1. Description

### 1.1. Name

Moxalactam disodium is a drug entity which was discovered at Shionogi and Company, Limited, Osaka, Japan, and codeveloped with Eli Lilly and Company. The drug is marketed under the trade names of MOXAM™ and LAMOXAM™. The chemical entity is the disodium salt of (6R,7R)-7-[[carboxy(4-hydroxy-phenyl)-acetyl]amino]-7-methoxy-3-[[1-methyl-1H-tetrazole-5-yl]thio]-methyl]-8-oxo-5-oxa-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid.

### 1.2. Structure



As seen in the structure, moxalactam disodium has three asymmetric centers. Two centers in the ring system, C6 and C7, are stereospecifically defined during the biosynthesis of the penicillin used to produce the compound. A third asymmetric center exists adjacent to the amide carbon on the side chain of the antibiotic. The configuration of this center is free to equilibrate and thus a pair of diastereoisomers is possible for moxalactam disodium. The rate of interconversion between the isomers increases with increasing acidity, with the maximum rate occurring at a pH of about 2.5. For solutions with pH lower than 2.5, the rate of interconversion is decreased only slightly from the maximum rate.

### 1.3. Molecular Formula

The molecular formula for moxalactam disodium is  $C_{20}H_{20}N_6O_9SNa_2$ .

### 1.4. Molecular Weight

The molecular weight for moxalactam disodium is 564.44.

### 1.5. Appearance

Moxalactam disodium is a white to slightly cream-colored amorphous powder.

## 2. Physical Properties

### 2.1. Infrared Spectrum

The infrared spectrum of moxalactam disodium in a potassium bromide pellet is presented in Figure 1. The assignments for the spectral peaks are given in Table 1.

Table 1

IR Absorption Band ( $\text{cm}^{-1}$ )	Assignment
3600-2500 (broad multiple bands)	$\text{O}-\text{H}$ , $\text{H}_2\text{O}$ , $\text{OH}$ stretching, strong hydrogen bonding
1770	$\beta$ -lactam, $\text{C}=\text{O}$ stretching
1680	Amide I, $\text{C}=\text{O}$ stretching
1610	$\text{CO}_2^-$ , asymmetrical stretching
1515	Amide II
1410	$\text{CO}_2^-$ , symmetrical stretching
1380, 1350	Tetrazole, $\text{N}-\text{CH}_3$ , $\text{CH}_2$ ( $\text{C}-\text{H}$ bending)
1250 (broad)	$\text{O}-\text{H}$ $\text{C}-\text{O}$ stretching
820	$p$ -disubstituted phenyl ( $\text{C}-\text{H}$ bending)

### 2.2. Nuclear Magnetic Resonance Spectrum

Figure 2 shows the proton magnetic resonance spectrum for moxalactam disodium. The spectrum was recorded on a 60 MHz instrument. An interpretation of the spectrum is presented in Table 2. The spectrum is complex, for the configuration at site  $\alpha$  undergoes rapid equilibrium and the proton at  $\alpha$  is exchanged with deuterium. At high resolution such as at 360 MHz every resonance is split except for that of  $\text{H}_0$ .

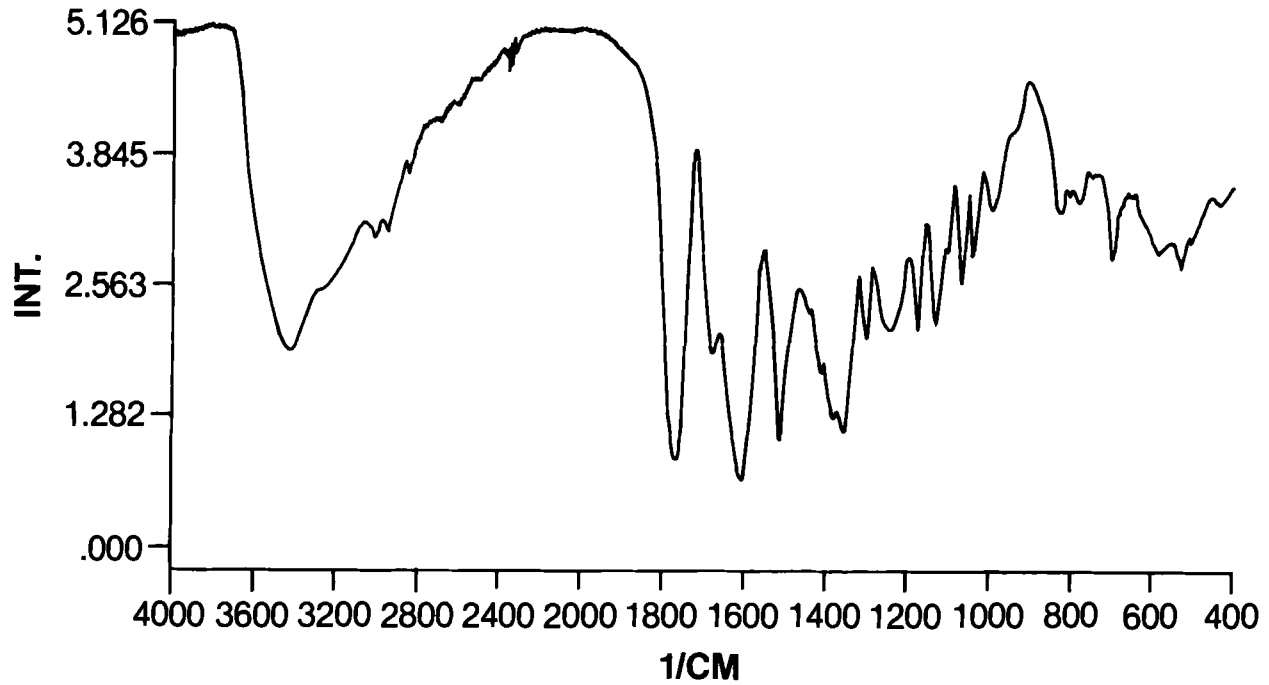


Figure 1. The Infrared Spectrum of Moxlactam Disodium in a KBr Pellet

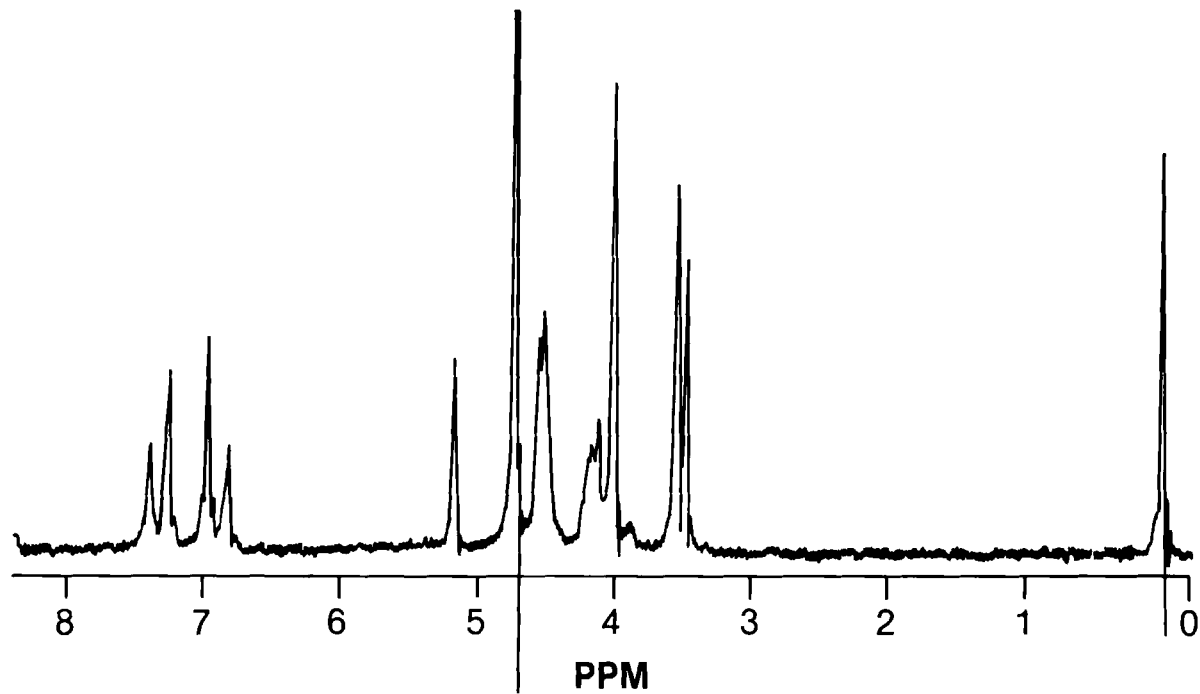
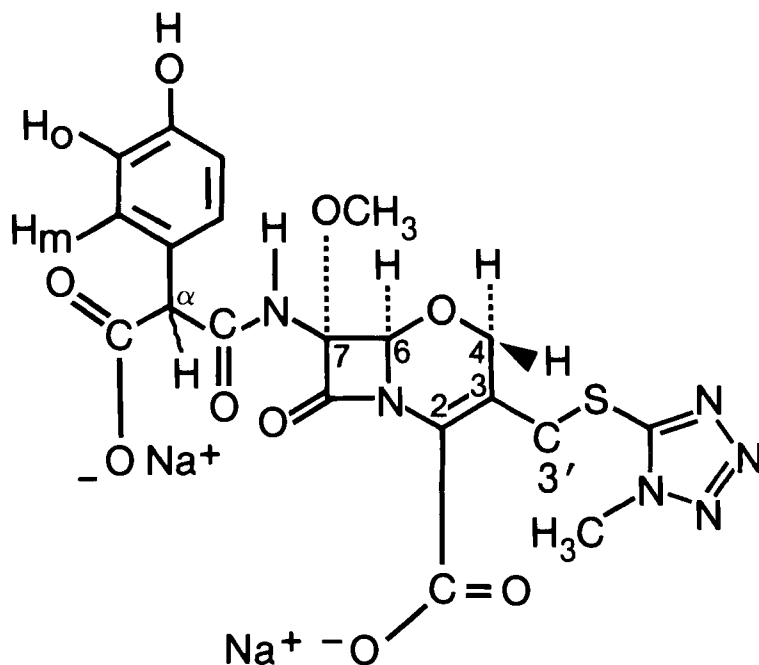


Figure 2. The Proton NMR Spectrum of Moxalactam Disodium in  $D_2O$

Table 2  
Proton Magnetic Resonance Spectrum



<u>ppm</u>	<u>Multiplicity</u>	<u>Assignment</u>
3.5	two singlets	OCH <sub>3</sub>
4.0	two singlets	NCH <sub>3</sub>
4.1	complex AB patterns	CH <sub>2</sub> (3')
4.5	complex AB patterns	CH <sub>2</sub> (4)
5.1	two singlets	Ho
6.83	doublet j-8Hz	Hm
	not visible	H <sub>α</sub>
	fully deuterium exchanged	

Figure 3 shows the carbon-13 nuclear magnetic resonance spectrum obtained for moxalactam disodium. The spectrum was run in deuterium oxide with dioxane present as a spectral marker. Moxalactam again has a complex spectrum, for the configuration at the C(11) carbon undergoes inversion. This tends to cause doubling of peaks; therefore, assignments for the aromatic resonances are difficult. Table 3 provides the assignments for the carbon-13 spectrum.

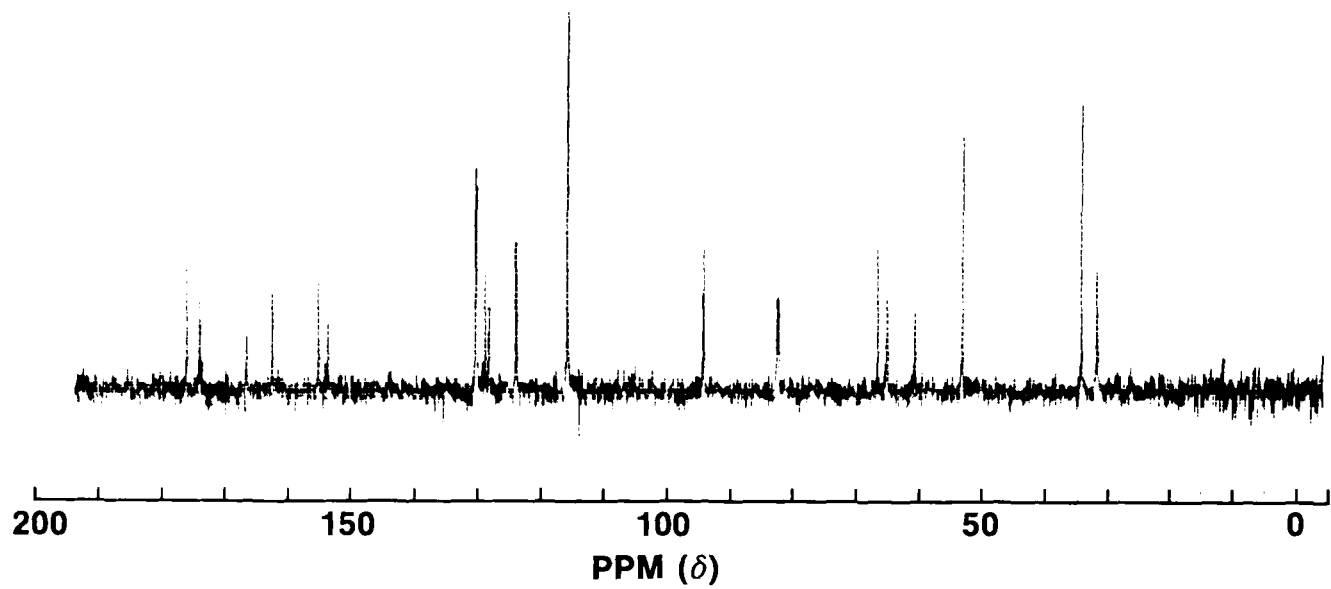


Figure 3. The Carbon-13 Nuclear Magnetic Resonance Spectrum for Moxalactam Disodium in D<sub>2</sub>O Plus Dioxane

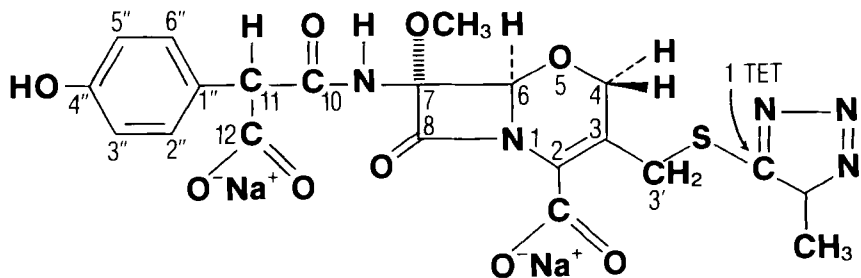


Table 3

ppm	Assignment
32.4	N-CH <sub>3</sub>
34.8	C(3')
53.8	OCH <sub>3</sub>
61.5	C(11)
65.9	C(4)
67.5	Dioxane marker
83.5	C(6)
94.9	C(7)
116.3	aromatic carbons C(2''), C(3''), C(5''), C(6''), C(2), and C(3)
124.4	
124.6	
129.5	
130.8	
130.9	
154.8	C(1-TET)
155.8	C(4'')
163.2	C(8)
167.2	2CO <sub>2</sub> <sup>-</sup>
174.6	C(12)
176.7	C(10)

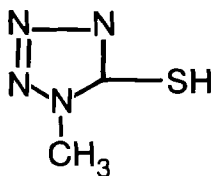
### 2.3. Mass Spectrum

Moxalactam disodium can yield a mass spectrum when run in the field desorption mode. The major ions for moxalactam disodium are at  $m/z$  of 116 and 130. These are attributed to



derived from the tetrazine moiety arising from pyrolytic processes.

The field desorption mass spectrum of moxalactam diacid gives ions at  $m/z$  of 543, 521, 477, and 361. The major ion is at  $m/z$  of 521 which is the quasi-molecular ion  $[M+H]^+$ . A small ion is observed at  $m/z$  of 543 which is the cationized molecular ion of moxalactam  $[M+Na]^+$ . The ion at  $m/z$  of 477 is the result of the decarboxylation of the parent compound. The ion observed at  $m/z$  of 361 is likely caused by the elimination of  $CO_2$  and



from the parent compound.

#### 2.4. Ultraviolet Spectrum

Figure 4 shows the ultraviolet spectrum for moxalactam disodium. The major chromophore contributing to the ultraviolet absorbance spectrum is the 3-oxacephem nucleus. This entity leads to peaks at 271 nm ( $\epsilon=12,100$ ) which is assigned to a  $\pi \rightarrow \pi^*$  transition and at 229 nm ( $\epsilon=18,000$ ) which is a  $n \rightarrow \pi^*$  transition.



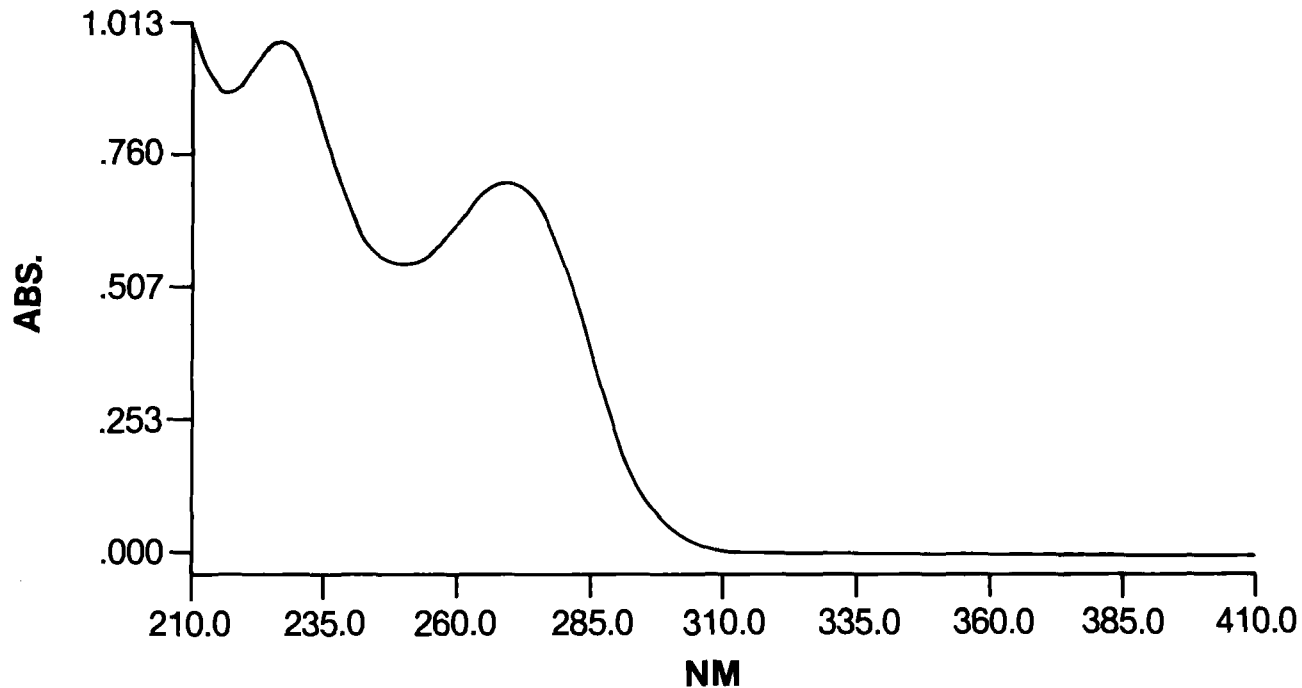


Figure 4. The Ultraviolet Spectrum of Moxalactam Disodium in Water

## 2.5. Optical Rotation

In section 1.2. moxalactam disodium is described as consisting of a pair of diastereoisomers which can interconvert in solution. The observed value for optical rotation is therefore a function of the isomer distribution in solution at the time of measurement. No experimental data are available for the rotation of the pure isomers.

## 2.6. Differential Thermal Analysis

The thermogram for moxalactam disodium shows an exotherm at about 170°C. This exotherm indicates the decomposition of the substance.

## 2.7. Thermogravimetric Analysis

The thermogram for moxalactam disodium shows a weight loss throughout the curve beginning at about 30°C. A major loss in mass occurs near 170°C which corresponds to the exotherm in the DTA curve for moxalactam disodium. Moxalactam disodium is thermally labile and undergoes increased decomposition with increasing temperature.

## 2.8. Dissociation Constants

The following dissociation constants have been determined for moxalactam disodium:

<u>Solvent</u>	<u>pKa</u>		<u>Phenol</u>
	<u>Carboxyl 1</u>	<u>Carboxyl 2</u>	
H <sub>2</sub> O	2.4	3.5	9.95
66% DMF	4.9	6.1	12.9

## 2.9. Solubility Properties

The solubility properties of moxalactam disodium are described in Table 4.

Table 4

Solvent	Solubility (mg/ml)
Water	$\geq 100.$
pH 1.2 (USP XIX)	$\geq 100.$
pH 4.5 (USP XIX)	$\geq 100.$
pH 7.0 (USP XIX)	$\geq 100.$
Methanol	$\geq 50.$ but $< 100.$
Octanol	$< 5.$
Isopropanol	$< 5.$
Diethyl ether	$< 0.5$
Ethyl acetate	$< 0.5$
Chloroform	$< 0.5$
Benzene	$< 0.5$
Cyclohexane	$< 0.5$

### 2.10. Crystal Properties

Salts of moxalactam have been prepared with cations such as sodium, potassium, and ammonium. These salts tend to crystallize, with the preferred form of moxalactam having the R configuration.

### 3. Chemical Synthesis

Otsuka *et al.* (1) have provided several synthetic routes to moxalactam and other similar types of compounds. A flow sheet indicating a synthetic route to moxalactam is shown in Figure 5.

6-Aminopenicillanic acid (I) is acylated, oxidized and esterified to give the sulfoxide ester (II). Sulfoxide ester (II) is converted to its C-6 epimer (III) by treatment with trimethylchlorosilane and triethylamine. Rearrangement of III using triphenyl phosphine yields the epioxazoline (IV). The epioxazoline is successively treated with chlorine, sodium iodide, cuprous oxide and boron trifluoride to produce the exomethylene compound (V). Methoxylation using chlorine with a methoxide followed by reaction with 1-methyl-1H-tetrazole-5-thiol and partial de-blocking using phosphorous pentachloride converts exomethylene (V) into the nucleus (VI). Acylation of the nucleus with an appropriately blocked side-chain (VII) followed by final de-blocking using aluminum chloride gives moxalactam acid (VIII).

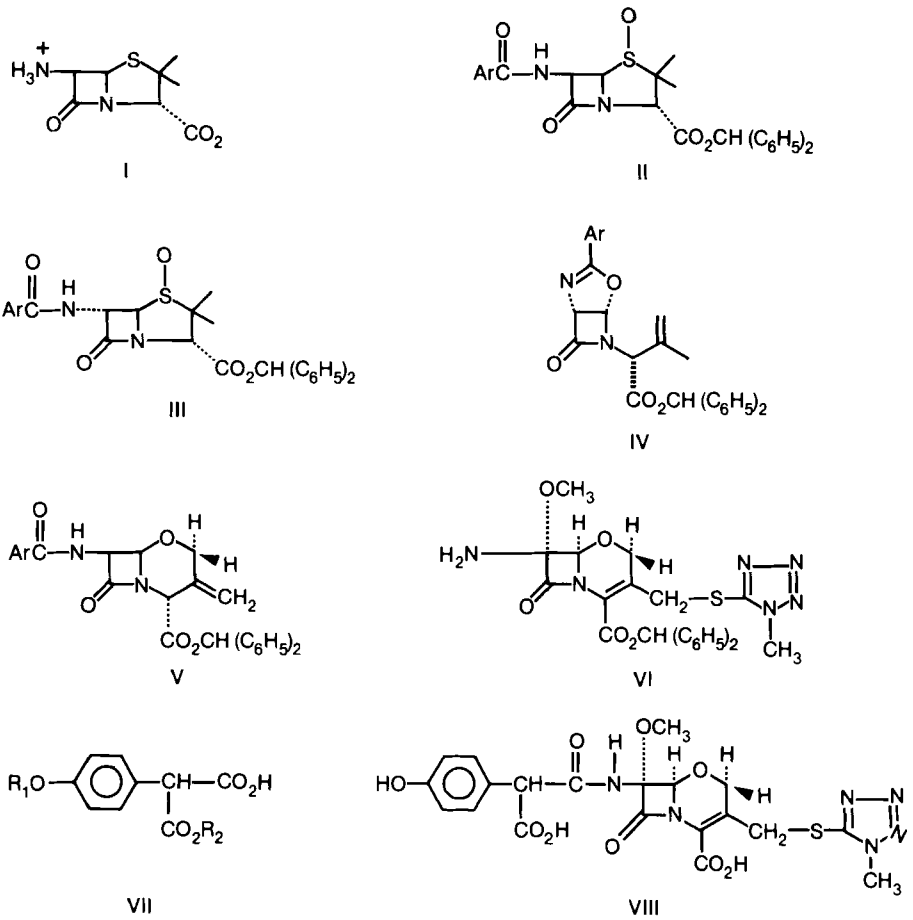
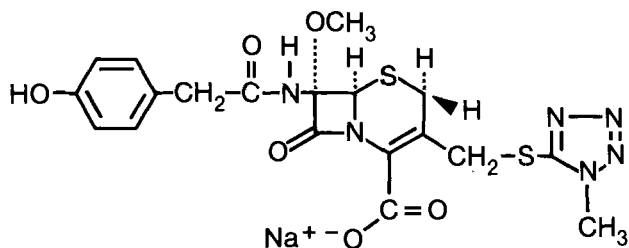


Figure 5. The Chemical Synthesis of Moxalactam Disodium

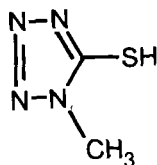
## 4. Stability

### 4.1. Bulk Stability

When moxalactam undergoes degradation, two major products are observed. These substances are the decarboxylated product of moxalactam and the thiotetrazole; the structures are given below.



**Decarboxylated Moxalactam**



**Thiotetrazole**

Decarboxylation of moxalactam is the dominant degradation pathway in the dry state. Elimination of the thiotetrazole sidechain is the dominant degradation pathway in solution.

### 4.2. Solution Stability

Moxalactam formulations in sterile vials remain stable for up to 24 months when stored at 25°C or below (38). MOXAM™ reconstituted in various commonly used intramuscular and intravenous diluents is stable for 96 hours at room temperature (2).

## 5. Bacteriology and Pharmacokinetics

The structural design of moxalactam includes four elements which contribute to its unique biological properties.

The oxygen atom, at a key position in the nucleus, gives moxalactam greater antibacterial activity than its cephalosporin analogue. The methyltetrazolethio moiety tends to maximize activity of the drug. The 7( $\alpha$ )-methoxyl substituent confers  $\beta$ -lactamase stability to the drug. The p-hydroxy-phenylmalonyl group positively influences the  $\beta$ -lactamase stability and antibacterial spectrum of the drug as well as influencing the pharmacokinetic parameters leading to a half-life without high serum binding (3,14,38).

## 5.1. Bacteriology

The bactericidal activity of moxalactam results from inhibition of cell wall synthesis.

### 5.1.1. Susceptibility Tests

Susceptibility testing for moxalactam disodium can be accomplished through the use of diffusion discs and solution MIC determinations which may include the use of systems such as the Autobac<sup>®</sup>. For moxalactam, susceptibility is defined as a minimum inhibitory concentration (MIC) of 16 mcg or less per mL; resistance is defined as an MIC value of 64 mcg or more per mL (55). Disc diffusion methods that require measurement of zone diameters give the most precise estimate of antibiotic susceptibility. A procedure has been recommended for use with discs to test the susceptibility of various gram-positive and gram-negative organisms to moxalactam (53). Highly susceptible organisms produce zones of 20 mm or greater while zones of 15-19 mm indicate organisms susceptible to higher dosages of moxalactam. Resistant organisms produce zones of 14 mm or less. Organisms should be tested against moxalactam discs since moxalactam is active against several strains found resistant to other  $\beta$ -lactam antibiotic discs in *in vitro* testing. The agar-dilution method of Sutter *et al.* (54) is recommended for susceptibility testing of anaerobic organisms.

### 5.1.2. In Vitro Susceptibility

Moxalactam is active against most commonly occurring gram-negative bacteria (4-34). Studies showed, for example, that 90-100% of Enterobacteriaceae were susceptible at 4 mcg or less per mL, including many strains resistant to the cephalosporins, cephamycins, semi-synthetic penicillins, or aminoglycosides (9,10,13,16,18,22,24,26). Moxalactam inhibited most strains of gram-positive aerobic organisms at concentrations of 8 mcg/mL or less (6,10,14,22,24-30).

Moxalactam is active against most commonly encountered anaerobic bacteria (6,10,14,23-25,29,30,35-37) and is more active against certain strains than cefotaxime, cefoperazone, and cefoxitin. For many isolates, the concentration of moxalactam required for bactericidal activity is the same as or two-fold greater than the MIC (23,27,28). For most bacterial strains, increasing the inoculum size has little or no effect on the MIC of moxalactam (6,9,10,13,16,21).

#### 5.1.3. Resistance to $\beta$ -lactamase

Because of its unique chemical structure, moxalactam is not only resistant to hydrolysis by  $\beta$ -lactamase, but acts as a potent inhibitor of several of these enzymes (29,39,40).

#### 5.1.4. Protein Binding

By the dialysis method, the protein binding of moxalactam was 43 percent and by the agar-diffusion method, 40 percent (6). By an ultrafiltration method, the binding of moxalactam in pooled fresh human plasma was 50.7 percent at physiologic temperature and pH (38).

#### 5.1.5. Synergism

Moxalactam in combination with several aminoglycosides, e.g. gentamycin or tobramycin, has been shown to be synergistic in vitro when tested against certain bacterial strains (16,19,21,24,26,38,41,42).

### 5.2. Pharmacokinetics

High concentrations are achieved in the serum and urine after intravenous and intramuscular administration of moxalactam. Significant concentrations also are obtained in certain body tissues and fluids, including the cerebral spinal fluid.

#### 5.2.1. Serum Concentrations - Intravenous and Intramuscular

After rapid intravenous infusion (2 minutes) of 500 mg and 1 g doses of moxalactam in normal adults, mean peak serum levels of 57 mcg/mL and 95 mcg/mL, respectively, were achieved (38). Peak serum concentrations at the end of single 20-minute infusions were: 24.1 mcg/mL after 250 mg; 47.8 mcg/mL after 500 mg; 101.2 mcg/mL after 1 g; and 204.3 mcg/mL after 2 g (38). After doses of 1 g or more, concentrations of moxalactam persist in the serum for 12 hours (38), but

intravenous administration of 4 g doses, every 8 hours for 7 doses, produced no evidence of accumulation. Following intramuscular administration of 250 mg, 500 mg, and 1 g doses of moxalactam to normal adults, the mean peak serum concentrations were 10, 16, and 27 mcg/mL, respectively, occurring at 60-120 minutes (38).

#### 5.2.2. Serum Half-life

The half-life after an intravenous dose is approximately 1.9 hours (114 minutes); after intramuscular injection, the half-life is about 2.1 hours (126 minutes) (38,43-45). In patients with reduced renal function, the serum half-life of moxalactam is significantly prolonged.

#### 5.2.3. Urine Concentrations, Excretions and Renal Clearance

Moxalactam is excreted primarily by the kidneys; small amounts are excreted in the stool via the biliary tract, but non-renal clearance appears to be low (46). Urinary concentrations of moxalactam were highest during the first 2-4 hours after intravenous administration: 170 mcg/mL for 250 mg; 446 mcg/mL for 500 mg; 1820 mcg/mL for 1 g; and 4220 mcg/mL for 2 g doses. The same dosages produced 24 hour recoveries ranging from 60-90 percent (38). Intramuscular administration also produced highest urinary concentrations during the first 2-4 hours with mean recoveries of 349 mcg/mL for 250 mg, 469 mcg/mL for 500 mg, and 586 mcg/mL for 1 g doses. From 55-65 percent of the drug was excreted in the first 24 hours after intramuscular injection (38). No detectable breakdown products or metabolites have been detected in the urine (46,48). The mean renal clearance of moxalactam was approximately 78 mL/minute, and it was found that probenecid did not significantly affect the elimination (38). In patients with renal impairment, drug clearance was significantly reduced (48).

#### 5.2.4. Body Fluid and Tissue Concentrations

Moxalactam readily diffuses into the cerebral spinal fluid (CSF) of patients with and without meningitis (49-52). Distribution of the drug following therapeutic dosages has been determined in CSF (49-52), bile (38,54,55), aqueous humor (53,54), peritoneal fluid, pleural fluid, prostatic fluid, sputum, and several other tissues and fluids. Body fluid and tissue analyses give primarily qualitative data as to the presence or absence of the drug at a particular site, and therapeutic efficacy cannot be predicted from these data.



### 5.2.5. Pediatric Pharmacology

Serum concentrations of moxalactam were determined in newborns, including prematures, and infants being treated for bacterial infections (49). After a 10 minute intravenous administration, individual peak serum concentration ranges were 28.6 mcg/mL (the lowest following the 25 mg/kg dose) to 260 mcg/mL (the highest following the 100 mg/kg dose). The mean half-life was 5.4 to 7.6 hours in neonates less than one week of age, 4.4 hours in those one to four weeks old, and 1.6 hours in infants over one month of age. The calculated plasma clearance rates, expressed in relation to body surface area were: from 16 to 31 mL/min/1.73m<sup>3</sup> in neonates and 137 mL/min/1.73m<sup>3</sup> in infants over one month of age. Serum values for moxalactam given intravenously and intramuscularly were similar for infants less than 7 days old. Intravenous infusion of moxalactam for 30 minutes in doses of 50 mg/kg or less to children and infants with bacterial meningitis produced mean serum concentrations and half-life values similar to those in adults following 1 g doses (50).

## 6. Methods of Analysis

### 6.1. Identification Tests

#### 6.1.1. Infrared

The infrared spectrum of a sample in a potassium bromide pellet may be used as an identity test. In such cases, the infrared spectrum of the sample should compare favorably with the moxalactam disodium reference spectrum over the range of 2.5 to 16 microns.

#### 6.1.2. Nuclear Magnetic Resonance

The NMR spectrum may be used as a means of identification for moxalactam disodium. In such cases, the NMR spectrum of the moxalactam sample dissolved in deuterium oxide should have chemical shifts and integrations over the range of 0 to 10 ppm which compare favorably to those of the moxalactam disodium reference spectrum.

#### 6.1.3. High Performance Liquid Chromatography

HPLC is a means for the identification of moxalactam materials. In such cases, the retention characteristics of both the R and S isomers of moxalactam must compare favorably to the elution of the two isomers present in a reference

sample of moxalactam.

#### 6.1.4. Thin Layer Chromatography

Thin layer chromatography is yet another means for determining identity of moxalactam materials. The mobility of the moxalactam sample must be identical to the mobility of the moxalactam reference standard which is run on the same TLC plate. The developing solvent for TLC consists of 42 parts ethyl acetate: 14 parts glacial acetic acid: 14 parts acetonitrile: and 18 parts water. A silica gel type F plate is used and is viewed under short wavelength UV light to detect the position of the components on the developed plate.

#### 6.2. Quantitative Tests

Amorphous disodium moxalactam and crystalline diammonium moxalactam salts have been used as standard materials in the control of moxalactam products. Special handling is required for all moxalactam standards used in quantitative testing of moxalactam. Amorphous moxalactam disodium is somewhat unstable and must be stored in a dry state. The dry material is hygroscopic and therefore difficult to weigh. The diammonium salt of moxalactam is a stable crystalline substance; however, it exists almost completely in the R configuration. For most assay procedures the required R to S ratio is about one; therefore, the material must be treated to equilibrate the ratio of the isomers.

Moxalactam disodium reference standards are equilibrated under controlled humidity conditions to raise the moisture content to a level where the material can be handled by ordinary means. This is accomplished by spreading a thin layer of material in a wide mouth weighing bottle which is placed in a 42% relative humidity chamber for about 16 hours before use. The 42% relative humidity chamber is prepared by storing a saturated aqueous solution of potassium carbonate in a desiccator or closed chamber held at room temperature. After a 16 hour equilibration period, the water content of the standard is determined by Karl Fischer titration. The activity of the standard is then corrected for the water content of the equilibrated reference material. Experience indicates that the water content of a properly equilibrated reference material should be in the range of 10 to 12%.

When the crystalline diammonium moxalactam is used as a standard, the R form of the substance must be equilibrated to yield a product which has nearly equal concentrations of both

the R and S isomers. This is achieved by equilibrating a weighed sample of moxalactam diammonium salt in a 0.04M solution of hydrochloric acid at room temperature for 90 to 120 minutes. The solution is then buffered to a pH of 6 with a potassium phosphate buffer.

#### 6.2.1. High Performance Liquid Chromatography

HPLC is the technique of choice for determining the purity of moxalactam disodium in raw materials, formulated products, and in body fluids. Moxalactam is determined in a system containing 0.05 to 0.1 M ammonium acetate with about 6 percent methanol present. An ES Industries Chromegabond™ C18 column or other alternative column with similar retention characteristics is used to determine the purity of the moxalactam sample. The substance may be monitored at 254 nm or, when available, a variable wavelength detector can be operated at 271 nm for assay. The sample is dissolved in water or in 0.1 M ammonium acetate solution. Under conditions of this method, the assay should be completed within 4 hours of sample dissolution.

The R isomer of moxalactam will be the first to elute with a  $k'$  of about 4.5. The S isomer of moxalactam will elute at a  $k'$  of about 7.0. For quantitative purposes, the responses of both peaks must be combined to provide a valid potency for the moxalactam standard or sample.

#### 6.2.2. Iodometric Assay

The iodometric assay for moxalactam involves the hydrolysis of moxalactam in aqueous base which results in the cleavage of the  $\beta$ -lactam ring. This is followed by oxidation of the hydrolysis products with iodine, and the titrimetric determination of the iodine consumed. Since the unhydrolyzed drug does not react with iodine, an unreacted sample is used as a blank to compensate for iodine consuming impurities. This assay is specific for an intact  $\beta$ -lactam ring and will give a result for any entity having such a moiety present. This procedure therefore may not be a stability indicating assay for the drug. This is a very real problem for moxalactam for one of the major degradation products is the decarboxylated product of moxalactam which retains the  $\beta$ -lactam moiety and is itself a compound with considerable antibiotic activity (61-63).

### 6.2.3. Hydroxylamine Assay

Moxalactam is also amenable to the popular hydroxylamine assay for  $\beta$ -lactam antibiotics. In this procedure the  $\beta$ -lactam is reacted with hydroxylamine to cleave the  $\beta$ -lactam moiety and form a hydroxamic acid. The hydroxamic acid will in turn react with acidified ferric ion to form a colored complex which is measured at 480 nm. A blank correction for non- $\beta$ -lactam impurities which react with hydroxylamine is incorporated by adding hydroxylamine to an acidified sample where the acid is used to destroy all  $\beta$ -lactam species. Again, this test is not specific for moxalactam and will react with all  $\beta$ -lactam moieties. This test would therefore include the decarboxylated product of moxalactam which may be present in the sample, and is not a good stability indicating assay for moxalactam (59,60).

### 6.2.4. Microbiological Assay

The microbiological assays recommended for moxalactam are performed with gram-negative organisms. Specifically, Escherichia coli is used for potency determinations with the bulk material and the final dosage forms, Providencia stuartii with biological fluids and tissues, and Pseudomonas aeruginosa for the assay of moxalactam in susceptibility discs.

#### 6.2.4.1. Turbidimetric Assay

A turbidimetric assay with E. coli ATCC 10536 may be used for the bulk drug substance and the dosage forms. On the day prior to assay, antibiotic medium 3 (64) is inoculated with the organism and the broth culture is incubated for approximately 16 hours on a rotary shaker at room temperature. Immediately prior to assay, a sufficient volume of medium 3 is inoculated with 0.05 mL of the broth culture for each 100 mL of medium. Samples are diluted with 0.1M, pH 6 potassium phosphate buffer and 0.1 mL of each sample is added to assay tubes containing 10 mL of inoculated medium. Dose response concentrations are in the range of 0.02 to 0.04 mcg/mL of assay medium. The test is incubated in a 37°C water bath until tubes containing no antibiotic have a light transmittance of 20% at 530 nm (usually 3 to 4 hours). The test is terminated by heating for 2 to 3 minutes in an 80°C water bath, and the percent light transmittance (530 nm) is determined for each tube after they are cooled and shaken.

#### 6.2.4.2. Agar Diffusion Method

For agar diffusion assays of bulk materials or final dosage forms, *E. coli* ATCC 10536 is used. With the agar plate system consisting of 10 mL of agar medium 2 (65) as base layer and 5 mL of medium 1 (66) as seed layer, dose response concentrations within the range of 1.0 to 20.0 mcg/mL are appropriate. The agar for the seed layer is inoculated with 0.5 mL of an overnight culture of *E. coli* per each 100 mL of medium. Tests are incubated at 30°C for 16 to 18 hours.

### 6.3. Related Substance Assays

#### 6.3.1. Decarboxylated Moxalactam

The decarboxylated product of moxalactam (see Section 4) is determined by an HPLC technique. A reverse phase HPLC system consisting of 80 parts of 0.1M ammonium acetate and 20 parts of methanol is used with a Dupont Zorbax™ C8 or other suitably similar column to determine the decarboxylated product. In this system, the decarboxylated moxalactam should elute with a  $k'$  of about 6.5. The decarboxylated moxalactam is quantitatively determined by comparing the peak response for the sample with a peak response calibration curve of the authentic decarboxylated moxalactam reference standard material.

#### 6.3.2. 1-Methyl-5-mercapto-1,2,3,4-tetrazole

1-Methyl-5-mercapto-1,2,3,4-tetrazole (see Section 4.1) is a possible impurity and degradation product of moxalactam which can be determined by polarographic techniques (67). It is oxidized at a dropping mercury electrode in a pH 5.8 buffer. The concentration of the impurity tetrazole is proportional to the sum of the currents for the adsorption prewave and the main wave (half-wave potentials of about -0.20 V and -0.05 V vs SCE, respectively).

#### 6.3.3. Analysis of Other Related Materials

Gradient HPLC procedures can be used for the determination of other impurities in moxalactam. This determination is usually run on a Spherisorb™ ODS column or other suitable similar column. The solvent system consists of 0.05M ammonium acetate in water for the weak solvent and 0.05M ammonium acetate in 10 parts water and 90 parts methanol for the strong solvent. A gradient is run from the weak solvent to the

strong solvent in two varying linear ramps. Most of the materials of interest elute in the weak end of the solvent gradient so a linear gradient is run with a change of 2% per minute for 25 minutes. To remove any strongly retained components, the second portion of the gradient curve is run from 50% to 100% of the strong solvent at a rate of change of 5% per minute.

Figure 6 shows a typical chromatogram obtained from such a procedure. In this chromatogram, the elution of the thio-tetrazole, the R and S isomers of moxalactam, and the moxalactam decarboxylated product are shown. In this test for other related substances, only those substances which are not dealt with by specific tests are considered. Quantitative information is obtained by summing the response for all the extraneous peaks and comparing them to the sum of the response for the R and S isomers of a diluted moxalactam reference standard. The assumption is made that all of these materials have similar spectral response characteristics at 254 nm.

## 7. Analysis of Biological Samples

### 7.1. Microbiological Assay

The microbiological assay for moxalactam in serum, urine, and tissues is with Providencia stuartii ATCC 33700 as the assay organism. The agar plate system is a 10 mL single layer plate consisting of trypticase soy agar which has been modified by the addition of 1 g of dextrose and 10 g of sodium acetate per liter of medium. The agar medium is inoculated at 0.1% (v/v) with a broth culture which has been adjusted to 25% light transmittance (530 nm). Standard curve concentrations are prepared in the same diluent as is used for samples and are within the range of 0.1 to 1.0 mcg/mL. Incubation is at 30°C for 16 to 18 hours.

### 7.2. Chromatography

HPLC techniques can be used to determine the levels of moxalactam in various biological fluids. Where appropriate, the protein is removed from the sample by addition of ice cold methanol and centrifugation. The resulting solutions are evaluated under conditions described in Section 6.2.1.

## 8. Analysis of Pharmaceutical Formulations

Pharmaceutical formulations of moxalactam are handled in a manner analogous to the substances described in Section 6.

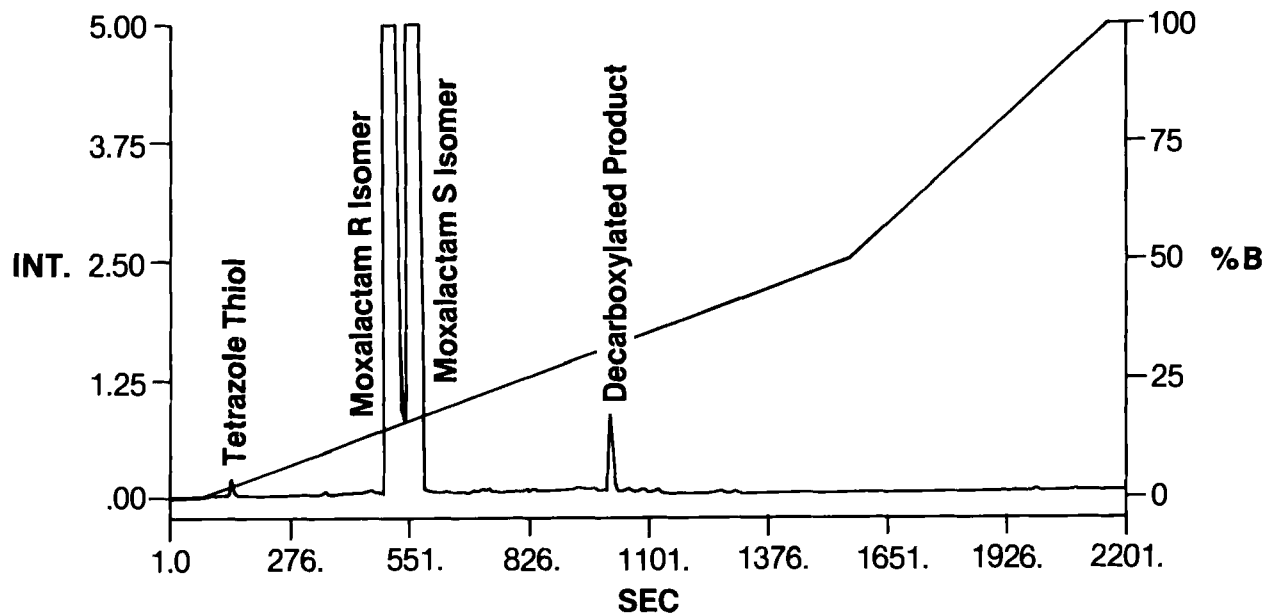


Figure 6. A Related Substances HPLC Chromatogram for Moxalactam Disodium on a Spherisorb™ ODS Column

The only difference is that the pharmaceutical formulation contains mannitol which is relatively innocuous to all of the test procedures described.

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# OXYPHENBUTAZONE

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## 1. Description

### 1.1 Nomenclature

#### 1.1.1 Chemical Names

4-Butyl-1-(4-hydroxyphenyl)-2-phenyl-3,5-pyrazolidinedione.  
 4-Butyl-1-(p-hydroxyphenyl)-2-phenyl-3,5-pyrazolidinedione.  
 4-Butyl-2-(4-hydroxyphenyl)-1-phenylpyrazolidine-3,5-dione.  
 4-Butyl-2-(p-hydroxyphenyl)-1-phenylpyrazolidine-3,5-dione.  
 4-Butyl-1-(p-hydroxyphenyl)-3,5-dioxo-2-phenylpyrazolidine.  
 4-Butyl-1-(4-hydroxyphenyl)-3,5 dioxo-2-phenylpyrazolidine.  
 3,5-Dioxo-1-phenyl-2-(p-hydroxyphenyl)-4-butylpyrazolidine.  
 1-(p-Hydroxyphenyl)-2-phenyl-4-butylpyrazolidine-3,5-dione.  
 1-Phenyl-2-(p-hydroxyphenyl)-3,5-dioxo-4-n-butylpyrazolidine.

#### 1.1.2 Generic Names

Oxyphenbutazone, G27202, Hydroxyphenylbutazone.

#### 1.1.3 Trade Names

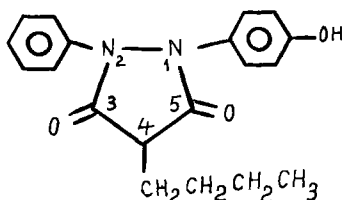
Crovaril, Flogitolo, Flogoril, Frabel, Neo-Farmadol, Oxalid, Tandacote, Tandearil, Tanderil, Visubutina.

## 1.2 Formulae

### 1.2.1 Empirical

$C_{19}H_{20}N_2O_3$  (anhydrous),  $C_{19}H_{20}N_2O_3 \cdot H_2O$  (mono-hydrate)

### 1.2.2 Structural



### 1.2.3 CAS no.

[129-20-4] (anhydrous), [7081-38-1] (monohydrate).

### 1.3 Molecular Weight

324.38 (anhydrous), 342.39 (monohydrate).

### 1.4 Elemental Composition

C 70.35%, H 6.22%, N 8.64%, O 14.80%.

### 1.5 Appearance, Color, Odor and Taste

White crystalline powder, odorless or almost odorless with bitter taste.

## 2. Physical Properties

### 2.1 Melting Point

Anhydrous crystals from ether and petroleum ether, m.p. 124-125° (1). Monohydrate crystals m.p. 96 (1,2).

### 2.2 Solubility

Practically insoluble in water, soluble in 3 parts of alcohol (95%), in 20 parts of chloroform, in 20 parts of solvent ether and in 6 parts of acetone; also soluble in solutions of alkali hydroxides (3).

### 2.3 Identification

#### 2.3.1 Infrared Spectroscopic Test

The infrared absorption characteristics of oxyphenbutazone have been used by the B.P. (3) and U.S.P. (4) as a means for identifying the drug. The infrared absorption spectrum exhibits maxima which are only at the same wavelengths as, and have similar relative intensities to, those in the spectrum of a similar preparation of a standard oxyphenbutazone. The infrared characteristics of oxyphenbutazone shall be discussed later in the spectral properties of the drug.

### 2.3.2 Ultraviolet Spectroscopic Test

The B.P. and U.S.P. also recommend the use of ultraviolet characteristics of oxyphenbutazone as an identification test. A solution of the drug in sodium hydroxide exhibits maxima and minima at the same wavelengths as that of a similar solution of a standard drug. The ultraviolet absorption characteristics of oxyphenbutazone shall be discussed later in the spectral properties of the drug.

### 2.3.3 Color Tests

Table 1 lists some of the color tests reported for the identification of oxyphenbutazone.

Table 1. Color Tests for Oxyphenbutazone.

Reagents	Color	Ref.
i) Hot solution of the drug in glacial acetic and hydrochloric acids. Cool and filter.		(3)
Filtrate plus sodium nitrite	Yellow color	
Addition of 2-naphthol solution	Bright orange ppt.	
Ppt + 95% alcohol	Orange solution	
ii) Drug in a mixture of sodium hydroxide and aminoacetic acid.	Solution clear with an extinction of 4-cm layer at 420 nm is not more than 0.40.	(3)
iii) Solution of drug in methanol plus Millon's Reagent, heat.	Cherry red ppt.	(4)
iv) Sulfuric acid - formaldehyde	Pale yellow (sensitivity : 1.0 µg).	(2)
v) Ammonium molybdate	Pale orange (sensitivity : 1.0 µg).	(2)
vi) Vitali's Test	Pale yellow or red (sensitivity: 0.25 µg).	(2)

#### 2.3.4 Crystal Tests

Crystal tests reported (2) include:

- a) Treatment with platinic iodide solution gives rosettes of prisms, forming overnight. The sensitivity of the test is 1 in 100.
- b) Treatment with potassium iodide solution gives shell-like crystals, forming overnight. The sensitivity of the test is 1 in 100.

#### 2.3.5 Chromatographic Tests

- a) Clarke (2) described a paper chromatographic method for the identification of the drug using three different solvent systems.

System I. Consists of citric acid: water: n-butanol (4.8 gm: 130 ml : 870 ml). Detection carried out using ultraviolet light absorption. p-Dimethylaminobenzaldehyde or potassium permanganate can be used as spray to locate the drug.  $R_f$  0.96.

System II. Acetate buffer (pH 4.58). Detection is effected by ultraviolet light absorption.  $R_f$  0.04.

System III. Phosphate buffer (pH 7.4). Detection is effected by ultraviolet light absorption.  $R_f$  0.58.

- b) An identification test for the drug by thin-layer chromatography was also reported by Clarke (2). The solvent system consists of: strong ammonia solution: methanol (1.5 : 100). Detection is effected by potassium permanganate spray.  $R_f$  0.77.



## 2.4 Spectral Properties

### 2.4.1 Ultraviolet Spectrum

The ultraviolet spectrum of oxyphenbutazone in neutral methanol using Cary, 219 Spectrophotometer is shown in Figure 1. The spectrum of the drug exhibits two maxima at 243 and 265 nm and a minimum at 221 nm. The ultraviolet absorption characteristics of the drug are used by the B.P. (3) and U.S.P. (4), as an identification test. A 0.001 % w/v solution of oxyphenbutazone in 0.01 N sodium hydroxide solution exhibits a maximum only at 254 nm with an extinction of about 1.5. Oxyphenbutazone in ethanol was also reported (2) to have a maximum at 242 nm (E1% 1 cm 564) and an inflexion at 275 nm.

### 2.4.2 Infrared Spectrum

The infrared spectrum of oxyphenbutazone in nujol mull is presented in Figure 2. An interpretation of the spectrum is given below. The infrared absorption spectrum of oxyphenbutazone is used as an identification test by the B.P. and U.S.P.

<u>Frequency (cm<sup>-1</sup>)</u>	<u>Assignment</u>
1288	C-N stretch
1702 1748	C=O stretch
3220	O-H stretch
1180	C-O stretch of phenol

Reported (2) principal infrared absorption peaks of oxyphenbutazone in potassium bromide discs are 1683, 1725, 1275 and 1518 cm<sup>-1</sup>.

### 2.4.3 <sup>1</sup>H Nuclear Magnetic Resonance (<sup>1</sup>H NMR) Spectrum

The <sup>1</sup>H NMR Spectrum of oxyphenbutazone is presented in Figure 3. The drug was dissolved in acetone-d<sub>6</sub> and its spectrum determined on a Varian-T60A NMR Spectrometer using TMS as the

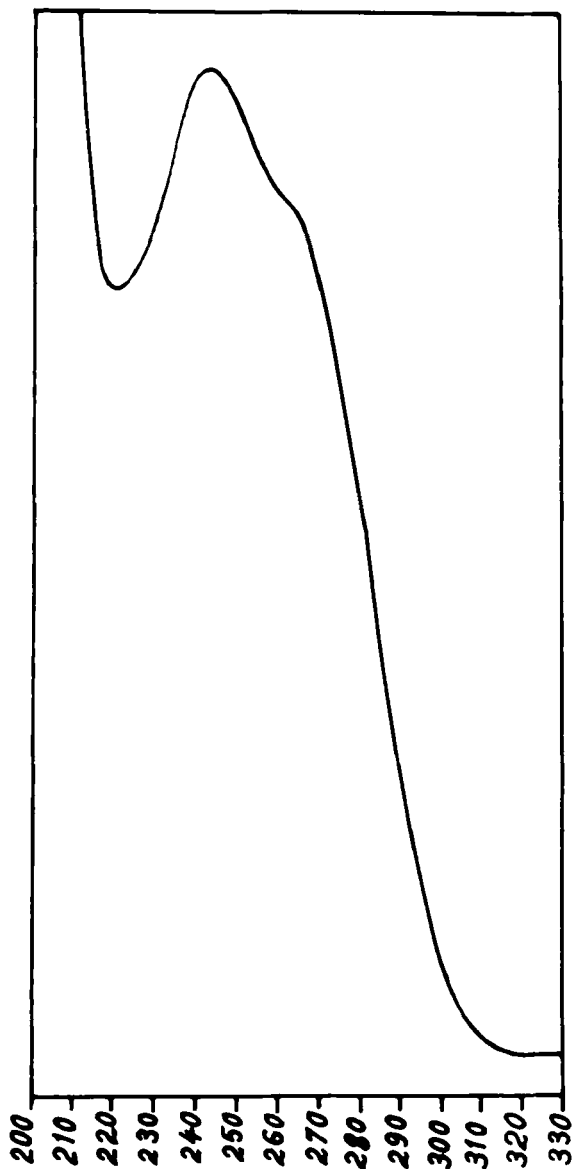


Fig. 1. Ultraviolet spectrum of oxyphenbutazone in neutral methanol.

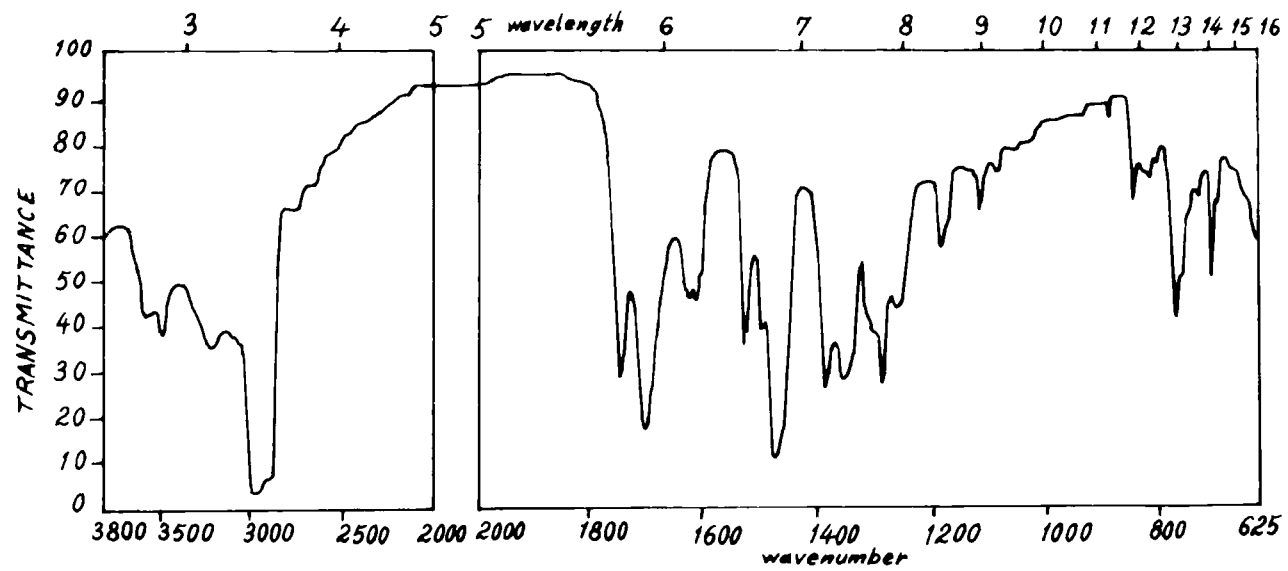


Fig. 2. Infrared spectrum of oxyphenbutazone, Nujol Mull.

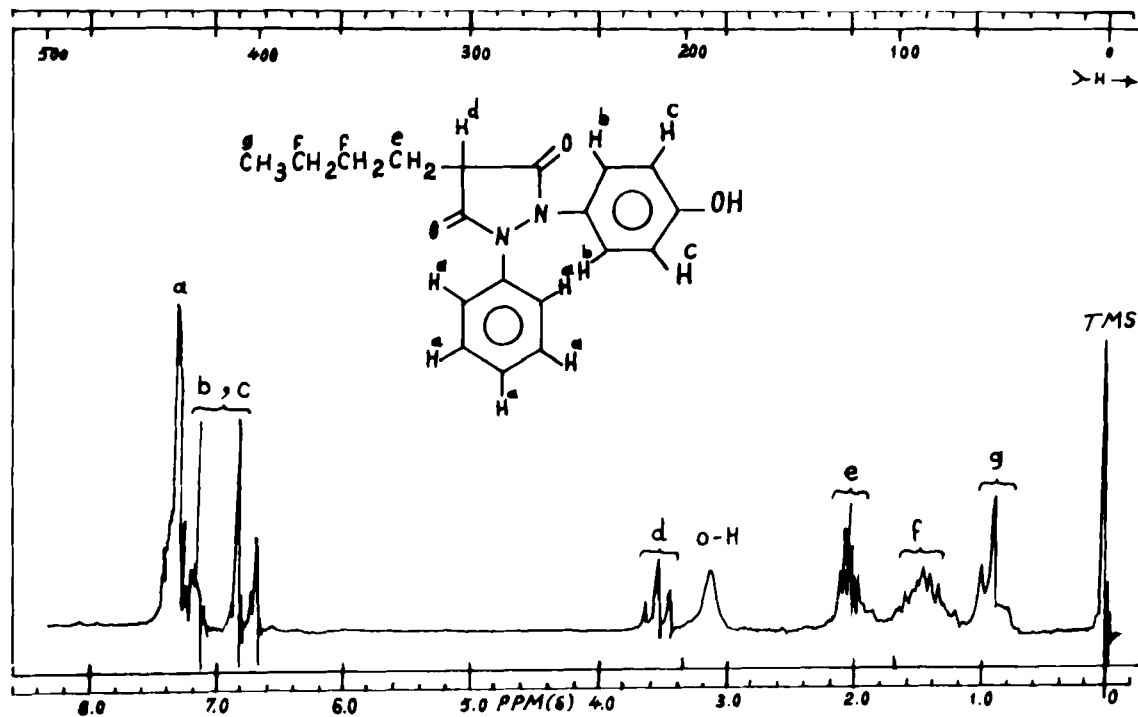


Fig. 3.  $^1\text{H}$  NMR spectrum of oxyphenbutazone in  $\text{acetone-}d_6$  with TMS as internal standard.

internal standard. The structural assignments are shown below.

<u>Proton Assignments*</u>	<u>Chemical Shifts (<math>\delta</math>)</u>	<u>Multiplicity</u>
a	7.33	broad singlet
b, c	7.00	AB quartet
d	3.57	triplet
e	2.04	multiplet
f	1.41	multiplet
g	0.92	quartet
O-H	3.14	broad singlet

\*See structure in Figure 3 for proton assignments.

#### 2.4.4 $^{13}\text{C}$ Nuclear Magnetic Resonance ( $^{13}\text{C}$ NMR) Spectrum

The  $^{13}\text{C}$  NMR spectrum of oxyphenbutazone in acetone- $d_6$  using TMS as an internal reference was obtained using a Jeol FX 100 MHz Spectrometer at an ambient temperature using 10 mm sample and is presented in Figure 4. The carbon chemical shift values are derived from the off-resonance spectrum and are listed below. The results are consistent with those reported earlier (5).

<u>Carbon No.*</u>	<u>Chemical Shifts (ppm)</u>	<u>Carbon No.</u>	<u>Chemical Shifts (ppm)</u>
1'	137.2	6	28.7
3	170.9	6'	126.4
3'	129.3	7	28.0
4	46.4	7'	116.1
4'	124.3	8	20.0
5	171.5	8'	157.1
5'	127.2	9	13.9

\*See structure in Figure 4 for carbon assignment.

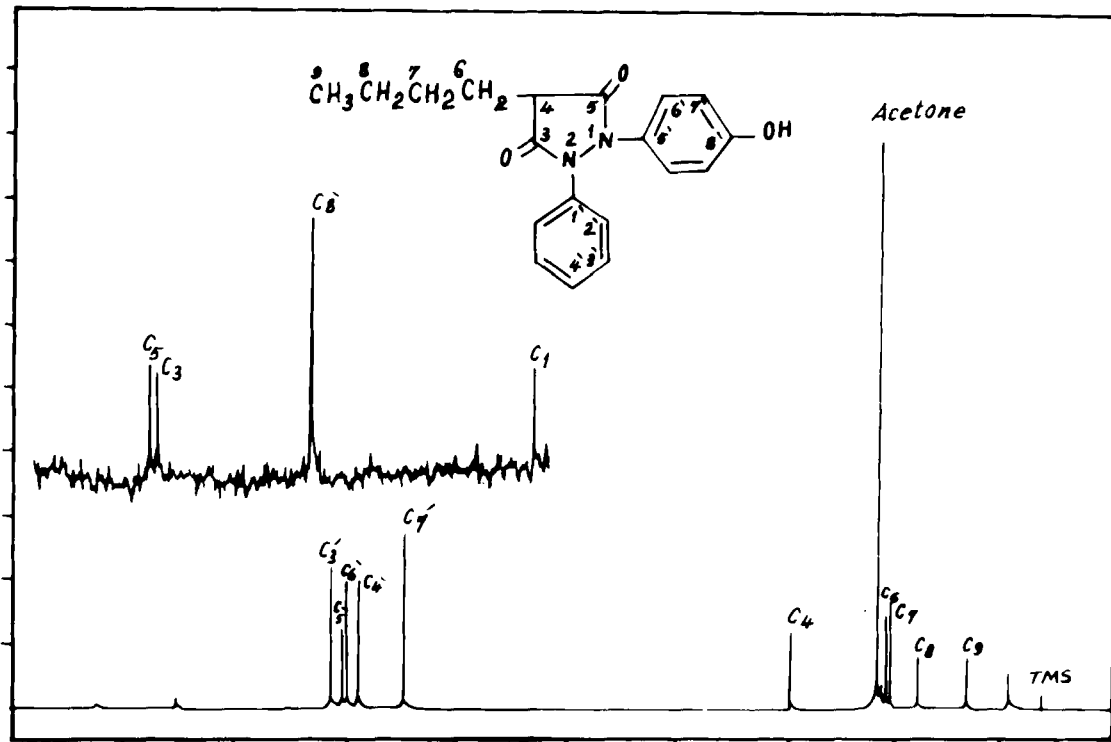


Fig. 4.  $^{13}\text{C}$  NMR spectrum of oxyphenbutazone in acetone  $-\text{d}_6$  with TMS internal reference.

#### 2.4.5 Mass Spectrum and Fragmentometry

The fragmentation patterns of oxyphenbutazone and other pyrazolidinediones were described (6) but the verification by evidence of metastable ions, accurate mass determinations or use of labeled derivatives was presented by Locock et al (7).

The mass spectra of oxyphenbutazone is reproduced in Figure 5. According to Locock et al (7) the drug undergoes McLafferty rearrangement to give a radical ion at m/e 268. A metastable ion is present in the spectrum to indicate that this ion is a direct fragmentation from the molecular ion with the loss of the elements of butene (Scheme I).

The loss of a propyl radical from the butyl side chain of the molecular ion represents a minor pathway to give an ion at m/e 281.

The most characteristic fragments in the mass spectrum of oxyphenbutazone were a series of peaks at m/e 198, 199 and 200. The peak at m/e 198 is due to the formation of the azobenzene radical ion  $(C_6H_5N_2C_6H_5)^\dagger$ . The remaining peaks at m/e 199 and 200 together with other peaks resulting from the fragmentation of oxyphenbutazone are explained by Scheme I.

Other peaks in the low mass range, below m/e 100, are common to substituted aromatic systems (Fig. 5).

### 3. Synthesis

Oxyphenbutazone was synthesized (8) by condensation of the protected aminophenol (I, Scheme 2) with nitrobenzene (II). Reduction of the resulting azo-compound (III) gave the hydrazobenzene (IV) which when condensed with diethylbutyl-

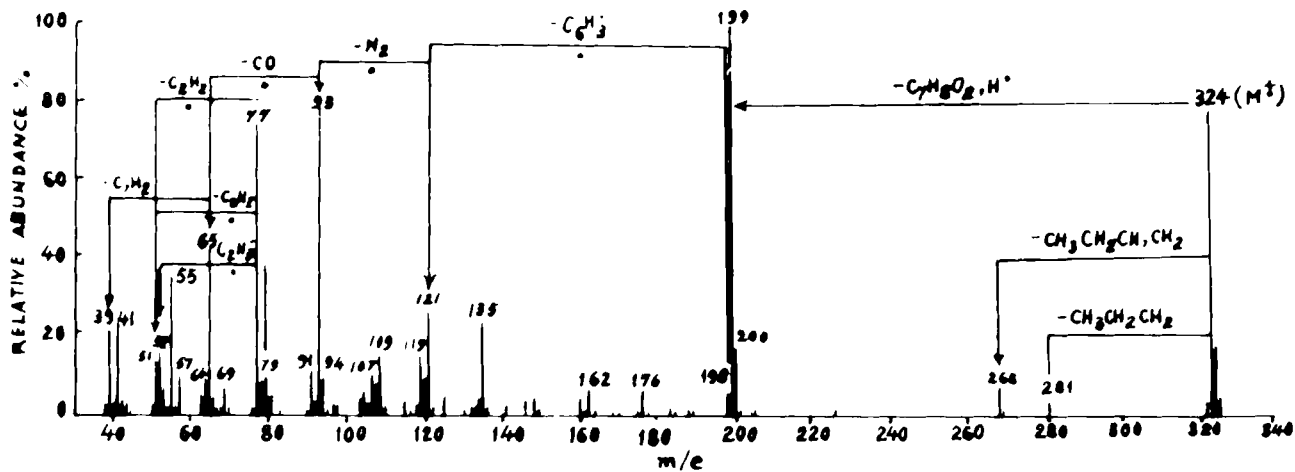
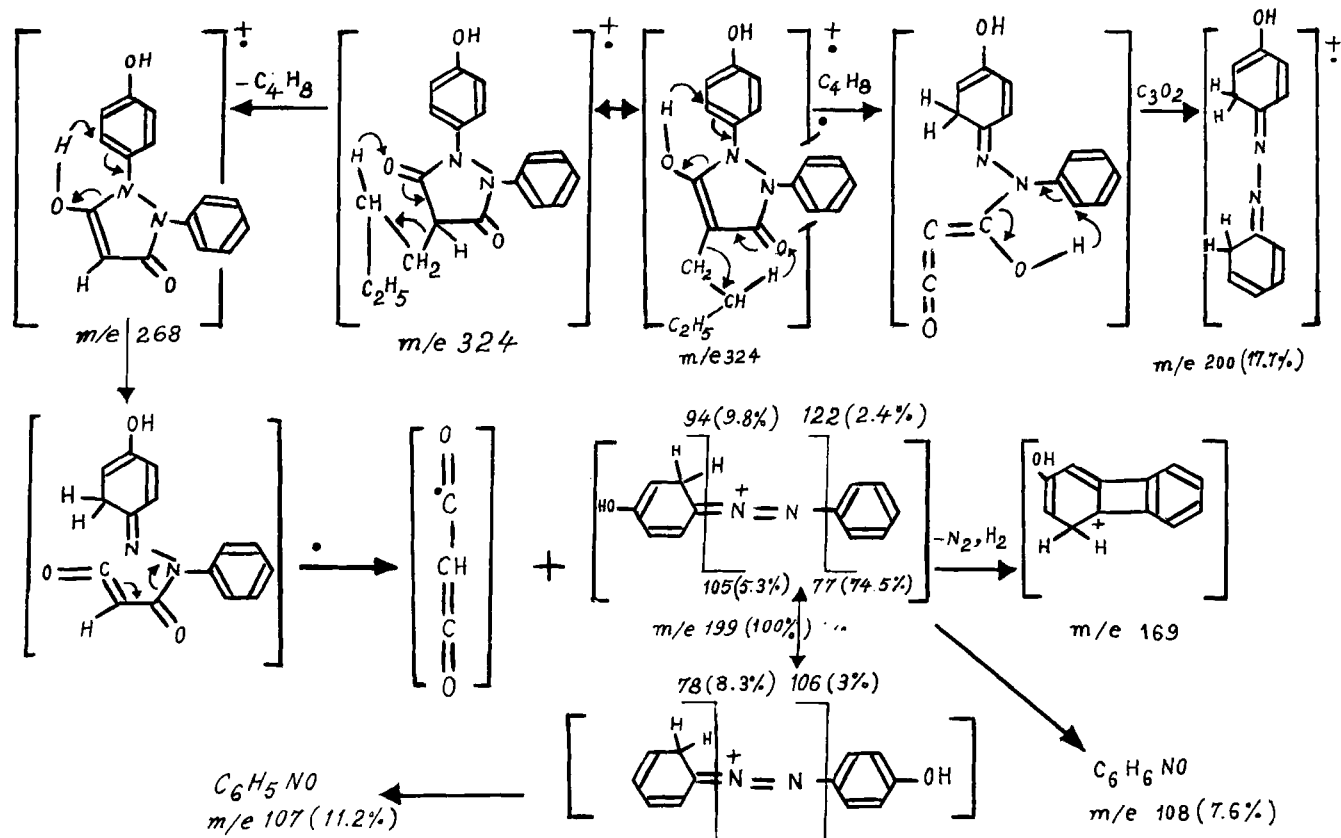


Fig. 5. Mass spectrum of oxyphenbutazone (7).

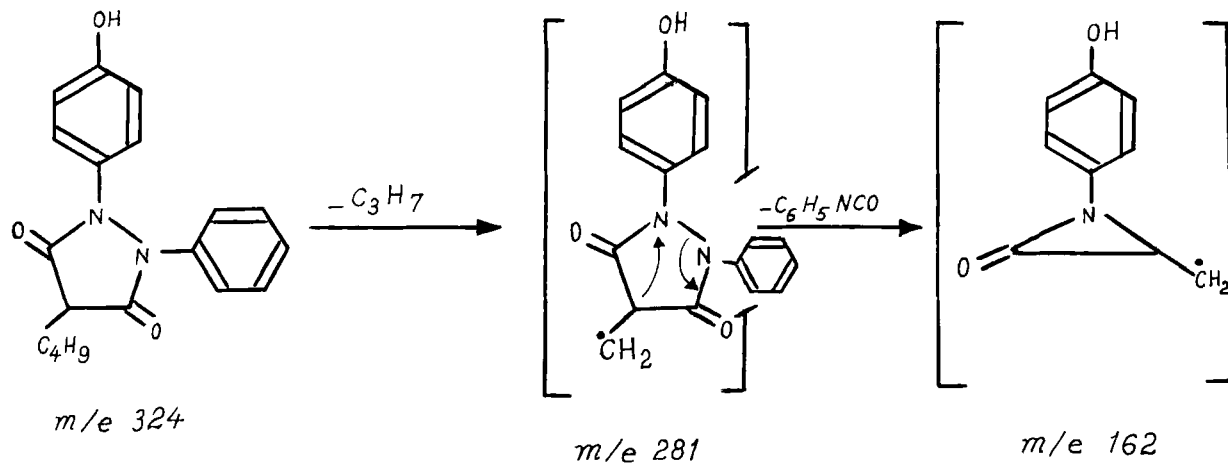




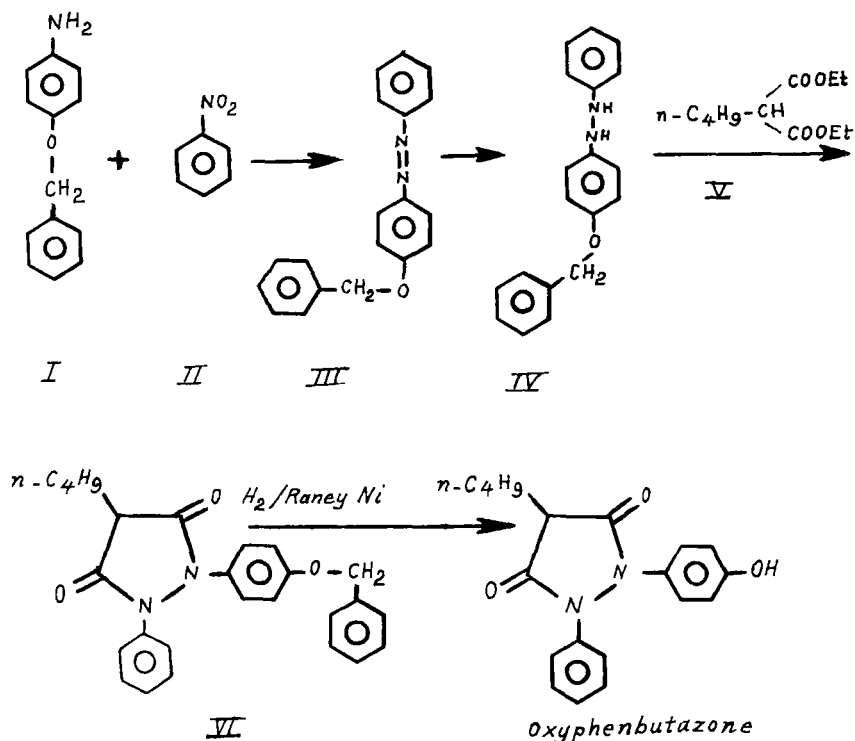
Scheme 1. Fragmentation of oxyphenbutazone 121 (26.6%) 93 (78.7%)

Scheme 1 Continued

347



malonate (V) gave the heterocycle (VI). Removal of the benzyl group was achieved by hydrogenolysis.



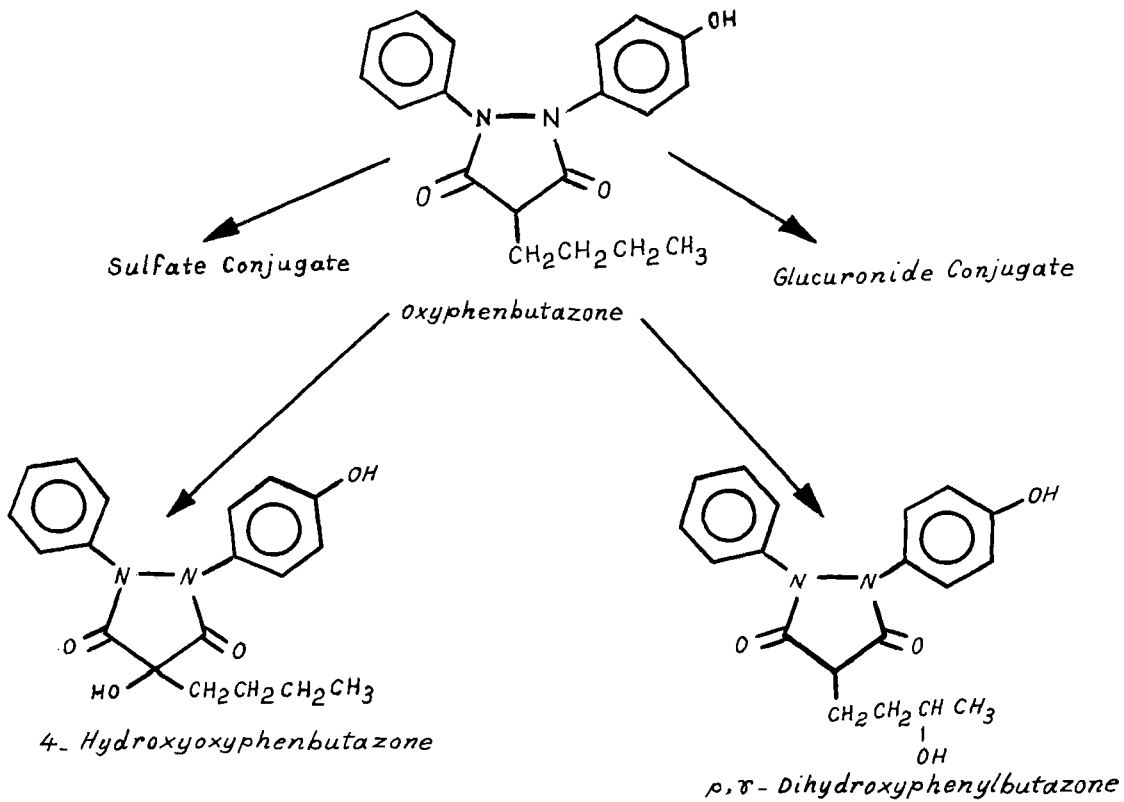
Scheme 2. Synthesis of oxyphenbutazone.

#### 4. Absorption, Metabolism and Excretion

Oxyphenbutazone is one of the main metabolites of phenylbutazone and is considered to be more toxic. It is rapidly absorbed from the gastrointestinal tract and slowly metabolized and excreted mainly in urine. The rate of its metabolism varies in different species as reflected by the great variance in half-lives.

In his study on the metabolism of butazolidine, Herrmann (9) measured the half-life of oxyphenbutazone in rats and rabbits after intravenous administration of the drug. They were 8 and 4-5 hrs respectively. Weiner et al (10) studied the drug disposition patterns in subhuman primates as compared to humans and other species. In the baboon, oxyphen-

butazone has a short half-life, while in man it has a long half-life. The plasma binding and apparent volumes of distribution of the drug are similar in the baboon and man. There is suggestive evidence that absorption may not be as complete in the baboon as it is in man. Studies in dogs indicated that the drug is not absorbed in the lymphatics. Burns (11) compared the rates of metabolism of oxyphenbutazone in man, rhesus monkey and dogs. The half-lives showed great variations. Previous studies indicated that metabolism of this and other compounds may be accomplished by different mechanisms so that species differences become both quantitative and qualitative. Perel et al (12) correlated the physicochemical properties of phenylbutazone analogs with their physiological disposition, in particular with reference to species differences. While in man there exists a direct relation between pKa and half-life, no such correlation was observed in dogs. The half-life in dogs appears to depend on factors such as fat/buffer partition coefficient, plasma protein binding, tissue distribution and drug metabolizing enzyme activity. The rate of metabolism of the analogs, based on plasma level decline, varied extensively in both species. Oxyphenbutazone has a half-life of 0.5 hr in the dog, whereas in man it is 72 hrs. The results of binding of oxyphenbutazone to human and dog plasma indicate that the drug is highly bound (98%). This higher binding of the drug to human plasma compared to dog plasma should be a significant factor in accounting for some of the species differences. In their study of the mechanism of hepatic drug oxidation and its relation to individual differences in rates of oxidation in man, Davies and Thorgeirsson (13) suggested that plasma half-lives of antipyrine determined following a single dose were a good measure of an individual's ability to metabolize phenylbutazone and oxyphenbutazone, if the half-lives of these two drugs were determined under conditions which did not change microsomal enzyme activity. Human liver microsomal protein and cytochrome P-450 concentrations did not show large individual differences and were similar to values for adult male rats. Hoffmann et al (14) carried out studies on the organ distribution and side effects of oxyphenbutazone and phenylbutazone using radioactive isotopes. No influence on the hemopoietic system was demonstrated by oxyphenbutazone or by phenylbutazone. A mild effect on oxyphenbutazone metabolism was seen. In direct relation of the concentration of oxyphenbutazone and phenylbutazone a decrease of oxyphenbutazone uptake by the thyroid was noted, with an increase in its protein binding. In the presence of fever, organs with a



Scheme 3. Metabolites of oxyphenbutazone

reticular endothelial cell system, especially the liver, had a higher uptake of oxyphenbutazone while muscle tissue showed a decrease at that time. Stierlin and Saubermann (15) studied the transport of locally administered radioactive oxyphenbutazone into the eye tissues of the rabbit. The results showed that the corneal-scleral barrier can be passed by the drug. The concentration of oxyphenbutazone locally administered in the tissue and fluids of the eye was higher than with a single 10 mg/kg i.v. dose. The direct application of oxyphenbutazone on the eye does not result in an accumulation of the drug was demonstrated by the fact that no oxyphenbutazone could be detected in the eye after 24 hrs.

The oxidation of oxyphenbutazone by sheep vesicular gland microsomes and lipoxygenase was studied by Portoghese et al (16). Oxyphenbutazone was oxidized when incubated with acetone powder prepared from sheep vesicular gland microsomes or with lipoxygenase at pH 4 or 5. Oxidation also occurred at pH 8 or 9, if arachidonate or linoleate was added to either of incubation mixtures. The oxygenated product was found to be identical with 4-hydroxyoxyphenbutazone (Scheme 3) which was synthesized and analysed by gas-liquid chromatography and mass spectrometry. The oxygenated compound was not an inhibitor of prostaglandin biosynthesis. Perel et al (12) found that oxyphenbutazone was slowly metabolized in man and rapidly metabolized in dogs. When the drug is administered intravenously to dogs, 10-15% of unchanged drug and 18-28% of its glucuronide were excreted in 24 hrs. When eight times this dose of the drug was given orally to human subjects, they excreted 1-5% of the dose as glucuronides in 24 hrs.

The sulfate of oxyphenbutazone is probably not a major metabolite. It hydrolyses in water at room temperature and since less than 2% of oxyphenbutazone is excreted in 24 hrs (17) this would represent the maximal conversion of oxyphenbutazone to sulfate.

## 5. Methods of Analysis

### 5.1 Titrimetric Methods

- a) The BP 1980 (3) describes a titrimetric method in which oxyphenbutazone solution in acetone is titrated with standard sodium hydroxide solution using bromothymol blue as indicator until a blue color persists for at least thirty seconds. The

operation is repeated without the drug; the difference between the titrations represents the amount of alkali required by oxyphenbutazone.

- b) The U S P 1980 (4) also recommends a standard sodium hydroxide titrimetric method but the drug solution is made in methanol and the end point is determined potentiometrically using a calomel-glass electrode system with a saturated salt bridge of potassium chloride in methanol. A blank determination is necessary.
- c) Walash and Rizk (18) determined oxyphenbutazone in certain dosage forms by a nonaqueous titration procedure in tetramethylurea using standard sodium methoxide as a titrant. Recovery from compound preparations of the analgesic was quantitative and the end point could be located either potentiometrically using glass-colomel electrode system or visually using thymol blue as indicator.

## 5.2 Electrochemical Methods

- a) Fogg and Ahmed (19) adopted a B.P. 1980 (3) test for the identification of oxyphenbutazone to the quantitation of the drug by differential pulse polarography. The drug solution in acetic and hydrochloric acids was treated with sodium nitrite and coupled with 1-naphthol. The azo-dye formed was determined by differential pulse polarography. Polarograms were reproduced for the determination of the drug at concentrations in the ranges 0 to 0.9  $\mu\text{M}$  and 0 to 70  $\mu\text{M}$ . The calibration graphs were rectilinear. The procedure, however, does not distinguish between oxyphenbutazone and phenylbutazone.
- b) Pelinard *et al* (20) studied the electrochemical behavior of antiinflammatory derivatives of pyrazolone and the application to determination in medicaments. Oxyphenbutazone and other 3,5-pyrazolinediones with mobile hydrogen atoms in position 4 were directly reduced electrochemically in neutral ethanol. Monoketones, phenazone and aminophenazone, were not reduced under similar conditions. The application of the method to the determination of 3,5-pyrazolidinediones, with labile hydrogen in the 4-position, gave a precision of about 3% when  $2 \times 10^{-6}$  mole of a substance were used.

### 5.3 Spectrophotometric Methods

#### 5.3.1 Ultraviolet Spectrophotometric Methods

- a) Herrmann (9) reported an ultraviolet method for the determination of phenylbutazolidine together with its metabolic products, chiefly oxyphenbutazone, in serum. The serum is shaken with 3N hydrochloric acid in 3% isoamyl alcohol in heptane. After centrifugation an aliquot of the organic phase is shaken with 2.5 N sodium hydroxide and the extinction of the alkaline extract is determined at 265 nm. A similar method has also been reported (21).
- b) Fuchs (22) described a method for the blood level determination of tanderil [oxyphenbutazone] using a single drop of capillary blood. The sample of the serum is mixed with water and 3N hydrochloric acid. The mixture is shaken with pure 1,2-dichloroethylene. After centrifugation, an aliquot of the organic phase is added to a separating funnel containing 1 ml of 2.5 N sodium hydroxide and shaken. The aqueous extract of Tanderil is separated by centrifugation and determined by measuring at 240, 245, 250, 252.5, 255, 257.5, 260, 265 and 270 nm. The error was < 4%.

#### 5.3.2 Colorimetric Methods

- a) Svatek and Hradkova (23) determined oxyphenbutazone and related keto derivatives in serum using a colorimetric method involving the reaction of the drugs with diazotized sulfanilic acid. According to the procedure, the diluted serum is shaken with N hydrochloric acid, heptane and isoamyl alcohol. The heptane phase is shaken with 0.1 N sodium hydroxide. The alkaline extract is shaken with a mixture of 1% sulfanilic acid, N hydrochloric acid, 1% sodium nitrite and methanol. The absorbance of this solution is measured at 525 nm against sodium hydroxide blank. The error was 3-5%. Oxyphenbutazone combines with diazotized sulfanilic acid in alkaline medium without need for preliminary hydrolysis. The methylene group of the side chain is activated by the



$\gamma$  keto group with the formation of a formazan derivative. The formazan derivative of sulfanilic acid is very soluble in water and has a maximum at 525 nm. The color is stabilized by the presence of 25% methanol. The method is sensitive to 0.5  $\gamma$ /ml and the linear range is 0-15  $\gamma$ /ml.

- b) Wahbi et al (24) used sodium cobaltinitrite as a reagent for determining some phenolic drugs. When oxyphenbutazone is reacted with the reagent in aqueous acetic acid a yellow color measurable at 320 nm is produced. Substituting sodium hydroxide for acetic acid gives a color measurable at 380 nm. The colored product is extractable with chloroform with a maximum absorption at 325 nm. The method is applied to the determination in tablet form. The results obtained are reasonably reproducible with a coefficient of variation < 2%.
- c) Sanghavi et al (25) treated oxyphenbutazone with a mixture of anhydrous acetic acid and hydrochloric acid. The product of the heated mixture is reacted with vanillin or 4-dimethylaminobenzaldehyde in acetic acid. The resulting color is measured absorptiometrically or at 424 nm with vanillin or at 406 nm with 4-dimethylaminobenzaldehyde. Beer's Law is obeyed for 8 to 12  $\gamma$ /ml when measured spectrophotometrically or 50 to 250  $\gamma$ /ml when measured absorptiometrically.

### 5.3.3 Spectrofluorometric Method

Miller et al (26) studied the luminescence properties of oxyphenbutazone and some other anti-inflammatory and antipyretic drugs. Although oxyphenbutazone showed no significant fluorescence at room temperature it was strongly fluorescent at 77K. Submicrogram quantities of the drug could be readily detected. The wavelength of excitation maximum in ethanol was 280 nm and that of the fluorescence maximum was 430 nm.

## 5.4 Chromatographic Methods

5.4.1 Thin-Layer Chromatography (TLC)

Several TLC methods are available for the separation, identification and determination of oxyphenbutazone and its metabolites.

Schmidt (27) analysed oxyphenbutazone together with other pharmaceuticals by staining on silica gel GF<sub>254</sub> using UV light visualization. An Avicel layer chromatography was developed by Lee (28) for the separation and identification of several kinds of drugs. Acidic materials were separated by acidic solvents and basic materials were separated satisfactorily by basic solvents using mixed Avicel kieselgel plates. The procedure was applied to the different pharmaceutical formulations. More TLC methods are included in Table 2.

Table 2. TLC Methods for the Determination of Oxyphenbutazone.

Adsorbent	Solvent System	Visualizing agent	R <sub>f</sub> value	Ref.
Silica gel G-0.06 M KH <sub>2</sub> PO <sub>4</sub> buffer (1:2) dry plate and activate at 110°.	Methanol/CHCl <sub>3</sub> /Aq. NH <sub>3</sub> (100:300:1)	I <sub>2</sub> /KI	-	(29)
Silica gel HF <sub>254</sub> + 366	Hexane/Acetone (1:1)	UV radiation	0.76	(30)
Silica gel HF <sub>254</sub> + 366	CHCl <sub>3</sub> /Ethylacetate	UV radiation	0.28	(30)
Silica gel	Strong NH <sub>3</sub> solution/Methanol (1.5:100)	KMnO <sub>4</sub>	0.77	(2)
Silica gel sheets	Cyclohexane/Acetone/Acetic acid (40: 50:1)	UV radiation at 254 nm	0.58	(31)

#### 5.4.2 Paper Chromatography

A paper chromatographic method was described by Clarke (2) and is outlined above under Chromatographic Tests.

#### 5.4.3 Gas Chromatography

A gas chromatographic method was reported by Hasegawa et al (32) for the quantitative determination of pyrazolidinedione analgesics present in blood plasma using an electron capture technique. The method is claimed to be simple and able to detect low concentrations. The gas chromatographic retention indexes of several compounds of toxicological interest including oxyphenbutazone were determined (33,34) isothermally at 180° on SE30, OV1, OV17 and QF4. The data for many of these substances were compared statistically among themselves when appropriate (SE30 and OV1) and with literature data. In general, no statistically significant deviations were found in the series compared. The values of the retention indexes were reproducible to a good degree which means that these indexes are of considerable diagnostic values in chemical toxicological investigations.

Bruce et al (35) determined oxyphenbutazone and phenylbutazone in plasma and urine by gas-liquid chromatography. While phenylbutazone was determined directly after extraction from blood plasma or urine, oxyphenbutazone was reacted with heptafluorobutyric anhydride prior to gas chromatography. Tanimura et al (36) described a gas-liquid chromatographic method for the determination of phenylbutazone and its metabolites, oxyphenbutazone and  $\gamma$ -hydroxyphenylbutazone in human and rabbit following administration of phenylbutazone. A modified Herrmann's extraction method has been used and coupled with the gas-liquid chromatographic procedure without derivative formation for phenylbutazone and using trimethylsilylation for the metabolites. The procedure was described as accurate and sufficiently sensitive for use in routine clinical assay and the estimation of the pharmacokinetic parameters of phenylbutazone and its metabolites.

#### 5.4.4 High Performance Liquid Chromatography (HPLC)

Pound and Sears (37) described a sensitive, specific, high-speed liquid chromatographic procedure for the simultaneous determination of phenylbutazone and its metabolite, oxyphenbutazone in blood plasma. According to this procedure, acidified plasma is partitioned with cyclohexane : ether (1:1) containing the 2,4-dinitrophenylhydrazone of 3,4-dimethoxybenzaldehyde as an internal standard. The organic extract is reduced to dryness, the resulting residue is redissolved in chloroform and aliquots of this solution are chromatographed on an adsorption column, using a mobile phase of 0.002% acetic acid and 23.0% tetrahydrofuran in n-hexane at 35°C. Use of a UV detector permits quantitative analysis of samples containing less than 0.25 µg/ml of phenylbutazone or oxyphenbutazone.

Harzer and Barchet (38) separated various combinations of analgesics by HPLC, using both reverse-phase and adsorption columns and UV detection at 254 nm. For the reverse-phase technique, various proportions of methanol-water were used as eluant, whereas the adsorption system requires a) chloroform-methanol-acetic acid and b) chloroform-C<sub>7</sub>H<sub>16</sub> - acetic acid. The advantage of the reverse-phase column was that it required changes only in column temperature and solvent proportions in order to effect all separations. Use of a variable wavelength detector enhanced the sensitivity by permitting adjustment to the absorption maximum of each drug.

Oxyphenbutazone, and other non-narcotic analgesics are screened by reverse-phase high-pressure liquid chromatography (39). The column is LiChrosorb RP8 and the mobile phase is a acetonitrile-water mixture. Blood and organs are homogenized in 4% perchloric acid before extraction with the solvents. Urine is extracted without primary treatment.

Dieterle et al (40) used a preparative reverse-phase chromatography for separation of polar and nonpolar metabolites, including those of phenyl-

butazone, on column packed with micronized XAD-2 resin. By equilibration with single-phase mixtures of water, a lower aliphatic alcohol and a hydrophobic solvent a reverse-phase partition system is formed in situ. The chromatographic technique is claimed to be characterized by a large capacity, a high power of resolution and a high degree of selectivity and reproducibility and versatile application in the isolation of polar and nonpolar drug metabolites from complex mixtures.

#### Acknowledgement

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# PENTAZOCINE

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1. Foreward

Pentazocine is a potent analgesic of the benzomorphan series with a 50 mg oral dose equivalent to 60 mg of codeine. It is also a weak narcotic antagonist with about one-fiftieth the activity of nalorphine. Pentazocine is indicated in the treatment of moderate to severe pain. It is also used as a preanesthetic and anesthetic supplement (1,2).

2. Description2.1 Nomenclature

Pentazocine

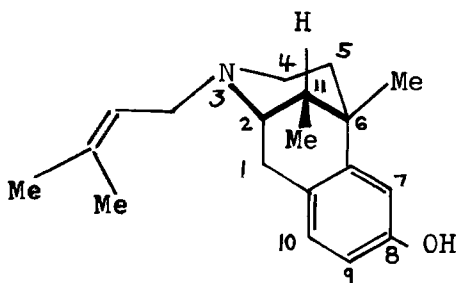
Talwin<sup>®</sup>

Fortral

(2 $\alpha$ , 6 $\alpha$ , 11R\*)-( $\pm$ )-1,2,3,4,5,6-hexahydro-6,11-dimethyl-3-(3-methyl-2-butanyl)-2,6-methano-3-benzazocin-8-ol

2.2 FormulaC<sub>19</sub>H<sub>27</sub>NO2.3 Molecular Weight

285.43

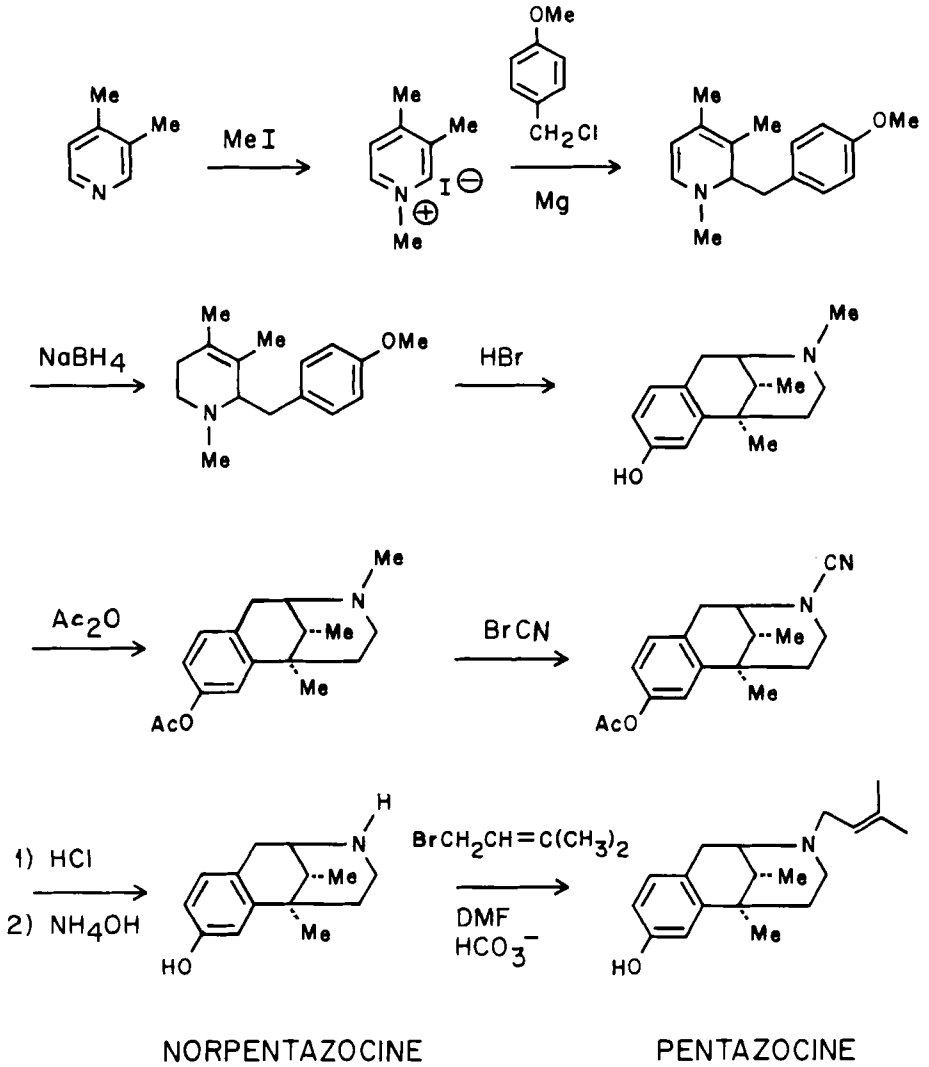
2.4 Structure2.5 CA Registry Number

base [359-83-1]

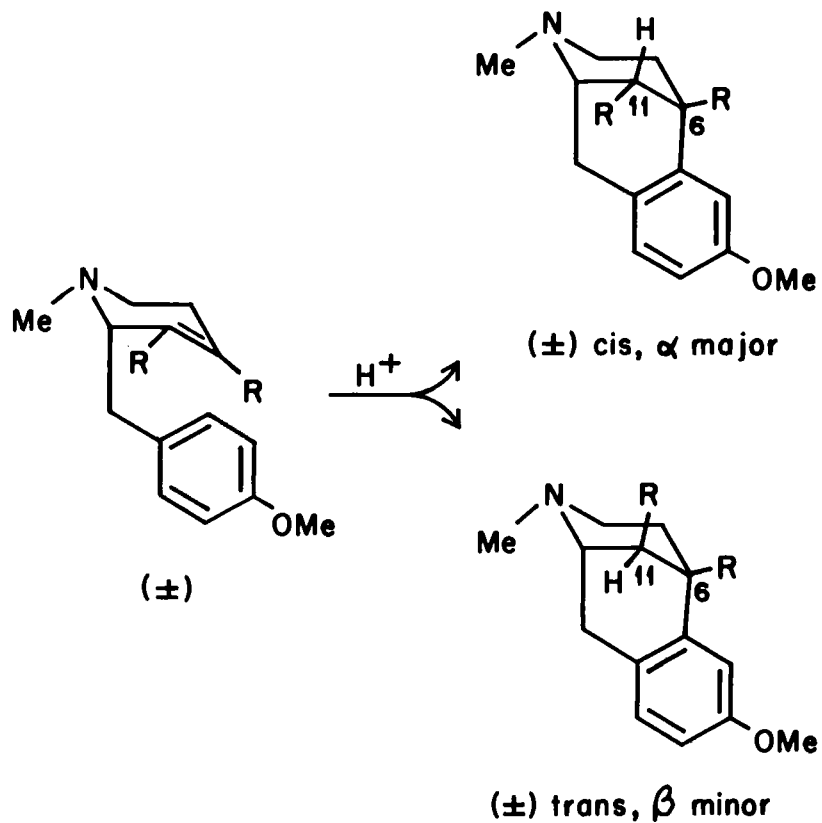
hydrochloride [68964-90-9]

2.6 Appearance, Color, Odor

Pentazocine is a white to pale tan, odorless, crystalline powder.



Scheme 1. Synthesis of pentazocine.



Scheme 2. Cyclization step in synthesis of pentazocine giving cis and trans isomers.

## 2.7 Recognized Dosage Forms

The forms of pentazocine recognized by the U.S.P. include pentazocine base, hydrochloride and lactate salts in the following formulations (1,3,4).

pentazocine hydrochloride tablets	50 mg base
pentazocine lactate injection	30 mg base/mL
pentazocine hydrochloride and aspirin tablets	12.5 mg base 325 mg aspirin

## 3. Synthesis and Resolution

### 3.1 Synthesis

The synthesis of pentazocine was originally accomplished by Archer and coworkers (5,6,7) and has been reviewed previously (8,9,10,11). The intermediate: 1,2,3,4,5,6-hexahydro-8-hydroxy-6,11-dimethyl-2,6-methano-3-benzazocine (norpentazocine) was synthesized earlier by May and Eddy (12) as shown in Scheme 1. Treatment of norpentazocine with 1-bromo-3-methyl-2-butene in DMF gave pentazocine. A variety of alternate routes to pentazocine have been demonstrated. Included are methods employing: a 3-benzyl intermediate (13,14), an initial reaction of 2-lithio-2-butene with formaldehyde (15), a thermal rearrangement of aminomethylcyclopropyl ketone (16), an abnormal Hofmann degradation of N-(4-hydroxybenzyl)-3-benzazocinium halides (17) and a Bischler-Napieralski reaction(18).

### 3.2 Isomers and Resolution

Geometrical (cis-trans) isomers of benzomorphans result from the cyclization step in their synthesis when the bond to C-6 is formed (Scheme 2). When this is considered a trans-addition to the double bond the preponderance of the cis or  $\alpha$  form is easily explained (10). Resolution of optical isomers from both cis and trans forms has been reported (7,19,20). The norpentazocine precursor in the cis form crystallized first as the (-) base with (+) tartaric acid which would give the twenty-fold more active (-) pentazocine. The (+) base (-) tartarate was recovered from resolution liquors. Quinic acid salts were formed for resolution of the trans isomers.

## 4. Physical Properties

### 4.1 Polymorphism

Two different interconvertible crystal polymorphs of pentazocine hydrochloride are known with melting points of  $218^{\circ}$  and  $254^{\circ}$  (2,21).

#### 4.2 Melting Point

The reported melting points of pentazocine base and hydrochloride salts along with those of resolved optical isomers are shown in Table 1.

#### 4.3 Differential Scanning Calorimetry

A D.S.C. curve for pentazocine is shown in Figure 1. It was carried out on a Perkin Elmer D.S.C. 1B at a scan rate of  $2.5^{\circ}$  per minute, a range setting of 4 and a nitrogen purge rate of 20 mL per minute (23).

#### 4.4 Optical Rotation

Table 2 includes results for optical rotation of resolved optical isomers of pentazocine carried out by Tullar *et al* (19). Also included is the value obtained by Nambara *et al* for (-) pentazocine (22).

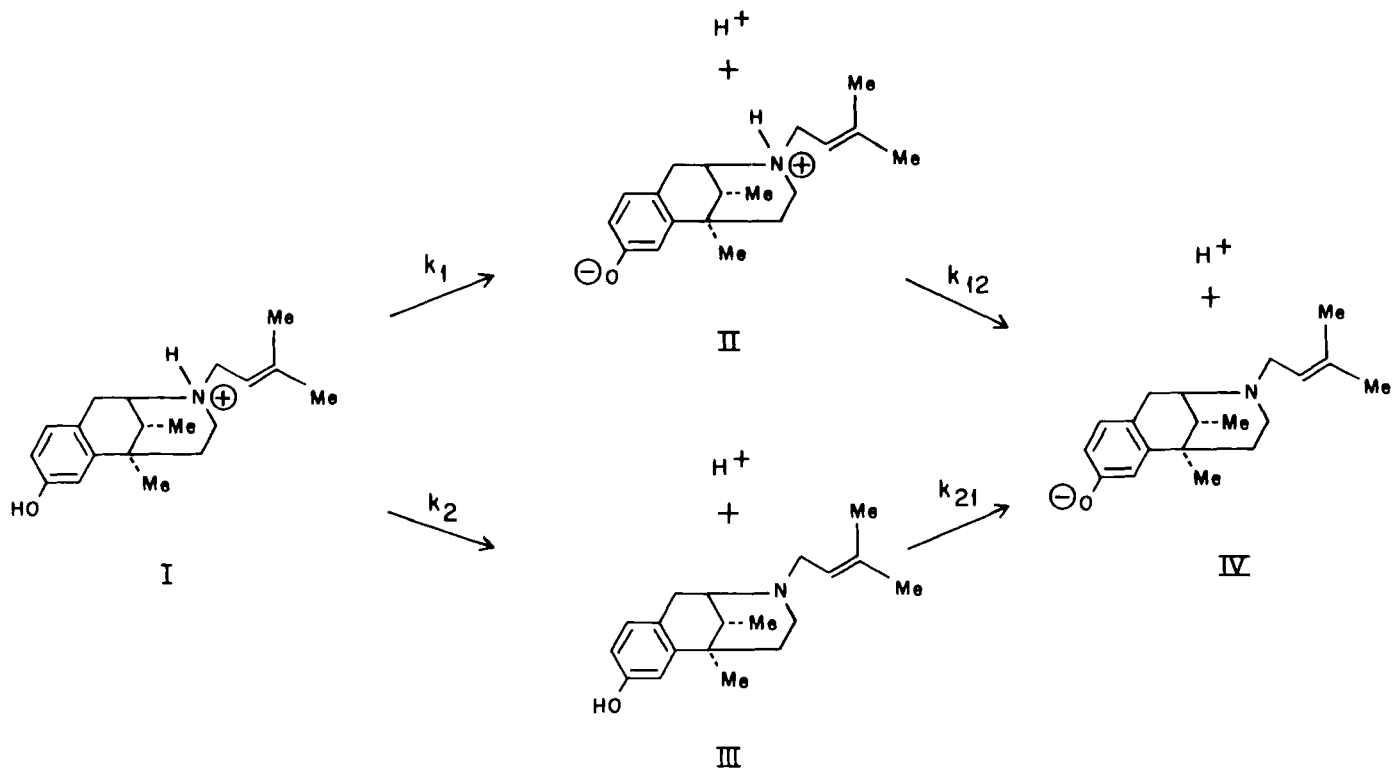
#### 4.5 Elemental Analysis

Results of elemental analysis for pentazocine and pentazocine hydrochloride are shown in Table 3.

#### 4.6 Ionization Constant

The  $pK_a$  value for pentazocine listed in general texts as 8.76 (24) or 8.95 (2) is the tertiary amine protonation constant. Borg and Mikaelsson however have shown that two ionizations for pentazocine should be considered. First the pentazocinium ion loses the proton from the nitrogen and then the phenolic proton is lost at higher pH. This is pictured in Scheme 3 where two separate intermediates are possible; the neutral pentazocine base (III) and a zwitterionic form (II).

The four microscopic dissociation constants were determined using 0.1 ionic strength carbonate/bicarbonate buffers with the values:  $pK_1 = 9.74$ ,  $pK_2 = 10.56$ ,  $pK_{12} = 11.17$  and  $pK_{21} = 10.35$ . From these the macroscopic dissociation constants were determined from the relations:



Scheme 3. Pentazocine ionization.

TABLE 1

## MELTING POINTS OF PENTAZOCINE

Isomer				
Form	Optical	Geometrical	Melting Point °C	Reference
base	(+)	cis	145.4-148.6	5
base	(+)	cis	179 -182	7
base	(-)	cis	176 -179	7
base	(-)	cis	164 -170	22
base	(+)	cis	180.4-182.0	19
base	(-)	cis	180.6-182.2	19
hydrochloride	(+)	trans	254.5-255.0	19
hydrochloride	(-)	trans	246.0-254.0	19

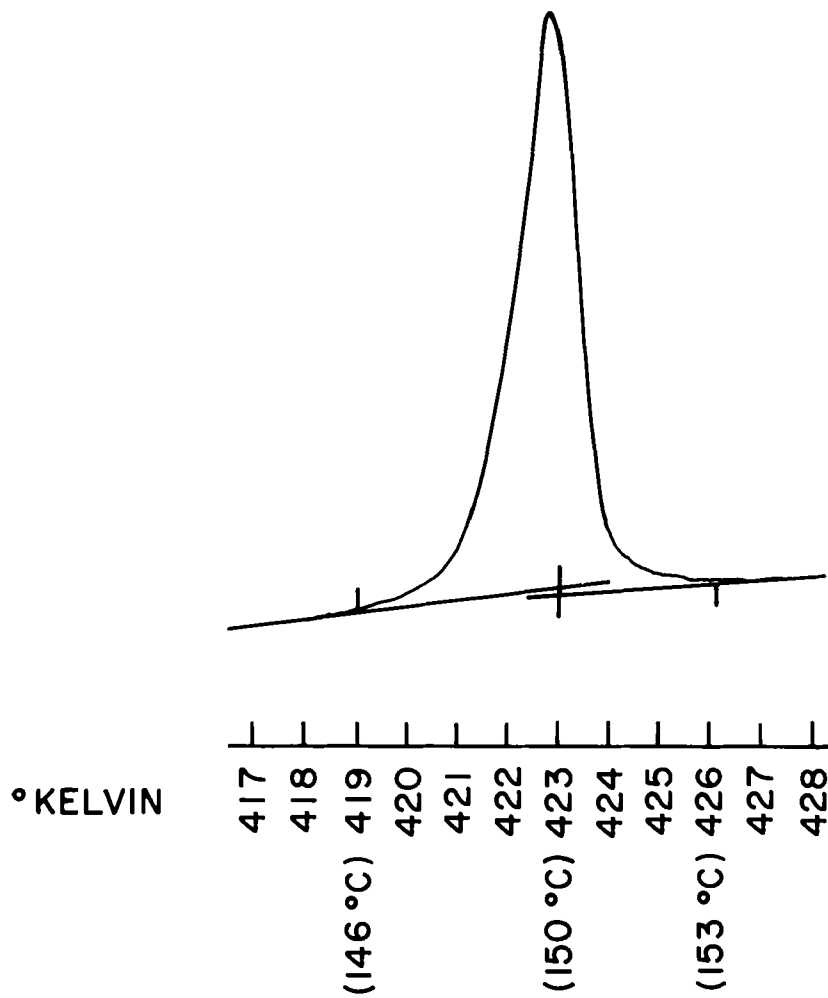


Fig. 1. Differential scanning calorimetry curve for pentazocine.



TABLE 2

## OPTICAL ROTATION OF PENTAZOCINE ISOMERS

Form	Isomer	$[\alpha]_D^{20}$	Solvent	Reference
base	cis (+)	+135.5	CHCl <sub>3</sub>	19
base	cis (-)	-138.0	CHCl <sub>3</sub>	19
base	cis (-)	-131	CHCl <sub>3</sub>	22
hydrochloride	trans(+)	+115.4	2% HOAc	19
hydrochloride	trans(-)	-116.3	2% HOAc	19

TABLE 3

## PENTAZOCINE ELEMENTAL ANALYSIS

Form	Isomer	% Carbon		% Hydrogen		% Nitrogen		% Chlorine*		Ref.
		Calc'd	Found	Calc'd	Found	Calc'd	Found	Calc'd	Found	
base	( <sup>+</sup> ) cis	79.95	79.84	9.34	9.28	4.91	5.23	-	-	5
HCl	( <sup>+</sup> )trans	70.89	70.62	8.77	8.46	4.35	4.25	11.01	11.25	19

\* (-) trans isomer

$$K_1 = \frac{a_{H^+} ([II] + [III])}{[I]}$$

$$K_2 = \frac{a_{H^+} [IV]}{[II] + [III]}$$

Thus  $pK_1$  was found to equal 9.68 and  $pK_2$  equals 11.23 (25).

#### 4.7 Partition Coefficient

The partition coefficient for pentazocine has been measured between various organic and aqueous phases. The results of four studies are shown in Table 4. The first value was obtained by a single extraction using 50 mL of each phase while the second value is from a countercurrent distribution study with pentazocine added to the aqueous phase and extracted eight times into benzene. The third value was obtained by the single extraction method with the additional definition of a distribution ratio,  $D_{NOH}$ , for pentazocine using the partition coefficient  $k_d(NO_H)$  in the relation:

$$\frac{1}{D_{NOH}} = \frac{1}{k_d(NO_H)} \left( 1 + \frac{a_{H^+}}{k_2} + \frac{k_1}{k_2} + \frac{k_{21}}{a_{H^+}} \right)$$

Here  $k_1$ ,  $k_2$ , and  $k_{21}$  are the microscopic ionization constants defined in section 4.6. From this it was possible to calculate and plot variation in  $D_{NOH}$  with pH for pentazocine as shown in Figure 2. The fourth partition coefficient was obtained by structural group contribution to the measured partition coefficient of a reference compound (morphine). This was done following the Hansch approach.

#### 4.8 Solubility

The solubility of pentazocine base is

TABLE 4  
PARTITION COEFFICIENTS FOR PENTAZOCINE

Organic	Aqueous	Coefficient	Reference
CHCl <sub>3</sub>	0.1 M pH 7.35 phosphate buffer	25.4	26
benzene	0.2 M pH 6.7 phosphate buffer	1.17	27
benzene	0.1 ionic strength pH 6.9-8.2 phos- phate buffer	630	25
octanol	water	1.07 x 10 <sup>4</sup>	28

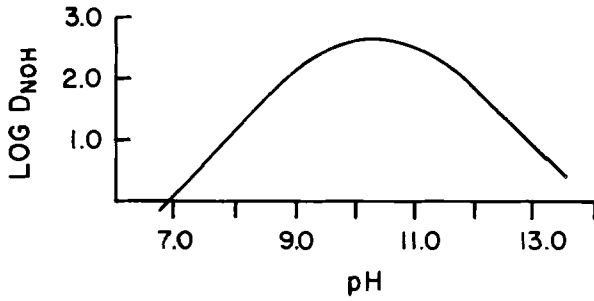


Fig. 2. Relationship between log (benzene/water) distribution ratio and pH for pentazocine (after Borg and Mikaelsson).

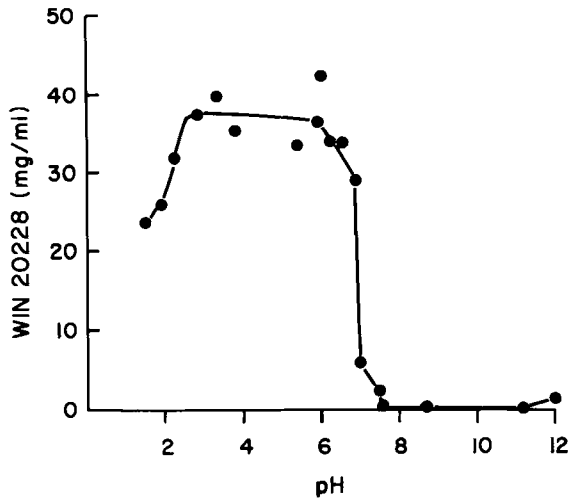


Fig. 3. Relationship between aqueous solubility and pH for pentazocine.

shown for various solvents in Table 5. In addition a pH/ equilibrium solubility study has been carried out in which saturated solutions of pentazocine were prepared using HCl and NaOH to adjust pH. A plot of the pH/ solubility relationship is shown in Figure 3(30).

#### 4.9 Spectral

##### 4.9.1 Mass Spectrum

A direct inlet electron impact mass spectrum of pentazocine (Sterling-Winthrop) obtained on a HP 5980 is shown in Figure 4(31). The electron energy, emission current and source temperature were 150 e.v, 0.42 mA and 185.6° respectively. The molecular ion is found at m/e of 285.1, with the base peak at 217.1 (100%).

G.C./M.S. results for pentazocine previously reported include both derivatization and nonderivatization procedures. Underivatized pentazocine gave a molecular ion at m/e 285 and the nor-ion at m/e 217 (32) while the 217 base peak and a 202 peak were observed by a multiple ion detector (33). Chemical ionization with methane gave a molecular ion of m/e 286 and fragments shown below (34).

m/e			
230	(M <sup>+</sup> - 55)	30%	(M <sup>+</sup> - CH-CH-(CH <sub>3</sub> ) <sub>2</sub> )
217	(M <sup>+</sup> - 68)	15%	(M <sup>+</sup> - CH <sub>2</sub> CH-C(CH <sub>3</sub> ) <sub>2</sub> )
270	(M <sup>+</sup> - 15)	10%	(M <sup>+</sup> - CH <sub>3</sub> )

The most frequently encountered derivative of pentazocine in GC/MS work has been the trifluoroacetyl with a molecular ion at m/e 381 (35,36,37). A pentafluorobenzyl bromide was run giving a molecular ion of m/e 465 (38). A trimethylsilyl derivative gave a M<sup>+</sup> of 357 with a base peak of 45 m/e for pentazocine (39), while a permethylated pentazocine glucuronide gave a molecular ion at 517, an aglycone at 217 and a base peak at m/e 201. Hydrolysis of the glucuronide followed by silylation gave the 357 monoTMS derivative (40).

##### 4.9.2 Nuclear Magnetic Resonance

The proton and <sup>13</sup>C nmr spectra of pentazocine (Sterling-Winthrop) are shown in Figures 5 and 6 respectively. Interpretations are given in Tables 6 and 7. The pmr spectrum was run on a Varian HA-100 on a 10% solution in

TABLE 5  
PENTAZOCINE SOLUBILITY

Solvent	Solubility (%w/v)	
	Reference: 29	2
CHCl <sub>3</sub>	25	50
95% ethanol	7.1-8.3	9.1
ethyl acetate	2.94	-
benzene	1.25	-
acetone	3.3	-
ether	3.3-5.0	2.4
water	0.005	0.1
1 N HCl	3	-
1 N lactic acid	15	-

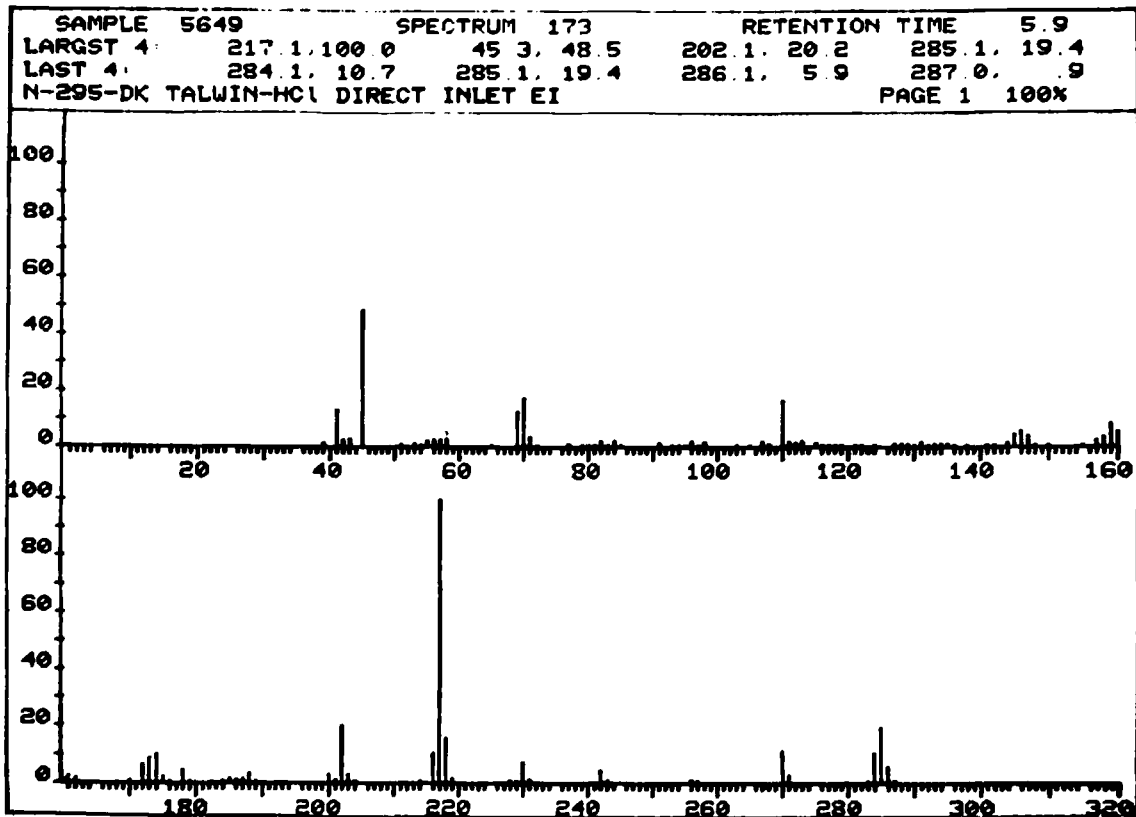


Fig. 4. Mass spectrum of pentazocine.



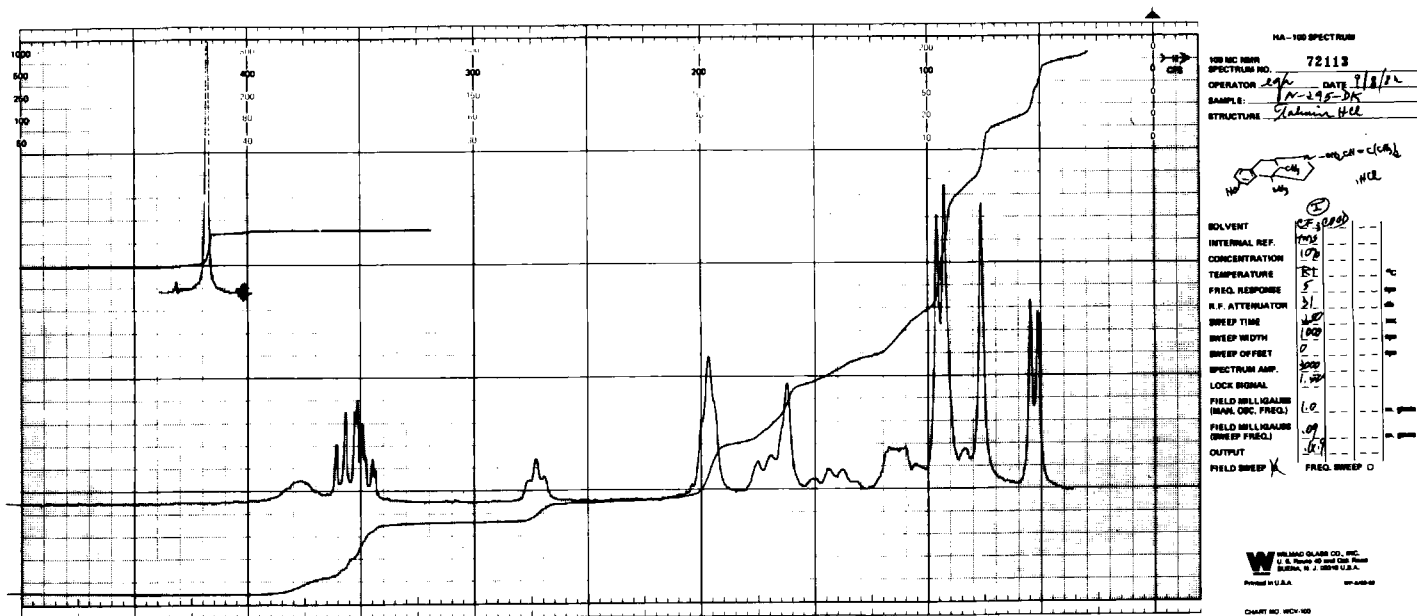


Fig. 5.  $^1\text{H}$  nuclear magnetic resonance spectrum of pentazocine hydrochloride.

379

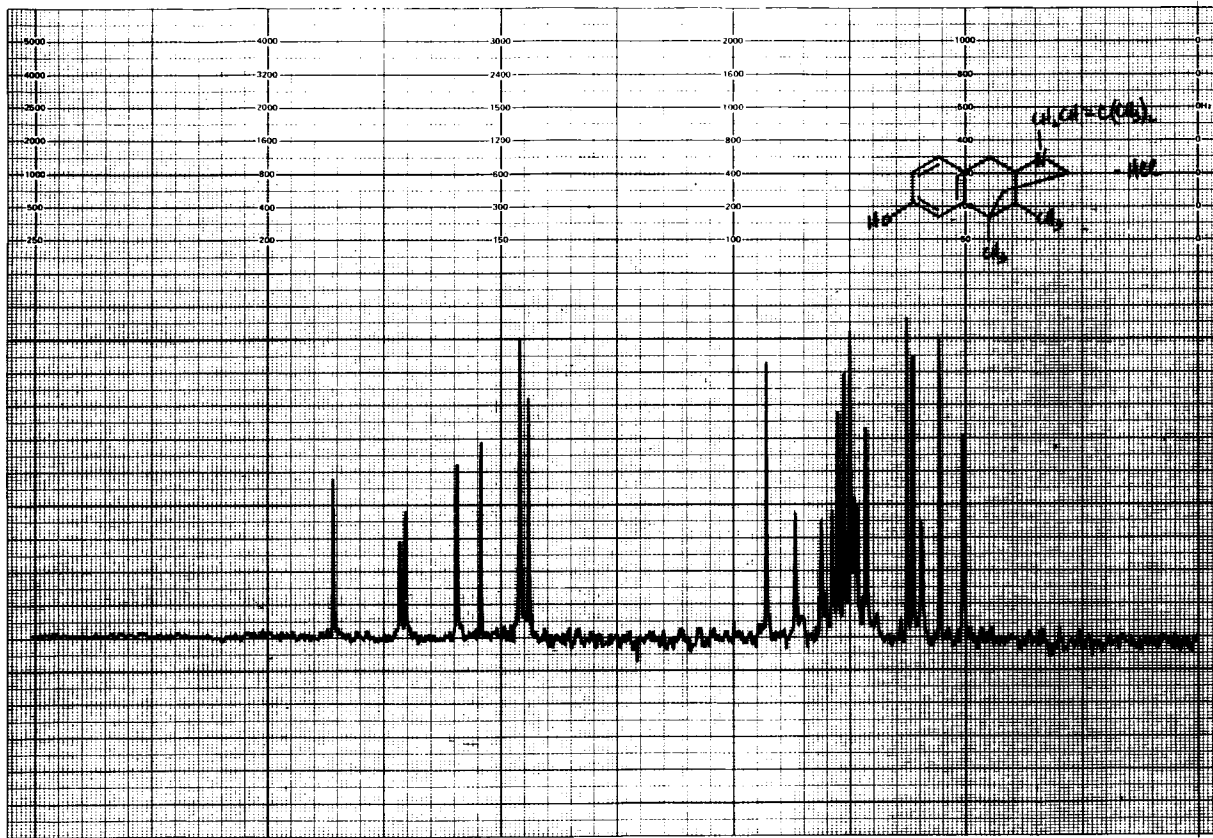


CHART NO. WCJFX3 Fig. 6.  $^{13}\text{C}$  nuclear magnetic resonance spectrum of pentazocine hydrochloride.

FX 60  
SPECTRUM NO. 72113  
SAMPLE

N-295-DK  
TALWIN Hydrochloride

SOLVENT  $\text{DMSO-d}_6$   
CONCENTRATION 10mg/15ml  
REFERENCE TMS  
TEMP 70 °C TUBE 10 min

NUCLEUS/OFFSET  
OBS  $^{13}\text{C}$  / 35.50 KHZ  
IRR  $^1\text{H}$  / 40.0 KHZ  
LOCK  INT  EXT  
PULSE  SINGLE  DOUBLE  
1ST 6 μSEC 190 °  
2ND μSEC 1 °  
INTERVAL SEC  
REPEITION 10 SEC  
ACQ TIME SEC  
NO of ACCUM 500

DATA POINTS 8K  
SPECTRAL WIDTH 4K Hz  
FILTER Hz  
WINDOW 20 Hz

RF / AMP OBS 5 23KHZ  
LOCK 4 2504  
IRR /

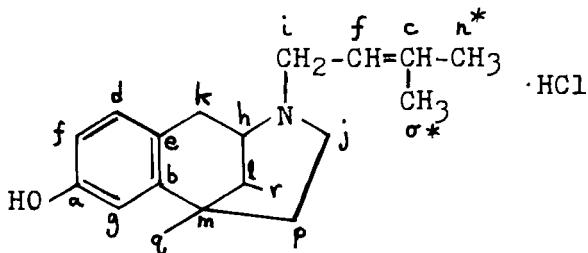
DECOUPLING MODE  
 NONE  HOMO  HETERO  
OTHER: \_\_\_\_\_  
POWER 60dB  
 CW  NOISE 1 KHZ

DATE 9/2/74  
OPERATOR JDS  
REMARKS

TABLE 6  
PENTAZOCINE  $^1\text{H}$  NMR ASSIGNMENTS

Chemical Shift (ppm)	No. H	Assignment
1.07	3 H	$\text{CH}_3\text{-CH}$
1.53	3 H	$\text{CH}_3\text{-C-}$
1.92, 1.85	6 H	$(\text{CH}_3)_2\text{C=}$
1.4-3.6	5 H	$\text{CH}_2\text{x}2$ , CH
3.8-4.0	4 H	$\text{NCH}_2\text{x}2$
5.45	1 H	N-CH
6.86-7.25	3 H	aromatics
7.5	1 H	OH, partially exch'd
11.36	2 H	exch'd H

TABLE 7  
PENTAZOCINE  $^{13}\text{C}$  NMR ASSIGNMENTS



Carbon	ppm/TMS	Multiplicity from partial off resonance decoupling
a	156.1	s
b	140.9	s
c	139.6	s
d	127.9	d
e	122.5	s
f	113.7	d, d
g	111.7	d
h	57.3	d
i	50.8	t
j	44.8	t
k	38.1	t
l	37.2	d
m	34.6	s
n	25.1	q*
o	23.7	q*
p	21.8	t
q	17.7	q
r	12.3	q

\*values may be interchanged

CF<sub>3</sub>COOD. The <sup>13</sup>C nmr was obtained on a Jeol FX 60 for a 10% DMSO d<sub>6</sub> solution (41). Previous proton nmr data has been published for pentazocine (32), N-benzylpentazocinium bromide (42) and pentazocine chloroacetic ether (22). These are summarized in Table 8.

#### 4.93 Infrared Spectrum

The infrared spectrum of pentazocine (Sterling-Winthrop), 1/2 % in KBr, taken on a Perkin Elmer 467 is shown in Figure 7 (43). The previously noted allylic (C = C) stretching at 1670 cm<sup>-1</sup> is also seen (32).

#### 4.94 Ultraviolet Spectrum

An ultraviolet spectrum of pentazocine (Sterling-Winthrop) was obtained on a Cary 15 and is shown in Figure 8. The relative  $\lambda_{\max}$  for this 0.0318 g/L solution in 95% alcohol is seen at 283 nm. The calculated absorptivity there is 2052 (44).

#### 4.10 X-Ray Diffraction

A normalized powder x-ray diffraction pattern for pentazocine (Sterling-Winthrop) is shown in Figure 9. It was recorded on a Rigaku X-ray Diffractometer Miniflex with Cu K $\alpha$ , Ni filtered radiation at 4000 cps, TC/1, ss/s, 0.5 2  $\theta$ /min. The scan was done from 40° to 3.5° 2  $\theta$  with I/I<sub>0</sub> peak height ratios relative to the 12.4° 2  $\theta$  100% peak. The calculated d-spacings associated with this pattern are listed in Table 9 (45).

#### 4.11 Dissolution

The dissolution method for pentazocine hydrochloride tablets utilizes a UV determination of pentazocine in filtered test samples at 278 nm. The apparatus used is USP number two at 50 rpm with water as the dissolution medium. The solutions are measured in hydrochloric acid diluted with dissolution medium to give 0.01 N HCl and quantitatively compared to a suitable standard (46).

A dissolution procedure for pentazocine hydrochloride and aspirin tablets is set forth in Addendum a, Second Supplement to the USP XX. The procedure is carried out with apparatus number one at 80 rpm using water as the dissolution medium.

TABLE 8

PENTAZOCINE  $^1\text{H}$  NMR DATA ( $\delta$ )

Reference:	32	42*	22**
Solvent:	<u>D<sub>2</sub>O</u>	<u>TFA</u>	<u>CDCl<sub>3</sub></u>
Proton			
11 CH <sub>3</sub>	0.9 <sup>d</sup> (J=6)	1.18 <sup>d</sup> (J=7)	0.83 <sup>d</sup> (J=6)
6 CH <sub>3</sub>	1.4 <sup>s</sup>	1.67 <sup>s</sup>	1.33 <sup>s</sup>
C=C(CH <sub>3</sub> ) <sub>2</sub>	1.9 <sup>d</sup> (J=4.5)	{1.82 <sup>s</sup> 2.06 <sup>s</sup> }	1.70 <sup>d</sup> (J=4)
4 CH <sub>2</sub>	3.15 <sup>m</sup>	-	-
1'CH <sub>2</sub>	3.8	-	-
CH=C	5.3 <sup>m</sup>	5.80 <sup>br</sup>	5.38 <sup>t</sup> (J=12)
aromatic	6.8 <sup>m</sup>	7.05-7.60 <sup>m</sup>	6.88 <sup>m</sup>

\* N-benzylpentazocinium bromide

\*\* chloroacetic ether of pentazocine

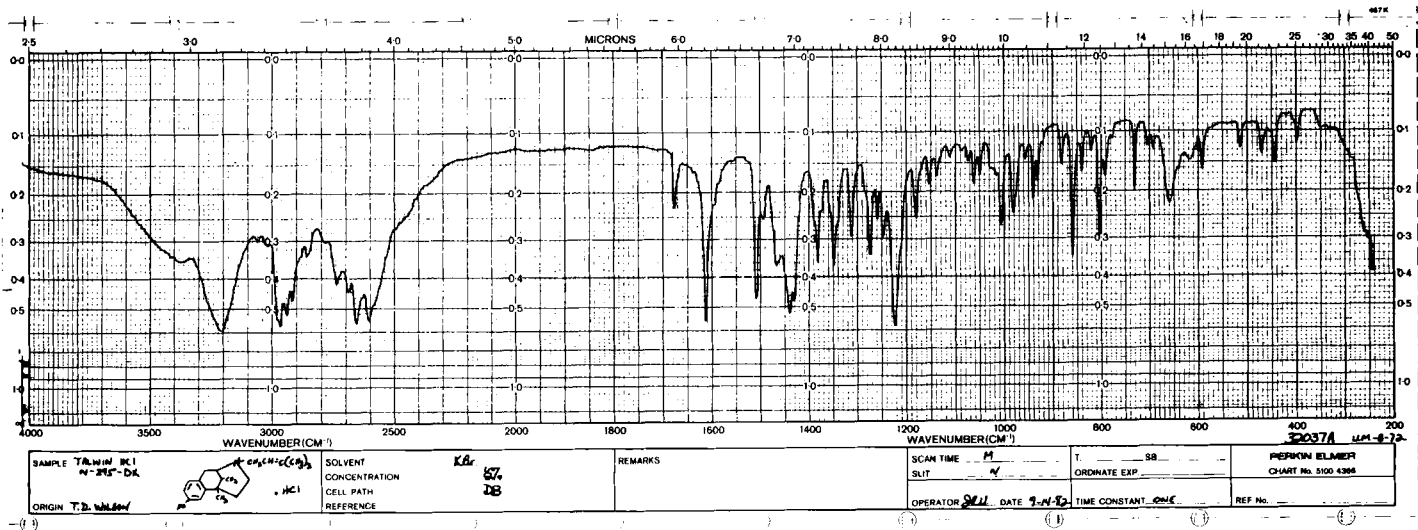


Fig. 7. Infrared spectrum of pentazocine hydrochloride.

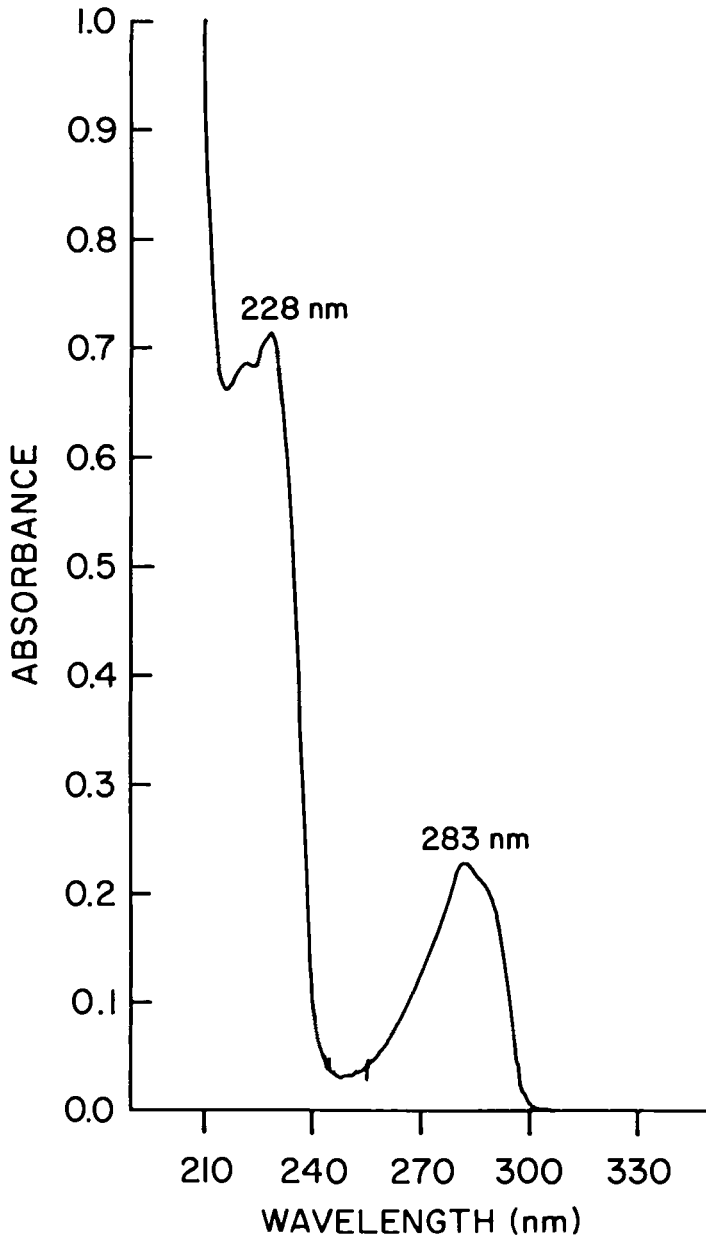


Fig. 8. Ultraviolet absorption spectrum of pentazocine.



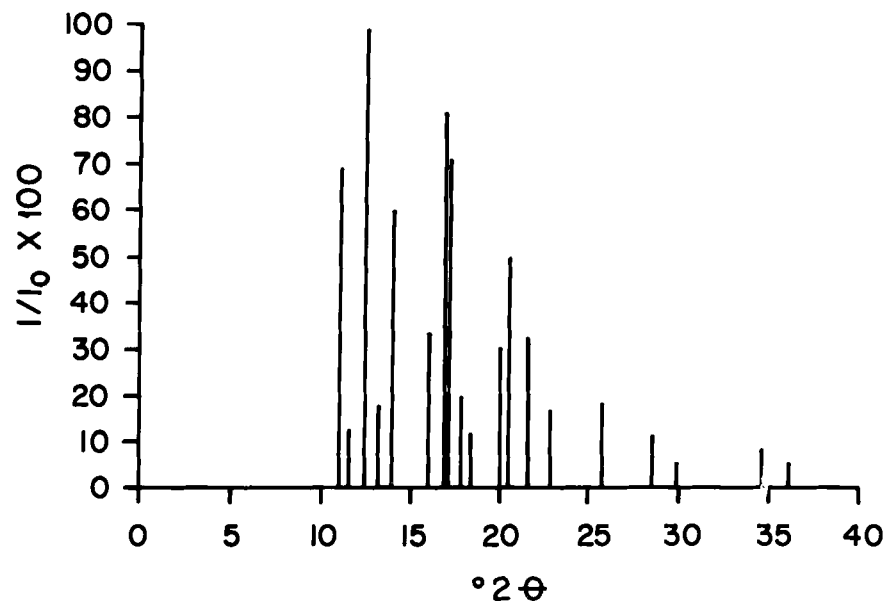


Fig. 9. Normalized x-ray powder diffraction pattern of pentazocine.

TABLE 9

## PENTAZOCINE d SPACINGS

$d(\text{\AA}) = \frac{\lambda}{2} \sin \theta = 1.54 \text{\AA}$		
$2 \theta^\circ$	$d(\text{\AA})$	$I/I_0$
11	8.03	69.69
11.5	7.68	13.13
12.4	7.12	100
13.15	6.72	18.18
13.95	6.34	60.6
15.95	5.54	34.34
16.85	5.25	81.81
17.1	5.17	71.71
17.75	4.99	20.2
18.35	4.82	12.12
20.0	4.43	31.31
20.45	4.33	50.5
21.55	4.11	33.33
22.85	3.88	17.17
25.75	3.45	19.19
25.85	3.44	15.15
28.5	3.12	12.12
29.9	2.98	6.06
34.6	2.58	9.09
36.05	2.48	6.06

Twenty-five mL of test solution is first shaken with a strongly basic anion exchange resin. Five mL of the supernatant is then extracted with a mixture of 20 mL chloroform and 10 mL of 1 in 4000 bromocresol purple in glacial acetic acid. A spectrophotometric determination of the ion pair formed between pentazocine and the indicator in the chloroform layer is then made at 408 nm and compared to a similarly extracted pentazocine standard (4).

#### 4.12 Identification

Pentazocine is identified according to USP XX by use of infrared or ultraviolet spectrophotometric methods. In the first a dispersion in KBr of the dried drug exhibits maxima only at the same wavelengths as a similar preparation of USP Pentazocine Reference Standard. In the ultraviolet method a 1 in 12,500 solution of pentazocine in 0.01 N HCl exhibits maxima and minima at the same wavelengths as reference standard. Calculated absorbances at 278 nm of dried drug and standard do not differ by more than 3.0 % (3).

Pentazocine is identified in the combination product: pentazocine hydrochloride and aspirin tablets by a method given in Addendum a, 2nd Supplement of USP XX. A silica gel TLC system is used with the solvent system: ethyl acetate : methanol : formic acid (90:5:5). Detection is by UV, iodine vapor and iodoplatinate spray (4).

### 5. Stability

#### 5.1 Hydrolysis

The hydrolysis reaction of pentazocine is the only chemically degradative pathway which has been observed. An early report described the reaction and characterized the product: 1,2,3,4,5,6-hexahydro-6,11-dimethyl-3-(3-hydroxy-3-methylbutyl)-2,6-methano-3-benzazocin-8-ol which was obtained when pentazocine was heated at 90° for two hours in 1 N HCl. Acidic hydrolysis of pentazocine glucuronide also yielded this product while pentazocine in biological fluids was found to be stable (32). This hydrolysis reaction was in fact used for the determination of pentazocine in human urine (34). The reaction was elucidated by a kinetics study which showed that a carbonium ion pseudo-first order mechanism was involved. The  $k_{obs}$  at 80° at a  $3.9 \times 10^{-2}$  M HCl concentration

was  $3.412 \times 10^{-4} \text{ hr}^{-1}$  with an activation energy of 35.9 kcal mol<sup>-1</sup> and an activation entropy of 27.3 cal (mol deg)<sup>-1</sup> (47).

### 5.2 Dosage Form Stability

The stability of repackaged pentazocine hydrochloride injection was studied to determine the expiration date. An extrapolation of the Arrhenius plot obtained using 40°, 50° and 60° rate constants gave a k of  $5.448 \times 10^{-5} \text{ day}^{-1}$  at 25°. This meant that a 10% loss of potency occurred in 1909 days at 25° (48).

## 6. Methods of Analysis

### 6.1 Titrimetric

Pentazocine can be determined by a titrimetric procedure in which the compound is dissolved in glacial acetic acid. Crystal violet is used as indicator and the solution is titrated to a green end-point with 0.1 N perchloric acid. Each mL of 0.1 N perchloric acid at this point is equivalent to 28.54 mg of pentazocine base (3).

### 6.2 Ultraviolet Spectrophotometry

Pentazocine can be determined in pentazocine hydrochloride tablets by a UV spectral method. The sample is extracted with sulfuric acid, basified, extracted with ether and back-extracted into 1 in 70 dilute sulfuric acid. The absorbance of the solution in 0.5 N H<sub>2</sub>SO<sub>4</sub> is determined at 278 nm and compared to that of the standard preparation. A cation exchange column isolation method can be utilized to determine pentazocine in pentazocine lactate injection. Pentazocine is eluted from the column with methanol: 6 N HCl (1:1). Sample absorbance is read at 278 nm and compared to a pentazocine standard at approximately 120 μg/mL (3).

### 6.3 Fluorescence Spectrophotometry

Highly sensitive spectrofluorometric measurements have been used extensively in pentazocine analysis especially in measurements from biological sources. The method developed early by Berkowitz, Way and coworkers (27,49,50,51,52) has been modified and extended by other investigators (53,54,55,56). Pentazocine levels in plasma, urine, brain, and intestine have been determined by this method. Briefly the tissue or fluid is basified with a

carbonate/bicarbonate buffer at pH 8-9 and the pentazocine is extracted into benzene. An aliquot is washed with 0.2 M sodium phosphate buffer, pH 6.7 and is then back-extracted into 0.2 N HCl. The fluorescence excitation wavelength used most commonly has been 278 nm with emission measured at 310 nm.

An extraction, ion-pair partition chromatography separation method was developed by Borg and Mikaelsson for pentazocine using fluorescence detection (25) and has been used by others (57,58). The tissue, plasma, brain or serum was extracted with benzene following basification with a carbonate/bicarbonate buffer at pH 10.5. An ethanolized cellulose column was used with a stationary phase of 0.1 M HCl and a mobile phase: cyclohexane: 1-pentanol (6:4) followed by extraction into 0.1 M phosphoric acid for fluorescence measurements.

Other fluorescence methods for pentazocine have been used on plasma (59) and for TLC quantitation (47). A fluorescent 2-p-chlorosulfophenyl-3-phenylindone (DIS-C1) derivative of pentazocine has been measured by TLC (60) while another extraction method using heptane with 0.5% isoamyl alcohol on basified plasma has been developed. In the latter, fluorescence was measured after back-extraction into 0.1 N HCl (61).

#### 6.4 Radioimmunoassay

Radioimmunoassay methods have been developed for pentazocine which were shown to be both sensitive and specific. An early method utilized tritium labeled pentazocine at 1.5 Ci/mmol. Antibodies were produced in rabbits to both an azobenzoic acid and a carboxymethyl derivative of pentazocine coupled by carbodiimide condensation to poly-L-lysine. No cross reactivity was found for metabolites and benzomorphan analogs to the former antiserum with only conjugated pentazocine cross-reacting with the latter antiserum (62).

In a second method rabbit antibodies were produced against a butanoic acid derivative of pentazocine which was conjugated to bovine serum albumin via a carbodiimide condensation. An <sup>125</sup>I labeled methyltyrosine pentazocine giving 25,000-

30,000 cpm/ 100  $\mu$ L was used in the assay. An approximate detection limit of 1 ng/mL was measured (63).

Antiserum specific for the biologically more active *l*-pentazocine has been produced in rabbits using an *l*-pentazocine-2'-carboxymethyl ether-bovine serum albumin conjugate. A low 0.084% cross-reactivity was found for *d*-pentazocine while 51.2% was measured for *dl*-pentazocine (22).

## 6.5 Chromatography

### 6.51 Thin Layer Chromatography

Extensive use has been made of TLC separation for pentazocine on both qualitative and quantitative levels. Table 10 indicates mobile and stationary phases used and  $R_f$  values obtained.

### 6.52 Gas Chromatography

Gas chromatography has been the most frequently used method for pentazocine analysis as indicated by published accounts. It has found especially wide spread application in the field of biological disposition of pentazocine. Table 11 summarizes column packing, detection, derivatization, percent recoveries and detection limits where applicable.

### 6.53 High-Performance Liquid Chromatography

Several reports of high-performance liquid chromatographic analysis of pentazocine have appeared. The separations have been made using both normal phase and reversed-phase systems. Detection was by ultraviolet, fluorescence and electrochemical means. UV measurements were generally made at 278-280 nm on underivatized pentazocine although a greater sensitivity was found using a 2-p-chlorosulfophenyl-3-phenylindone derivative (91). Recoveries found for particular processes described include 78% by extraction from blood (91) and 98.6 and 98.9% on pentazocine tablet extractions by normal and reversed-phase methods respectively (92).

Fluorescence measurements were made on a dimer of pentazocine produced by on-column oxidation with 0.04 M  $K_3Fe(CN)_6$ . This product was separated from a dihydromorphine fluorescent dimer and a pentazocine-dihydromorphine product (93).

TABLE 10

## THIN LAYER CHROMATOGRAPHY OF PENTAZOCINE

Stationary Phase	Mobile Phase	Visualization	R <sub>f</sub>	Reference
Silica gel glass microfiber sheets	EtOAc:cyclohexane:MeOH:NH <sub>4</sub> OH (56:40:0.8:0.4)	iodoplatinate and iodine/ KI	.35-.42	64
Silica gel	nBuOH:HOAc:H <sub>2</sub> O (4:1:2)	Folin-Ciocalteu and 20% Na <sub>2</sub> CO <sub>3</sub>	.60	50
	benzene:MeOH:28%NH <sub>3</sub> (79:21:1)		.35	
Silica gel G	EtOAc:triethylamine (9:1)	Dragendorff and iodine	.62	32
	HOAc:EtOAc:acetone (3:5:5)	vapor	.33	
Kieselgel 60 F254	EtOAc:ether:Et <sub>2</sub> NH (10:10:1)	fluorescence (280/380nm) iodine vapor	.4	47

Silica gel G	benzene:MeOH:28% NH <sub>4</sub> OH (79:20:1)	Folin-Ciocalteu 20% Na <sub>2</sub> CO <sub>3</sub>	.30	49
	nBuOH:HOAc:H <sub>2</sub> O (4:1:2)		.62	
Silica gel	MeOH:H <sub>2</sub> O (95:5)	2-p-chlorosulfo- phenyl-3-phenyl- indone derivative Na in MeOH:DMSO (8g:100:8)	.27	60
Silica gel G- 254	EtOAc:MeOH:NH <sub>4</sub> - OH (85:10:5)	ninhydrin	.68	34
	EtOAc:MeOH:H <sub>2</sub> O (80:15:5)	iodoplatinate fluorescamine		
	hexane:EtOAc:EtOH: NH <sub>4</sub> OH (45:50:5:2)		.57	
Silica gel	EtOAc:MeOH:formic acid (90:5:5)	iodine vapor iodoplatinate	-	4

(Continued)



TABLE 10 (Continued)

## THIN LAYER CHROMATOGRAPHY OF PENTAZOCINE

Stationary Phase	Mobile Phase	Visualization	R <sub>f</sub>	Reference
Silica gel G	MeOH:NH <sub>3</sub> (100:1.5) benzene:MeOH:28%NH <sub>3</sub> (79:20:1)	radiochromatog. Folin-Ciocalteu diazotized p-nitro- aniline, naphtha- resorcinol	.67 .53	65
Silica gel F-254	benzene:MeOH:isopropylamine (95:5:3)	liquid scintillation	-	66
Preparative Silica gel G	dioxane:MeOH:formic acid (100:5:5) benzene:MeOH:isopropylamine (95:5:3)	radiochromatog.	-	67
Silica gel F-254	benzene:MeOH:isopropylamine (95:5:3)	liquid scintillation	-	68

Silica gel	benzene:MeOH:NH <sub>4</sub> - OH (80:20:1)	radiochromatog.	.52	26
	EtOAc:Et <sub>3</sub> N (90: 10)	-	-	
Silica gel glass microfiber sheets	EtOAc:cyclohex- ane:NH <sub>4</sub> OH:MeOH: H <sub>2</sub> O (70:15:2: 8:0.5)	ninhydrin 0.5% H <sub>2</sub> SO <sub>4</sub> iodoplatinate ammoniacal AgNO <sub>3</sub>	-	69
Silica gel G and GF	benzene:MeOH:iso- propylamine(95:5: 3)	UV iodine vapor Dragendorff	.65	70
	dioxane:H <sub>2</sub> O:HOAc (100:2:5)		.45	
	dioxane:H <sub>2</sub> O:NH <sub>3</sub> (100:15:15)		.87	
	dioxane:MeOH:formic acid (100:5:5)		.70	
	EtOAc:Et <sub>3</sub> N (90:10)		.57	

(Continued)

TABLE 10 (Continued)

## THIN LAYER CHROMATOGRAPHY OF PENTAZOCINE

Stationary Phase	Mobile Phase	Visualization	R <sub>f</sub>	Reference
Silica gel glass microfiber	EtOAc:cyclohexane:MeOH:NH <sub>3</sub> (70:15:10:5) develop to 9 cm then: EtOAc:cyclohexane:NH <sub>3</sub> (50:40:0.1) to 14.5 cm	ninhydrin iodoplatinate iodine/KI	.60	71
Kieselgel 60/ Kieselgur F <sub>254</sub>	benzene:MeOH:isopro-UV pylamine (95:5:3) dioxane:MeOH:formic acid (100:5:5) EtOAc:Et <sub>3</sub> N (90:10) benzene:MeOH:NH <sub>3</sub> (79:20:1)	iodoplatinate Dragendorff Folin-Ciocal- teau	.73 .79 .65 .72	39

TABLE 11

## GAS CHROMATOGRAPHY OF PENTAZOCINE

Column	Detection	Derivatization	Col. Temp.°C	Detection Lim.	%Recovery	Ref.
1.5% OV-1 3% OV-17	mass frag- mentography	trifluoroacet- yl	-	-	-	37
3% OV-17	nitrogen	-	220	0.5 $\mu$ g/mL	81	72
1.5% OV-101	F.I.D. mass spect- ral	trimethylsilyl permethyl	100-320	-	-	40
3% OV-17	mass spect- ral	trifluoroacet- yl	180-260	10 ng/mL	89	36
OV-1	<sup>63</sup> Ni E.C.	pentafluoropro- pionyl	220	-	-	73
3% OV-1	F.I.D.	-	210	25 ng	96-100	74
2.5%SE-30	F.I.D.	-	200	-	93-98	75
2.5%SE-30	F.I.D.	-	220	.5 $\mu$ g/mL	97	76
5% QF-1	nitrogen	-	206	2.4 ng	100.3	57

(Continued)

TABLE 11(Continued)

## GAS CHROMATOGRAPHY OF PENTAZOCINE

Column	Detection	Derivatization	Col. Temp.°C	Detection	Lim.	%Recovery	Ref.
5% OV-17	<sup>63</sup> Ni E.C. F.I.D.	pentafluoro- benzyl	235 220	3 pg	-	-	38
3%Dexsil 300	<sup>63</sup> Ni E.C.	pentafluoro- benzyl	265	5 ng	-	-	77
10% OV-1	<sup>3</sup> H E.C. F.I.D.	heptafluorobutyryl trimethylsilyl	215 245	5 ng 0.5 μg/mL	66.4-95	-	78
2.5%SE-30	F.I.D.	acetyl	200	-	-	-	32
4%Apiezon L	F.I.D.	-	250	5 ng/mL	65-95	-	79
5% QF-1	nitrogen	-	206	2.4 ng	100.3	-	65
3% OV-17	F.I.D.	trimethylsilyl	90-220	10 ng	79.9	-	80
3% OV-1	F.I.D.	trimethylsilyl	225	-	-	-	81
3% SE-30	mass frag- mentography	-	210	2 ng/mL	92-94	-	33

5% OV-17	<sup>63</sup> Ni E.C.	pentafluorobenz- yl	250	125 pg	-	82
3% OV-1	F.I.D.	-	210	150 ng	-	83
5% OV-17	<sup>63</sup> Ni E.C.	pentafluorobenz- yl	235	5 ng/mL	-	84
2.5%SE-30	F.I.D.	-	200	-	-	85
2.5%SE-30	F.I.D.	-	200	.05 μg/mL	93-98	86
3% OV-1 3% OV-17	F.I.D.	trimethylsilyl	230-250	-	-	70
OV-17	F.I.D.	-	210	-	-	49
3% OV-17	mass spectral	trifluoroacet- yl	235	20 ng/mL	80-85	35
3% OV-17 2% OV-17	F.I.D. mass spectral	-	230 150-250	-	-	34

(Continued)

TABLE 11(Continued)

## GAS CHROMATOGRAPHY OF PENTAZOCINE

Column	Detection	Derivatization	Col. Temp.°C	Detection Lim.	%Recovery	Ref.
3% SE-30	F.I.D.	-	130-290	-	-	87
3% OV-7			150-290			
3% OV-17						
3% OV-1	F.I.D.	trimethylsilyl	230	-	-	39
	mass spect- ral					
5% SE-30	F.I.D.		260			
OV-101	nitrogen	pentafluoropro- pionyl	220	-	-	88
3% Dexsil 300	<sup>63</sup> Ni E.C.	pentafluorobenz- yl	265	5 ng	-	89
3% SE-30	F.I.D.	-	130-290	-	-	90

Glassy carbon working electrodes were used in electrochemical detection of pentazocine while no response was measured in one study at an operating potential of 0.8 V (94). Table 12 summarizes the HPLC column and mobile phase conditions utilized in reported investigations.

## 7. Biological Disposition and Pharmacokinetics

### 7.1 Disposition

The absorption, distribution, metabolism and excretion of pentazocine have been widely studied in both man and animal species. Early work was carried out by three major groups: Pittman and coworkers, Beckett and coworkers and Berkowitz, Way and coworkers. Reviews of metabolism (51) and pharmacokinetics (97) of pentazocine have been published. The effect of route of administration on the disposition of pentazocine has been explored (27,49,75,85,86). The metabolic pathways available to pentazocine are outlined in Figure 10. Also shown are urinary excretion data for the metabolites expressed as percent of administered dose. A rhesus monkey study is also included (66) which species shows the trans-alcohol metabolite excreted as well. Urinary excretion consists of unchanged pentazocine and metabolites plus phenolic glucuronides of each. Fecal excretion consists of less than 1% administered dose (86).

Differences in pentazocine disposition in smokers and nonsmokers has been observed with the former metabolizing 40% more rapidly(98).The effect of cirrhosis on pentazocine bioavailability has also been studied. A 46% decrease in clearance and a 233-278% increase in bioavailability of pentazocine for these patients was found (84,89). Other disposition studies have been done on post-operative patients (59), mother and infant at time of birth (99) and normal subjects with circadian variation (36).

### 7.2 Pharmacokinetics

The pharmacokinetics of pentazocine have been investigated in humans (11,76,86). Table 13 shows values for half-life, volume of distribution, bioavailability and clearance calculated by various authors. The plasma half-life values obtained range from 2 to 5.7 hours. The possibility that



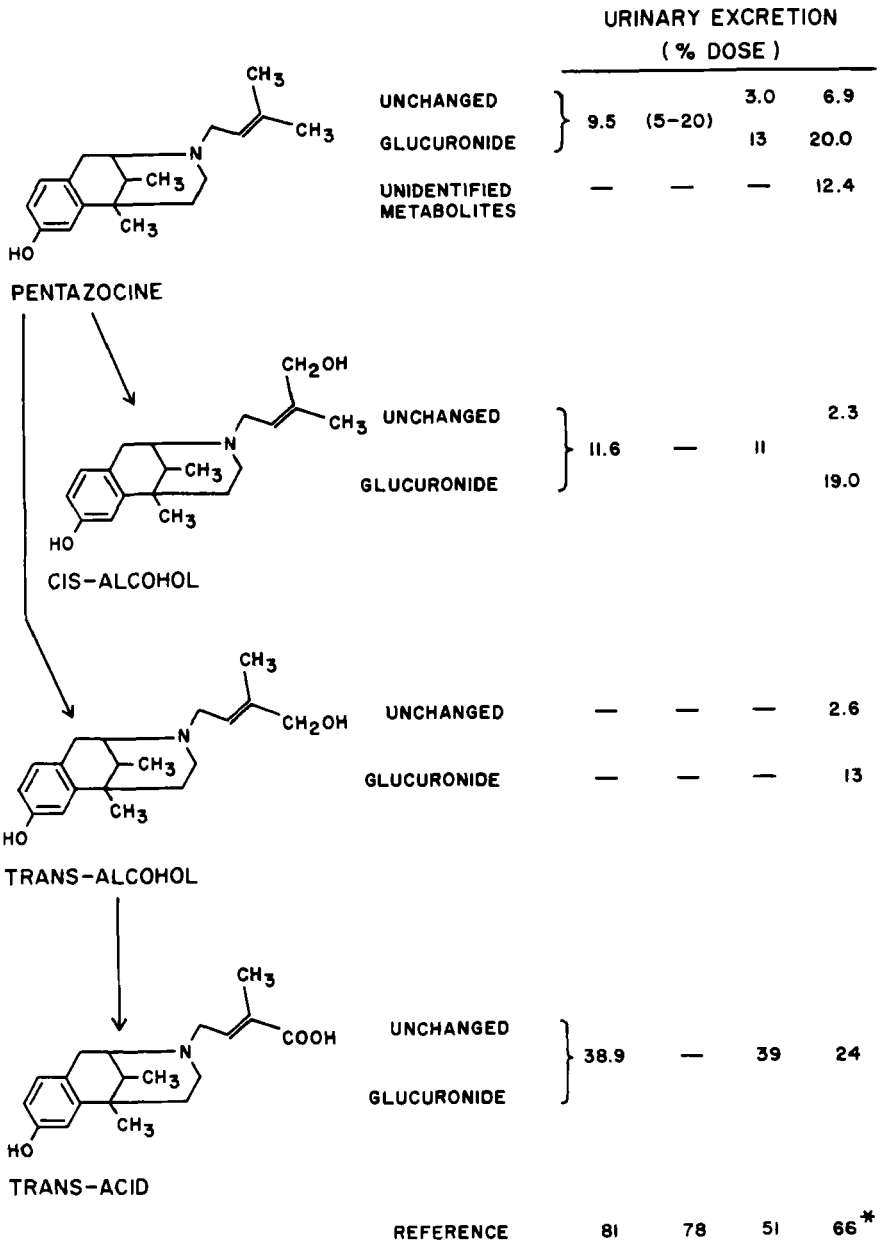
TABLE 12

## HPLC ANALYSIS OF PENTAZOCINE

Detection	Column	Mobile Phase	Mobile Phase pH	Reference
UV-278 nm	$\mu$ Bondapak C <sub>18</sub>	methanol:water (66: 34) PIC B7	3.5	48
UV-280 nm	$\mu$ Bondapak C <sub>18</sub>	acetonitrile:0.7% NH <sub>4</sub> Cl (80:20)	8.0	91
UV-254 nm	$\mu$ Bondapak C <sub>18</sub>	methanol:water (28: 12) (0.0048 M K <sub>2</sub> HPO <sub>4</sub> , 0.0077M KH <sub>2</sub> PO <sub>4</sub> )	7.0	28
UV-254 nm	$\mu$ Bondapak C <sub>18</sub>	methanol:water (20: 80), 0.05 M tetra- butylammonium hydrox- ide, H <sub>3</sub> PO <sub>4</sub> pH adjust- ment	6.1	94
UV-280 nm	Partisil 5 $\mu$ Silica	CHCl <sub>3</sub> :methanol:iso- propylamine (960:40: 2)	-	92
UV-280 nm	$\mu$ Bondapak C <sub>18</sub>	sodium octanesul- fonate (0.005 M): methanol: H <sub>3</sub> PO <sub>4</sub> (600: 400:1)	2.4	92

fluorescence 320/436 nm	Partisil porous silica	methanol: 2N NH <sub>4</sub> OH: 1 N NH <sub>4</sub> Cl (30:20: 10)	9.5	93
electrochem. 0.6 V, 50 nA/V	Syloid 74 silica	methanol:ammonium nitrate buffer (9: 1)	10.2	95
electrochem. 0.8 V, 5-20 nA/ V	$\mu$ Bondapak C <sub>18</sub>	methanol:water (20: 80), 0.05 M tetra- butylammonium hydrox- ide, H <sub>3</sub> PO <sub>4</sub> pH adjust- ment	6.1	94
electrochem. 1.0 V	LiChrosorb ODS	methanol:0.01 M KH <sub>2</sub> PO <sub>4</sub> (85:15)	4.6	96

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\* Rhesus Monkey

Fig. 10. Pentazocine metabolic pathways and urinary excretion products in man and monkey.

TABLE 13

## HUMAN PENTAZOCINE PHARMACOKINETICS PARAMETERS

Half-life (hr)	Volume of Distribution (L)	Oral Bioavail- ability (%)	Clearance (L/min)	Reference
3.4	396	18.4	1.38	100
3.8	342	18	1.25	84
2.41	258	-	1.741 day	36
2.20	265	-	1.672 night	
5.7	5.53 L/kg	21	0.768	89
3.7	-	-	-	75
3.2	-	-	-	85
2	-	-	-	49
2.2	208	-	-	33
2	-	-	-	59

the intestine metabolizes pentazocine to account for the low bioavailability was shown in a rabbit jejunum study. Here sulfation and glucuronidation occurred by saturable processes (101). Data in reference 76 was fit to a three compartment open model with a determination of metabolic, absorption and elimination rate constants from urinary excretion rates. Data calculated to fit the equation:

$$C_p = A e^{-\alpha t} + B e^{-\beta t}$$

for a two compartment open model for pentazocine is shown in Table 14. It was shown that circulating pentazocine is distributed with 48% in blood cells, 33% bound to plasma protein and 19% in plasma water (67).

Studies of pentazocine biological disposition in animals consist of tissue uptake (26,52,58,65,68,79), blood level (50,61,74) and urine level determinations (88). While animal metabolic pathways identified (39,40,70) are similar to those found in man, additional products have been demonstrated. In the rat 9-methoxypentazocine (or its 8-methoxy-9-hydroxy isomer), 8,9 (or 9,8)-methoxyhydroxy metabolite of the cis-alcohol product and 8,9 (or 9,8)-methoxyhydroxy metabolite of the trans-alcohol product were found. Pharmacokinetic studies have been done in rhesus monkey (66) and in the dog (35,54,63). Typical plasma half-lives measured in dog plasma are 1.2-1.6 hours. A day-night variation was also observed in the calculated parameters (35).

#### 8. Determination in Body Tissues and Fluids

Pentazocine and its metabolites have been determined in human and animal tissues and fluids by a variety of analytical methods. References for these techniques are shown in Table 15A for humans and Table 15B for animal studies.

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TABLE 14

## TWO COMPARTMENT OPEN MODEL PARAMETERS FOR PENTAZOCINE

A (ng ml <sup>-1</sup> )	B (ng ml <sup>-1</sup> )	$\alpha$ (hr <sup>-1</sup> )	$\beta$ (hr <sup>-1</sup> )	$k_{12}$ (hr <sup>-1</sup> )	$k_{21}$ (hr <sup>-1</sup> )	$k_e$ (hr <sup>-1</sup> )	Ref.
553	102	8.82	0.0942	5.81	1.638	1.668	33
495	108	11.39	0.357	7.357	2.74	1.653	36*
324	98	10.58	0.349	6.65	2.98	1.32	36**

\* day  
 \*\* night

TABLE 15

## REFERENCES FOR PENTAZOCINE DETERMINATION IN BODY TISSUES AND FLUIDS

## A) HUMAN STUDIES

Method:	GC	GC/MS	TLC	HPLC	fluorescence	RIA
<u>Tissue/Fluid</u>						
Blood	72,75,77, 84,99,85, 67,86,89, 90	-	60	91	-	-
Plasma	73,77,78, 67,7,84, 99,49	36,38,33	49,60	-	59,100	62,63
Urine	75,76,77, 81,67,85, 86,89,90	34	64,102	93	27	62
Feces	75,86	-	-	-	-	-
Cerebro- spinal Fluid	-	33	-	-	-	-

B) ANIMAL STUDIES

Method:		GC	GC/MS	TLC	fluorescence	RIA
<u>Species</u>	<u>Tissue/Fluid</u>					
baboon	brain	79	-	-	-	-
	plasma	79	-	-	-	-
cat	bile	-	-	26	-	-
	plasma	-	-	-	61	-
	urine	-	-	26	-	-
dog	CSF	-	-	-	54	-
	plasma	-	36	-	54	63
goat	plasma	-	-	-	61	-
horse	urine	88	-	-	-	-
monkey	brain	66	-	68	-	-
	plasma	66	-	68	-	-
	urine	-	-	66	-	-

(Continued)



TABLE 15 (Continued)

## REFERENCES FOR PENTAZOCINE DETERMINATION IN BODY TISSUES AND FLUIDS

## B) ANIMAL STUDIES

Method:		GC	GC/MS	TLC	fluorescence	RIA
<u>Species</u>	<u>Tissue/Fluid</u>					
mouse	bile	65	-	-	-	-
	brain	-	-	-	25, 58	-
	gut	65	-	-	-	-
	plasma	-	-	-	25	-
	serum	-	-	-	58	-
pony	plasma	-	-	-	61	-
rabbit	blood	57	-	-	-	-
rat	bile	-	40	50	50	-
	brain	74	-	-	52	-
	gut	-	-	-	53	-
	plasma	74	-	-	52	-
	urine	80	39	39	-	-
	wbc	103	-	-	-	-
swine	plasma	-	-	-	61	-

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The present literature review includes material published through July, 1982.

# PHENYTOIN

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## 1. General Information

### 1.1 Nomenclature

#### 1.11 Chemical Names

5,5-Diphenyl-2,4-imidazolidinedione  
5,5-Diphenylhydantoin

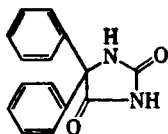
#### 1.12 Generic Names

Phenytoin, diphenylhydantoin

1.13 Trade Names - Dantoin, Diphentyn, Divulsan, Novodiphenyl (all Canad.); Difhydan, Fenantoin (both Swed.); Di-Hydan, Pyoredol, Solantyl (all Fr.); Dilabid, Ekko, Kessodanten (all USA); Dilantin (Austral., Canad., USA); Ditoin, Phentoin (both Austral.); Phenhydan, Zentropil (both Ger.); Toin Unicelles (S. Afr.)

### 1.2 Formulas and Molecular Weight

$C_{15}H_{12}N_2O_2$  Molecular Weight = 252.26



### 1.3 Description

White or almost white crystalline powder.

### 1.4 Forms in Official Compendia

The USP contains Phenytoin, Phenytoin Sodium and the following formulations:

Phenytoin Oral Suspension

Phenytoin Tablets

Extended Phenytoin Sodium Capsules

Prompt Phenytoin Sodium Capsules.

USP reference standards for Phenytoin and Phenytoin Sodium are available.

## 2. Physical Properties

### 2.1 Spectra

2.11 Infrared: The infrared spectrum of phenytoin is presented in Figure 1. The band assignments<sup>1</sup> are summarized in Table 1.

Table 1: Infrared Band Assignments for Phenytoin.

<u>Wavenumber</u> ( $\text{cm}^{-1}$ )	<u>Assignment</u>
3275, 3205	N-H stretching
3064	aromatic C-H stretch
1774, 1740, 1719	carbonyl stretching vibrations of hydantoin ring
1719, 1599, 1496, 1450	phenyl ring breathing vibrations
1403	C-N stretching vibration
747, 690	C-H out of plane vibrations of monosubstituted phenyl

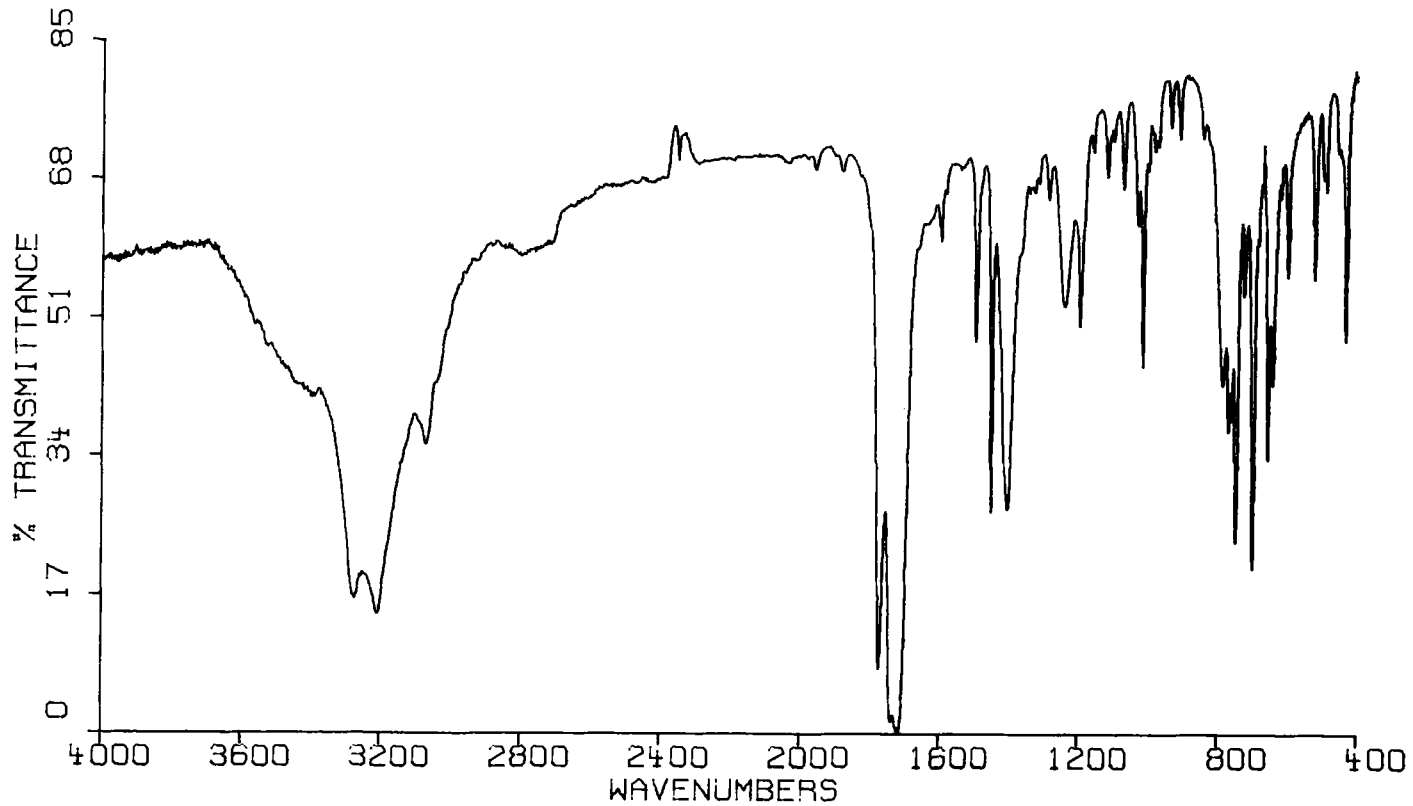


Fig. 1. Infrared spectrum of phenytoin in KBr.

### 2.12 Nuclear Magnetic Resonance

The Proton NMR spectrum of phenytoin is shown in Figure 2<sup>1</sup> and the spectral assignments are in Table 2.

Table 2: NMR spectral assignments for Phenytoin

<u>Resonance (PPM)</u>	<u>Multiplicity</u>	<u>Integration</u>	<u>Assignment</u>
7.3	singlet	10	phenyl protons
9.2	singlet	1	amide protons
10.9	very broad singlet	1	

### 2.13 Mass Spectrum

The mass spectrum is shown in figure 3<sup>1</sup>. These are the principal ions observed.

<u>Table 3:</u>	<u>m/e</u>	<u>Fragmentation</u>
	252	molecular ion
	223	M-CHO
	209	M-HNCO
	180	M-HNCO-CHO
	175	M-C <sub>6</sub> H <sub>5</sub>
	165	C <sub>13</sub> H <sub>9</sub>
	147	M-C <sub>6</sub> H <sub>5</sub> -CO
	104	C <sub>6</sub> H <sub>5</sub> CN
	77	C <sub>6</sub> H <sub>5</sub>

### 2.14 Ultraviolet Spectrum

The UV spectrum of phenytoin in methanol is given in Figure 4<sup>1</sup>. The absorptivity,  $a$ , at 258 nm is 2.93.

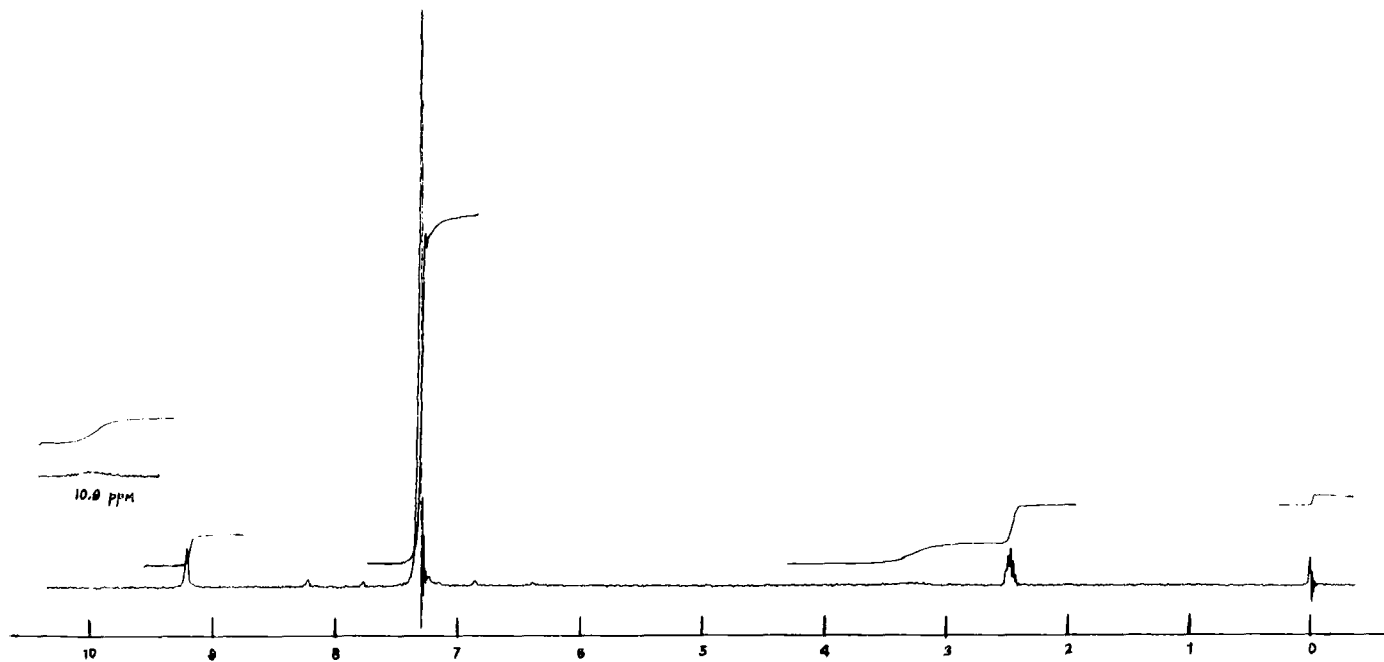


Fig. 2. PMR spectrum of phenytoin in DMSO d<sub>6</sub>.

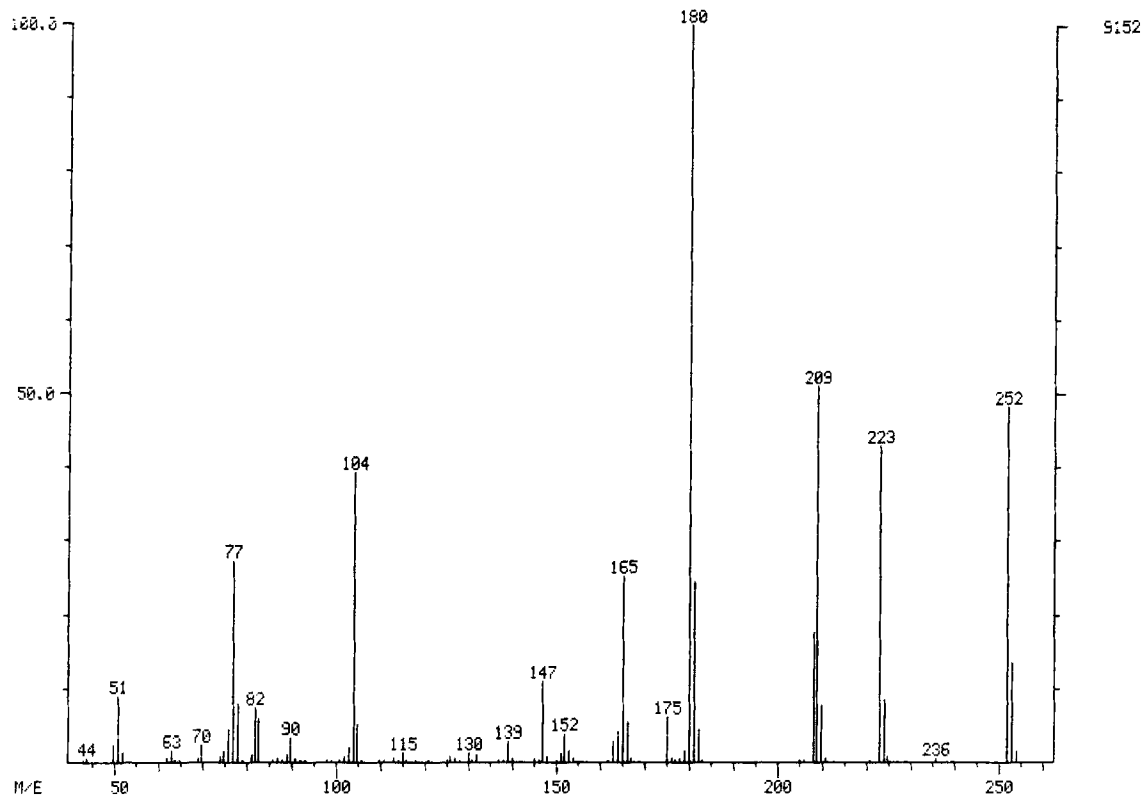


Fig. 3. Electron impact mass spectrum of phenytoin.

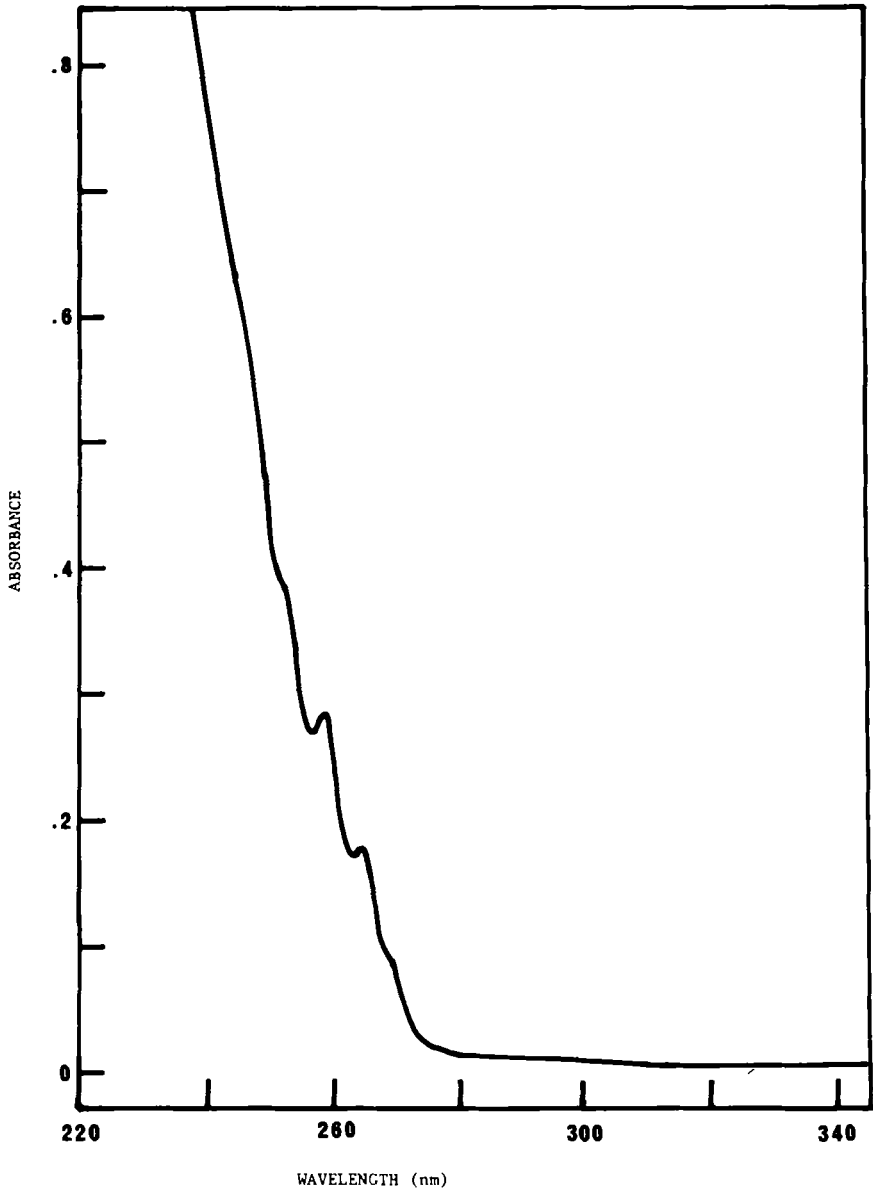


Fig. 4. Ultraviolet absorbance spectrum of phenytoin in methanol.

## 2.2 Crystal Properties

### 2.21 Crystal Morphology

Microcrystallographic characteristics of Phenytoin were determined and reported by G. L. Keenan<sup>2</sup>. Rodlike plates and fine needles of Phenytoin were examined with a polarizing microscope. In parallel polarized light the extinction is parallel and the sign or elongation negative. In convergent polarized light no interference figures are observed. Refractive indices:  $\alpha = 1.600 \pm 002$ ;

$$\gamma = 1.635 \pm 0.002.$$

### 2.22 Melting Range

Phenytoin melts at 295-298°.

2.3 Solubility - Phenytoin is practically insoluble in water, 1 g. dissolves in about 60 ml alcohol, about 30 ml acetone. The compound is soluble in alkali hydroxides.

Hong et al<sup>3</sup> studied the solubility of phenytoin in buffers of different pH values.

<u>pH</u>	<u>Solubility (mg/ml)</u>
1.6	0.02
4.4	0.02
5.0	0.01
5.9	0.02
6.9	0.02
8.0	0.01
9.0	0.10
10.0	0.96
11.0	9.6
12.0	96.0



## 2.4 Dissociation Constant

The ionization constant, pKa, of phenytoin was determined to be 8.31 by ultraviolet spectrophotometry and 8.33 by potentiometric titration<sup>4</sup>. A value of 8.06 was reported by Schwartz et al.<sup>5</sup>

## 2.5 Partition Coefficients

Riedel et al<sup>6a</sup> determined partition coefficients (K) for phenytoin between a few selected organic solvents and aqueous solutions at three different pH values. The results are tabulated in Table 4.

Table 4. Partition coefficients (K) of phenytoin (DPH), between aqueous layers pH 4-10 and organic solvents; DCE = dichloroethane,  $K = \frac{C_{\text{organic}}}{C_{\text{aqueous}}}$ .

pH	K (DPH) in:		
	CH <sub>2</sub> Cl <sub>2</sub>	CHCl <sub>3</sub>	DCE
4	26.0	10.1	13.5
5	26.0	9.9	13.5
6	15.7	3.2	10.1
7	4.6	4.3	9.0
8	3.0	2.0	9.0
9	1.0	1.0	3.2
10	0.37	0.56	0.70

K values obtained by Philip<sup>6b</sup> between chloroform and aqueous buffers of pH 4, 5 and 6 are given below:

<u>Solvent</u>	<u>K</u>		
	<u>pH = 4.0</u>	<u>pH = 5.0</u>	<u>pH = 6.0</u>
Chloroform	28.9	24.1	18.9

In Table 5, the partition ratios of phenytoin with six solvents and aqueous buffers at three different pH values determined by Godhart et al<sup>6c</sup> are given.

Table 5. Partition ratios K (concentration in solvent/concentration in aqueous phase).

Solvent	K		
	pH 3.4	pH 7.4	pH 12.4
Hexane	0.008	0.004	0.0001
Toluene	4.11	2.59	0.001
Chloroform	28.8	25.4	0.007
Dichloroethane	28.7	26.0	0.02
Ether	40.8	30.3	0.009
Ethylacetate	197	178	-

Similar results were obtained by Goldberg and Todoroff<sup>6d</sup> in 0.1 M phosphate buffer of pH 7.0, (Table 6).

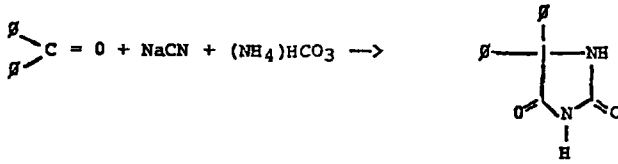
Table 6. Partition coefficients(K) in pH 7.0.

Chloroform	25.5
Dichloroethane	24.1
Toluene	4.3
Hexane	0.02

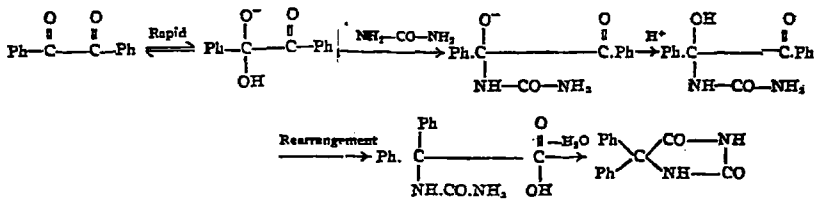
### 3. Synthesis

Phenytoin can be synthesized in several ways:

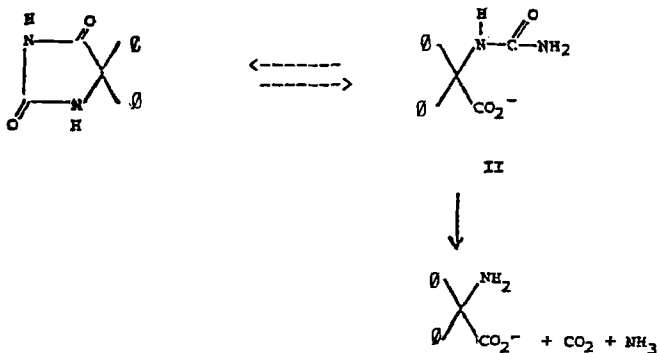
U.S. Patent 2,409,754 (1946) to H. R. Henze, Parke-Davis and Co. involves reacting benzophenone, potassium cyanide and ammonium carbonate in 60% ethanol. It can also be prepared by reacting benzophenone, sodium cyanide and ammonium bicarbonate according to Scheme 1.



SCHEME 1 - Synthesis of Phenytoin (Henze)



SCHEME 2 - Synthesis of Phenytoin (Biltz)



SCHEME 3 - Hydrolysis of Phenytoin

Biltz<sup>7</sup> prepared phenytoin by heating urea with a previously prepared alkaline alcoholic solution of benzil. Sikdar and Ghosh<sup>8</sup> studied the mechanism of the Biltz technique and has shown the reaction to take place as shown in Scheme 2.

#### 4. Stability and Degradation

Phenytoin is very stable even under conditions of extreme stress. After refluxing phenytoin in 2.5 N hydrochloric acid for seven hours, essentially complete recovery of starting material was obtained by Fusari.<sup>9</sup> Duschinski<sup>10</sup> reported that phenytoin, heated 24 hours at 170-180° in 20% sodium hydroxide (5N) gave an 82% yield of diphenylglycine. Hong<sup>3</sup> determined a 75% conversion to diphenylglycine for a solution of phenytoin sodium heated at reflux in 2N sodium hydroxide for five days. He found detectable amounts of benzhydrol in phenytoin solutions heated in 0.2 N sodium hydroxide in ampules in a 105° oven after 75 days. Stability studies on Dilantin® Infusion concentrate, a solution of phenytoin sodium in tetraglycol tromethamine-water, have shown the presence of diphenylhydantoic acid as a decomposition product along with diphenylglycine. Hydrolysis studies of phenytoin in 0.1M phosphate buffer of pH 11 and 12 at 70, 80 and 90° showed decomposition to follow pseudo-first order kinetics. Extrapolation of the Arrhenius plots at 25°C indicated that the times for decomposition of 10% of the drug at room temperature,  $t_{0.9}$  would be about 61 years at pH 11 and 35 years at pH 12.<sup>11</sup>

Alkaline degradation of phenytoin was inferred to proceed via hydrolysis to diphenylhydantoic acid, a reversible reaction, and irreversible hydrolysis of the latter to diphenylglycine (Scheme 3).

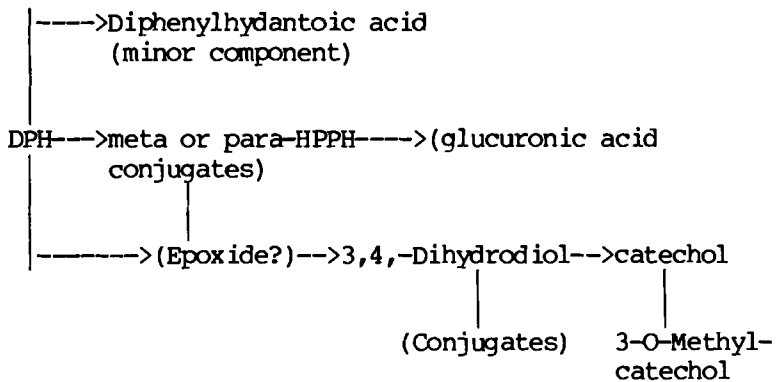
Alkaline permanganate oxidation of phenytoin will result in quantitative yield of benzophenone.

## 5. Metabolism and Pharmacokinetics

Phenytoin is partially metabolized and partially excreted unchanged in the urine. About 4% of the dose is excreted in the urine unchanged; the excretion of unchanged drug is increased when the urine is alkaline.<sup>12</sup>

The principal metabolic products of phenytoin are p-hydroxy-phenyl derivative (HPPH) and a conjugate of HPPH with glucuronic acid (13-21).

The most likely pathways involved in the metabolic disposition of DPH are summarized below:



Effective serum concentrations are in the range 10 to 20 ug/ml; at concentrations in the range 20 to 40 ug/ml, side effects may occur.

Plasma half life varies considerably and is in the approximate range of 7 to 40 hours; at doses above 100 mg, the half life is dose dependent. Phenytoin is widely and rapidly distributed throughout the body; it is secreted in the milk, accumulates in red blood cells and amounts secreted in saliva show a linear relationship to non-protein bound concentrations in serum.

## 6. Identification

1. The infrared absorption spectrum of a potassium bromide dispersion of phenytoin is identical to that of a reference standard, similarly prepared (Figure 1).
2. Dissolve about 100 mg in a mixture of 0.5 ml of 1N sodium hydroxide and 2 ml of a 10% solution of pyridine, add a further 8 ml solution of pyridine, shake, add 1 ml of copper sulfate with pyridine solution and allow to stand for 10 minutes; a blue precipitate is produced<sup>12</sup>. Pennington, et al have described identification data, using microcrystalline, chromatographic, spectrophotometric methods and techniques for color tests.<sup>22</sup>.

## 7. Methods of Analysis

### 7.1 Differential Thermal Analysis

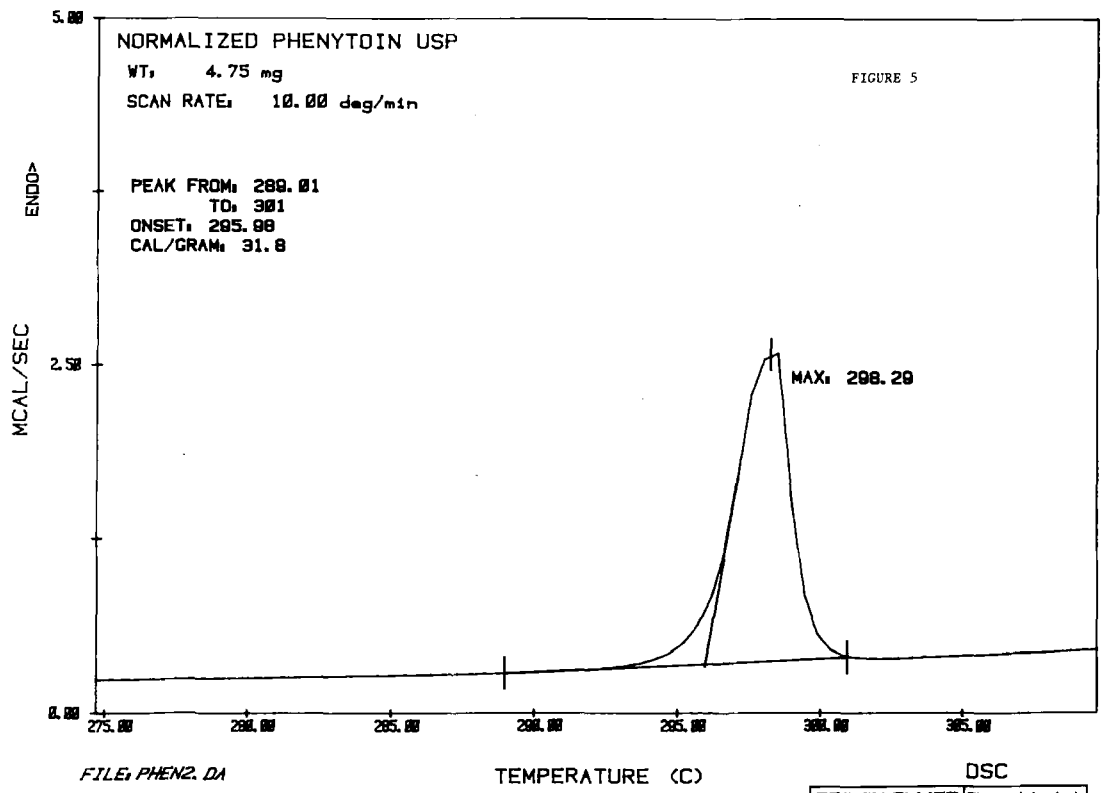
Differential thermal analysis was carried out on a Perkin-Elmer differential scanning calorimeter, model DSC-2C, at a heating rate of 10°/min, in an atmosphere of nitrogen. It gave a single sharp melting endothermic peak at 298.2°C, followed by decomposition (Figure 5).<sup>23</sup>

### 7.2 Thermo-Gravimetric Analysis

TGA analysis<sup>23</sup> was carried out on a Perkin-Elmer Thermogravimetric analyzer, model TGS-2 at a heating rate of 10°/min. It showed no weight loss up to 200°C, indicating non-volatility and absence of solvent of crystallization.

### 7.3 Elemental Analysis

<u>Element</u>	<u>% Calculated</u>
C	71.42
H	4.80
N	11.10



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#### 7.4 Colorimetric

A colorimetric procedure for phenytoin developed by Dill et al<sup>24</sup>, provided the first reliable means for determining blood and tissue levels of this drug. The procedure involves quantitative nitration, reduction of the nitro group to a primary amine, diazotization and coupling with the Bratton-Marshall reagent.

#### 7.5 Spectrophotometric

##### 7.51 Ultraviolet

Ultraviolet absorption in methyl alcohol: maximum at 258 nm;  $a = 2.93$ .

The lack of a well defined UV absorption spectrum for phenytoin makes its determinations by direct UV spectrophotometry difficult; nevertheless, the literature describes several methods which depend upon the spectrum of the unchanged drug<sup>25</sup>. Wallace et al<sup>26</sup> and Fellenberg et al<sup>27</sup> reviewed the use of UV spectrophotometric methods for the analysis of phenytoin. A spectrophotometric method for the quantitative determination of phenytoin has been described by Bgorge et al<sup>28</sup> based on the difference in absorbance of an alcoholic solution at 233 nm before and after conversion of phenytoin to the sodium salt. Methods that require conversion of the drug to benzophenone have been reported<sup>29,30,31,32</sup>. Amer et al<sup>33</sup> have described a single ultraviolet spectrophotometric method for the determination of phenytoin in pharmaceutical preparations. The mean was 100.9% (six determinations) and the relative standard deviation was 1.2%. The method was applied successfully to the determination of phenytoin in capsules and suspensions.



Cunningham and coworkers<sup>34</sup> have described a column chromatographic method for the determination of phenytoin in capsules. The method is based on the acidic nature of phenytoin and permits the separation and direct UV quantitation of the drug. The sample is dissolved in dimethyl sulfoxide and hydrochloric acid, and then diluted to volume with sodium carbonate. An aliquot of this is mixed with Celite and added to a chromatographic column consisting of 0.5 M sodium carbonate adsorbed on Celite. A prewash of 30 percent chloroform-isooctane removes interfering substances and 75 ml chloroform completely removes the drug from the column. The eluate is evaporated and the residue is dissolved in ethanolic HCl and determined spectrophotometrically.

Fusari and coworkers<sup>35</sup> have developed a similar method for the assay of phenytoin and phenobarbital in dosage forms. They have used Silanized Celite 545 as the support material, 25% amyl alcohol in chloroform as the stationary phase and 0.05M borate buffer of pH 9.5 as the mobile phase. Phenobarbital was eluted with the mobile phase and phenytoin eluted with absolute ethanol. Ultraviolet absorption was used to quantitate the substances.

#### 7.52 Fluorometric

A simple fluorometric assay for phenytoin in plasma was reported by Dill et al<sup>36</sup>.

The assay was done directly on 0.2 ml of plasma by treating with alkaline potassium permanganate to form benzophenone, extracting with heptane and shaking the heptane layer with sulfuric acid. The fluorescence of the reaction product was measured with excitation at 360 nm and emission at 440 nm. Detection of 1 ug phenytoin per ml of plasma was reported.

Abrahamsson et al<sup>37</sup> described a fluorimetric microdetermination of phenytoin in plasma. The phenytoin is extracted from acidified plasma and oxidized to benzophenone which is then condensed with N'-methylnicotinamide. The resulting product is strongly fluorescent with excitation at 440 nm and emission at 500 nm.

#### 7.53 Phosphorimetric

The method<sup>38</sup> is based on Wallace method for phenytoin, conversion to benzophenone in an alkaline permanganate medium, exciting at 260 nm and emitting at 446 nm. The limit of detection is 0.05 ug/ml.

#### 7.6 Polarographic

Brooken et al<sup>39</sup> described a sensitive differential pulse polarographic assay for phenytoin in blood. It involves extraction of phenytoin into chloroform followed by nitration. The analysis of the nitro derivative is then accomplished.

#### 7.7 Titrimetric Assay

Weigh accurately about 500 mg of phenytoin and transfer to a 125-ml conical flask. Dissolve in 50 ml of dimethylformamide, add 3 drops of a saturated solution of azoviolet in benzene, and titrate with 0.1N sodium methoxide to a blue end-point. Perform a blank determination, and make any necessary correction. Each ml of 0.1 N sodium methoxide is equivalent to 25.23 mg of  $C_{15}H_{12}N_2O_2$ .<sup>40</sup>

Numerous assay procedures for phenytoin in pharmaceutical preparations have been devised, based on the separation of phenytoin from phenobarbital by ion exchange chromatography or solvent extraction procedure, followed by non-aqueous titration.<sup>41</sup>

## 7.8 Chromatographic

### 7.81 Thin-Layer (TLC)

The TLC system for identification of phenytoin in capsules and tablets<sup>42</sup> is composed of silica gel G plate (Analtech) and chloroform-isopropyl alcohol-ammonia (45-45-10).  $R_f = 0.6$ . Mason<sup>43</sup> has given the  $R_f$  values for Phenytoin in the following systems on silica gel GF plate:

Chloroform-ether-methanol-NH <sub>4</sub> OH (75-25-5-1)	0.48
Ethylacetate-1-propanol-NH <sub>4</sub> OH (40-30-3)	0.83
Methanol-NH <sub>4</sub> OH (100-1.5)	0.86
Ethanol-Acetic acid-H <sub>2</sub> O (60-20-10)	0.95
Benzene	0

Detection of phenytoin on the plate can be achieved by UV light, iodine exposure and Ehrlich's spray followed by Iodoplatinate spray.

Wad et al<sup>44</sup> described a rapid quantitative method for the determination of phenytoin in serum by TLC.

Perhaps the most exacting TLC studies were carried out by Simon et al<sup>45</sup>. Phenytoin was extracted from plasma, concentrated by evaporation, and subjected to TLC on silica gel plates. After the phenytoin spot was scraped off, the colorimetric procedure of Dill et al<sup>46</sup> was applied directly to the scrapings. Phenytoin levels of 5 ug/ml or more could be recognized by visual inspection of the plates under ultraviolet light, and serum levels of 1 ug/ml were detectable with the colorimetric procedure.

### 7.82 Gas Chromatography (GLC)

Glazko<sup>47</sup> reviewed some early studies on the gas chromatographic assay of phenytoin. These included derivatization with diazomethane<sup>48</sup>, the use of trimethylsilyl derivative<sup>49,50</sup>, on column methylation using tetramethyl ammonium hydroxide<sup>51</sup> and trimethylanilinium hydroxide<sup>52</sup>. A large number of GLC procedures have been reported in which phenytoin is chromatographed directly at high temperatures without derivative formation.<sup>53,54,55</sup>

In general the direct GLC methods without derivative formation tend to produce asymmetric peaks which increase the difficulty of quantitation by measurement of peak heights. The on-column methylation techniques appear to be convenient to run and sharper peaks are obtained at lower temperatures.

Cramers et al<sup>56</sup> described high resolution gas chromatography for the quantitative determination of underivatized anti-convulsant drugs with support coated open tubular columns. A siliceous support material (Cab-O-Sil) is deactivated with benzyltriphenyl phosphonium chloride and deposited on the inside wall of a glass capillary column. After additional coating with OV-225, a number of anticonvulsant drugs were run.

Vandemark et al<sup>57</sup> described a procedure for determining phenytoin in 50 ul volume of serum by use of GLC and a detector with heightened sensitivity and selectivity for nitrogenous compounds. Total analysis time for a single sample is 15 minutes.

### 7.83 High Performance Liquid Chromatography (HPLC)

Holcomb et al<sup>58</sup> developed a stability indicating HPLC procedure for the simultaneous determination of phenytoin and phenobarbital in formulations. They used a Waters Associates, 2 ft x 1/8 in. O.D. stainless steel column packed with u Bondapak C<sub>18</sub> on Corasil II. The mobile phase was a mixture

of methanol-water-acetic acid in the ratio 25-75-0.1. With the development of new columns, this procedure was later revised by Fusari et al.<sup>59</sup> The current method uses a Waters Associates, 10 u, 3.9 mm x 30 cm u Bondapak C<sub>18</sub> column; UV detection at 254 nm, the mobile phase is a mixture of methanol-water-acetic acid in the ratio 40-59.5-0.5. At a flow rate of 1.5 ml per minute, phenobarbital eluted at 6.6 minutes and phenytoin at 13.8 minutes.

Atwell et al<sup>60</sup> described an HPLC procedure for the simultaneous analysis of phenytoin and phenobarbital in plasma.

Slonek et al<sup>61</sup> developed a rapid and simple HPLC microanalytical method for the determination of clinically encountered plasma phenytoin levels. This method is accurate down to about 1 ug of phenytoin/ml of plasma and requires as little as 10 ul of sample.

Chafetz et al<sup>11</sup> used an HPLC method to study the hydrolysis of phenytoin sodium in aqueous solutions at pHs 11 and 12. They used an Altex Ultrasphere® reverse-phase bonded octadecylsilane column, 4.6 mm x 25 cm; the mobile phase was a mixture of 550 ml of methanol with 450 ml of an aqueous solution at pH 3.2 containing 30 ml of 0.067 M potassium phosphate and 1 g of hexanesulfonic acid sodium salt; the UV detector was set at 220 nm. Under these conditions, phenytoin eluted at 10.3 min, diphenylglycine at 4.0 min, diphenyldydantoic acid at 5.6 min, benzhydrol at 21.6 min and benzophenone at 37.0 min.

## 7.9 Immunoassay

Immunologic methods for quantitating the plasma concentrations of many hormones and a few drugs have been described<sup>62,63,64</sup>. Tigelaar et al<sup>65</sup> have developed a radioimmunoassay for phenytoin. Cook et al<sup>66</sup> studied the saliva and plasma levels of phenytoin in a series of epileptic patients by means of radioimmunoassay that required only 10 ul of saliva or plasma. The radio immunoassay is very sensitive, quantitation of substances present in concentrations as low as a few picograms per milliliter of a biological fluid can be achieved. The development of simple radioimmunoassay is reviewed by Cook et al<sup>67</sup>. Pippenger et al<sup>68</sup> reported a homogenous immunoassay system which has the advantages of radioimmunoassay without the use of radioactive compounds. Quantitative correlation to GLC was obtained. Phenytoin in human serum was determined by spinimmunoassay technique by Montgomery et al<sup>69</sup>. A specific radioimmunoassay for phenytoin, that will measure therapeutic levels directly in 1 ul of plasma is described by Christensen<sup>70</sup>.

Castro et al<sup>71</sup> made comparative determinations of phenytoin by various methods. Serum from patients being treated with phenytoin were analyzed for the drug by spectrophotometry, gas chromatography, radioimmunoassay, enzyme immunoassay and liquid chromatography. The assay values obtained were compared statistically. Enzyme immunoassay and liquid chromatography appear to be attractive alternatives to the more traditional methods of spectrophotometry and gas chromatography.

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# PYRIDOXINE HYDROCHLORIDE

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## 1. Description

### 1.1 Nomenclature

#### 1.1.1 Chemical Names

5-Hydroxy-6-methyl-3,4-pyridinedimethanol hydrochloride;  
2-Methyl-3-hydroxy-4,5-bis (hydroxymethyl) pyridine hydrochloride;  
5-Hydroxy-6-methyl-3,4-pyridinedicarbino1 hydrochloride;  
3-Hydroxy-4,5-dimethyl-ol- $\alpha$ -picoline hydrochloride (1);  
3-Hydroxy-4,5-di(hydroxymethyl)-2-methylpyridine hydrochloride (2);  
4,5-Bis (hydroxymethyl)-3-hydroxy-2-methylpyridinium chloride (3);  
3,4-Pyridinedimethanol, 5-hydroxy-6-methyl-, hydrochloride (4);  
3-Hydroxy-4,5-bis (hydroxymethyl)-2-methylpyridinium chloride (5).

#### 1.1.2 Generic Names

Pyridoxine hydrochloride (5, 6); Pyridoxini-chloridum; Pyridoxini hydrochloridum; Pyridoxinium chloride; Pyridoxol hydrochloride; Vitamin B<sub>6</sub>; Adermine hydrochloride; Piridossina chlorid-rato (2); Vitamin B<sub>6</sub> hydrochloride (1); Hexabione hydrochloride.

#### 1.1.3 Trade Names

Beadox; Benadon; Hexa-Betalin (7); Hexavibox, Hexabion; Pyrivel; Pyridipca; Becilan, Hexermin; Campoviton 6 (1); Comploment; Ancoloxin (with meclozine hydrochloride) (3).

## 1.2 Formulae

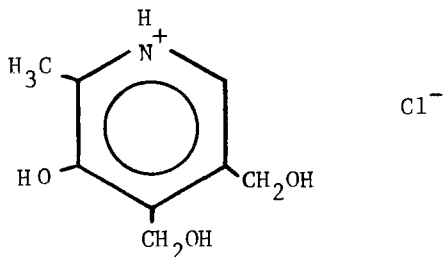
### 1.2.1 Empirical

$C_8H_{11}NO_3$ , HCl.

### 1.2.2 Structural

Pyridoxine is only one of three similar compounds

that may be referred to as vitamin B<sub>6</sub>, the other two are pyridoxal and pyridoxamine (8). Only pyridoxine hydrochloride, however, is used in pharmaceutical preparations (9).



### 1.2.3 Wiswesser Line Notation

T6 NJBCQDI  
QEIQ and GH (10)

### 1.2.4 Chemical Abstract Registry Number

58-56-0 (5 , 10)

### 1.3 Molecular Weight

205.64.

### 1.4 Elemental Composition

C, 46.72%; H, 5.88%;  
Cl, 17.24%; N, 6.81%, O, 23.34%.

### 1.5 Appearance, Color, Odor and Taste

A white or almost white, crystalline powder, platelets or thick birefringent rods (from alcohol + acetone); odorless, with a slightly bitter saline taste, acid.

### 1.6 Dissociation Constants

pKa 5.0, 9.0 (25°) (3).

## 2. Physical Properties

### 2.1 Melting Point

About 205° (3); 202-206° (5); 205-212° (1), with decom-



position. It sublimes. The free base melts at 160° (1).

## 2.2 Solubility

Freely soluble in water (1 gm : 5 ml), slightly soluble in alcohol (1 gm : 100 ml), soluble in propylene glycol; sparingly soluble in acetone; insoluble in ether and in chloroform.

## 2.3 X-Ray Diffraction

X-ray diffraction patterns of vitamin B<sub>6</sub> and other vitamins by a precision x-ray camera, have been determined. Elementary cell-dimension studies have been reported (11).

## 2.4 Stability

Pyridoxine, HCl is more stable than pyridoxamine and pyridoxal. Acidic aqueous solutions of vitamin B<sub>6</sub> are stable definitely at room temperature and may be heated at 120° for 30 minutes without decomposition (13).

Pyridoxine is destroyed by UV radiation in neutral or alkaline solutions, but not in acidic solution (9).

Pyridoxine is unstable toward various food, drug, and cosmetic colors, because of the possibility of the formation of complex addition products between the colours and pyridoxine (14).

Several stability studies of pyridoxine, when added to bread, flour (15), cereals (16) and during canning and freezing of sweet corn (17), have been published.

Shiroishi and Hayakawa (18) have described the effect of sunlight irradiation on pyridoxine and related compounds in aqueous solution at various pH's. Pyridoxine and pyridoxamine are relatively stable in acidic medium, while pyridoxal decomposes independently of pH. The authors suggested that the aldehydic O participates in the photolysis of pyridoxal. The presence of Cu<sup>++</sup> had no effect on the stability of these substances. A borate complex of pyridoxine is stable to sunlight irradiation, heating, or autoclaving. Acetic acid and a reddish brown substance were separated as the decomposition products from an irradiated solution of pyridoxine (18).

The effect of shelf-life on the stability of vitamin B<sub>6</sub>, in oral pharmaceutical preparations, has been reported (19).

## 2.5 Identification

The following tests have been described for the identification of pyridoxine hydrochloride:

- a. To 0.1 ml of a 5% w/v solution add 10 ml of a 5% w/v solution of sodium acetate, 1 ml of water and 1 ml of a 0.5% w/v solution of 2,6-dichloroquinone-4-chloroimine in ethanol (96%) and shake; a blue color is produced which rapidly fades and turns to brown. Repeat the operation adding 1 ml of a 3% w/v solution of boric acid in place of the 1 ml of water; no blue color is produced (3-6, 12).
- b. To 2 ml of a solution (1 in 200) add 0.5 ml of phosphotungstic acid, a white precipitate is formed (4).
- c. A 5% w/v solution acidified with 2M nitric acid yields reactions characteristic of chlorides.
- d. pH of a 1% solution, 2.5-3.2 (12); pH of a 5% w/v solutions, 2.3-3.5 (7).

### 2.5.1 Micro-Color Tests

- a. Ammonium molybdate test gives faint blue color (sensitivity : 0.25 µg) (7).
- b. Ammonium vanadate test produces blue then grey-green color (sensitivity : 0.25 µg).

### 2.5.2 Micro-Crystal Tests

- a. Platinic iodide solution forms rectangular crystals (sensitivity : 1 in 1000) (7).
- b. Potassium bismuth iodide solution gives bunches of plates or blades (sensitivity : 1 in 200) (7).

## 2.6 Spectral Properties

### 2.6.1 Ultraviolet Spectrum

The UV spectrum of pyridoxine, HCl in neutral ethanol is shown in Fig. 1. It exhibits a maximum at about 291 nm (E1%, 1 cm = 523 nm).

In a basic solution, pyridoxine gives two maxima at 245 nm (E1%, 1 cm = 219), and at 307 nm as shown in Fig. 2. These are in agreement with previously published data (7, 20-21). In phosphate buffer of pH = 7, two maxima are observed at 324 and 254 nm (E1%, 1 cm = 345 and 183, respectively) (9).

Metzler and Snell (22) have reported the UV absorption spectra and ionisation constants of vitamin B<sub>6</sub> group. The authors have calculated the equilibrium constants between dipolar ionic and uncharged neutral forms of the 3-hydroxypyridines investigated.

### 2.6.2 Infrared Spectrum

The IR spectrum of pyridoxine, HCl in KBr-disc is shown in Fig. 3. Major band assignments are given in Table 1.

Table 1. IR Spectral Assignments of Pyridoxine, HCl.

Frequency, cm <sup>-1</sup>	Assignment
3340	OH phenolic
3250	OH alcoholic
1630 } 1550 }	C=C and C=N aromatic ring

Other finger-print bands characteristic of pyridoxine, HCl are: 1275, 1220, 1100, and 1570 cm<sup>-1</sup>. These data are in agreement with previously reported ones (7, 20, 23).

### 2.6.3 Nuclear Magnetic Resonance Spectrum

The PMR spectrum of pyridoxine, HCl in deuterated water was recorded on a Varian T-60A, 60-MHz NMR spectrometer. The spectrum is shown in Fig. 4. The following structural assignments have been elicited from Fig. 4.

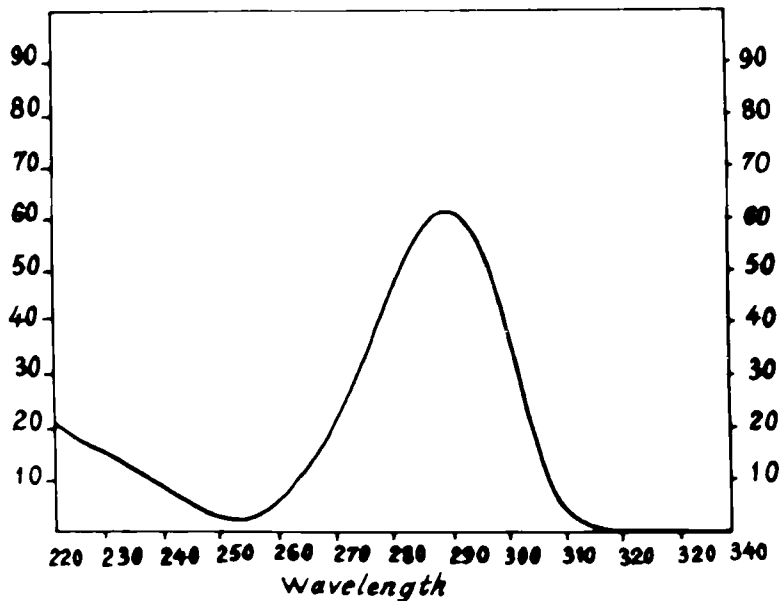


Fig. 1. Ultraviolet spectrum of pyridoxine hydrochloride in ethanol solution.

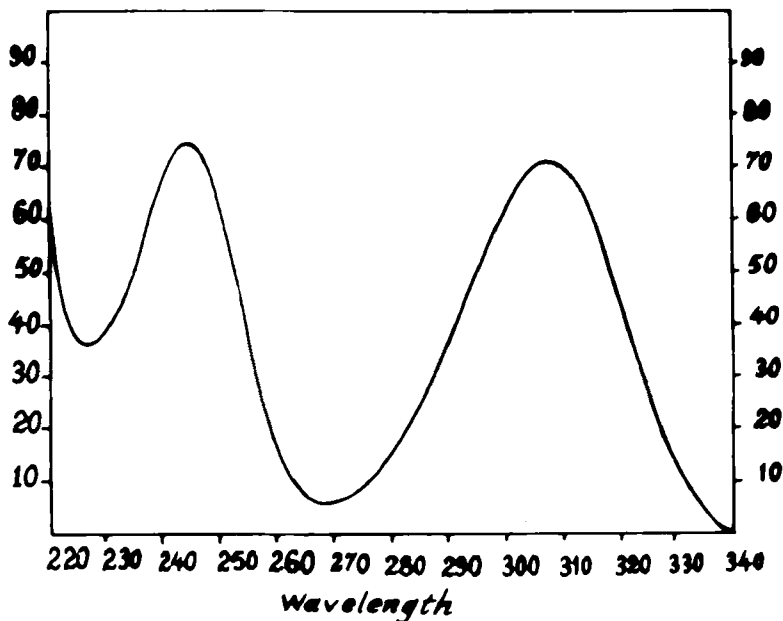


Fig. 2. Ultraviolet spectrum of pyridoxine in basic ethanol solution.

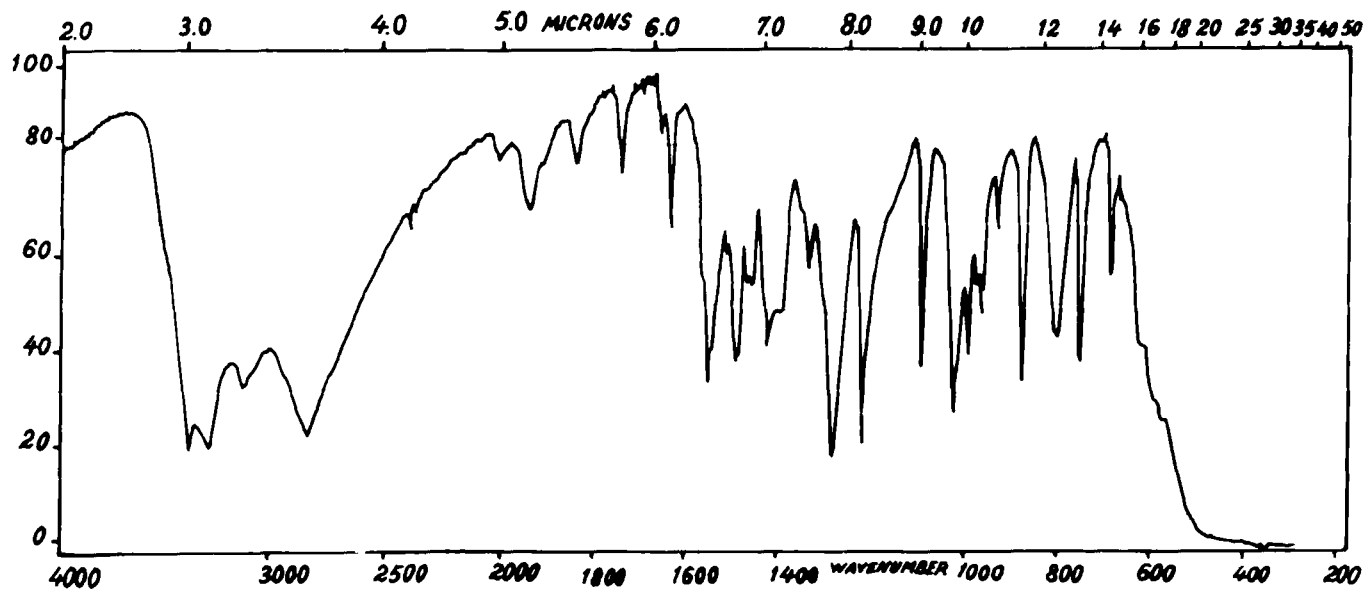


Fig. 3. Infrared spectrum of pyridoxine hydrochloride (KBr-pellet).

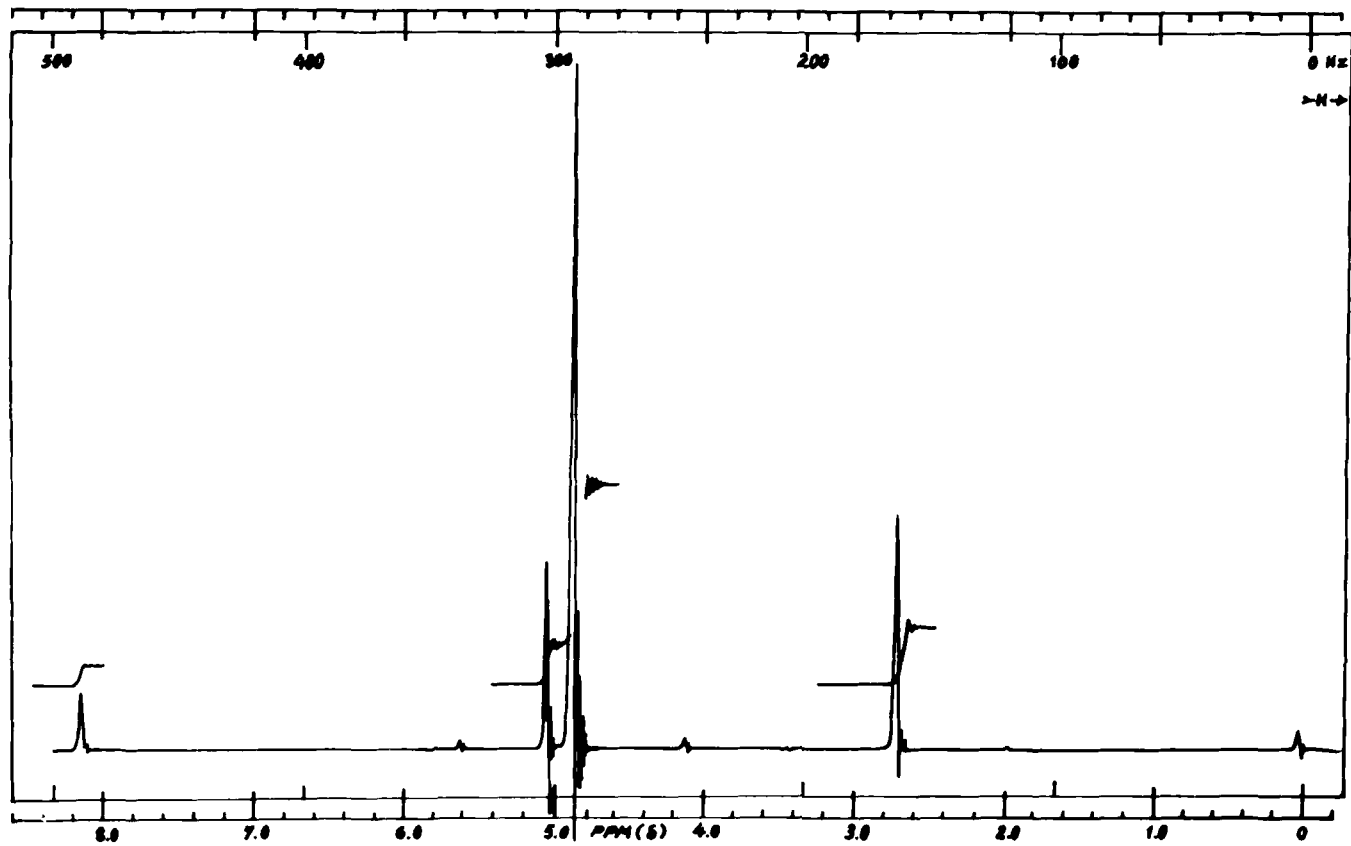


Fig. 4.  $^1\text{H-NMR}$  (60MHz) spectrum of pyridoxine hydrochloride in  $\text{D}_2\text{O}$ .

<u>Chemical Shift (<math>\delta</math>)</u>	<u>Proton Assignments</u>
2.73 singlet	CH <sub>3</sub> at C <sub>2</sub>
5.06 singlet	CH <sub>2</sub> at C <sub>4</sub> and C <sub>5</sub> .
8.13 singlet	aromatic proton at C <sub>6</sub> .

When the spectrum is determined in DMSO-d<sub>6</sub>, the hydroxymethyl groups are resolved into two singlets at 4.7  $\delta$  (for CH<sub>2</sub>OH at C<sub>5</sub>) at 4.8  $\delta$  (for CH<sub>2</sub>OH at C<sub>4</sub>).

These spectra are in agreement with previously published data (24).

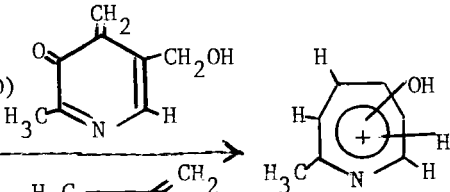
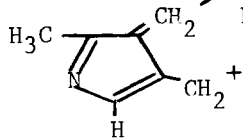
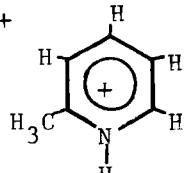
The electronic properties of various ionic forms of vitamin B<sub>6</sub> group have been studied and rationalized using PMR (25).

Furthermore, the PMR spectroscopy of vitamin B<sub>6</sub> compounds has been studied by Korytnyk and Paul (26).

#### 2.6.4 Mass Spectrum

The mass spectrum of pyrdoxine is shown in Fig. 5. The most prominent ions are shown in Table 2.

Table 2. Mass Spectral Fragmentation of Pyridoxine.

<u>m/e</u>	<u>Fragment</u>
169	Molecular ion M <sup>+</sup> .
151	(M <sup>+</sup> - H <sub>2</sub> O)
122	
106	
94	

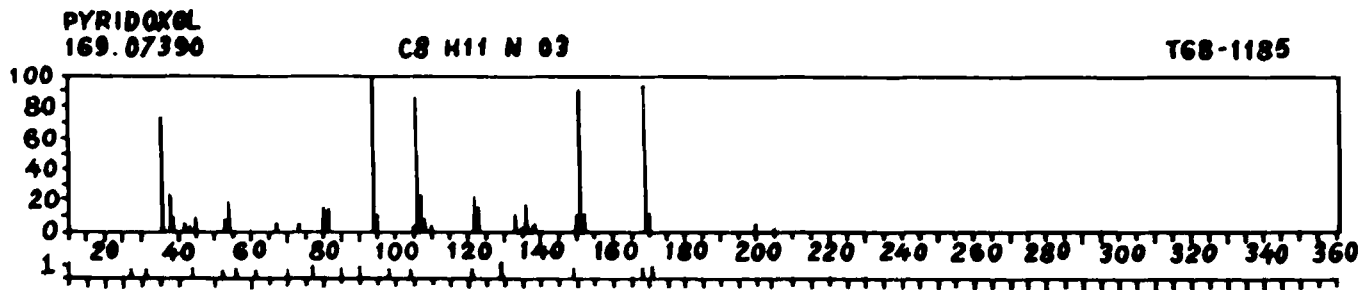
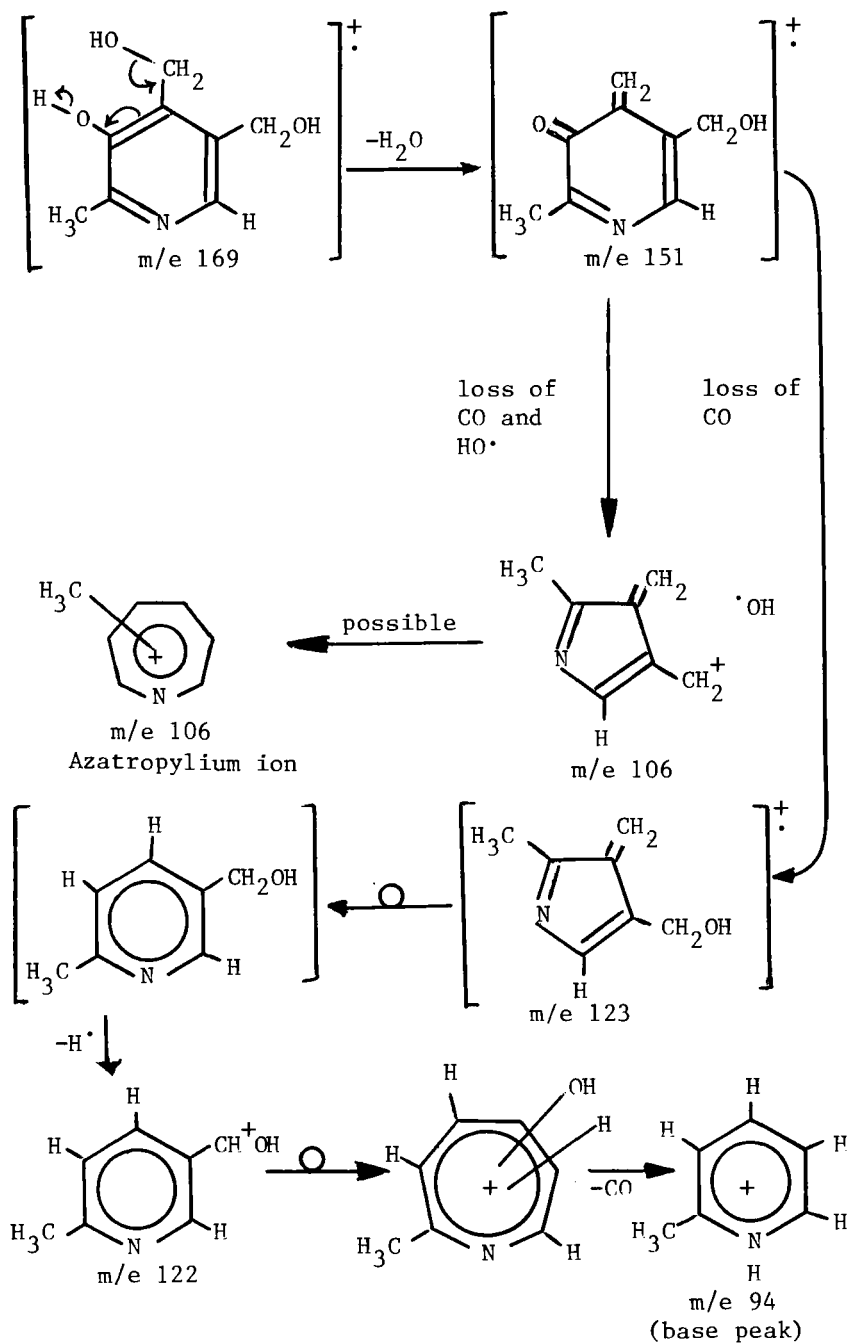


Fig. 5. Low resolution mass spectrum of pyridoxine (EI).





Scheme 1. Mass Spectral Fragmentation of Pyridoxine.

The mechanism by which the major fragments are formed, is shown in Scheme 1. The detailed mechanism is reported by De Jongh et al. (27) with the aid of metastable peaks, deuterium labeling, and analysis with similar system.

### 2.6.5 $^{13}\text{C}$ -NMR

The  $^{13}\text{C}$ -nuclear magnetic resonance spectra of pyridoxine hydrochloride, obtained in  $\text{D}_2\text{O}$  at ambient temperature using dioxane as reference line, is shown in Fig. 6 (off-resonance) and Fig. 7 ( $^1\text{H}$ -decoupled).

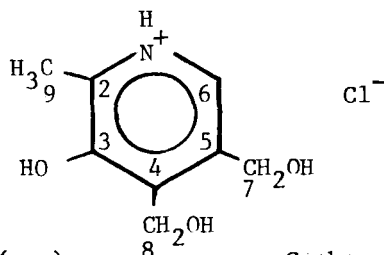
The spectra have been recorded at 20 MHz for carbon-13 on a Varian FT80A spectrometer. The chemical shifts and spectral assignments are given in Table 3.

Table 3.  $^{13}\text{C}$ -NMR of Pyridoxine, HCl in  $\text{D}_2\text{O}$ .

Chemical Shift (ppm)		Carbon No.
153.52	s	3
143.42	s	2
141.32	s	4
137.62	s	5
130.57	d	6
58.92	t	7 and 8.
57.88	t	
15.23	q	9

s = singlet  
t = triplet

d = doublet  
q = quartet



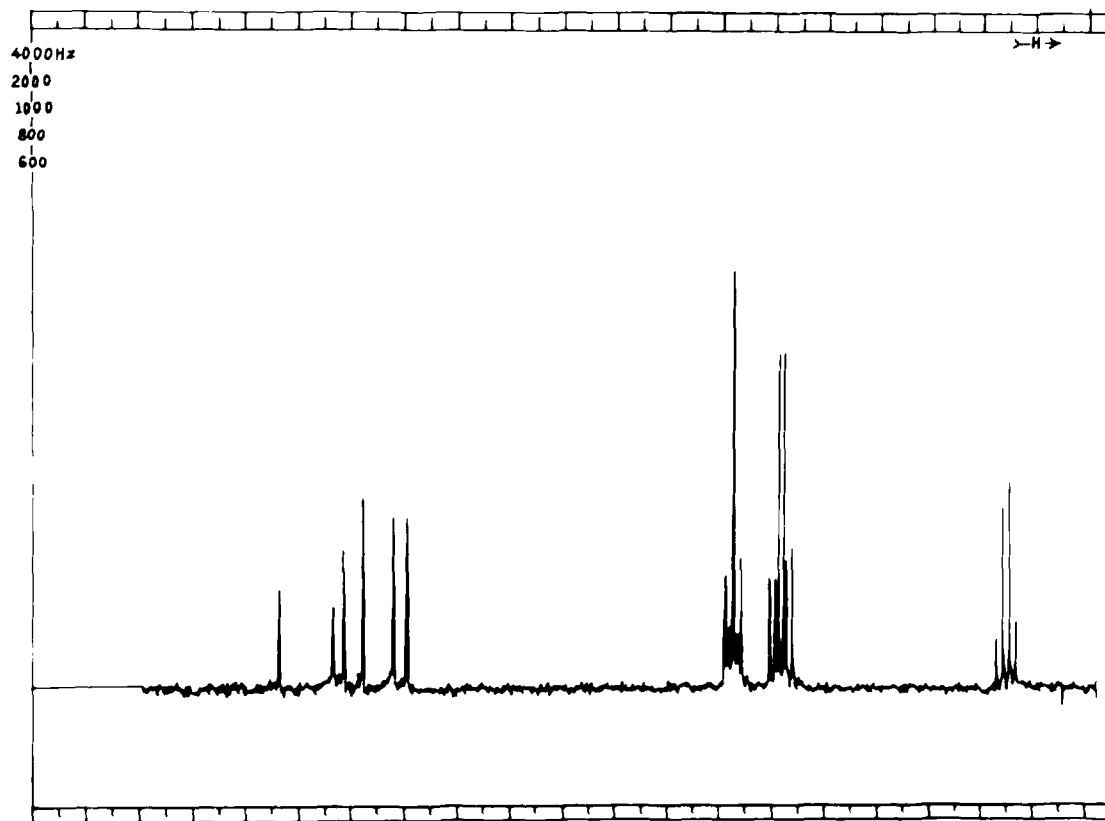


Fig. 6.  $^{13}\text{C}$ -NMR Off-Resonance Spectrum of Pyridoxine Hydrochloride in  $\text{D}_2\text{O}$ .

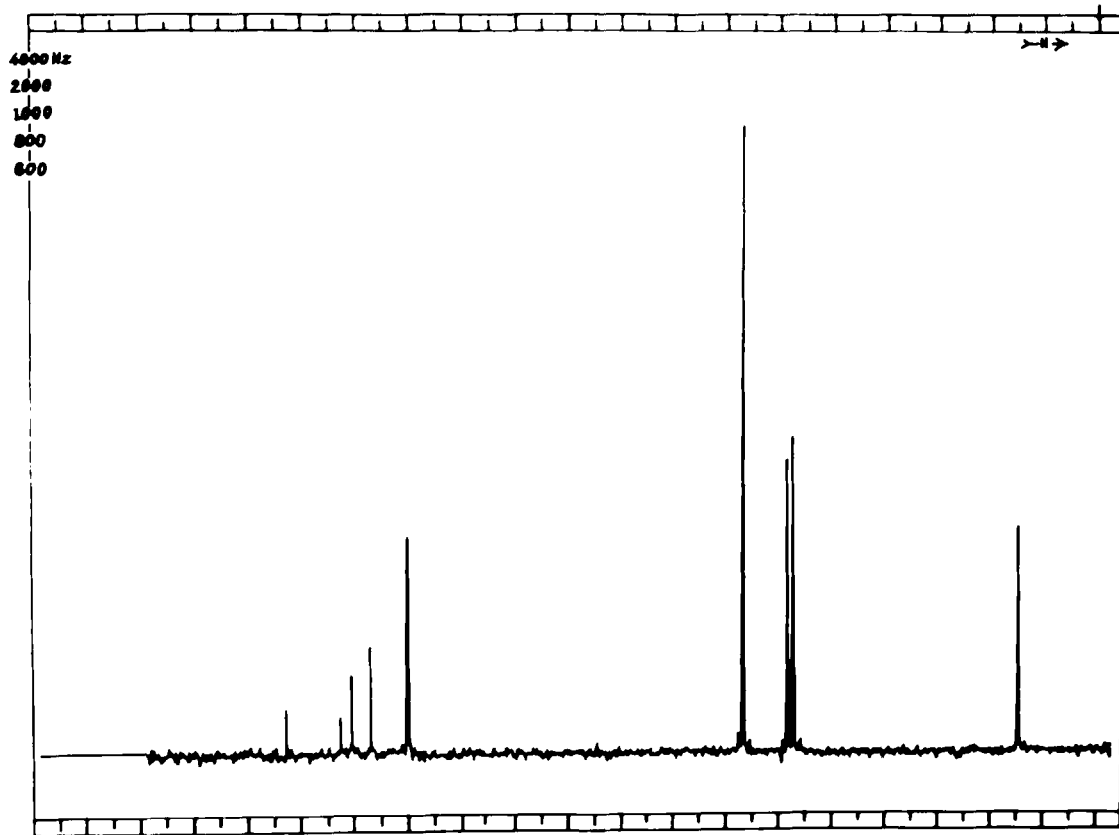


Fig. 7.  $^{13}\text{C}$ -NMR-Noise Decoupled of Pyridoxine Hydrochloride in  $\text{D}_2\text{O}$ .

These data were referenced from dioxane which appears at 67.39 ppm. The assignments were based on published data of several pyridine derivatives (28).

### 3. Biosynthesis

Yoshiki (29) has studied the biosynthetic pathways of vitamin B<sub>6</sub>, including the discovery of the precursor glycolaldehyde, glycolaldehyde dehydrogenase, and isolation of microorganisms deficient in regulation mechanism of B<sub>6</sub> biosynthesis.

The status of glycolaldehyde in the biosynthesis of vitamin B<sub>6</sub> has been also described by Vella, et al. (30). Competition experiments, employing <sup>14</sup>C-labeled samples of glycerol and glycolaldehyde, indicate that in *E. Coli* B ware 2 independent pathways leading to pyridoxal. In mutant WG2 the major pathway utilizes glycerol and related trioses as the sole C source in the construction of the C<sub>8</sub> N skeleton of pyridoxol. In the minor pathway glycolaldehyde, and not glycerol, supplies C-5 and C-5' of pyridoxol, while glycerol is the source of the other 6 carbon atoms.

Pope and Smith (31) have reported the synthesis of pyridoxine by tubercle bacilli when grown on synthetic media. Pyridoxine has been synthesized, together with other B-complex vitamins, by H<sub>37</sub> and Ravenel strains of the tubercle bacillus.

Tani, et al. (32) have described a paper disc assay method to screen the biosynthetic intermediates of vitamin B<sub>6</sub>.

Dempsey (33) has published the biosynthesis and control of vitamin B<sub>6</sub> in *Escherichia coli*.

Yamada, et al. (34) have studied the vitamin B<sub>6</sub> biosynthesis of a pdx B mutant, *Escherichia coli* B WG3, in amino acid - supplemented medium. An amino acid mixture and glycolaldehyde, which satisfy the vitamin B<sub>6</sub> requirement of *E. Coli* B WG 3, a pdx B mutant, have been examined for their effect on growth of the mutant in liquid culture. The amount of B<sub>6</sub> synthesized by the mutant in the amino acid - supplemented medium is lower than the amount synthesized in the glycolaldehyde - supplemented medium.

#### 4. Synthesis

Several routes have been described for the synthesis of pyridoxine hydrochloride.

##### Route 1:

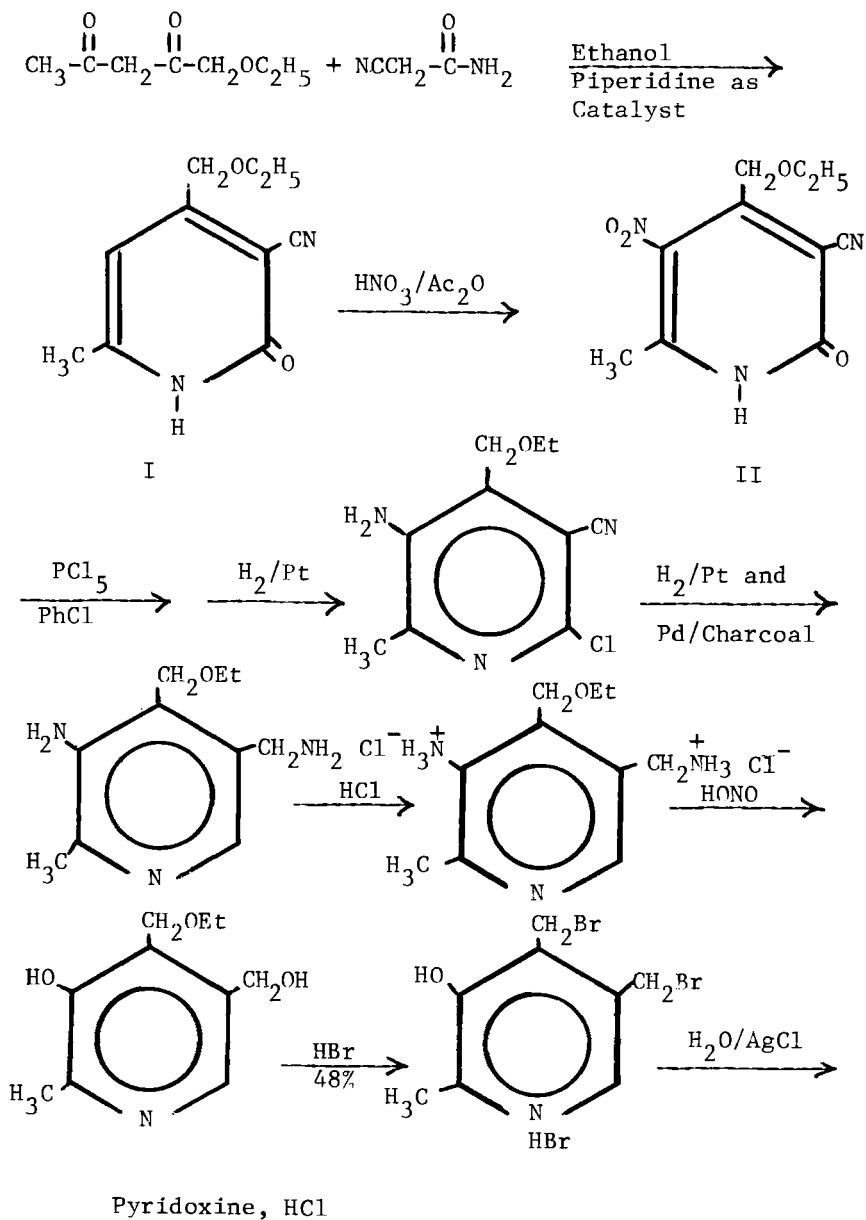
By the condensation of cyanoacetamide and ethoxyacetylacetone (35-38). The resulting 3-cyano-4-ethoxymethyl-6-methyl-2-pyridone (I) was nitrated to give nitro pyridone (II). The sequence of reactions of this route may be represented graphically (Route 1).

Some variations and improvements in this route have been reported (37).

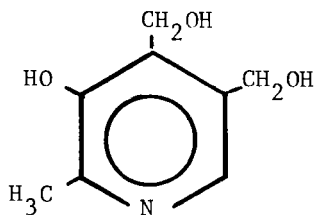
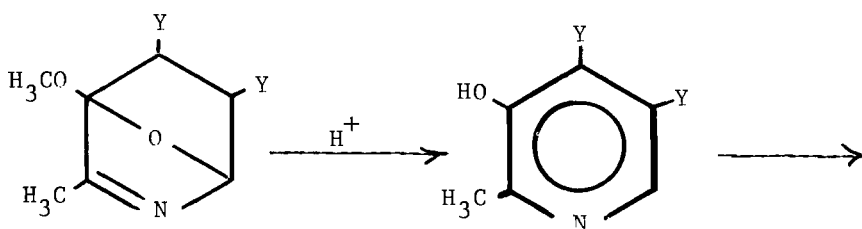
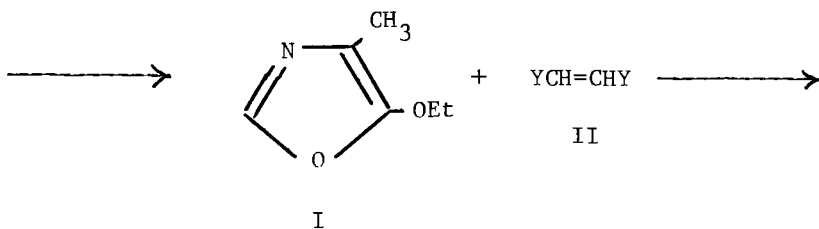
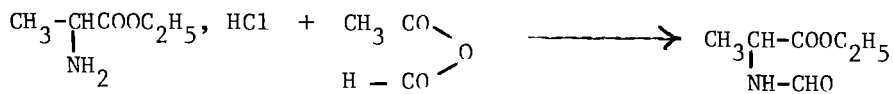
##### Route 2:

Alkyl-substituted oxazoles have been found to react with maleic acid or its anhydride in a diene synthesis to yield substituted pyridine readily converted to pyridoxine (39). In this route, ethyl d, l-alaninate hydrochloride is treated with formic-acetic anhydride to yield ethyl N-formyl d,l-alaninate (78%). This compound is refluxed in chloroform with phosphorous pentoxide (40), quenched with aqueous potassium hydroxide, and the organic layer distilled to give 4-methyl-5-ethoxyoxazole (I) (60%). The resulting oxazole (I) is condensed readily with a number of appropriate dienophiles to form 2-methyl-3-hydroxy-4,5-disubstituted-pyridines containing substituents (III, a, b, c) which could be converted to pyridoxine as follows:

1. A mixture of the oxazole (I) with two moles of diethyl maleate (IIa) is heated and the adduct cleaved with ethanolic hydrogen chloride to form the diethyl ester of 2-methyl-3-hydroxy-pyridine-4,5-dicarboxylic acid hydrochloride (IIIa) (85%). This diester is reduced with lithium aluminum hydride to pyridoxine (41-42) isolated as its hydrochloride.
2. A mixture of the oxazole (I) with one mole of fumaronitrile (IIb) is refluxed in methanol and the adduct cleaved with concentrated hydrochloric acid to give crystalline 2-methyl-3-hydroxy-4,5-dicyanopyridine (IIIb) (75%). Hydrogenation of IIIb in methanolic hydrogen chloride over 5% palladium on charcoal gives 2-methyl-3-hydroxy-4,5-diaminomethylpyridine trihydrochloride (III,  $Y=CH_2NH_2$ ) (70%), which on diazotisation



Route 1. Synthesis of Pyridoxine Hydrochloride.



Pyridoxine

- a. Y = - COOC<sub>2</sub>H<sub>5</sub>
- b. Y = - CN
- c. Y-Y = - CH<sub>2</sub>-O-CH<sub>2</sub>-

Route 2. Synthesis of Pyridoxine.



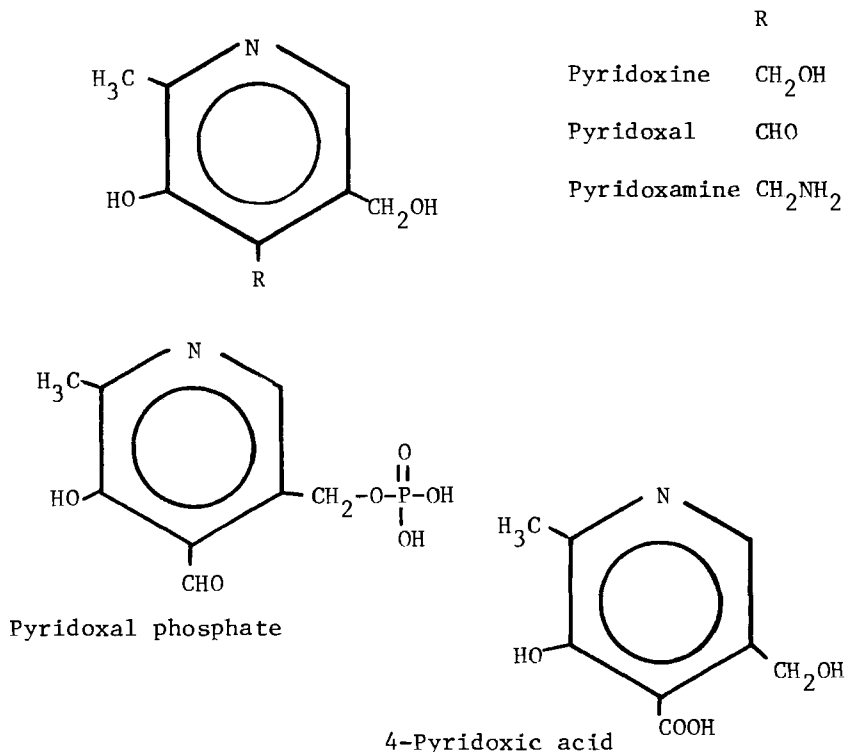
in water at 85° yields pyridoxine hydrochloride (79%).

3. A solution of the oxazole (I) in a 20-mole excess of 2,5-dihydrofuran (IIc) containing 1% of trichloroacetic acid is heated to give 2-methyl-3-hydroxy-4,5-epoxy-dimethylpyridine (IIIc) of 95% purity (58%). The cyclic ether (IIIc) is cleaved with 48% hydrobromic acid to the dibromide hydrobromide (III, Y=CH<sub>2</sub>Br) and hydrolysed to pyridoxine (35-36).

Several reports and patents are listed in the literature for the synthesis of the drug (43-54).

### 5. Metabolism

Pyridoxine, pyridoxal, and pyridoxamine, which occur in foodstuffs, are collectively known as vitamin B<sub>6</sub>. In the body, all three are converted to pyridoxal phosphate which is the coenzyme for amino-acid decarboxylase and for transaminase. The structures of the three active forms of vitamin B<sub>6</sub> and the pyridoxal phosphate, are shown below (55).



Although, owing to the wide distribution of vitamin B<sub>6</sub> in nature, clinical deficiency symptoms are seldom observed, there is little doubt that pyridoxine is essential in human nutrition. Pyridoxine is absorbed from the gastrointestinal tract and is converted to the active form pyridoxal phosphate. Absorption is decreased in gastrointestinal diseases and also in subjects taking isoniazid (3). It is excreted in the urine as 4-pyridoxic acid (2). The metabolism of vitamin B<sub>6</sub> in human beings has been investigated (56).

Moller (57) has recognized pyridoxine, pyridoxal, pyridoxamine, and 4-pyridoxic acid as the excretion products of vitamin B<sub>6</sub>. Complete balance studies have been made in pigs and in babies on all the known vitamin B<sub>6</sub> metabolic compounds. In babies, the total output exceeded the intake. The assumption that a limited synthesis of vitamin B<sub>6</sub> occurs seems justifiable.

Lumeng et al. (58) have reported the plasma content of B<sub>6</sub> vitamers and its relationship to hepatic vitamin B<sub>6</sub> metabolism. Orally ingested pyridoxine is rapidly metabolized in liver and its products are released into the circulation in the form of pyridoxal phosphate, pyridoxal, and pyridoxic acid.

Johansson et al. (59) have studied the metabolism of labeled pyridoxine in man. 15-20% of the labeled administered pyridoxine is excreted in the urine during the first day. The rate of elimination of vitamin B<sub>6</sub> from the body reservoir is 2.3% /day.

Wozenski et al. (60) have reported the metabolism of small doses of vitamin B<sub>6</sub> in men. It has been found that responses to pyridoxamine are generally slower than for pyridoxine or pyridoxal, suggesting that pyridoxamine is absorbed more slowly or metabolized differently, or both, than pyridoxal or pyridoxine. A dose of at least 1 mg of B<sub>6</sub> is necessary to obtain measurable changes in vitamin B<sub>6</sub> metabolism.

Cox, et al. (61) have described the metabolism of tritium-labeled pyridoxine in rats. Urine, blood, and fecal samples from rats injected intravenously with labeled pyridoxine are collected for excretion and chromatographic studies. Approximately 70% of the label is excreted in the urine in 5 hours. Fecal excretion is less than 0.03 % in

96 hours. Chromatographic studies of urine have showed that pyridoxine is excreted primarily unchanged with a small amount of only one metabolite, probably 4-pyridoxic acid.

Johansson, et al. (62) have reported the metabolism of tritium-labeled pyridoxine in rats and humans. 4-pyridoxic acid accounted for 25% of the total isotope in the urine of rats and humans.

Hamm (63) has described the transport and interconversion of B<sub>6</sub> vitamers in the perfused intestine and kidney of the rat.

It has been reported that vitamin B<sub>6</sub> metabolism becomes altered with age (64).

Some abnormalities of vitamin B<sub>6</sub> metabolism in human beings have been described (65-66).

The metabolic significance of vitamin B<sub>6</sub> and its role to the growth and functional development have been published (67-78).

Several other in vitro and in vivo metabolic studies of vitamin B<sub>6</sub> have been published (79-86).

## 6. Methods of Analysis

### 6.1 Titrimetric

#### 6.1.1 Non-Aqueous

Pyridoxine, HCl has been assayed by non-aqueous titrations, using 0.1 N perchloric acid in the presence of mercuric acetate and crystal violet indicator (4,6, 10, 87).

#### 6.1.2 Complexometric

The drug has been determined by complexometric titration of the Cu<sup>++</sup> liberated by passing the solution through a column of cation exchange resin in the copper form. A solution of 0.005 or 0.0025 M of disodium EDTA is the titrant and using murexide as an indicator (88).

### 6.1.3 Amperometric

Lemahieu, et al. (89) have reported an amperometric method for the determination of hydrochlorides of several organic bases, including pyridoxine. Titration, with  $\text{AgNO}_3$  solution in dimethyl sulphoxide, of such hydrochlorides in dimethyl sulphoxide gives an end-point corresponding to the formation of  $\text{AgCl}_2^-$  and a less sharp end-point for the precipitation of  $\text{AgCl}$ . Use of the first end-point and two platinum indicator electrodes with a potential difference of 100 mV allows titration down to a 0.2 mM concentration of pyridoxine, HCl.

### 6.1.4 Polarographic

Manousek and Zuman (90) have described a direct polarographic determination of pyridoxal in the presence of pyridoxal 5-phosphate. The simultaneous determination of both compounds is possible only when the concentrations are comparable. The same authors also have reported the polarography of pyridoxine and several analogues in buffered solutions. A 2-electron wave was observed which corresponds to a diffusion-controlled reduction of the activated C-OH bond in position 4.

Nakaya (91) has described polarographic studies of pyridoxine, HCl and several pyridine derivatives at various pH's.

Another polarographic method for the evaluation of vitamin  $\text{B}_6$  and its metabolites has been reported (92).

## 6.2 Chromatography

### 6.2.1 Paper Chromatography

Several solvents systems (93-97) have been used for the identification, separation, and quantitation of pyridoxine in pharmaceuticals and biological fluids. These are presented in Table 4.

Table 4. Paper Chromatography of Pyridoxine.

Solvent System	Localising agent	R <sub>f</sub>	Ref.
1. Citric acid: water: n-butanol (4.8 gm : 130 ml: 870 ml).	UV light (blue fluorescence), iodoplatinate spray (white), bromocresol green spray (strong reaction).	0.18	(7)
2. Acetate buffer (pH=4.58)	UV light (blue fluorescence)	0.90	(7)
3. Phosphate buffer (pH=7.4)	UV light (blue fluorescence).	0.86	(7)
4. Two dimensional chromatography consists of:			
a. Collidine: lutine (1:1) saturated with water.	-	0.88	(98)
b. Butanol: acetic acid: water (40:10:50)	-	0.63	(98)
5. Amyl alcohol acetone: water (2:1:2).	-	-	(99)
Isoamyl alcohol: pyridine: water (2:1:2).	-	-	
6. Two dimensional chromatography consists of:			
a. Propanol: 10% formic acid (80:20).	UV light	-	(100)
b. Butanol saturated with 1% ammonia.	-	-	

The separation of water-soluble vitamins, including vitamin B<sub>6</sub>, on ion-exchange paper has been described by Klotz and Huettenrauch, (101) using Amberlite SB-2, Amberlite WB-2, and Amberlite WA-2.

For the separatory determination of pyridoxine in multi-vitamin preparations, other vitamins are separated by paper chromatography in which the upper layer of butanol-ammonium hydroxide-water (4:1:5) are used as the developer, and by the one-dimensional ascending method (102).

#### 6.2.2 Thin-Layer Chromatography

Clarke (7) has described a solvent system consisting of strong ammonia solution-methanol (1.5:100) and potassium permanganate spray as a detecting agent.

Lyle and Tehrani (103) have separated several vitamins, including vitamin B<sub>6</sub>, by TLC, using anhydrous acetic acid - acetone - methanol - benzene (1:1:4:14).

A rapid TLC method for the routine identification of vitamin B<sub>6</sub>, and other drugs, in biological fluids, has been described (104).

#### 6.2.3 Electrophoresis

A rapid electrophoretic method for the simultaneous separation and estimation of vitamin B<sub>6</sub> and derivatives from various tissues has been described (105). The method is based on high voltage electrophoresis of small aliquots of tissue extracts, applied on cellulose-coated plates. The method is sensitive upto 1 µg of vitamin B<sub>6</sub>.

#### 6.2.4 Column Chromatography

Several methods have been reported for the separation of water soluble vitamins including vitamin B<sub>6</sub>. Some of these systems are described as follows:

Table 5. Gas-Chromatography of Pyridoxine, HCl.

Column	Conditions	Comment	Ref.
1. 3% SE-30	100-270° temperature programming.	Derivatized using NO-bis(trimethylsilyl) acetamide.	(108)
2. 15% DOW - Corning high-vacuum silicone grease on Gas-Chrom Q (60-80 mesh).	Column temperature 175°, with thermal conductivity detection and He as carrier gas.	Derivatized as above.	(109)
3. 3% SE-52 on silanized Gas Chrom P (60-80 mesh).	Column temperature 165°, N <sub>2</sub> as carrier gas.	Derivatized as above.	(110)
4. 3% OV-101 on Gas Chrom Q (100-200 mesh).	Column temperature 160°.	Derivatized with diazomethane and butaneboronic acid as pyridoxine O-methyl ether cyclic butaneboronate.	(111)

reaction has been utilized for the quantitation of the drug (122-126).

Recently, Moussa (122) has described a modified procedure to improve the stability of the color, the speed, and the sensitivity of assay. In this procedure, the reaction is carried out in 2-propanol medium (instead of water), triethanolamine is used as a buffer, and iodine is used as an oxidant. The detection limit of this method is 10 µg of vitamin B<sub>6</sub>.

- a. Amberlite WA-2, carboxylic acid resin, using acetate buffer (pH=4.62) as an eluent (106).
- b. SD cationite, using concentrated aqueous ammonia as an eluent (107).

Another system consisting of Amberlite IRA-400, using 80% acetic acid as an eluent, has been also described (107).

#### 6.2.5 Gas-Chromatography

Several gas-chromatographic procedures have been reported for the determination of vitamin B<sub>6</sub> in pharmaceutical formulations and in biological fluids. Table 5 summarises some of these methods.

Application of pyrolysis-gas chromatography for the determination of pyridoxone, HCl and other water-soluble vitamins, has been reported (103).

Identification and semi-quantitative estimation of vitamin B<sub>6</sub>, using microphase extraction and gas-chromatography, has been described (104).

#### 6.2.6 High-Performance Liquid Chromatography

Several HPLC procedures have been published for the determination of pyridoxine, HCl and other water-soluble vitamins, in biological fluids and in feeds (112-116).

Table 6 summarises, some of the HPLC systems used for the analysis of vitamin B<sub>6</sub>.

### 6.3 Spectrophotometry

#### 6.3.1 Colorimetric

Several colorimetric assays have been developed for pyridoxine. The U.S.P. method (4) for the determination of pyridoxine HCl, is based on coupling of pyridoxine with 2,6-dichloroquinone chloroimide to give a blue color, according to the method described by Kuhn and Low (121). The absorbance of the blue color is measured at 650 nm and compared with standard solutions. The



Table 6. HPLC systems of pyridoxine.

Column	Mobile Phase	Detection	Comment	Ref.
1. LiChrosorb RP-8	Methanol-phosphate buffer (1:1) containing 0.002 M C TAB.	UV	The method is applicable in analysis of vitamin B <sub>6</sub> in urine but not in blood.	(116)
2. TSK-Gel LS-160	Sodium perchlorate - potassium dihydrogen phosphate - Sodium chloride.	UV	Applicable in urine and blood analysis. Detection limit 30 ng.	(117)
3. Octadecyl silica	Acidic potassium phosphate	Fluorescence	Recovery 93.9 ± 7.6%	(118)
4. LiChrosorb NH <sub>2</sub>	Acetonitrile - phosphate buffer	UV	Applicable for the analysis of vitamin B <sub>6</sub> in i.v. solutions, and beverages. Standard deviations 1-2%.	(119)
5. Ultrasphere IP	0.033 M potassium phosphate buffer (pH=2.2) containing 2.5% acetonitrile.	Fluorescence	Recovery 82.4-105.1%. Applicable for the analysis of vitamin B <sub>6</sub> in animal tissues and in milk.	(120)

Among other colorimetric methods for the determination of vitamin B<sub>6</sub>, are the following:

- a. Pyridoxine and other derivatives give a stable, alcohol-soluble, red-violet color with zinc chloride-stabilized diazotised sulphathiazole at pH 6.5-7.0 (127).
- b. Murai (102) has determined vitamin B<sub>6</sub> by its reaction with dimethyl-p-phenylenediamine, HCl and sodium hypochlorite in a buffered solution (pH=8). The resulting color is extracted with isobutyl alcohol and the absorbancy of the solution is measured at 625 nm.
- c. A colorimetric method similar to that described in b, using N,N-diethyl-p-phenylenediamine, HCl and potassium ferricyanide at pH 7 (128).

Other colorimetric methods for the analysis of vitamin B<sub>6</sub> have been reported (9, 129).

### 6.3.2 Ultraviolet

Bosch (130) has published a spectrophotometric determination of vitamin B<sub>6</sub>, and other vitamins, in pharmaceutical preparations with N-bromo-succinimide, in methanol at 370-380 nm. The method allows determination of 100-1000 µg of vitamin B<sub>6</sub>. Vitamin B<sub>1</sub>, B<sub>12</sub>, A, and menadione bisulphite interfere with this method.

Vitamin B<sub>6</sub> has been spectrophotometrically determined at 290 nm, after chromatographic separation from other related analogues (131).

A survey of normal and derivative spectra of numerous vitamins, including vitamin B<sub>6</sub>, has been reported. The second derivative spectra of these vitamins are especially suitable for their quantitative determinations (132). Quantitative determination by derivative spectroscopy is superior to direct spectrophotometric measurement in many problem situations.

Barrosa (133) has reported the analysis of a mixture of pyridoxine and isoniazid in tablet formulation. Pyridoxine is determined by differential spectrophotometry at 324 nm.

### 6.3.3 Infrared

Pyridoxine, HCl has been determined by IR spectrophotometry, using the region 6-11  $\mu$ , and water as a solvent (134).

### 6.3.4 Nuclear Magnetic Resonance

A PMR spectrometric method has been described (135) for simultaneous determination of vitamin B<sub>1</sub> and B<sub>6</sub>. The relationship of the peak areas of the signals at  $\delta$  2.55 ppm (vitamin B<sub>1</sub>) and at  $\delta$  2.65 ppm (vitamin B<sub>1</sub> + B<sub>6</sub>) to that at  $\delta$  6.6 ppm (maleic acid) are derived, whereby the compounds of the mixtures can be readily quantitated.

Recently, another PMR method for the simultaneous analysis of vitamin B<sub>6</sub> and B<sub>1</sub>, utilising simpler equation for calculation, has been developed (136). The mean percent recovery of vitamin B<sub>6</sub> and B<sub>1</sub> in their dosage forms is  $97.42 \pm 2.99$  and  $97.57 \pm 1.52$ , respectively. This method avoids overlap in the peak areas used in the former method.

### 6.3.5 Absorption-Luminescence

The technique of absorption-luminescence spectroscopy has been recently applied for the analysis of vitamin B<sub>6</sub> and related compounds (137-138).

Bazhulina and Morozov (137) have investigated the equilibrium constants between various ions and the tautomeric forms of vitamin B<sub>6</sub> together with the effect of temperature, substitution, and medium on the absorption properties of these forms. The experimental spectroscopic data are in good agreement with quantum calculation.

### 6.3.6 Spectrofluorimetric

AleKseichik, et al (139) have published qualitative analysis of vitamin B<sub>6</sub> and other drug preparations. The spectra are determined in ethanol, hydrochloric acid, and sodium hydroxide, using vitamin B<sub>6</sub> concentrations from 0.01-0.001 mg/ml.

Fujita, et al. (140) have reported fluorometric determination of vitamin B<sub>6</sub>. The authors have made extensive study in this respect (141).

Fujino (142) has improved a method for the determination of vitamin B<sub>6</sub>, by utilising the fluorescence of 4-pyridoxic acid which is produced by oxidation of vitamin B<sub>6</sub> with permanganate. This method is applied for the determination of vitamin B<sub>6</sub> in biological fluids (86, 143-144).

### 6.4 Microbiological

Several microbiological methods for the analysis of vitamin B<sub>6</sub> in biological materials and other products have been reported (145-147).

The growth response of some microorganisms, e.g., *Streptococcus faecalis* (148) and *Saccharomyces carlsbergensis* (99, 149), provide a reliable estimate of vitamin B<sub>6</sub> content in natural products. The vitamin B<sub>6</sub> content is measured nephelometrically after the proper treatment of the sample.

Gregory (118) has determined vitamin B<sub>6</sub>, using *Saccharomyces uvarum*. The method is used for the estimation of vitamin B<sub>6</sub> in fortified breakfast cereals.

### 6.5 Enzymatic

Wada and Snell (150) have developed a method for the assay of pyridoxine and pyridoxamine phosphate, based on enzymatic oxidation to pyridoxal which is allowed to react with phenylhydrazine.

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# SACCHARIN

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## 1. Description

### 1.1 Nomenclature

#### 1.1.1 Chemical Names

- a. 1,2-Benzisothiazole-3 (2H)-one 1, 1-dioxide.
- b. 1,2-Benzisothiazol-3-one 1, 1-dioxide.
- c. 2,3-Dihydro-2-ketobenzisosulfonazole.
- d. 1,2-Dihydro-2-ketobenzisosulfonazole.
- e. Benzosulphimide.
- f. o-Benzoicsulphimide.
- g. o-Sulfobenzimide.

#### 1.1.2 Generic Names

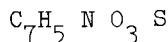
Benzoic sulfimide, benzosulfimide, benzoic sulfimide, o-sulfobenzimide, o-sulfobenzoic acid imide, saccharin, saccharin insoluble.

#### 1.1.3 Trade Names

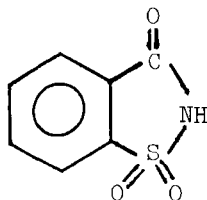
Garantose, Glucid, Gluside, Hermesetas, Sacarina, Sacarina, Saccharinol, Saccharinose, Saccharol, Saxin, Sykose, Zaharina.

### 1.2 Formula

#### 1.2.1 Empirical



#### 1.2.2 Structural



1.2.3 CAS' No.

81-07-2

1.2.4 Wiswesser Line Notation

T56 BSWMVJ

1.3 Molecular Weight

183.18

1.4 Elemental Composition

C, 45.89%; H, 2.75%; N, 7.65%;  
O, 26.20%; S, 17.50%.

1.5 Appearance, Colour, Taste, Odor

White odorless or faintly aromatic crystals or crystalline powder (1). In dilute aqueous solution it is 500 times as sweet as sugar, the sweet taste is detectable even at 1:100,000 dilution. (2)

2. Physical Properties2.1 Crystal Properties2.1.1 Crystallinity

The crystal structure of saccharin was determined (3) by 3-dimensional integrated intensity data collected on a computer-controlled diffractometer operated by an IBM 1620, in a closed-loop manner. The crystals were found to be monoclinic with a  $9.55_2 \pm 3$ , b  $6.91_9 \pm 3$ , c  $11.80_3 \pm 4$  Å, and  $\beta 103.9^\circ$ , and the space group is 'p2<sub>1</sub>/c. The H atoms were also located and are included in the refinement. The centrosymmetric dimer (C<sub>6</sub>H<sub>4</sub>CO NHSO<sub>2</sub>)<sub>2</sub> mols. are formed by hydrogen bonds between the N of imide and O of the carbonyl atoms, (NH...OC), of the five membered ring system of saccharin. The six-sided ring formed by the hydrogen bonds around the center of symmetry is completely planar. The location of the H atom eliminates the possibility of the lactim structure for



the molecule. The contact mode of phenyl rings is normal. A characteristic feature of the molecular configuration is the narrow C-S-N angle of  $92.2^\circ$  in the five-membered ring.

The narrow angle diminishes strain from the ring and thus makes it possible for the entire molecule to assume a planar shape. Other bond angles and bond distances were found to be normal.

Luigi et al (4) have also studied the crystal and molecular structures of saccharin, and observed some  $\pi$ delocalization in the C-O and C-N bonds [triclinic ( $P_1$ ),  $\alpha=82^\circ 45'(20')$ ,  $\beta=82^\circ 17'(10')$ ,  $\gamma=65^\circ 40'(12')$ ,  $Z=4$ ]. The C-C, C-S and S-N bonds in the isothiazole ring are single, and strong hydrogen bonding (NH...O) give rise to centrosymmetrical dimers, as was observed while determining the crystal structure of saccharin.

### 2.1.2 Melting Range

The melting ranges of saccharin are

(a) 228.8-229.7 (2,5) and (b) 226-230(1,6,7) dec. 228.8-229.7 (8).

## 2.2 Solubility

Saccharin is soluble at  $20^\circ$ , in 290 parts of water, in 30 parts of alcohol, in 12 parts of acetone, in 50 parts of glycerol, in about 25 parts of boiling water. It is very slightly soluble in ether and chloroform; soluble in dilute ammonia solution, and in solutions of alkali bicarbonates with evolution of carbon dioxide (9). Its aqueous solution is acid to litmus (2). On alkaline and acid hydrolysis saccharin gives rise to O-sulphamoyl-benzoic acid and ammonium O-sulphobenzoic acid respectively (2).

## 2.3 pKa, Dissociation Constant

The dissociation constant of saccharin at  $25^\circ$  has been reported to be pKa 1.6.

## 2.4 Spectral Properties

### 2.4.1 Infrared Spectrum

The IR spectrum of saccharin as KBr disc was recorded on a Perkin Elmer 580-B Spectrophotometer and shown in Fig 1 (10). The structural assignments have been correlated with band frequencies and are given in Table 1.

Table 1. IR Characteristics of Saccharin

Frequency $\text{Cm}^{-1}$	Assignment
3410, 3100	-CO-NH-
2970, 2700	-C-H frequencies
1722	$\begin{array}{c} \text{O} \\ \parallel \\ \text{-C-NH-} \end{array}$
1595	aromatic- C=C-
1340	
1165, 1180, 1188	-SO <sub>2</sub> - N -
700-900	C-H out of plane deformation showing 1,2-disubstituted benzene ring.
760	Four adjacent hydrogens, ortho-disubstituted benzene.

Other characteristic bands are:

2010, 1980, 1870, 1462, 1300, 1260, 1200, 1140, 1122, 1055, 1020, 1010, 975, 902, 688, 632.

Other IR studies were also reported (11,12).

### 2.4.2 Ultraviolet Spectrum

The UV spectrum of saccharin in ethanol was scanned from 200 to 400 nm using Varian DMS 90 spectrophotometer. It exhibits a characteristic UV spectrum Fig 2 with maxima at 283, 275, 225 and 208 nm (13).

Other UV spectral data of saccharin have also been reported (14).

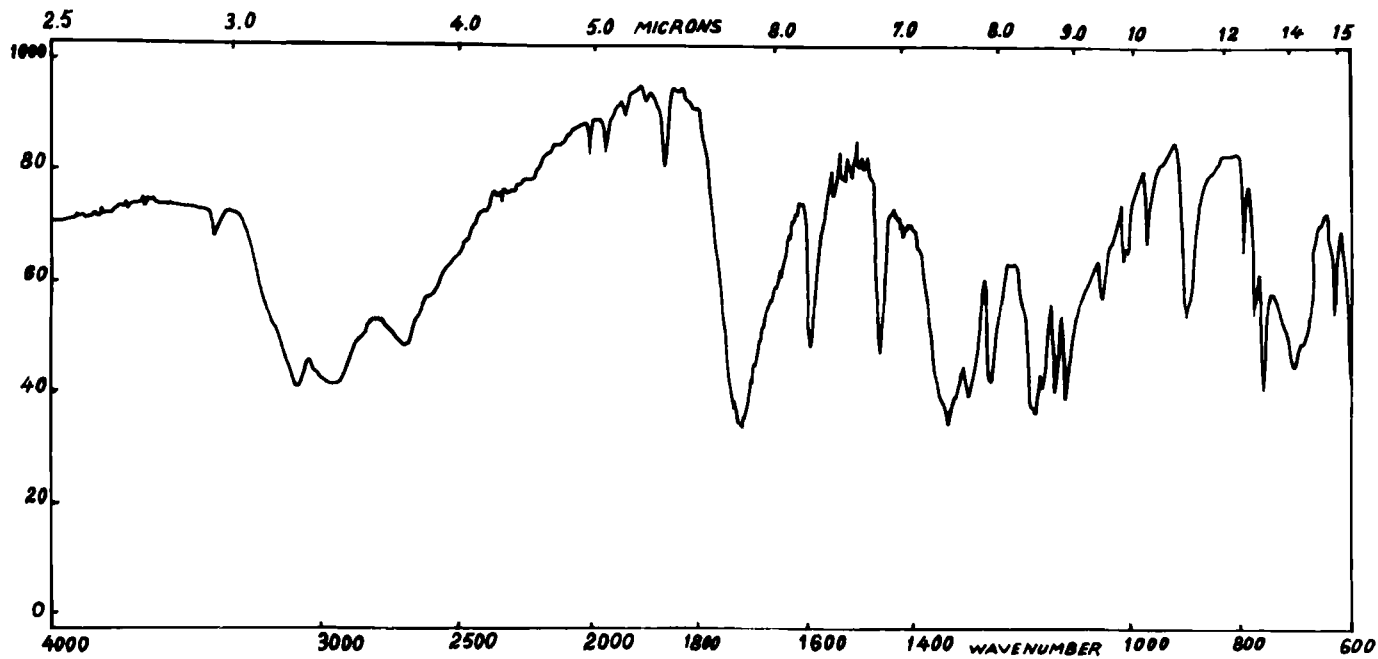


Fig. 1. IR spectrum of saccharin as KBr disc.

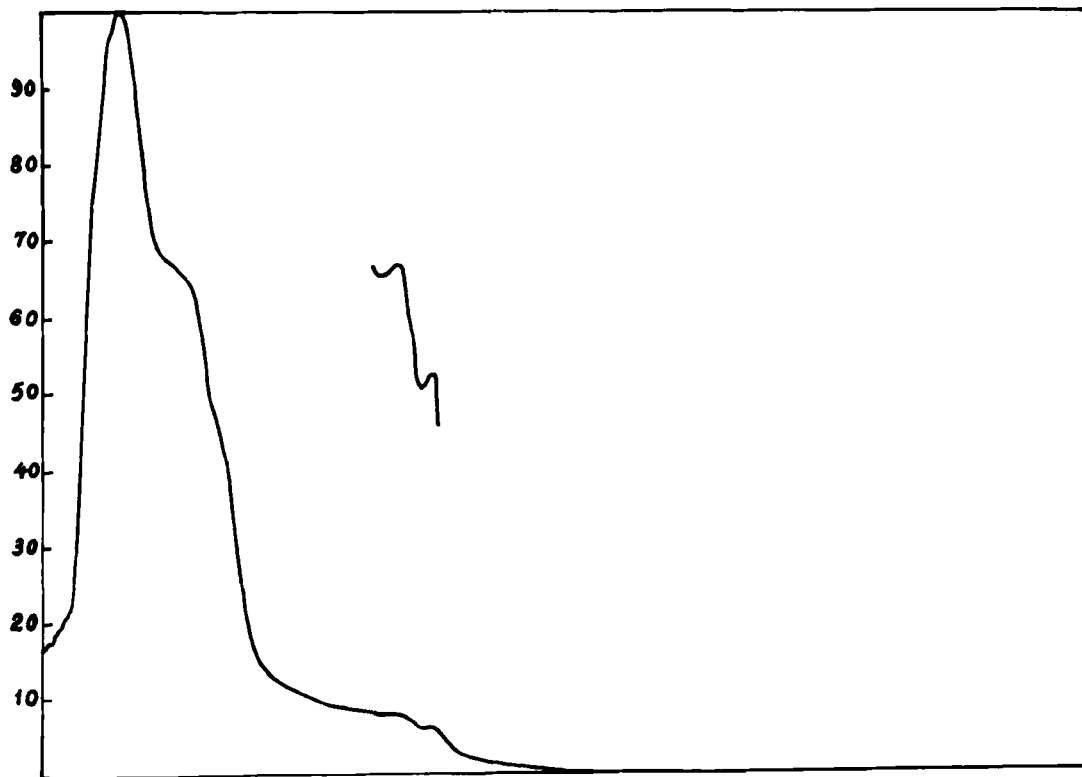


Fig. 2. UV spectrum of saccharin in ethanol.

<u><math>\lambda_{\max}</math></u>	<u><math>\epsilon</math></u>
285	775
276	951
226	8570 in methanol.
<u><math>\lambda_{\max}</math></u>	<u><math>E_{1\text{cm}}^{1\%}</math></u>
234.5	351 in 0.01 N NaOH (5, 9)
268	89
284	Inflexion
<u><math>\lambda_{\max}</math></u>	<u><math>E_{1\text{cm}}^{1\%}</math></u>
267.5	69
244.5	minima saccharin sodium in water (5)

### 2.4.3 Nuclear Magnetic Resonance Spectra

#### 2.4.3.1 Proton Spectrum

The PMR spectrum of saccharin in DMSO- $d_6$  was recorded on a Varian FT80 NMR spectrometer using tetramethylsilane as a reference standard, Fig 3 (15). The following structural assignments have been made.

Table 2. PMR Characteristics of Saccharin

<u>Assignments</u>	<u>Chemical Shifts</u>
Aromatic protons	8.11 (m)
- NH	12.45 (s)

m = multiplet; s = singlet

Natural abundance  $^{15}\text{N}$  NMR data has been reported (16). N-H chemical shift is 215.4 ppm.

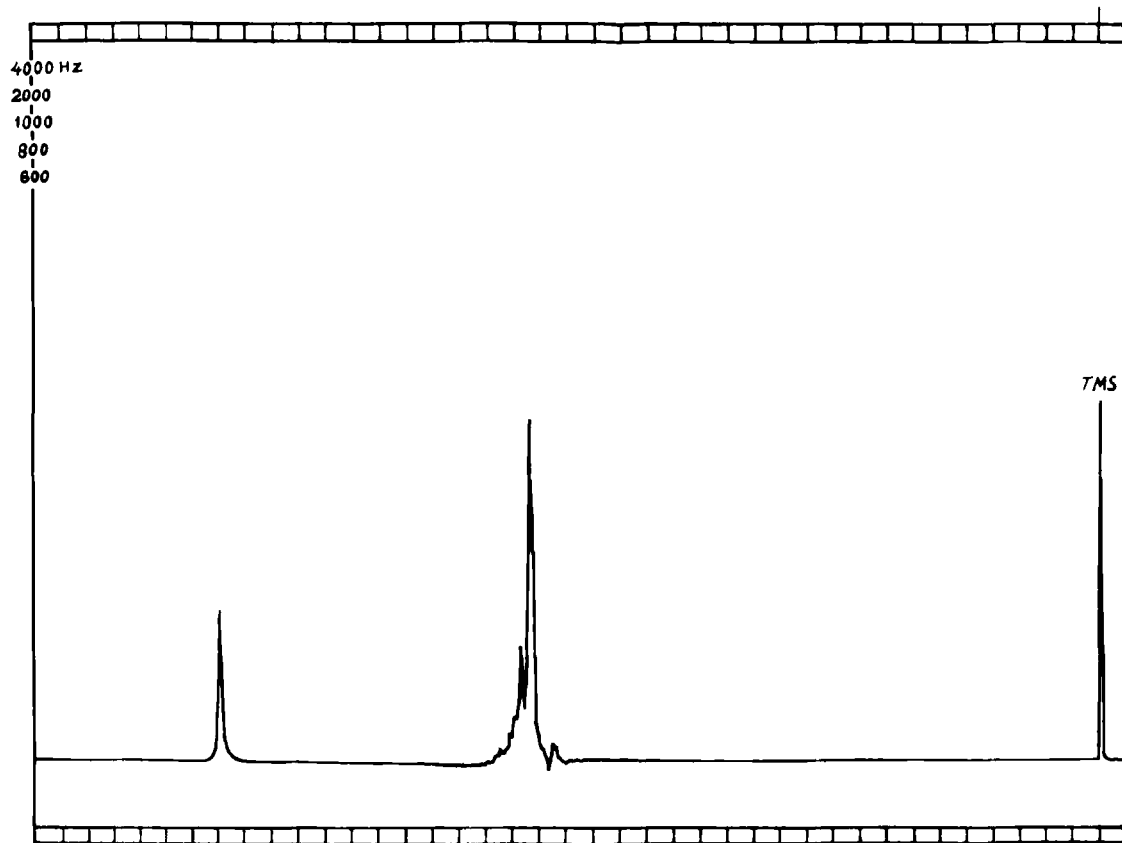


Fig. 3. FT-80 NMR spectrum of saccharin in DMSO D<sub>6</sub>.

### 2.4.3.2 $^{13}\text{C}$ NMR Spectra

The  $^{13}\text{C}$ -NMR noise decoupled and off-resonance spectra are shown in Fig 4 and Fig 5 respectively (17).

Both were recorded over 5000 Hz range in DMSO- $d_6$  on FT 80, 80 MHz NMR spectrometer, using 10 ml sample tube, and tetramethylsilane as reference standard at ambient temperature. The carbon chemical shifts assigned on the basis of additivity principle and off-resonance splitting pattern are given in Table 3.

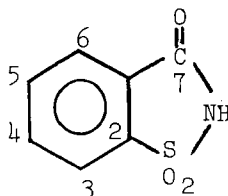


Table 3. Carbon Chemical Shifts of Saccharin

Carbon no.	Chemical Shift (ppm)
C-1	127.57(s)
C-2	139.75(s)
C-3	124.53(d)
C-4	134.72(d)
C-5	135.55(d)
C-6	121.18(d)
C-7	160.79(s)

d = doublet; s = singlet

### 2.4.4 Mass Spectrum

The mass spectrum of saccharin obtained by electron impact ionization and which was recorded on a Ribermag R-10-10 mass spectrometer equipped with direct inlet probe and a data

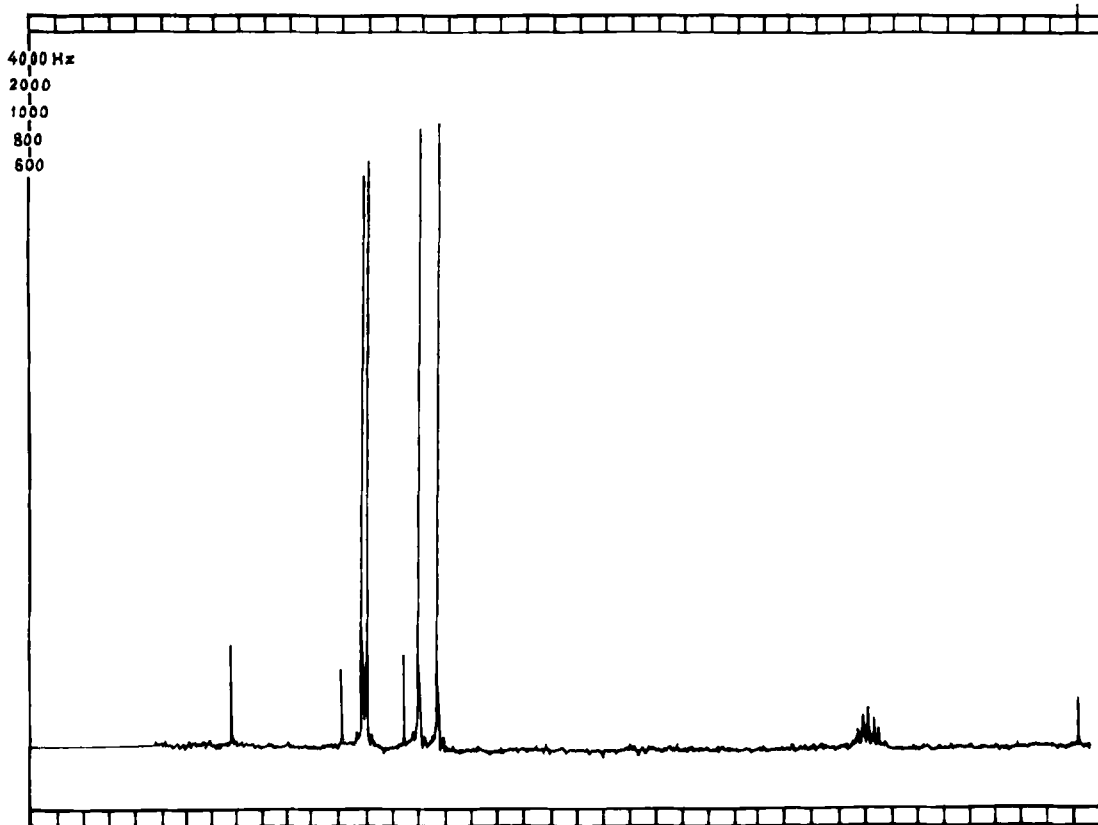


Fig. 4.  $^{13}\text{C}$ -NMR noise decoupled spectrum of saccharin in  $\text{DMSO-}d_6$ .



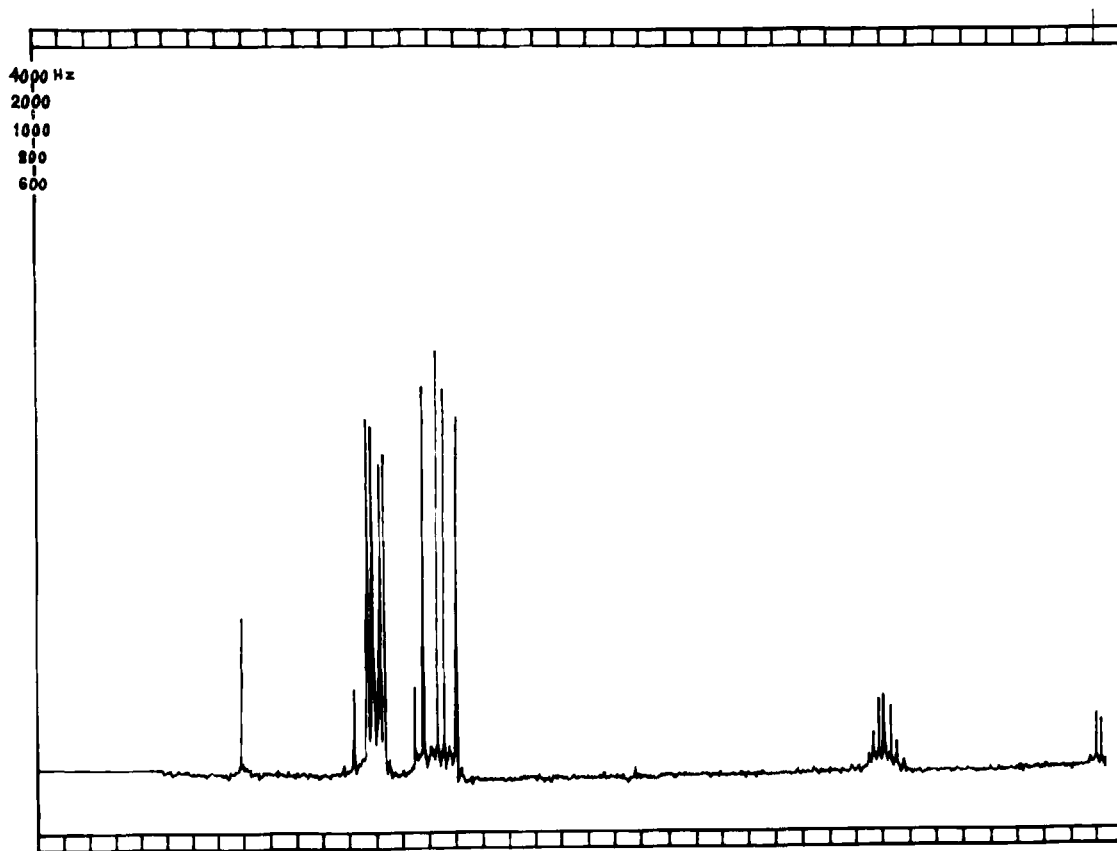
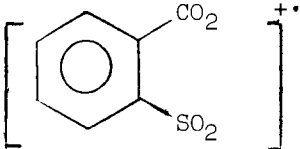
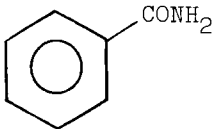
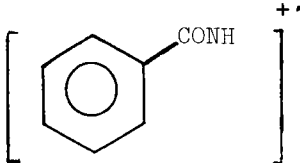
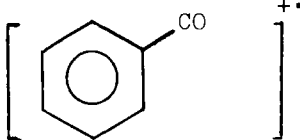


Fig. 5.  $^{13}\text{C}$ -NMR off-resonance spectrum of saccharin in  $\text{DMSO-}d_6$ .

system (18). The spectrum (Fig 6) shows a molecular ion peak at  $m/e$  183 and a base peak  $m/e$  184 ( $M+1$ ). The most prominent fragment, their relative intensities and possible structures are listed in Table 4.

Table 4. Mass Fragments of Saccharin

$m/e$	Relative Intensity	Fragment
184	100	$M+1$
183	30.7	$M^+$
168	6.9	
		
120	54	
		
119	59	
		
104	25.6	
		

(Continued)

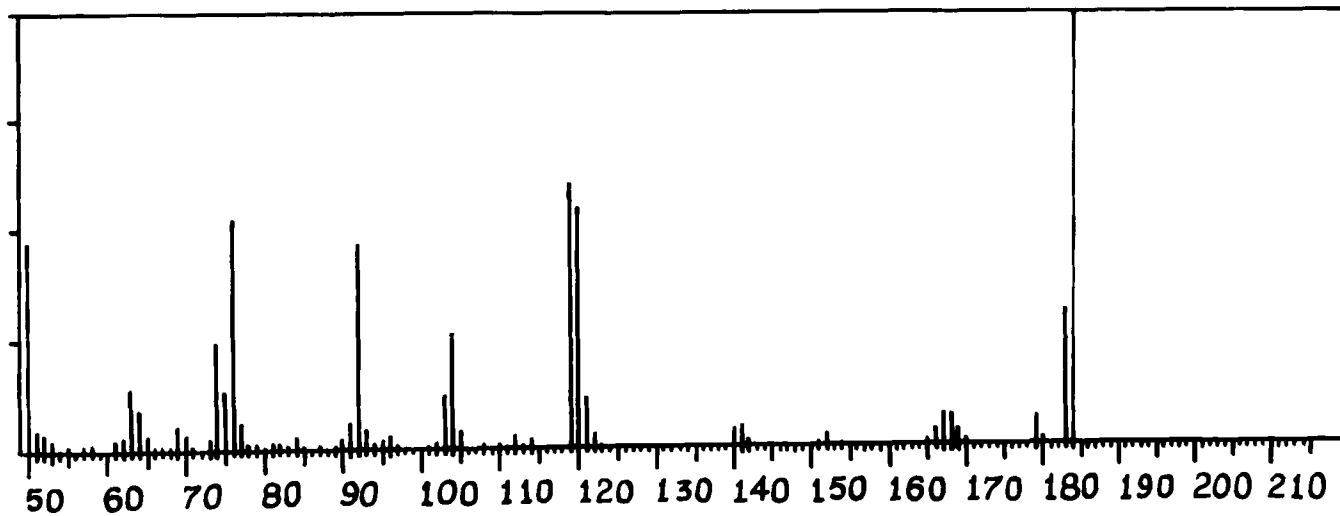
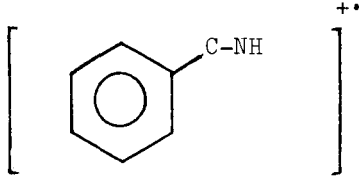
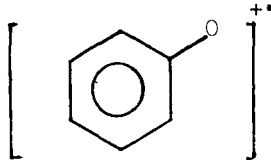
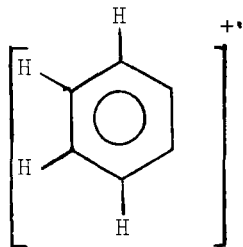


Fig. 6. Mass spectrum of saccharin (EI).

Table 4 (continued)

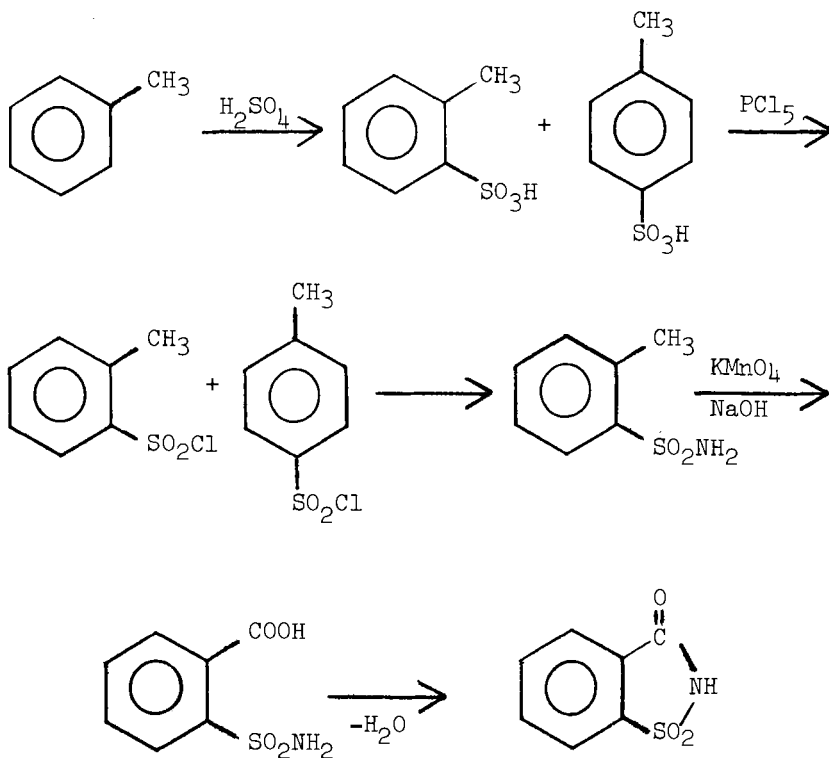
m/e	Relative Intensity	Fragment
103	11.8	
92	46	
76	51	

Other mass spectral data are also reported (19).

### 3. Synthesis

#### Route 1

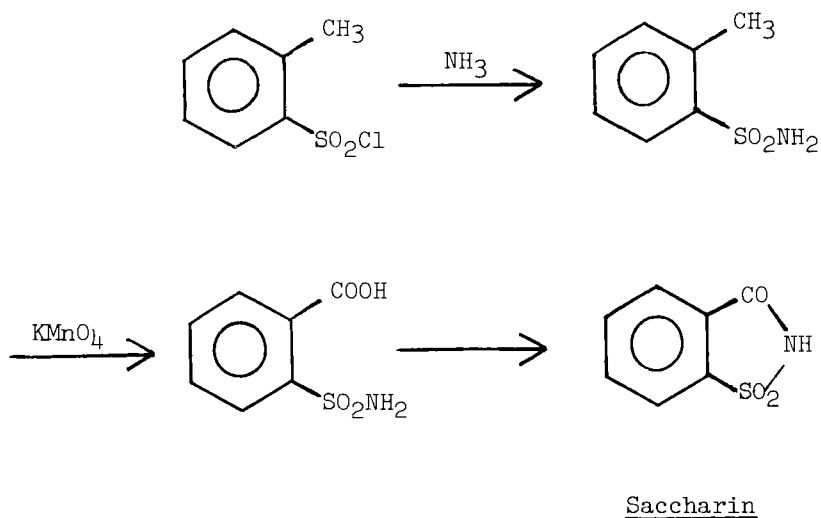
Saccharin was first synthesised in 1879 from o-sulphamoylbenzoic acid (20). The starting material is obtained from the mixture of o- and p-sulfonic acids, resulting from sulfonation of toluene. The sulfonic acids are then converted to the sulfonylchlorides by the action of phosphorous pentachloride. The p-toluenesulfonyl chloride is largely removed by freezing, and the liquid residue containing the compound is treated with ammonia to give the o-toluenesulfonamide which on oxidation with alkaline potassium permanganate solution give o-sulfonamidobenzoic acid which undergoes spontaneous loss of water to yield saccharin which can be obtained on acidification of the reaction mixture.



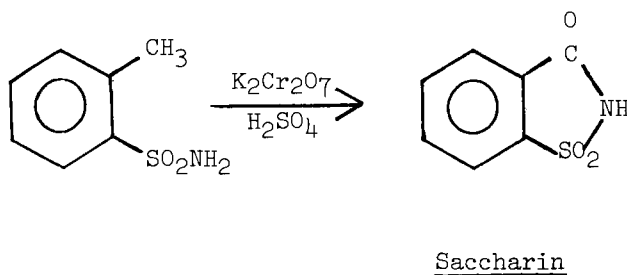
Saccharin

### Route II

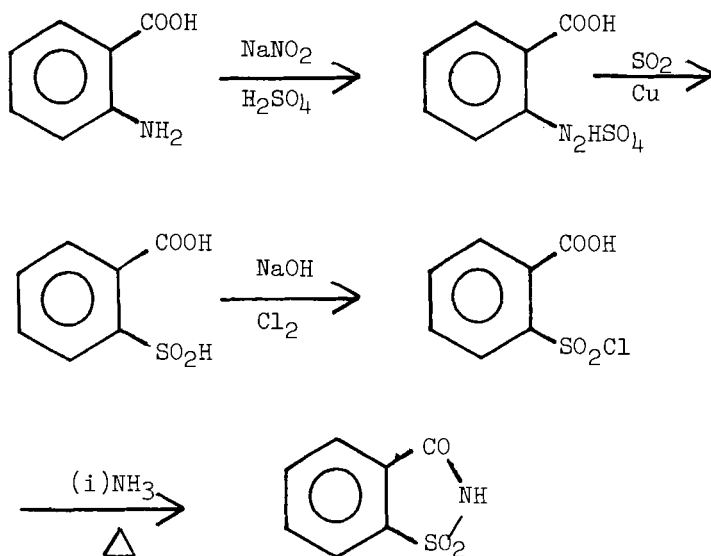
It may be prepared (21) by treating toluene with chlorosulfonic acid and separating the *o*- and *p*-toluenesulfonylchloride. The *o*-compound is then treated with ammonia and the resulting sulfonamide is oxidized with potassium permanganate in alkaline solution yields a salt of *o*-sulfamoylbenzoic acid. The free acid which is liberated on acidification loses water spontaneously to give saccharin.

Route III

Saccharin in high yield may be obtained by oxidising o-toluenesulfonamide with dichromate and sulfuric acid.

Route IV

Many other industrial methods are now employed e.g. one starts (22) with anthranilic acid which is diazotised and treated with liquid sulfur dioxide in presence of copper as catalyst. The sulphinic acid derivative thereby obtained is treated with chlorine in alkaline solution and sulfonyl chloride so obtained is treated with ammonia and heated.



#### 4. Metabolism

Early studies reported (23) that 90-95% of an i.v. dose of saccharin could be recovered from the urine of a catheterized dog. Staub (24) reported that orally administered saccharin was excreted unchanged in the urine. Herter (25) demonstrated the presence of saccharin in various organs of a rabbit after an oral dose. In recent studies utilizing radio-labelled saccharin (26-28) the compound was shown to be metabolised to a slight extent in rats and other animals. Lethio and Wallace (29) have used (3- $^{14}\text{C}$ ) saccharine (I) in rat, dog, rabbit, guinea pig and hamster, indicated that there was little difference in pattern of metabolic profiles due to animal species or dose level. They also showed that labelled  $\text{CO}_2$  in the expired air indicated that saccharin was decarboxylated to a slight degree (II), and DEAE chromatographic separation and isolation of labelled compounds indicated that more than 99% of the urinary  $^{14}\text{C}$  was unchanged

saccharin and up to 1% of  $^{14}\text{C}$  was a metabolite identified as o-sulfamoylbenzoic acid (III) (Fig 1, Scheme ).

Scheme

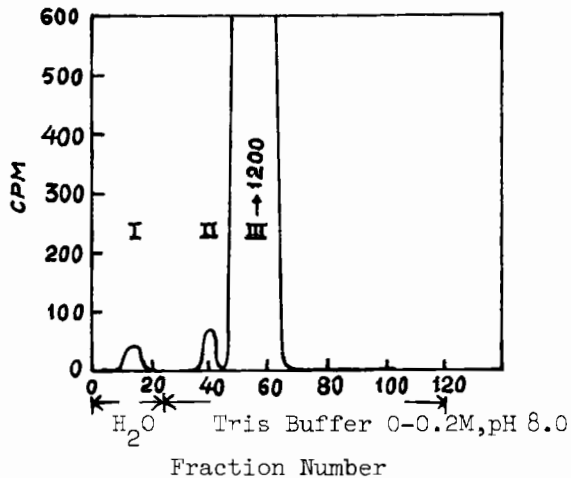
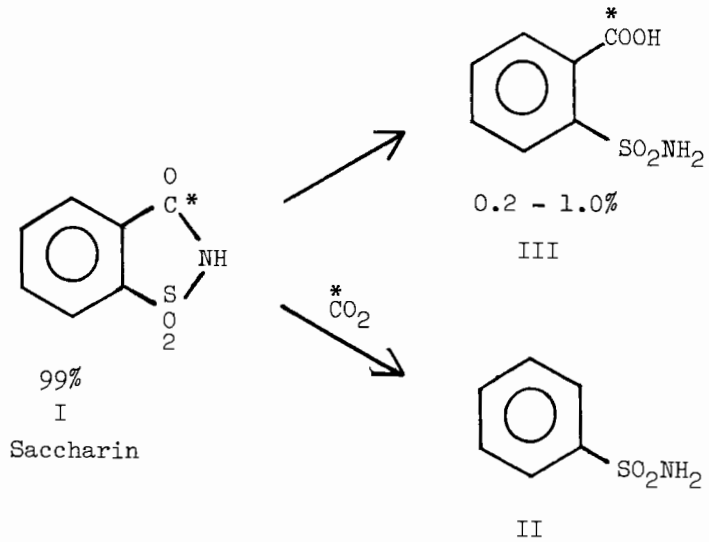


Fig. 1. Elution diagram of the chromatography on DEAE cellulose of urine from a rat which received  $[3\ ^{14}\text{C}]$  saccharin at 50 mg/kg. Each fraction contained 10 ml. Peak I: carbonate; Peak II: sulfamoylbenzoic acid; Peak III: saccharin.



In man saccharin is rapidly absorbed from the gastrointestinal tract and excreted unchanged mostly in the urine within 24 hours (5).

## 5. Methods of Analysis

### 5.1 Detection and Identification

- a. Saccharin is heated with resorcinol and sulphuric acid over a low flame; on dilution and addition of sodium hydroxide solution, a fluorescent green liquid is obtained (5,30,31).
- b. Saccharin is fused with sodium hydroxide till the evolution of ammonia ceases. Neutralised solution on addition of ferric chloride gives rise to violet colour (30,31).
- c. Thermal analyses (32) and microchemical reactions of saccharin with Mn, Co, Ni and Cu have been employed for the characterisation of saccharin (33).
- d. Saccharin can also be identified by its reaction with  $H_2O_2$  and  $NaNO_2$  and subsequent basification with alkali affords dark brown-red colour (34).
- e. Identity tests for saccharin present in food have also been based on column and thin layer chromatography (35), mass spectrometry (36), spectrophotometry (37) and microanalytical methods (38).

### 5.2 Titrimetric Methods

#### 5.2.1 Aqueous

The USP XIX(7) and B.P 1980 (6) methods consist of dissolving an accurately weighed sample of saccharin in hot water and titrating with 0.1 N sodium hydroxide, using phenolphthalein as indicator.

Another official procedure (30) involves digestion of an accurately weighed sample of saccharin in hydrochloric acid. The resulting ammonium salt is reacted with sodium hydroxide and the liberated ammonia is distilled into 0.1 N sulphuric acid. The excess of the acid is determined by titrating with sodium hydroxide using methyl red solution as indicator.

### 5.2.2 Non-aqueous

A non-aqueous differentiating titration procedure (39) involves passage of the artificial sweeteners mixture through a cationic exchange resin, Dowex 50-X8, to separate and convert saccharin to its acid form. The solution obtained is titrated potentiometrically in methyl isobutyl ketone with 0.1 N sodium methoxide. The method has been applied in presence of cyclamate and benzyl alcohol.

### 5.2.3 Ion Selective Electrode

Hazemoto et al (40) developed an ion-selective electrode sensitive to saccharin, by establishing an ion association between  $\text{Fe}^{2+}$ -bathophenanthroline chelate and saccharin in nitrobenzene. The electrode developed could measure saccharin ion in presence of other sweetening agents e.g., sucrose, glucose, sodium cyclamate and sorbitol in the concentration range of  $10^{-1}$  to  $10^{-5}$  M.

### 5.2.4 Polarography

Polarographic methods of analysis have been applied to samples of foods containing saccharin (41-44). In a procedure (44) saccharin is extracted into organic solvents in an acidic medium. Further purification is achieved by column chromatography. The residue obtained is dissolved in 0.1 N NaOH and an aliquot is polarographed in a supporting electrolyte of 0.1 N HCl, 0.1 N KCl and 0.1%  $\text{Bu}_4\text{N Br}$ .

Nasierowska (45) has applied alternate-current oscillopolarographic method to pharmaceuticals containing saccharin and sodium saccharin. Both forms a well developed oscillopolarographic curve in  $\text{MH}_2\text{SO}_4$  in air with mercury anode and dropping-mercury cathode, and the results are comparable to those obtained by UV spectrometry.

### 5.3 Gravimetric

Saccharin has been determined gravimetrically by the oxidation of its sulfur to sulfate ion (46). An accurately weighed amount of the sample is boiled with concentrated nitric acid and the vapours evolved are reacted with sodium hydroxide in a tube. The sulfate content is then determined gravimetrically.

### 5.4 Spectrophotometric

#### 5.4.1 Colorimetric

A method involving salt formation between saccharin and methylene blue has been developed (47) and its concentration as low as 10  $\mu\text{M}$  can be determined by this method.

Taneka et al (48) developed another procedure in which saccharin is reacted with phenothiazine and copper acetate in 50% aqueous ethanol. The resulting complex is extracted into xylene and its absorbance is measured at 510 nm.

Belagy (49) analysed saccharin by forming a complex with safranin and measuring its absorbance at 510 nm.

#### 5.4.2 Ultraviolet

Several procedures have been developed for the extraction and isolation of saccharin when present in beverages (50-53), and food (54-57) before its estimation spectrophotometrically. The absorbance was measured in chloroform at 278 nm (50), in ethanol at 271 nm (55) and in 1% aqueous sodium carbonate at 235 and 244 nm (52).

#### 5.4.3 Infrared

A procedure has been developed (58) for the determination of saccharin by IR spectrophotometric method. Saccharin is separated from cyclamate by extracting it into a mixture of benzene and ether from an acid medium.

The IR absorption at  $1721\text{ cm}^{-1}$  is then measured. Saccharin up to 0.2 mg can be determined by this method.

#### 5.4.4 Spectrofluorometric

Nakamura (59) had developed a procedure for the isolation and determination of saccharin in foods by measuring fluorescence at 410 nm (excitation at 277 nm). However recovery of saccharin added to 23 types of food ranged from 82 to 98%.

#### 5.4.5 PMR Spectrometry

A PMR procedure was described (60) involving the integration of the aromatic protons singlet of benzisothiazoline ring occurring at 8.00 ppm and that singlet due to protons of the internal standard maleic acid occurring at 6.25 ppm. The method was employed for saccharin sodium tablets resulted in an average of  $97.1\% \pm 3.15$  and for saccharin-lactose mixture with an average of  $98.92 \pm 3.5$  for saccharin.

### 5.5 Chromatographic Methods

#### 5.5.1 Paper Chromatography

Saccharin has been detected and estimated after extraction from food samples on Whatman No. 1 filter paper (61,62). The sample in solution form, such as carbonated water can be used for paper chromatography. The spotted paper is developed in  $\text{BuOH-AcOH-H}_2\text{O}$  (40:10:22) for 18 hours; and sprayed with a solution of phthalic acid and aniline for spot development. Saccharin with an  $R_f$  0.17 can be estimated colorimetrically after elution of the spot with 60% AcOH (62).

#### 5.5.2 Thin Layer Chromatography

Thin layer chromatography has been used in qualitative and quantitative analysis of saccharin, when present in artificial sweetening agents, beverages, food and pharmaceuticals. Several systems have been used and are listed in table 5.

Table 5

## Thin Layer Chromatography Systems for Saccharin Analysis

Stationary Phase	Developing Phase	Detection	Reference
10%-Acetylated Cellulose-Polyamide (3:2)	Shellsol A-propanol-acetic acid-formic acid (45:6:7:2)	U.V. and multiple colorimetric methods	63
Silica gel	chloroform-acetic acid (9:1)	U.V.	64
Kieselgel G	acetone-ammonia, ethanol-ammonia and benzene	silver nitrate, peroxide-ferric chloride and 2,6-dichloroquinone chloroimide spray reagent	65
Wakogel B-5 plates	-	multiple colour reaction	66
Acetyl cellulose-polyamide powder (3:2)	xylene-propanol-acetic acid-formic acid (45:6:7:2)	-	67
Silica gel (Adsorbosil-1)	butanol-ethanol (95%)-ammonia (28%)-water, (40:4:1:9)	254 nm irradiation	68
Avicel SF	ethanol-ammonia-acetone, (1:1:8) dimethyl formamide-ethanol-water, (5:4:1) dioxane-pyrimidine-water. (7:2:1)	spray with 0.1% pina crystal yellow in 95% ethanol and irradiate at 3650A°.	69
Polyamide	benzene-ethyl acetate-formic acid (5:10:2)	methyl red bromocresol green	70

(Continued)

Table 5 (continued)

Stationary Phase	Developing Phase	Detection	Reference
Al <sub>2</sub> O <sub>3</sub> /Polyamide	toluene- propionic acid- formic acid (5:4:2)	multiple colour reactions	71
Silica gel G	butanol- ethanol- 25% aqueous ammonia (40:4:5)	U.V.	72

### 5.5.3 Gas Chromatography

Saccharin has been detected and analysed by gas chromatography, when present in pharmaceuticals and food. It is usually, first extracted from the sample with an organic solvent and then converted to its ester, such as methyl or silalyl to make it more volatile. Table 6 shows various systems that have been used.

### 5.5.4 High-Performance Liquid Chromatography

High-Performance Liquid Chromatography has been used extensively for analysing saccharin, present in pharmaceuticals, beverages and food. The different HPLC systems used for the analyses are given in Table 7.

Table 6 Gas Chromatography of Saccharin

Column	Support	Mesh	Length	Temp.	Flow (ml/min) Carrier gas	Ref.
20% of SE 30	Chromosorb W	60-80	1.8 meter	172°	N <sub>2</sub> ; 60 ml min <sup>-1</sup>	73
Carbowox 20M	Silica gel	-	-	-	-	74
30% of OV-7 and 1.5% of OV-22	Chromosorb G	-	1.75 meters x 4 mm	180°	N <sub>2</sub>	75
0.5% Carbowox 20M	Chromosorb G AW-DMCS	80-100	-	185	N <sub>2</sub> ; 45 ml min <sup>-1</sup>	76
-	-	-	-	-	-	77
10% silicone	DC 200	-	-	200	-	78
3% of OV-17	Gas-ChromQ	80-100 mesh	1.5 m x 2 mm	180°	N <sub>2</sub> ; 30 ml min <sup>-1</sup>	79
DEGS 3% or 2.5% and H <sub>3</sub> PO <sub>4</sub> 1%	Gas-ChromQ	80-100	1.5 m x 3 or 2.6 mm	200° or 175°		80
3% of OV-17	Chromosorb G HP-AW	100-120	180 cm x 4 mm	200	Ar; 45 ml min <sup>-1</sup>	81
3% OV-17	Gas Chrom Q	-	-	210	N <sub>2</sub>	82

Table 7. HPLC of Saccharin

Analyte or Matrix	Parameters and Comments	Reference
Pharmaceuticals	Micronized silica gel treated with 3-amino-propyltriethoxysilane; 0.04 M phosphate buffer; 254 nm.	83
Beverages	$\mu$ Bondapak/C <sub>18</sub> (S/S 30 cm x 4 mm); 5% acetic acid, at 2000 p.s.i, (2 ml min <sup>-1</sup> ); 254 nm.	84
Alcoholic products	Partisil-10 ODS (S/S, 25 cm x 4 mm); 0.01 M KH <sub>2</sub> PO <sub>4</sub> at pH 2.2 (1.2 ml min <sup>-1</sup> ); 254 nm.	85
Food	Permaphase AA X or Zipax SAX; 10 nm-KClO <sub>4</sub> in 10 mM-Na <sub>2</sub> B <sub>4</sub> O <sub>7</sub> buffer of pH 8.0;	86
Pharmaceuticals and non-alcoholic drinks	ODS-Sil-X-1, 400-500 p.s.i.; aqueous 45% methanol or phosphate buffer solution (pH 3.0) and methanol (3:2); u.v. detection.	87
Pharmaceuticals	$\mu$ Bondapak C <sub>18</sub> ; acetic acid-methanol-H <sub>2</sub> O (1:60:139); 280 nm	88
Plasma and urine	Partisil PXS ODS-2 (10 $\mu$ m) (30 cm x 4 mm), 1.2 ml min <sup>-1</sup> ; H <sub>2</sub> O-methanol-anhyd. acetic acid (399:100:1); fluorimetric detection (excitation at 230 nm; emission at 350 nm)	89
Beverages	HC ODS Sil-X (25x0.26 cm); 1M CH <sub>3</sub> COOH	90,91



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# SALICYLAMIDE

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SALICYLAMIDE1. Description1.1 Nomenclature1.11 Chemical Names

o-Hydroxybenzamide, 2-Hydroxybenzamide.

1.12 Generic Name

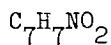
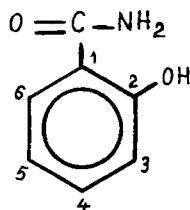
Salicylamide.

1.13 Trade Names

Afko-sal, Amid-sal, Doldram, Drosprin, Liquiprin, Raspberin, Salrin, Uromide, Salamide, Samid, Acket, Algiamida, Cidal, Oramid, Panithal, Salizell, Novecyl, Dolomide, Salipur, Salymid, Saliamin, urtosal, Algamon, Benesal (1,2).

1.14 Chemical Abstract Registry Number

[65-45-2].

1.2 Formulae1.21 Empirical1.22 Structural1.3 Molecular Weight

137.13

#### 1.4 Elemental Composition

C, 61.27%; H, 5.11%; N, 10.28%; O, 23.34%.

#### 1.5 Appearance, Color, and Odor

White or yellowish white crystalline powder or crystals darkens on exposure to air, odorless.

### 2. Physical Properties

#### 2.1 Crystal Properties

##### 2.11 Crystal System

Monoclinic, Rods from solvents or sublimation.

##### 2.12 X-Ray Diffraction

Cell Dimensions  $a = 12.93 \text{ \AA}$ ,  $b = 5.02 \text{ \AA}$ ,  
 $c = 24.80 \text{ \AA}$ .

Formula Weight per cell. 8 (8.05 calculated from X-ray data).

Density. 1.320 (flotation and pycnometer);  
1.308 (X-ray).

Optical Properties.

Refractive indices (5893  $\text{\AA}$ ,  $25^\circ\text{C}$ )  $a = 1.595 \pm 0.002$ .  $b = 1.637 \pm 0.001$ ,  $q = 1.727 \pm 0.005$ .  
Optic Axial Angles (5893  $\text{\AA}$ ,  $25^\circ\text{C}$ )  $2H = (+) 82.5^\circ$   
 $2V = (+) 76^\circ$  (calculated from  $b$  and  $2H$ ),  $2V = 75^\circ$  (calculated from  $a$ ,  $b$  and  $q$ ) (3).

##### 2.13 Melting Point and Fusion Data

Salicylamide sublimes before melting ( $142^\circ\text{C}$ ; hot stage). As the melt cools, broad and unstable form appears. This form is metastable below the melting point. When seeded or on standing the stable form grows slowly through the unstable form. The rate of transformation is very slow at room temperature (3).

#### 2.2 Solubility

One gram is soluble in about 500 ml of water, about 15 ml of alcohol, about 35 ml of ether, about 100 ml

of chloroform and about 20 ml of propylene glycol.

Insoluble in benzene, carbon tetrachloride and xylene. Soluble in solution of alkalies. A saturated solution of salicylamide in water has a pH 5.2-6(1,4,5).

### 2.3 pKa

8.1 (6).

### 2.4 Identification

Salicylamide gives a blue violet color with ferric chloride solution, a color reaction indicative to salicylate derivatives.

Trinder's reagent give purple color with salicylamide (5).

Matta and Nunes (7) identified salicylamide from chemically related analogues by the determination of its refractive index at the melting point which was found to be 1.55.

Furthermore, salicylamide could be characterized through the formation of diphenylmethyl derivative prepared by refluxing with diphenylmethanol and p-toluene sulphonic acid in acetic acid (8)

A full report for the identification of salicylamide through melting point, reactions of the amide group, the phenolic group, the benzene ring, and the formation of chelate compounds has been published (9).

Salicylamide can be detected microchemically by means of the formation of the dibromo-derivative (9). Furthermore, the identification of salicylamide by chromatographic analysis are discussed further in the monograph.

### 2.5 Spectral Properties

#### 2.51 Ultraviolet spectrum

The ultraviolet spectrum of salicylamide in methanol and water shows maxima at 235 nm and 302 nm. The ultraviolet spectrum in ethanol is shown in Figure [1] and is recorded on Varian AG UV-VIS spectrophotometer model DMS 90 with maxima at

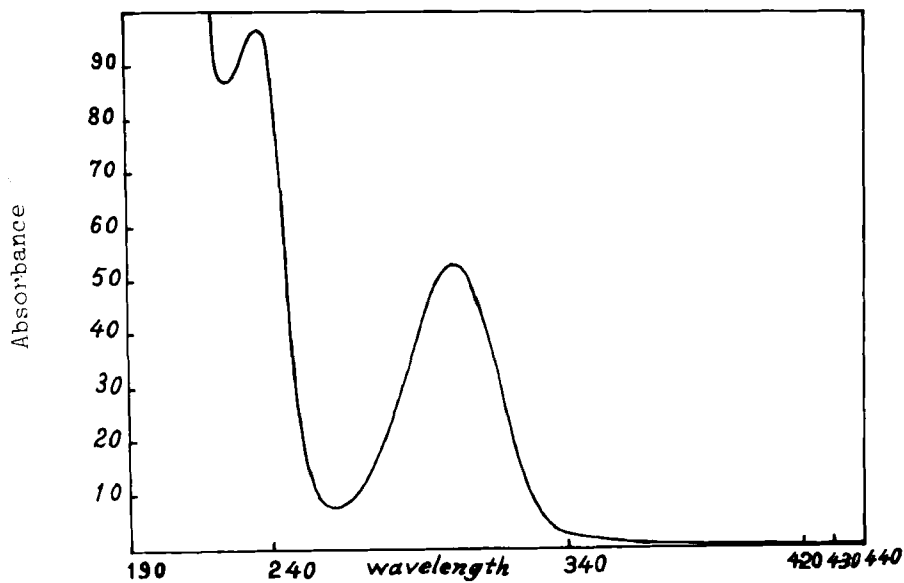


Fig. 1. The UV spectrum of salicylamide in ethanol.

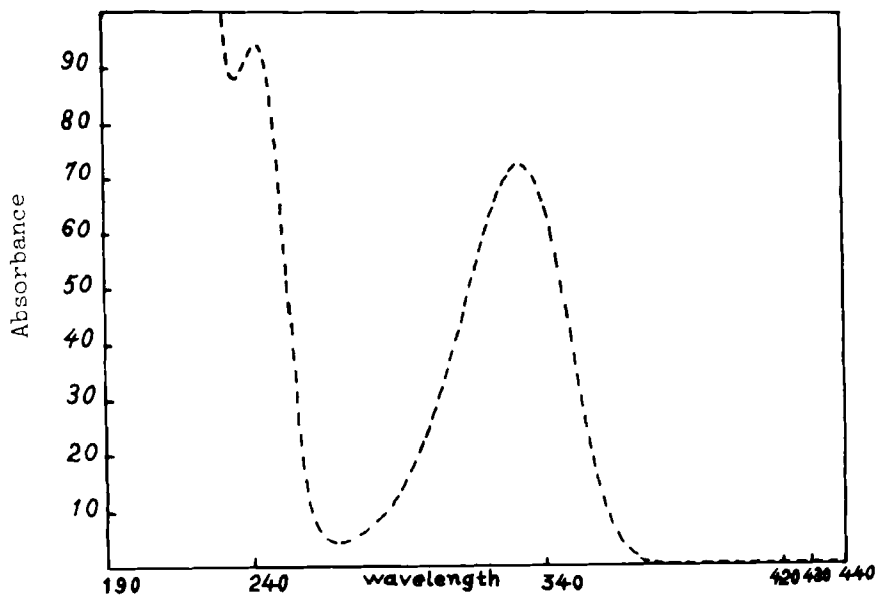


Fig. 2. The UV spectrum of salicylamide in 0.1 N NaOH solution.

235 nm ( $E_{1\%}^{1\text{ cm}}$ , 543) and 302 nm ( $E_{1\%}^{1\text{ cm}}$ , 295). In alkaline medium, the spectrum exhibits two maxima at 242 nm ( $E_{1\%}^{1\text{ cm}}$ , 536) and 328 nm ( $E_{1\%}^{1\text{ cm}}$ , 435) as shown in figure [2].

### 2.52 Infrared spectrum

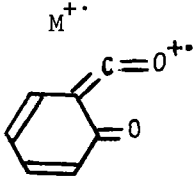
The infrared of salicylamide in KBr disc is presented in figure [3] and is recorded in Perkin-Elmer spectrophotometer model 580 B. The frequencies and their structural assignment are shown below:

<u>Frequency (cm<sup>-1</sup>)</u>	<u>Assignments</u>
3400	OH stretch
3200	NH stretch
1680	CO (amide)
1630 } 1590 }	C=C aromatic
760	CH out-of-plane
750	bending aromatic.

Other characteristic fingerprint bands are 1500, 1450, 1430, 1360, 1310, 1250 cm<sup>-1</sup>.

### 2.53 Mass Spectrum

The mass spectrum of salicylamide is shown in figure [4]. The spectrum was carried out on Varian MAT 311 spectrometer with an ionising energy of 70 eV. The sample was introduced by direct probe at 120°C. A molecular ion peak was observed at m/e 137. Some characteristic peak observed are listed below:

<u>Mass (m/e)</u>	<u>Species or Fragment</u>
137	M <sup>+</sup>
120	 <p>M-17 (NH<sub>3</sub>) base peak.</p>

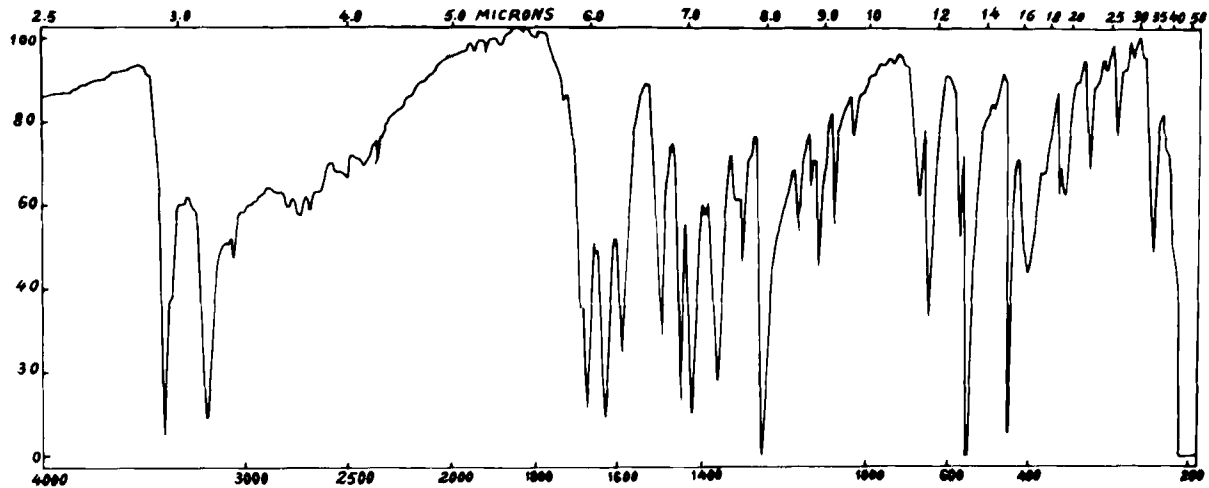


Fig. 3. The IR spectrum of salicylamide in KBr disc.

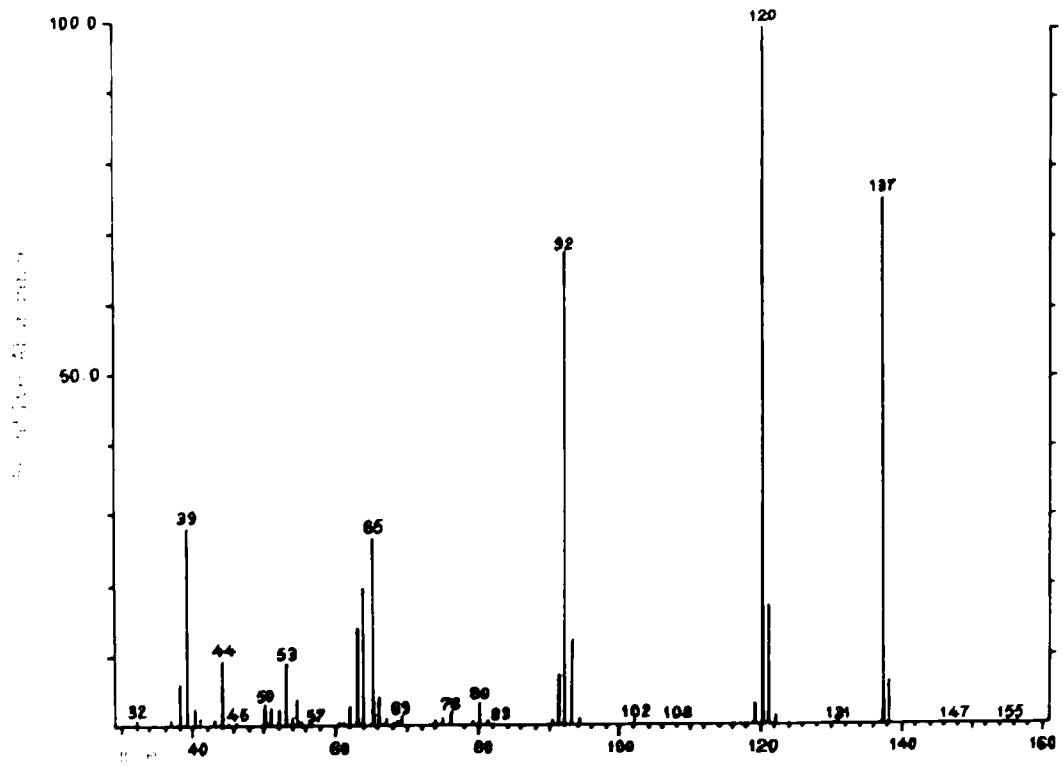
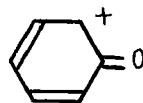


Fig. 4. The mass spectrum of salicylamide determined by electron impact.

92

65 }  
39 }aromatic  
fragmentations.2.54 Nuclear Magnetic resonance spectrum2.541 <sup>1</sup>H-NMR Spectrum

The 60 MHz <sup>1</sup>H-NMR spectrum of salicylamide in DMSO-D<sub>6</sub> is shown in figure [5]. The spectrum was recorded on Varian T60-A NMR spectrometer using TMS as the internal standard. The following structural assignments have been elicited from figure [5].

<u>Chemical shift (ppm)</u>	<u>Assignments</u>
Multiplet centered at 6.83	Aromatic protons at C <sub>3</sub> and C <sub>5</sub>
Multiplet centered at 7.36	Aromatic proton at C <sub>4</sub>
Multiplet centered at 7.90	Aromatic proton at C <sub>6</sub>
Broad singlet at 8.33	NH <sub>2</sub> amide(exchangeable with D <sub>2</sub> O).
Singlet at 13.00	OH phenolic (exchangeable with D <sub>2</sub> O, Fig. [6]).
Long range coupling between protons at C <sub>3</sub> , C <sub>5</sub> , C <sub>4</sub> and C <sub>6</sub> is observed.	

2.542 <sup>13</sup>C-NMR Spectrum

The Carbon-13 NMR spectrum of Salicylamide have been determined on Varian FT 80 Spectrometer. The



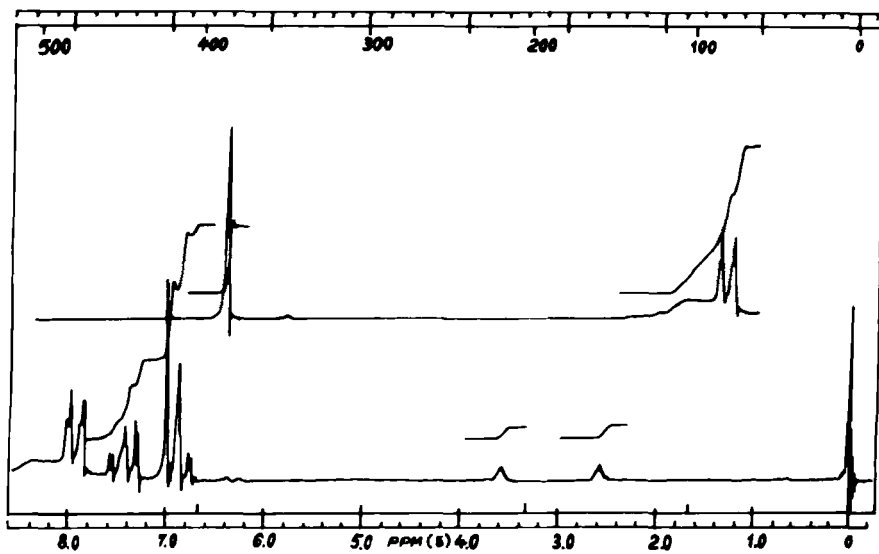


Fig. 5.  $^1\text{H-NMR}$  spectrum of salicylamide in  $\text{DMSO-D}_6$ .

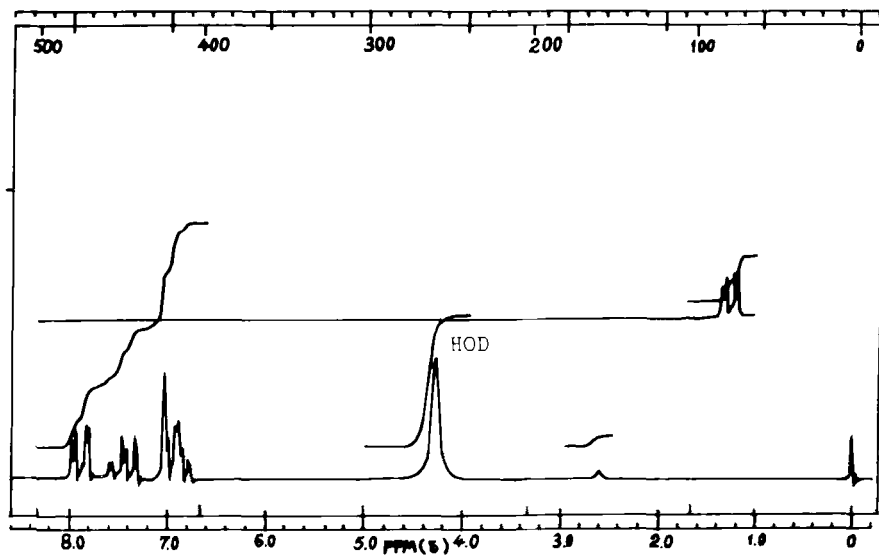
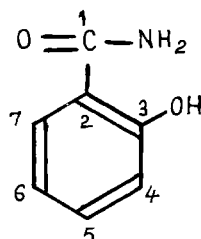


Fig. 6.  $^1\text{H-NMR}$  spectrum of salicylamide in  $\text{DMSO-D}_6$ , showing  $\text{D}_2\text{O}$  exchange.

sample was dissolved in DMSO-D<sub>6</sub> in a tube with a diameter of 10 mm. Spectral width: 4000 Hz, acquisition time: 1.023 seconds, pulse width 6 μ seconds, and number of data points 8192. The noise-decoupled spectrum is shown in figure [7] shows seven singlets. The complete off-resonance spectrum is shown in figure [8] and spectral assignments are listed below:



Chemical shift (in ppm relative to TMS)	Assignment
114.60 (s)	C <sub>2</sub>
161.31 (s)	C <sub>3</sub>
117.39 (d)	C <sub>4</sub>
134.16 (d)	C <sub>5</sub>
118.49 (d)	C <sub>6</sub>
128.31 (d)	C <sub>7</sub>
172.39 (s)	C=O

s = singlet.  
d = doublet.

The assignments are in agreement with recently published data (11).

### 3. Synthesis

Although several methods were published for the synthesis of salicylamide, yet the main route is through the aminolysis of methyl or ethyl salicylate (12, 13, 14, 15, 16, 17) as shown in the following scheme:

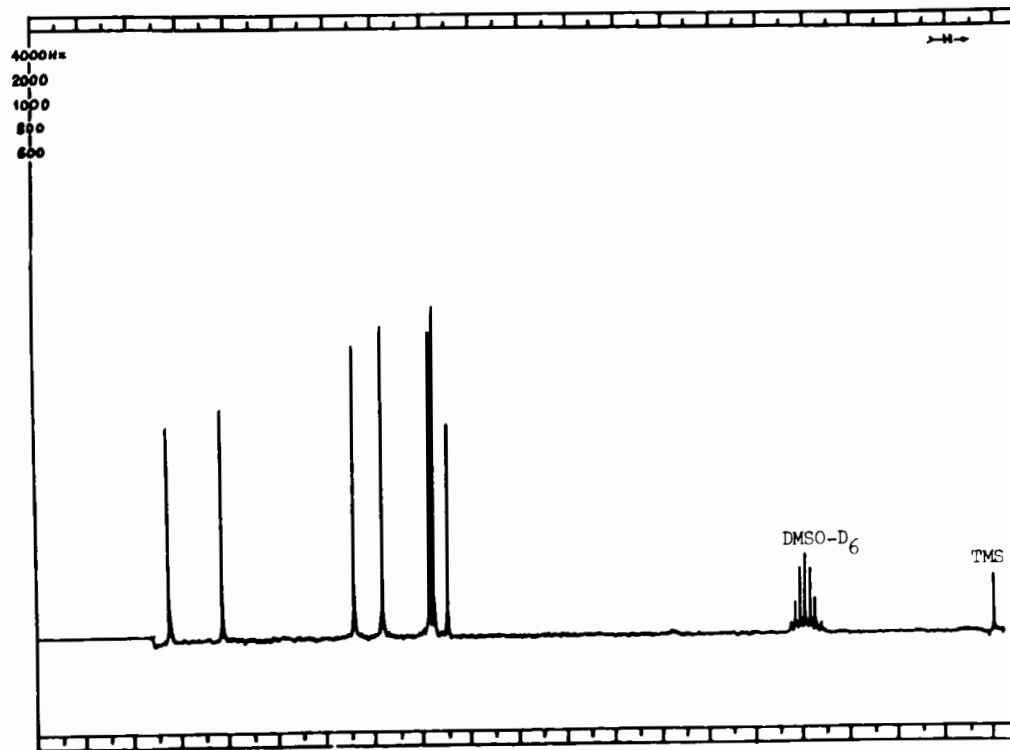


Fig. 7. Carbon-13 NMR noise-decoupled spectrum of salicylamide.

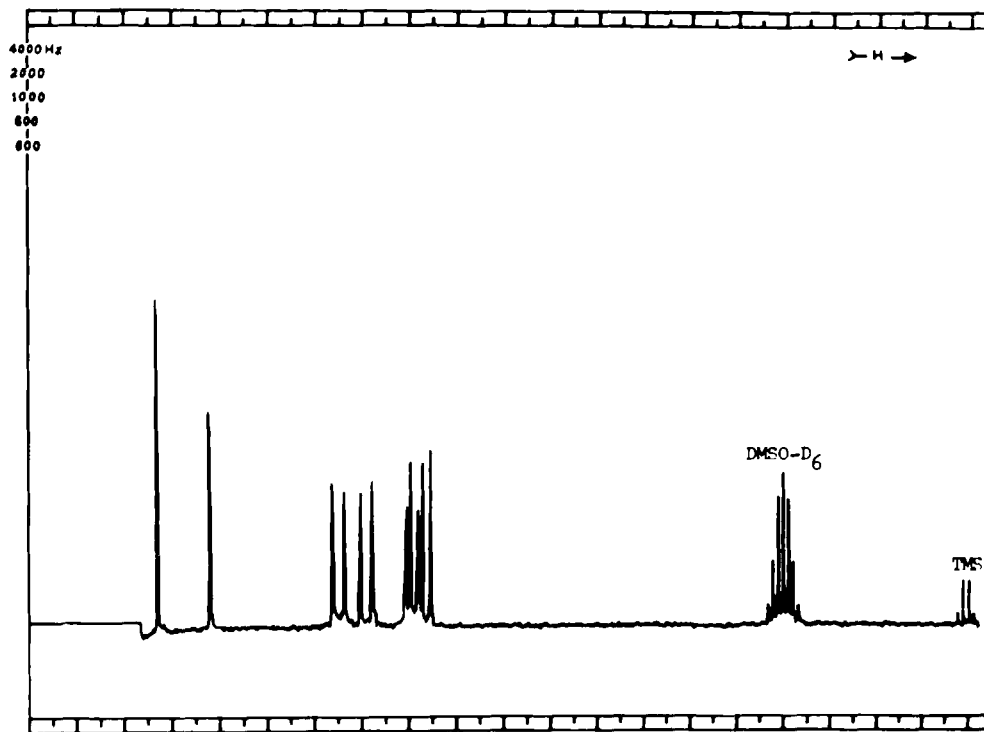
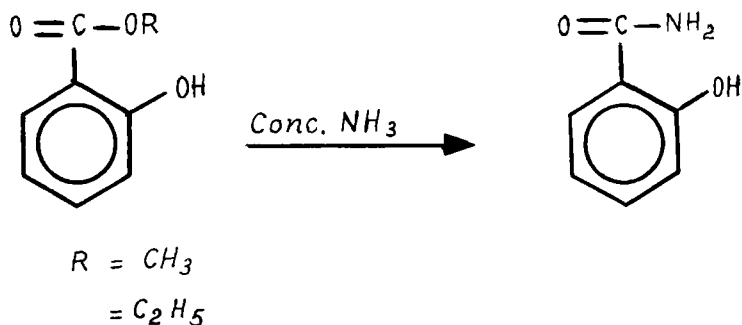


Fig. 8. Carbon-13 NMR off-resonance spectrum of salicylamide.



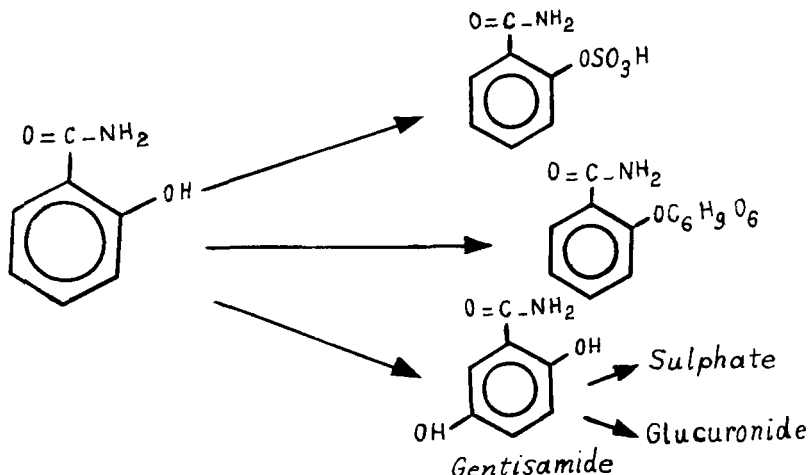
#### 4. Stability

Salicylamide is stable in solid state, however, it is recommended to be stored at room temperature in a dry-dark container. The hydrolysis of salicylamide under experimental conditions designed to simulate the gastrointestinal environment proved that the amide bond is resistant to acid cleavage (18). The kinetics and mechanism of alkaline hydrolysis was reported and found to follow pseudo first order kinetics (19).

#### 5. Metabolism and pharmacokinetics

Salicylamide is readily absorbed from the gastrointestinal tract and distributed to all body tissues but the drug does not bind appreciably to plasma proteins. Several studies had been reported in literatures dealing with the absorption of salicylamide and its plasma concentration (20), (21), (22), (23). It is rapidly excreted in the urine mainly as the glucuronide and sulphate conjugates. Salicylamide is metabolized in human entirely as shown in Scheme 1.

The percentage of the urinary conjugated metabolites varies according to animal species (24), (25), age (26) and route of administration (27) and (28).



Scheme 1, Metabolic pathways of salicylamide in humans.

The proportion of a dose of salicylamide excreted as sulphate decreased with increased doses (29). Peak serum levels are reached between 30 minutes and 1 hour after oral administration (5). Investigation has revealed that salicylamide inhibits the acylation of sulphonamides leading to increase in their concentration (30).

## 6. Methods of Analysis

### 6.1 Non-aqueous Titration

Rhodes *et al* (31) determined a mixture containing acetylsalicylic acid, acetaminophen and salicylamide, potentiometrically by non-aqueous titration. The titrant was 0.1 N tetrabutylammonium hydroxide in benzene/methanol mixture and the titration solvent was dimethylformamide. The difference in the  $\text{pK}_a$  values for these weak acidic drugs (aspirin  $\text{pK}_a$  3.5, acetaminophen  $\text{pK}_a$  8.31) was sufficient to permit useful differentiation.

Walash and Rizk (32) reported a non-aqueous titration of several analgesics in dosage form including salicylamide in tetramethylurea using 0.1 N sodium methoxide as the titrant, the end point was measured either potentiometrically or with thymol blue as indicator. The results were comparable with those obtained using dimethylformamide as a solvent.

## 6.2 Colorimetric Analysis

Rutkowski (33) reported the simultaneous colorimetric assay of salicylamide, salicylic acid and genetic acid in pharmaceutical preparations. These compounds give blue color complexes with ferric salt (nitrate in ethanol solution). The blue color complex of salicylamide is extracted with ether while the others are not.

Another method reported by Souza (34) for the determination of several phenols including salicylamide and aminopyrazoles in pharmaceutical products, where a solution of 2% potassium ferricyanide gives a colored indo-phenol derivative which is measured at 500 nm.

## 6.3 Spectrophotometric Analysis

Salicylamide had been estimated spectrophotometrically as a single component, in complex pharmaceutical mixture and analgesic tablets by several procedures among them are the following:

- a) The treatment of salicylamide with chloramine at pH 8.00 in the presence of thiobromine sodium salicylate, caffeine, salicylic acid, acetaminophen, and methanamine. The method could be used to determine as low as 0.1 mg of salicylamide in the mixture (35).
- b) The mixture of salicylamide, papaverine HCl, phenobarbital and glyceryl guaiacolate in glycol-glycerol aqueous solution was determined in 0.1 M H<sub>2</sub>SO<sub>4</sub> at 250, 273 and 299 nm (36).
- c) Soliman and Salaheldin (37) reported a simple rapid method for routine analysis of salicylamide in analgesic tablet containing acetaminophen, phenobarbital, caffeine, codeine phosphate, ascorbic acid, chloramine phosphate and prednisone. The method does not require preliminary separation of salicylamide from other constituents prior to determination. The absorbance was linear for concentration of salicylamide up to 4 mg/100 ml solution at 308 nm.
- d) Salicylamide may be estimated as salicylic acid in biological fluids following the addition of Trinder's reagent (38) or after extraction into 0.4% malonic acid in butyl ether (39).

#### 6.4 Spectrophotofluorimetric Analysis

The spectrophotofluorimetric determination of salicylamide in biological fluids (blood, serum and urine) was reported by Vercsh et al (40). The procedure involves the simultaneous determination of salicylamide and salicylic acid at pH 11. The method is sensitive and capable of estimating a concentration of salicylamide and/or its metabolites as low as 0.1 µg/ml blood, serum or other biological fluids. The method may also be used in monitoring the bioavailability of salicylamide provided in different dosage forms. The fluorescence of salicylamide was measured directly at maximum activation and emission wavelengths of 340 and 430 nm respectively while for salicylic acid at 310 and 435 nm respectively. Lange et al (41) employed the fluorescent property of salicylamide in basic solution to determine the drug and other salicylates following the gel filtration separation.

Barr and Riegelman (42) detailed a spectrofluorimetric procedure for the determination of salicylamide and its metabolites in biological fluids following hydrolysis as described by Levy and Matsuzawa (29). The method involves the hydrolysis of all metabolites to salicylate and determining them at emission to excitation wavelength of 350 and 430 nm respectively. Total salicylamide was determined by hydrolysing all metabolites to salicylic acid in aqueous basic solution at a maximum activation and emission wavelengths of 350 and 430 nm respectively.

Recently, Street and Schenk (43) reported a spectrofluorimetric analysis of salicylamide in the presence of acetylsalicylic acid and salicylic acid as impurities by direct and indirect methods. Salicylic acid is determined in the range of  $10^{-7}$ M concentration after separation from salicylamide. The instrumental conditions for the spectrofluorimetric determination of salicylamide, acetylsalicylic acid and salicylic acid is shown in Table 1.



Table 1

Ingredient determined	Wavelength nm		Slit Width nm	
	Excitation	Emission	Excitation	Emission
Salicylamide				
in ethanol.	300	425	10	10
in chloroform	315	445	10	20
Acetylsalicylic acid	280	335	10	20
Salicylic acid	315	445	10	10

The presence of other medicinals such as phenacetin, ascorbic acid, methapyreline hydrochloride among other analgesics does not affect the determinations of salicylamide, acetylsalicylic acid and salicylic acid.

### 6.5 Infrared Spectrophotometric Analysis

Salicylamide in mixed pharmaceutical preparations was determined by infrared spectrophotometry using the amide absorption at  $3425\text{ cm}^{-1}$  as the key band (44). The absorbance measured with a base line at  $3300\text{ cm}^{-1}$  or  $3470\text{ cm}^{-1}$ . The calibration curves were linear in the concentration range between 0.5 to 2 mg/ml and the results were in good agreement with those determined colorimetrically. The determination was not interfered with the coexistence of a limited amount of 40 possible drug constituents e.g., phenacetin and pyrabital (44).

Another IR spectrophotometric method for the determination of salicylamide among other medicinally used amides was reported (45). The amide carbonyl absorption band at  $1732\text{ cm}^{-1}$  was used as the basis of quantitative assay.

### 6.6 Chromatographic Analysis

#### 6.6.1 Paper Chromatography

- 1) Clarke (5) described a method for the separation of salicylates using the following two systems:

- a) Strong ammonia: n- butanol: water 33:100:66 by descending technique. The localizing agent used was ferric chloride spray or ultraviolet light at 254 nm..
  - b) Phosphate buffer (pH 7.4) and detecting agent bromine-starch potassium iodide.
- 2) Other method was described by Wagner (46) for the analysis and detection of salicylic acid and derivatives including salicylamide. Development was effected by using color reactions, with ferric ammonium sulphate, ammoniacal silver nitrate solution, potassium nitrate, HCl and 5% KOH through diazotization and coupling with, 2-naphthol and coupling with diazotized sulphanilic acid.
- 3) It was reported (47) that salicylamide and other aromatic hydroxy carboxylic acid amides undergo autooxidation, when chromatographed in alkaline media. This autooxidation products hinder the development of the complex coloration with ferric ion. However, paper chromatographic separation of salicylamide and analogs in alkaline buffer media can be accomplished by ionophoresis (48).

#### 6.62 Column Chromatography

Column chromatography has been applied to separate and analyse a mixture of salicylamide and acetaminophen in tablets and capsules (49). Strongly acidic and basic celite columns are used to separate acetaminophen and salicylamide respectively. Quantitation is performed using ultraviolet spectrophotometry.

Cataionic and anionic ion-exchange resins (50) were used as stationary phases for the separation of several analgesic drugs including salicylamide using aqueous ethanol as the mobile phase.

#### 6.63 Thin Layer Chromatography

Several reports have been published on the identification and separation of salicylamide which are summarized in Table 2.

Table 2: Thin Layer Chromatography of Salicylamide.

Stationary Phase	Solvent System	Detecting Agent	References
1) Silica gel	Butyl acetate : Chloroform : 85% formic acid (6:4:2)	5% solution of ferric chloride in acetone followed 1% alcoholic o-phenanthroline.	(51)
2) Polyamide resin	a) Isopropanol : Water : 90% formic acid. (1.5 : 6 : 0.1) b) Chloroform : benzene : 90% Formic acid. (5 : 1 : 0.1). c) Chloroform : 90% Formic acid. (20 : 0.1). d) Cyclohexane : Chloroform : acetic acid. (4 : 5 : 1).	-	(52)
3) SGF plates	Benzene : Acetic acid : Methanol Chloroform : Petroleum ether (30-70°) (10:5:5:10:70).	Ultraviolet light 254 nm.	(53)

- |  |   |  |      |
|--|---|--|------|
| 4) Silica gel G.                         | Methanol  | 10% Copper sulphate, 2% ammonium hydroxide, dark yellow color. | (54) |
| 5) Silica gel                            | a) Chloroform : Ether : Acetone (60 : 20 : 20).                       | Ferric chloride and 2,6-dibromoquinone-4-chloroimide.          | (55) |
|  | b) Chloroform : Benzene : Acetone (60 : 20 : 20).                     |  |      |
| 6) Silica gel G.                         | Acetic acid : Carbon tetrachloride: Chloroform : Water (100:60:90:50) |  | (56) |
| Kieselguhr G impregnated with formamide. | a) Benzene : Chloroform (30:120) saturated with formamide.            |  | (56) |
|  | b) Carbon tetrachloride saturated with formamide.                     |  | (56) |
-

### 6.64 Gas Liquid Chromatography

Various gas liquid chromatographic methods have been developed for the identification and determination of salicylamide in pharmaceuticals and biological fluids. It is of interest to mention that salicylamide could be gas chromatographed without derivatization. Some of the data obtained from the literature are summarized in Table 3.

The analysis of several drugs including salicylamide using semiautomated gas liquid chromatography has been reported (57).

Nieminen (58) described a gas chromatographic method for simultaneous separation and quantitation of some antipyretics including salicylamide containing phenobarbital, caffeine and codeine phosphate. Codeine phosphate was first separated from the samples as the base. Although the separation was excellent for all components, yet acetylsalicylic acid interferes with the separation of salicylamide.

### 6.65 High-Pressure Liquid Chromatography

High-pressure liquid chromatography has been utilized for the determination of salicylamide in blood, serum and other biological fluids as well as pharmaceutical formulations. Some of recently reported methods are summarized in Table 4.

### 6.7 Radiochemical Analysis

The radiochemical analysis of salicylamide in plasma using the ring-labelled tritiated compound was reported by Stella *et al* (59). The tritiated salicylamide was synthesized by reacting cold salicylamide with tritium oxide in the presence of heptafluorobutyric acid. This procedure is effective for the analysis of salicylamide and its metabolites (the sulphate and glucuronide) in the presence of similar phenolic compounds.

### 6.8 Flame Emission Spectrometry

Dialonzo and Siggia reported a method for determining several compounds including salicylamide based on the use of Hofmann reaction for the synthesis of amines

Table 3: Gas Chromatography of Salicylamide

Column	Carrier Gas	Detector	Remarks	Reference
27% OV-225 +1% OV-17 Gas Chrom-Q	Helium	Flame ionization	Quantitation was carried out by combined GC/MS. Sensitivity ranges from 1 to 25 µg/ml (whole blood plasma and saliva) and 5-100 µg/50 ul urine.	(60)
1% Silicon Gum SE-30 on Chromosorb-G	Helium	Thermal conducting	Applied for simultaneous determination of dextromethorphan, chlorpheniramine nore- phedrine and salicylamide.	(61)
8% OV-101	-	Flame ioni- zation	Simultaneous analysis of salicylamide, phenylpropanolamine HCl, caffeine, chlor- pheniramine maleate phenylephrine HCl and pyrilamine maleate, the sample should be treated with 4-(dimethylamino)-pyridine in pyridine-acetic anhydride 1:1.	(62)
2% SP-2501 DA	Helium	Flame ioni- zation.	Detection limits 5 mg/l.	(63)

(continued)

Table 3 (continued)

Column	Carrier Gas	Detector	Remarks.	Reference
Mixture of 9:1 2% methyl/phenyl silicon (SP 2110) and 1% SP 2510 on 100-120 mesh acid washed dimethyldichlorosilane treated diatomite support.	Helium	Flame ionization.	Detection limits 5 mg/l.	(63)

Table 4: HPLC for Salicylamide

Column	Mobile Phase	Detection	Remarks	Reference
RPz	Methanol : Water (60:40)	UV	Limit of detectability is 0.2 µg/ml. Caffeine and paracetamol do not interfere in the determination of salicylamide.	(64)
RP-5C <sub>18</sub>	Methanol:Buffer pH 3 (10:50)	UV	Method is suitable for clinical determina- tion and toxicological use.	(65)
µ-Bonda- pak C <sub>18</sub>	0.01 M potassium acid phosphate in water with 19% v/v methanol, pH was adjusted to 2.3 with 80% aq. phosphonic acid solution.	UV	Simultaneous quantitation of acetaminophen, aspirin, caffeine, codeine phosphate, phenacetin and salicylamide is affected.  Solvent system with pH 2.3 affects better separation.	(66)
,,	Same as above except pH was adjusted to 4.85		Reverse-HPLC technique was used.	(66)
µ-Bonda- pak C <sub>18</sub>	1% acetic acid in 25% v/v methanol/water solution.	UV	Low concentration of unconjugated salicyla- mide in human serum and saliva were assayed.	(67)
	3 mM tetrabutyl- ammonium hydroxide in a mixture of 8 parts methanol and 92 parts v/v acetic acid 7%.	UV	Reverse phase ion-pair chromatography is used.  Salicylamide and its conjugated metabolites were assayed.	(67)

(continued)



Table 4 (continued)

Column	Mobile Phase	Detection	Remarks	Reference
$\mu$ -Bondapak C <sub>18</sub>	1% Acetic acid in 15% v/v methanol/Water/ solution.	UV	Gentisamide and its conjugates is assayed.	(67)
-Bondapak ODS-C <sub>18</sub>	0.15 M Sodium dibasic phosphate pH 8 : Methanol (70 : 30).	Flourometric detector	Can detect salicylamide as low as 5 mg/ml in plasma.	(68)
LFS Pelli- cuar anion- exchange resin.	One molar Tris (pH 9) at 60°	UV	Applied for routine separation of common components of analgesic tablets.	(69)

with one less of carbon atom than the starting amide. Barium nitrate is used as reagent. Barium carbonate formed in the reaction is dissolved in nitric acid and is determined by flame atomic emission spectroscopy; the precision was  $\pm 1.5$  to 6.5% in the range 1 to 4 mM (4 to 9 replicate results for each compound). The mean recoveries were generally between  $\approx 95$  and 105% (70).

#### 6.9 Thermomicroscopic Method

Analysis of ternary mixtures of certain drugs including salicylamide were studied by plotting the refractive index isotherms of the fused mass and the isotherms of the primary crystals on triangular concentration diagrams. The influence of each factor upon the quantitative determination could be found. In each case there was an area unsuitable for measurement where the isotherms were practically parallel; when this was not the case, quantitative determination was possible. Precaution should be taken to prevent loss of highly volatile components (71).

#### 6.10 The ring-oven Technique.

Salicylamide was determined at concentrations of 10-400 ng with a maximum error of 7.6% by the ring-oven technique. Drugs such as paracetamol and caffeine did not interfere in the determination (72).

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# SILVER SULFADIAZINE

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## 1. HISTORY

Silver sulfadiazine was first described in 1943 by Wruble (1) and was found to be mildly antiseptic. Fox (2) rediscovered the compound in 1968 in his search for a suitable substance for topical treatment of burns. His philosophy was to combine the oligodynamic effect of the silver ion with the antibacterial activity of sulfadiazine. The compound has been in clinical use in the USA since 1973 and appears to be active against a wide range of micro-organisms (3). The usual application form is a 1% w/w oil-water cream.

## 2. DESCRIPTION

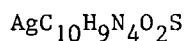
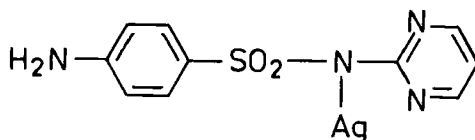
### 2.1 Name, Formula, Molecular Weight

Generic name - Silver sulfadiazine

Nomenclature - The following nomenclature is used in Chemical Abstracts:  
monosilver(I) salt of 4-amino-N-2-pyrimidinylbenzenesulfonamide [22199-08-2]

Synonyms - Silver compound (salt) of N<sup>1</sup>-2-pyrimidinyl-sulfanilamide

Structure



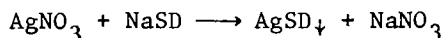
Molecular Weight: 357.14

### 2.2 Appearance, Colour, Odour

White or creamy-white micro-crystalline powder which is odourless or almost odourless. It is stable in air but slowly becomes yellow on exposure to light.

## 3. SYNTHESIS

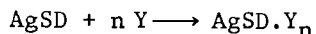
The synthesis of silver sulfadiazine is based on the following reaction scheme (1,4):



Mixing of equimolar amounts of silver nitrate and sodium sulfadiazine (NaSD), both dissolved in water, results in the almost

quantitative precipitation of silver sulfadiazine. An alternative procedure makes use of ammoniacal solutions of silver nitrate and sulfadiazine (5). Recrystallization of the compound can take place from 25% ammonia solution (4).

Derivatives of silver sulfadiazine can be prepared according to:



Examples of such derivatives are: Y = morfoline,  $n = 1$  (6) and Y = imidazole,  $n = 2$  (7).

#### 4. PHYSICAL PROPERTIES

##### 4.1 Infrared Spectrum

The infrared spectrum given in Figure 1 was determined for a KBr pellet preparation of silver sulfadiazine with the use of a Beckman IR 10 spectrometer. The assignments for important absorption bands are presented - as far as possible - in Table I. The assignments are based on various literature references (4, 8,9). In practice it is found that different batches of silver sulfadiazine do produce slightly different IR spectra, while the batches assay closely to the theoretical values (4,5,9). The differences occur in the regions above  $3000 \text{ cm}^{-1}$ , between  $1630$  and  $1660 \text{ cm}^{-1}$ , near  $1600 \text{ cm}^{-1}$  and  $1360 \text{ cm}^{-1}$ , and between  $1200$  and  $1300 \text{ cm}^{-1}$  and  $650$  and  $750 \text{ cm}^{-1}$  (9). The  $\text{SO}_2$  stretching vibrations are known to show a multiple structure with sub-bands on the sides of the main peaks usually (10). The origin of these sub-bands is not clear. Wysor and Scovill (5) distinguished two forms of silver sulfadiazine based upon differences in the IR spectra. Both forms of the compound were active

Table I. Infrared Assignments for Silver Sulfadiazine

Wavenumber ( $\text{cm}^{-1}$ )	Assignment
3390	$\nu_{\text{asym}}$ ( $\text{NH}_2$ )
3340	$\nu_{\text{sym}}$ ( $\text{NH}_2$ )
1655	$\delta$ ( $\text{NH}_2$ )
1595	phenyl skeletal vibrations
1500	phenyl skeletal vibrations
1560	pyrimidine skeletal vibration
1230	$\nu_{\text{asym}}$ ( $\text{SO}_2$ )
1125	$\nu_{\text{sym}}$ ( $\text{SO}_2$ )
1070	aromatic vibration

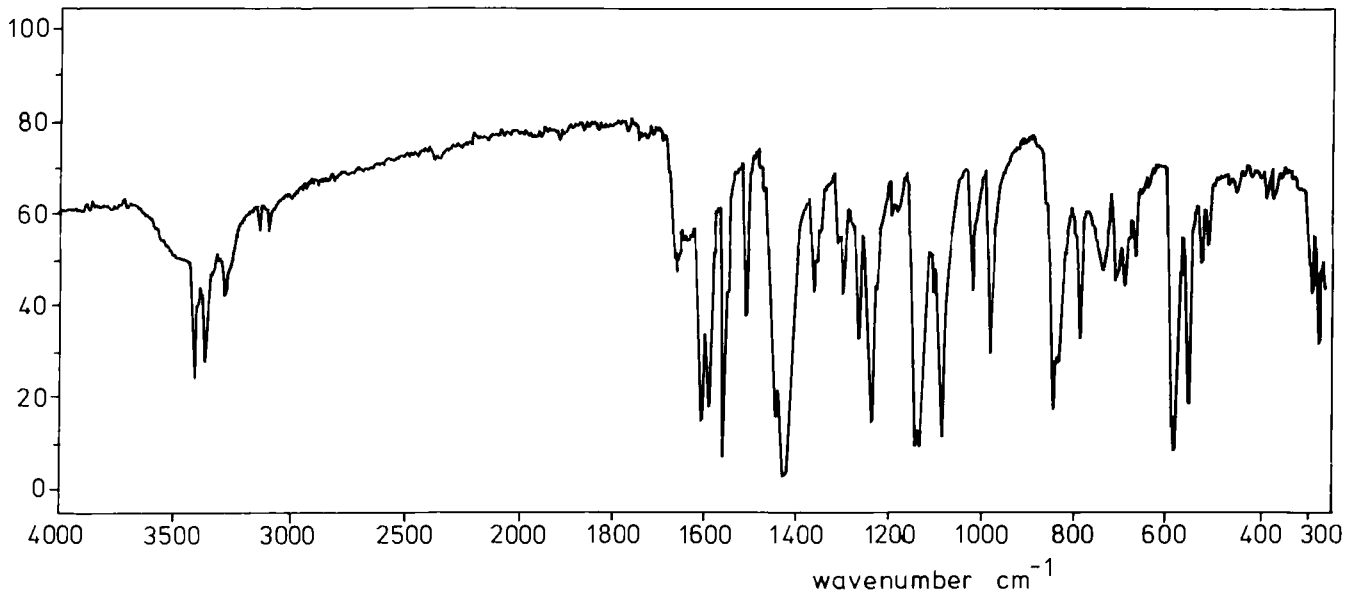


Figure 1  
IR spectrum of silver sulfadiazine

against bacteria, but only one form was active against trypanosomes. Evidence of polymorphism was not found with the usual physical methods. The two types of silver sulfadiazine resulted from synthesis according to apparently identical methods.

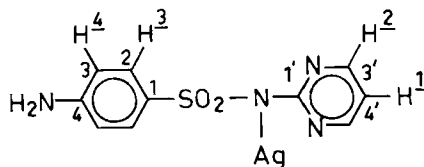
#### 4.2 Nuclear Magnetic Resonance (NMR) Spectrum

The proton NMR spectrum was recorded in DMSO- $d_6$  containing tetramethylsilane as internal reference and with the use of a Bruker WM-300 spectrometer at frequency 300.13 MHz. The spectrum is presented in Figure 2 and the spectral assignments are summarized in Table II (11). The chemical shifts roughly agree with those reported for sulfadiazine (12). The change in chemical shifts (to high field) for silver sulfadiazine compared to sulfadiazine is 0.3 ppm ( $NH_2$ ) or less (11).

The natural abundance carbon-13 NMR spectrum was recorded under the same experimental conditions except that the frequency was 75.46 MHz. Because of the low solubility of silver sulfadiazine in DMSO- $d_6$  an incomplete spectrum was obtained. The chemical shifts observed in the proton-noise decoupled spectrum are given in Table II (11). Again the chemical shifts of silver sulfadiazine compared to sulfadiazine are shifted slightly to high field, e.g. 4.5 ppm and less (11). Carbon-13 NMR data of sulfadiazine are reported by Chang et al. (13).

Table II.  $^1H$  and  $^{13}C$  NMR Assignments for Silver Sulfadiazine

Chemical shift $\delta$ (ppm)	Multiplicity	Number of atoms	Assignment
5.69	singlet	2	$NH_2$
6.52	doublet	2	H-4
6.81	triplet	1	H-1
7.65	doublet	2	H-3
8.42	doublet	2	H-2
111.2		1	C-4'
111.8		2	C-3
129.6		2	C-2
151.4		1	C-4
159.0		2	C-3'



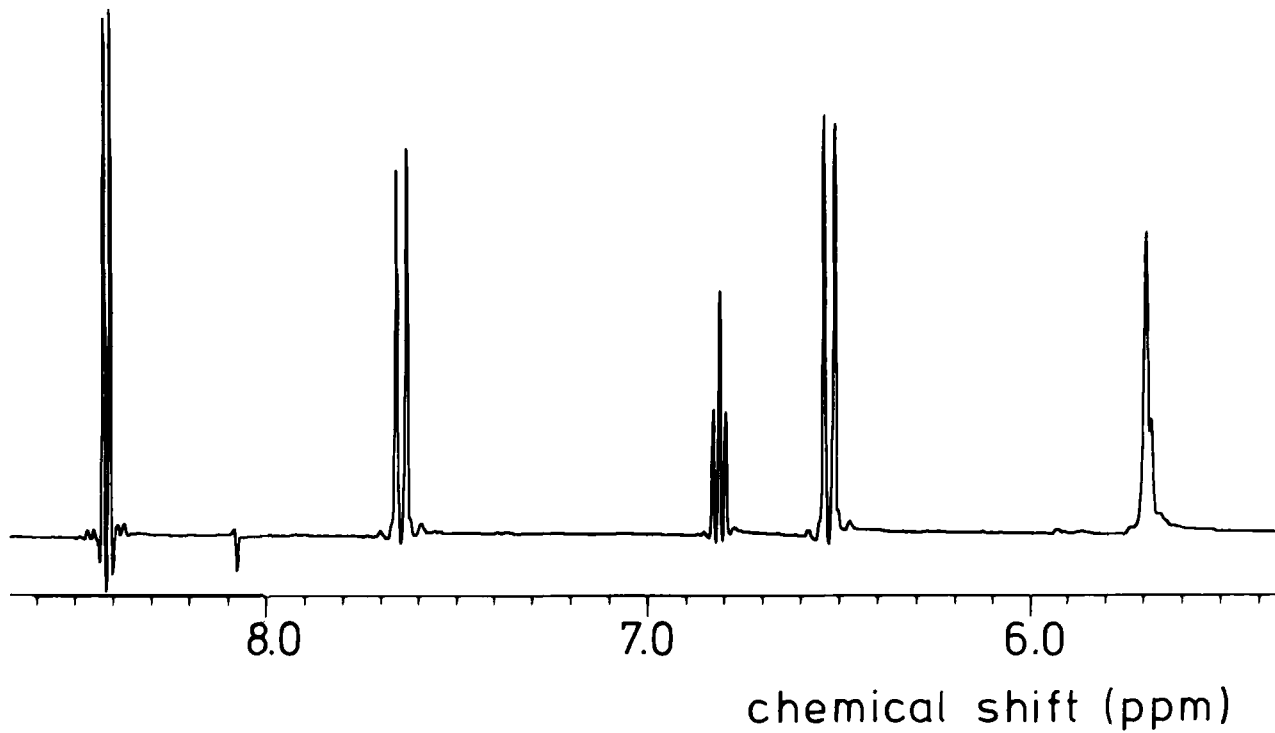


Figure 2  
Proton NMR spectrum of silver sulfadiazine (detail)

### 4.3 Ultraviolet Spectrum

The ultraviolet spectra for silver sulfadiazine were determined in very dilute aqueous ammonia solutions. One set of data was obtained with 0.0010% silver sulfadiazine in 0.005%  $\text{NH}_3$

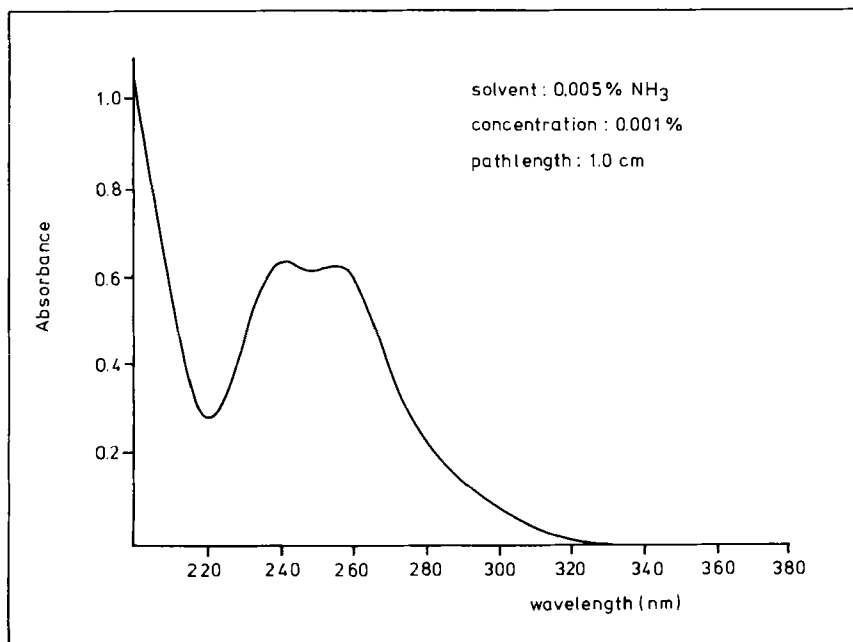


Figure 3  
UV spectrum of silver sulfadiazine

solution and recorded with an Uvikon 810/820 spectrophotometer (14); the second set of data was obtained with 0.0015% silver sulfadiazine in 0.05%  $\text{NH}_3$  solution and recorded with an Unicam SP 800 spectrophotometer (9).

A summary of the data is presented in Table III. The data in 0.005%  $\text{NH}_3$  solution are the average values of three samples of silver sulfadiazine from different origin and/or batches (14).

The ultraviolet spectrum for 0.001% silver sulfadiazine in 0.005%  $\text{NH}_3$  solution is presented in Figure 3 (14).

Table III. Ultraviolet Spectral Values for Silver Sulfadiazine

Solvent	$\lambda_{\max}$ (nm)	$A_{1\text{ cm}}^{1\%}$	$\epsilon$ ( $1.\text{mol}^{-1}.\text{cm}^{-1}$ )
0.005% $\text{NH}_3$	241	616	22,000
	254.5	614	21,930
0.05% $\text{NH}_3$	240	621	22,170
	254	617	22,050

#### 4.4 Mass Spectrum

The mass spectrum of silver sulfadiazine (Figure 4) was obtained with a Kratos MS 9/50 mass spectrometer in the electron impact mode with an ionizing energy of 70 eV and equipped with a direct inlet probe, probe temperature 250°C (15).

The base peak is found at m/e 185, while the molecular ion peak is very weak, viz. m/e 250, relative intensity 0.3%. The mass spectrum of silver sulfadiazine corresponds in peak positions and relative intensities with the spectrum of sulfadiazine (12). A peak corresponding with  $\text{Ag}^+$  (m/e 108), if present, is masked by a relatively intensive peak of sulfadiazine.

#### 4.5 Melting Range

Silver sulfadiazine melts with decomposition. A summary of the data obtained from the literature is listed below.

<i>Melting Range/Point (°C)</i>	<i>Reference</i>
271	(8)
277	(5)
286-288	(6)
286-290	(9)
290	(16)

#### 4.6 Differential Scanning Calorimetry

The DSC thermogram for silver sulfadiazine (Figure 5) was determined with a Mettler TA 3000 equipped with a DSC 30 measuring cell and alumina pan with pierced lid sample holder. The sample size was about 3 mg and the heating rate was 5 K/min (17).

In air an exothermic double-peak appears at about 290°C. In nitrogen or helium atmosphere an endothermic peak appears between about 283 and 300°C, but again an exothermic peak

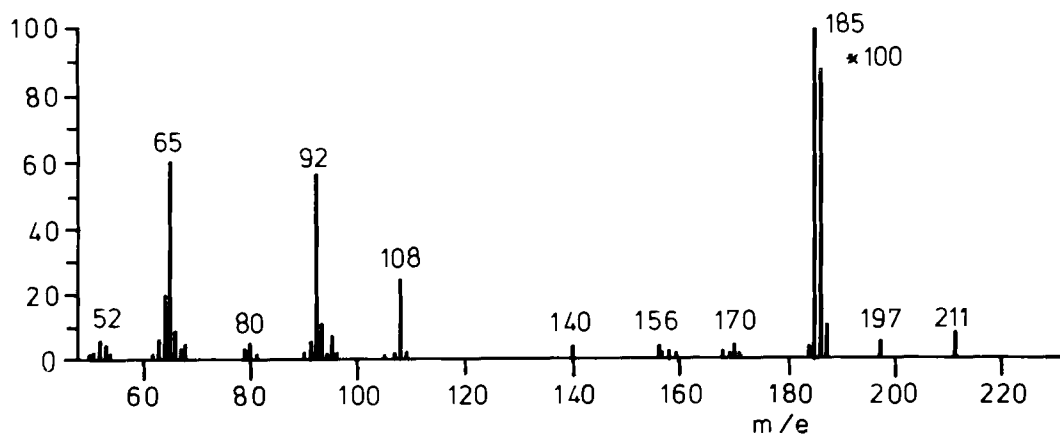
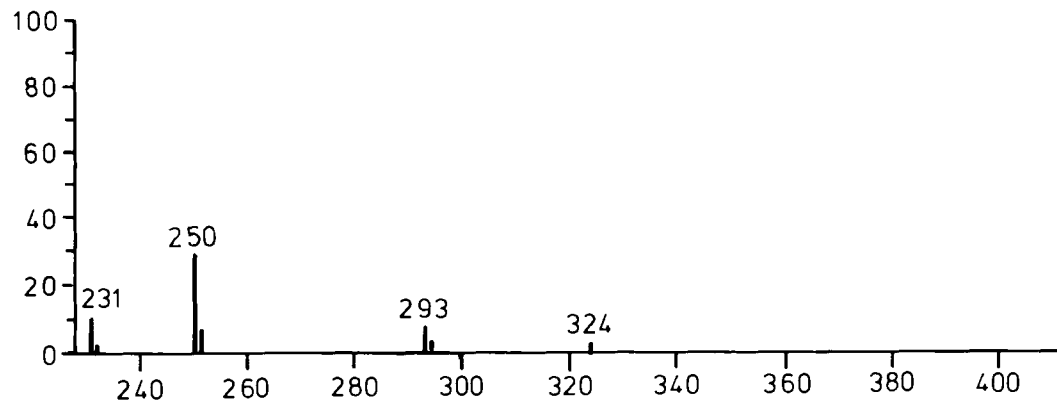


Figure 4. Mass spectrum of silver sulfadiazine: the peaks with  $m/e$  values higher than 190 are multiplied by a factor 100 times.



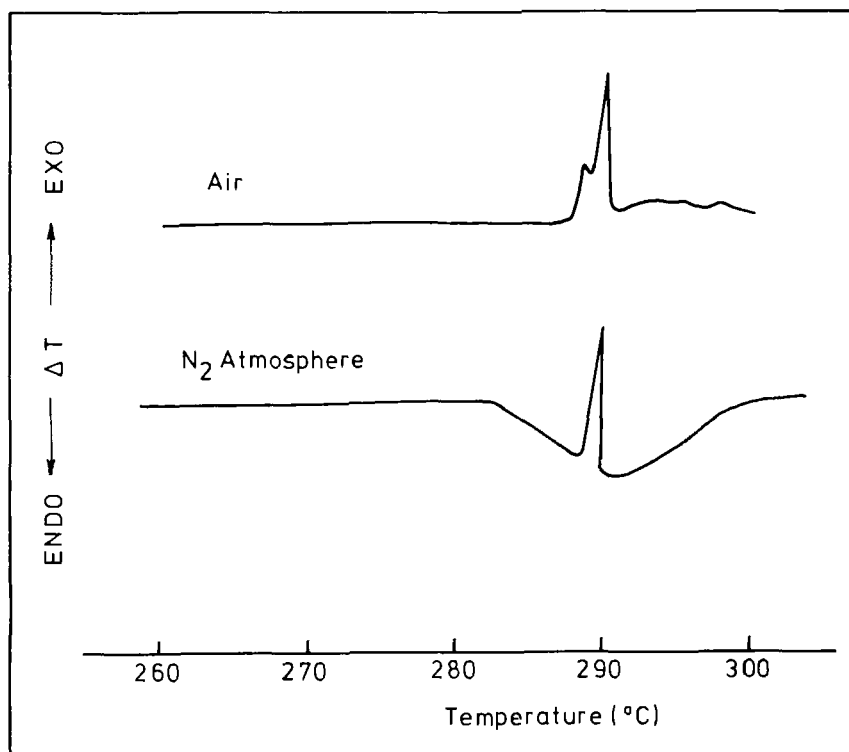


Figure 5  
DSC thermogram of silver sulfadiazine

occurs at about 290°C. The exothermic effect is not found with sulfadiazine or sodium sulfadiazine and is most probably connected with a chemical reaction of silver or catalyzed by silver. The endothermic process coincides with the melting range of silver sulfadiazine.

#### 4.7 Crystal Structure and X-ray Powder Diffraction

Two independent single-crystal structure determinations of silver sulfadiazine have been reported (18,19) which mutually agree in the description of the main features of the structure. Each silver atom is coordinated by three separate sulfadiazine moieties, resulting in a distorted tetrahedral coordination by

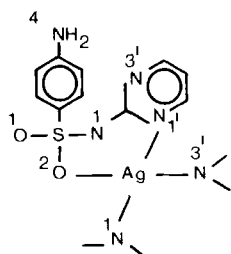


Figure 6  
Crystal structure of silver  
sulfadiazine (18)

three N-atoms and one O-atom (Figure 6). The crystal data are given in Table IV.

Table IV. Crystal Data for Silver Sulfadiazine

Space group	$P2_1/c$ (ref. 19)	$P2_1/c$ (ref. 18)
a (Å)	6.173(2)	6.172(5)
b (Å)	9.600(5)	9.605(8)
c (Å)	20.30(2)	20.33(2)
$\beta$ ( $^\circ$ )	96.22(8)	96.60(8)

The X-ray powder diffraction pattern was recorded by means of a Philips diffractometer, type PW 1025/25, using Cu-K $\alpha$  radiation ( $\lambda = 1.5418$  Å). The results are in good agreement with the reported single-crystal data. A strong reflection was observed at  $2\theta = 10.2^\circ$ . All other reflections have an intensity less than 20% relative to this strong (011) reflection. The data for the major lines in the diffraction pattern of silver sulfadiazine are given in Table V.

#### 4.8 Stability Constant

The thermodynamic stability constant of silver sulfadiazine in water has been measured by Boelema et al. (20). They made use of a microcomputer-controlled titrator and measured simultaneously the pH value with a Radiometer G 2040 C glass electrode and the silver ion concentration with an Orion research 941600 ion-selective silver sulfide electrode. The measured stability constant and standard deviation were  $\log K = 3.62 \pm 0.05$ ;  $n = 9$ , temperature  $25^\circ\text{C}$ , ionic strength 0.1 (sodium nitrate). The calculated conditional stability constant  $\log K'$  at pH: 7.4 was 3.57.

Table V. X-ray Powder Diffraction Pattern of Silver Sulfadiazine

$2\theta$ Degrees	$d$ (Å)	$I/I_0$ *
8.80	10.05	20
10.21	8.664	100
16.20	5.505	6
18.17	4.882	3
18.49	4.798	10
19.91	4.459	7
20.05 (s)	4.43	2
20.6 (s)	4.32	4
20.66	4.299	6
23.67	3.759	5
24.28	3.666	12
25.62	3.477	2
26.69	3.340	2
27.91	3.197	8
28.11	3.174	5
28.85	3.094	4
30.91	2.893	2
31.51	2.839	4
33.20	2.698	9
33.60	2.667	2
34.63	2.590	2
36.06	2.491	2
36.33	2.473	3
37.17	2.419	5
37.44	2.402	5
38.53	2.337	6

\*Relative intensity in percentage of the strongest signal. Reflections with intensities less than 2% have been excluded.

(s) Shoulder or poorly resolved from adjacent peak.

#### 4.9 Conductivity

Bult and Klasen (4) have performed conductivity measurements of silver sulfadiazine with a Radiometer conductivity meter, type CDM<sup>2d</sup>, and conductivity cell, type CDC 104. The molar conductivity  $\Lambda_M$  in DMSO was  $2.2 \Omega^{-1} \cdot \text{cm}^2 \cdot \text{mole}^{-1}$  (measured at  $1.10^{-3}$  M). Considering the standard range of an 1:1 electrolyte in DMSO,  $\Lambda_M = 23-42$  (21), the conclusion was

drawn that the compound is almost undissociated in this solvent.

#### 4.10 Solubility

The solubility of silver sulfadiazine has been determined by equilibrating the compound in doubly distilled water at 25°C and the measurements of the saturated solution for sulfadiazine by UV spectrometry and for silver by atomic absorption spectroscopy (7). Nesbitt and Sandmann (22) measured the equilibrated solution for silver using a silver-ion selective electrode. Their measurements were performed at 25°C in nitric acid-potassium nitrate buffers (pH 2-3) and ionic strength 0.1. The data are summarized in Table VI.

Table VI. Solubility of Silver Sulfadiazine

Solvent	Solubility (mg/100 ml)	pH	Reference
water	0.34	6.8	(7)
water	0.2	--	(23)
water, $\mu$ : 0.1	20	2.13	(22)
water, $\mu$ : 0.1	2.3	3.85	(22)
water*	0.19	6	(22)
water*	0.11	7	(22)
dimethyl sulfoxide	>35		(4)
10% w/v NH <sub>3</sub> solution	>2.10 <sup>3</sup>		(14)
25% w/v NH <sub>3</sub> solution	>5.10 <sup>3</sup>		(24)

\* calculated

As can be seen from the data, the solubility of silver sulfadiazine increases with the decrease of pH. A solubility of 0.34 mg/100 ml corresponds with a  $1.10^{-5}$  M solution. The degree of dissociation,  $\alpha$ , can be calculated from the equation  $K.c = (1-\alpha)/\alpha^2$ , where K is the stability constant and c is the molar concentration. For silver sulfadiazine the calculated  $\alpha$  is about 1 at pH 7, which means that the dissolved fraction of silver sulfadiazine in water is almost completely dissociated into the ions silver and sulfadiazine. This complete dissociation has been confirmed by experiment (22).

The solubilities of silver sulfadiazine in various solvents are only known approximately. The compound is very slightly soluble in acetone, practically insoluble in ethanol, chloroform and diethyl ether (14), freely soluble in 30% ammonia solution, and decomposes in moderately strong mineral acids (9).

Broad information about the solubility of silver sulfadiazine in a number of solvents may be derived from identification and quantitative determination procedures. Some of the solubilities collected in this way are presented in Table VI.

## 5. METHODS OF ANALYSIS

### 5.1 Elemental Analysis

The results from an elemental analysis of silver sulfadiazine are listed below (19).

#### *Elemental Analysis of Silver Sulfadiazine*

<i>Element</i>	<i>% Theory</i>	<i>% Found</i>
C	33.63	33.3
H	2.54	1.9
N	15.69	15.7
Ag	30.20	30.22

The assay limits demanded in a draft of a monography on silver sulfadiazine for the Dutch Pharmacopoeia Ed. IX (14) are: Ag 29.6 - 30.3% and sulfadiazine 69.1 - 71.2%. Indemans (14) assayed three different samples of silver sulfadiazine and found contents within the ranges Ag 29.8 - 30.1% and sulfadiazine 69.3 - 69.8%. Horlington (9) reported the results of five batches of the compound: Ag 29.6 - 30.08% and sulfadiazine 69.3 - 70.0%.

### 5.2 Identification Tests

The identification of silver sulfadiazine is based on the detection of the silver ion and the sulfadiazine moiety.

#### *5.2.1 Silver*

The compound is dissolved in 35% ammonia solution or in diluted nitric acid. Upon addition of hydrochloric acid a curdled, white precipitate is formed. The precipitate dissolves on addition of 10% ammonia solution.

#### *5.2.2 Sulfadiazine*

The identification can be based on the following tests:

- a) Detection of the primary aromatic amine group: the compound is (partly) dissolved into dilute hydrochloric acid. Upon addition of a 10% sodium nitrite solution, followed after two minutes by the addition of an alkaline 5% 2-naphtol solution, an orange or red colour is formed (25).
- b) Detection of the 2-aminopyrimidine moiety: the compound is suspended in a 5% resorcinol solution in ethanol 94% v/v. Upon addition of 95% sulphuric acid a deep red colour is formed (26).

- c) Detection of sulfadiazine: an ammoniacal solution of the compound shows two absorption maxima at 255 nm and 240 nm. The specific absorbance ( $A_{1\text{ cm}}^{1\%}$ ) at both maxima is between 610 and 660. The ratio of  $A_{1\text{ cm}}^{1\%}$  (255 nm)/ $A_{1\text{ cm}}^{1\%}$  (240 nm) is 0.97 to 1.00 (14).

Other useful identification methods are the IR spectrum and thin layer chromatography. These methods demand the availability of a reference sample of silver sulfadiazine.

### 5.3 Purity Tests

In a draft of a monography on silver sulfadiazine for the Dutch Pharmacopoeia Ed. IX (14) the following purity tests are included.

2.5 gram of the finely ground silver sulfadiazine is shaken for 5 min. with 50 ml of water. The solution is filtered off and the residue is washed with 50 ml of water. The combined filtrate is used for the tests on pH, nitrate and free silver. *pH*: 4.5 to 6.5. *Nitrate*:  $\leq 0.1\%$ ; based on colour formation with a 0.05% solution of the sodium salt of chromotropic acid in sulphuric acid. *Free silver*:  $\leq 100$  ppm; turbidity measurement in a hydrochloric acid solution.

Other purity tests are:

*Loss on drying*: not more than 0.5% at 100 to 105°C.

*TLC*: the thin layer chromatogram of the tested compound may not differ from the chromatogram of the reference compound; stationary phase, silica gel GF<sub>254</sub>R, mobile phase: chloroform-methanol-ammonia 25% (70:40:10 vol. parts), saturated chamber, detection: 254 nm.

*Light-absorbing impurities*: the absorbance of a 5% solution of silver sulfadiazine in 35% ammonia, measured at 400 nm, may not exceed 0.20. This demand seems to be sharp because Indemans (14) reported for three batches an absorbance between 0.199 and 0.273, while the three batches meet the other requirements.

*Particle size*: the particle size of the highly insoluble silver sulfadiazine is thought to be important for its availability under wound conditions in the topical treatment of burns. Therefore, the micronized form is an important quality criterion. A current demand is that 90% of the particles should measure 10  $\mu\text{m}$  or less in greatest dimension, 99% not over 20  $\mu\text{m}$  and 100% not over 50  $\mu\text{m}$  (24).

### 5.4 Volumetric Methods

#### 5.4.1 Silver Content

Silver sulfadiazine is dissolved in 65% nitric acid and the solution is diluted with water to a tenfold volume. The silver is assayed by the Volhard procedure: titration with thiocyanate, indicator  $\text{Fe}^{3+}$ .

#### 5.4.2 *Sulfadiazine Content*

Silver sulfadiazine is dissolved in dilute hydrochloric acid and determined by titration with sodium nitrite solution (assay of the primary aromatic amine function). The endpoint detection is commonly biamperometric (27).

### 5.5 Spectroscopic Methods

Both silver and sulfadiazine of silver sulfadiazine have been determined by spectroscopic methods. An appropriate quantity of the compound is dissolved in concentrated ammonia and diluted with water to the desired concentration.

#### 5.5.1 *Silver Content*

Determination with atomic absorption spectroscopy with the use of an acetylene-air flame and hollow-cathode lamp, e.g. 3 UAX/Ag Cathodeon Ltd.; measurement at 328.1 nm (7,9).

#### 5.5.2 *Sulfadiazine Content*

- a) Determination with UV absorption spectrophotometry at 254 nm (9,28).
- b) Application of the Bratton-Marshall reaction (29): sulfadiazine is diazotized with sodium nitrite and then coupled with *N*-(1-naphthyl)-ethylenediamine dihydrochloride. The pink colour is measured at 545 nm (9).

### 5.6 Thin Layer Chromatography

The sulfadiazine moiety of silver sulfadiazine has been analyzed by thin layer chromatography. The compound is dissolved in 35% ammonia solution and the solution is diluted, if necessary, with methanol. The separation is performed on silica gel F<sub>254</sub> plates with one of the following mobile phases:

- A. Ethanol-25% ammonia (95:5);  $R_f$  about 0.5 (28)
- B. Chloroform-methanol-25% ammonia (70:40:10);  $R_f$  0.3 (14,24)
- C. Chloroform-methanol-25% ammonia (30:10:2);  $R_f$  0.12 (24)
- D. Ethyl acetate;  $R_f$  0.42 (24)
- E. 2-Propanol-2-butanol-ethyl acetate-35% ammonia (1:1:2:1);  $R_f$  about 0.25 (9)

The spot can be located by one of the following methods:

- 1) UV detection at 254 nm
- 2) I<sub>2</sub> vapour (28)
- 3) 5% potassium dichromate in sulphuric acid (40% w/w) (24)
- 4) 0.1% *N,N*-dimethylaminobenzaldehyde in an ethanol-conc. hydrochloric acid (99:1) mixture (9)

The detection limit with spray reagent (4) is about 0.2 µg (9).

### 5.7 High Performance Liquid Chromatography

The only reported separation of silver sulfadiazine is based on reverse phase high performance liquid chromatography

(9). A C<sub>18</sub>-type bonded column, e.g. Partisil 10 ODS, was used as stationary phase and 1% acetic acid-methanol (80:20) as mobile phase. The detection was performed at 254 nm and o-cresol was used as internal standard. This quantitation is based on detection of the sulfadiazine moiety of silver sulfadiazine.

## 6. STABILITY

Silver sulfadiazine in the solid state turns into slightly yellow within one day upon exposure to light and remains in that state for at least two years (24). No subsidiary spots were found in TLC separation, nor changes in silver and sulfadiazine contents. The compound remains unchanged for four years at 20°C in the dark (24). Preservation in the dark at 20°C and at a high relative humidity (90%) results in a light yellow powder after two years (24). Exposure to a temperature of 50°C in the dark results in a light yellow-brown product after two years (24). The extent of colour formation increases with rise of the temperature, but no subsidiary TLC spots or changes in silver and sulfadiazine contents were found.

## 7. PHARMACOLOGY

Silver sulfadiazine is more or less active against both gram-positive and gram-negative bacteria, fungi, treponema and viruses. The compound is in use as a 1% w/w oil-water cream in topical burn treatment and has favourable clinical results (first choice drug).

In the environment of the burn the compound slowly dissociates into silver and sulfadiazine (30). The silver ion is the antimicrobially active part of the compound and acts by interaction with the micro-organism. Possibly sulfadiazine has a supportive antibacterial effect, but the concentration seems to be subinhibitory (30). The role of sulfadiazine may be to create a sustained delivery of silver ions into the wound environs.

Almost the total concentration (>99%) of silver remains located in the wound environment (31). The sulfadiazine is absorbed to about 10% into the general circulation (32). Depending on the burnt surface, blood levels up to about 3 mg/100 ml and renal excretion to 2 g / 24 hours have been reported (32). Details about the metabolism of sulfadiazine have been reported before (12).

## 8. IDENTIFICATION AND DETERMINATION IN BODY FLUIDS AND PHARMACEUTICALS

### 8.1 Determination in Body Fluids



From the topically applied silver sulfadiazine only sulfadiazine is absorbed to some extent (about 10%). The analysis of sulfadiazine in biological fluids can be based on the methods described by Stober and De Witte (12). Delaveau and Friedrich-Noue (33) employed the Bratton-Marshall reaction for the measurement of sulfadiazine levels in plasma, serum and urine after topical application of silver sulfadiazine cream.

## 8.2 Identification and Determination in Pharmaceuticals

The common application form of silver sulfadiazine is a 1% w/w oil-water cream. Prior to the identification and determination of silver sulfadiazine the cream basis can be removed by dissolution into a suitable organic solvent (or mixture of solvents). The following extraction media are described:

- a) a mixture of ethanol 94% and chloroform (1:1) and
- b) the successive extraction with n-butanol and diethyl ether (9,28). The identification and/or determination of the isolated silver sulfadiazine can be based on the methods of analysis described in sections 5.2 - 5.7.

Direct treatment of the whole cream with 2 M hydrochloric acid by refluxing for 30 minutes followed by filtration results in isolation of sulfadiazine. The sulfonamide present in the filtrate is determined by biamperometric titration (24).

Treatment of the cream with concentrated ammonia followed by acidification with 1% acetic acid is suitable as a sample preparation method for the high performance liquid chromatographic determination (9). The clear supernatant is used for the analysis.

## ACKNOWLEDGEMENT

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# SULINDAC

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## 1. Foreword, History, Therapeutic Category.

Sulindac is a new nonsteroid anti-inflammatory drug with analgesic and antipyretic activity<sup>1,2</sup>.

Sulindac and indomethacin had similar pharmacologic profiles in laboratory animals but sulindac showed less gastrointestinal bleeding and ulcers<sup>2,3</sup>.

## 2. Description

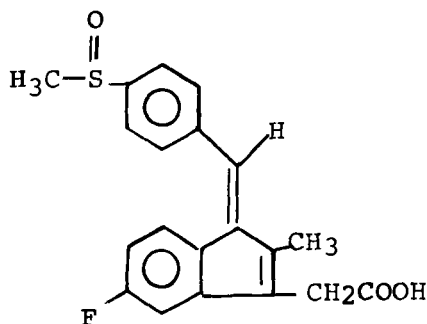
### 2.1 Name, Formula, Molecular Weight

1H-Indene-3-acetic acid, 5-fluoro-2-methyl-1{4-(methyl-sulfinyl) phenyl}methylene -, (2)-; CIS-5-fluoro-2-methyl-1- {(p-methyl-sulfinyl)benzyl-idene} indene-3-acetic acid.

Empirical formula C<sub>20</sub>H<sub>17</sub>FO<sub>3</sub>S

Molecular weight 356.41

The CAS Registry number is CAS-38194-50-2

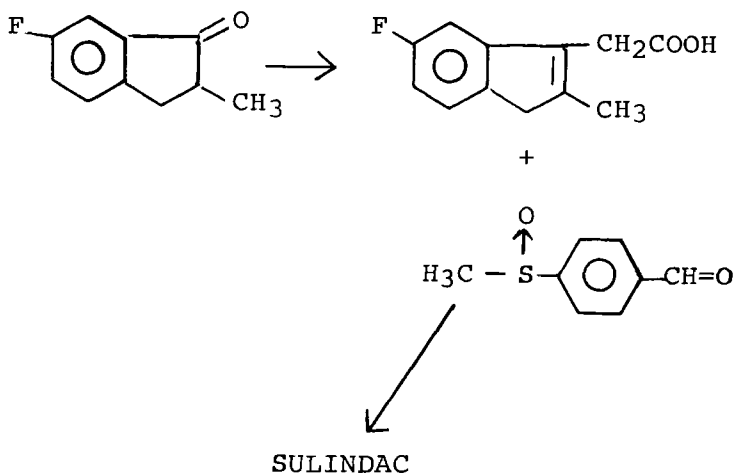


### 2.2 Appearance, Color, Odor

Sulindac is an odorless, yellow, crystalline powder.

### 3. Synthesis

A synthesis<sup>4,5</sup> of sulindac is summarized as follows:



### 4. Physical Properties

#### 4.1 Infrared Spectrum

The infrared spectrum of sulindac, run as a nujol mull, is reproduced in Figure 1 and is consistent with the structure. Band assignments which may be made with confidence are tabulated below:

TABLE I

<u>Band Assignment Infrared Spectrum of Sulindac</u>	
$\nu$ (cm <sup>-1</sup> )	Assignment
2500-2650	-CO H dimer
1700	$\nu$ C=O
1600	Aromatic ring modes
1580	
1270	
1155	-CO H dimer
1000-1020	$\nu$ C-F
	$\nu$ S-O

In addition to these, sharp bands between 800

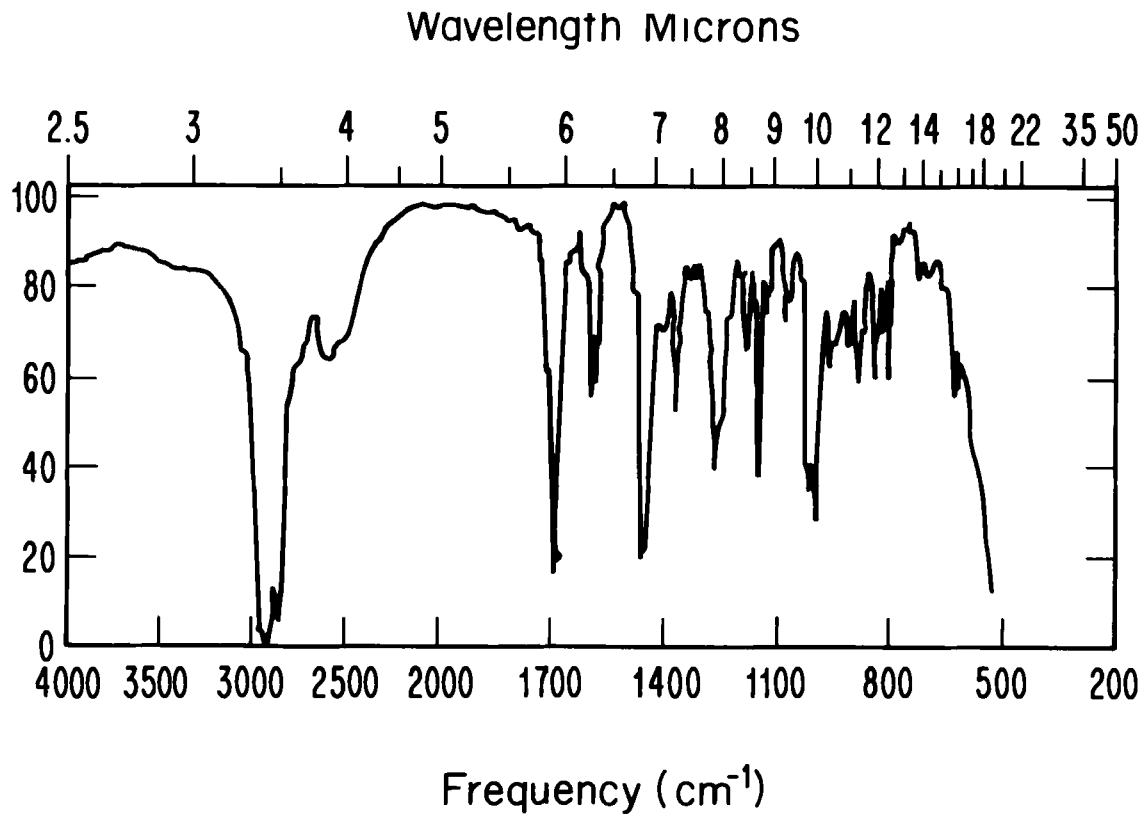


Figure 1. Infrared Absorption Spectrum of Sulindac in Nujol Mull

and  $900\text{ cm}^{-1}$  are expected from the ArH out-of-plane modes in both rings. The nujol mulling agent, of course, accounts for the very strong band from 2825 to  $2975\text{ cm}^{-1}$ , as well as for all or part of the bands at  $1460$  and  $1375\text{ cm}^{-1}$ .<sup>(6)</sup>

## 4.2 Mass Spectrum

The electron impact ionization spectrum is given in Figure 2. A LKB model 9000 mass spectrometer was used with ionization potential of 70eV and accelerating voltage of 3.5kv. The mass spectrum shows a molecular ion at 356 (m/e) with two relatively intense peaks at 341 and 233 (m/e). Loss of a methyl group from the parent molecule will account for the 341 m/e fragment. Loss of CH COOH, CH SO and hydrogen will account for the other fragment.<sup>(7)</sup>

## 4.3 Nuclear Magnetic Resonance Spectra

### 4.3.1 Proton Spectrum

The proton NMR spectrum is found in Figure 3 and assignments to the characteristic protons are recorded below. The complex pattern located at 6.4 to 7.2 ppm is consistent with aromatic 1,2,4 substitution with fluorine splitting. The singlet with weak satellites ( $\sim 7.74$  ppm) is consistent with an aromatic AA'BB' pattern which is nearly collapsed into a singlet. A solvent (DMSO- $d_6$ ) resonance appears at approximately 2.5 ppm<sup>(6)</sup>.



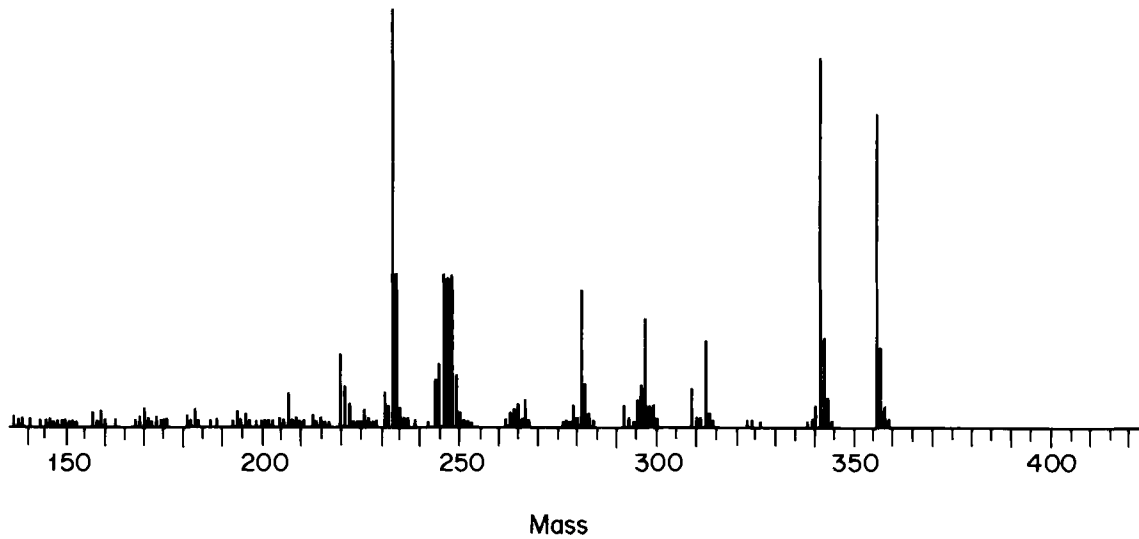
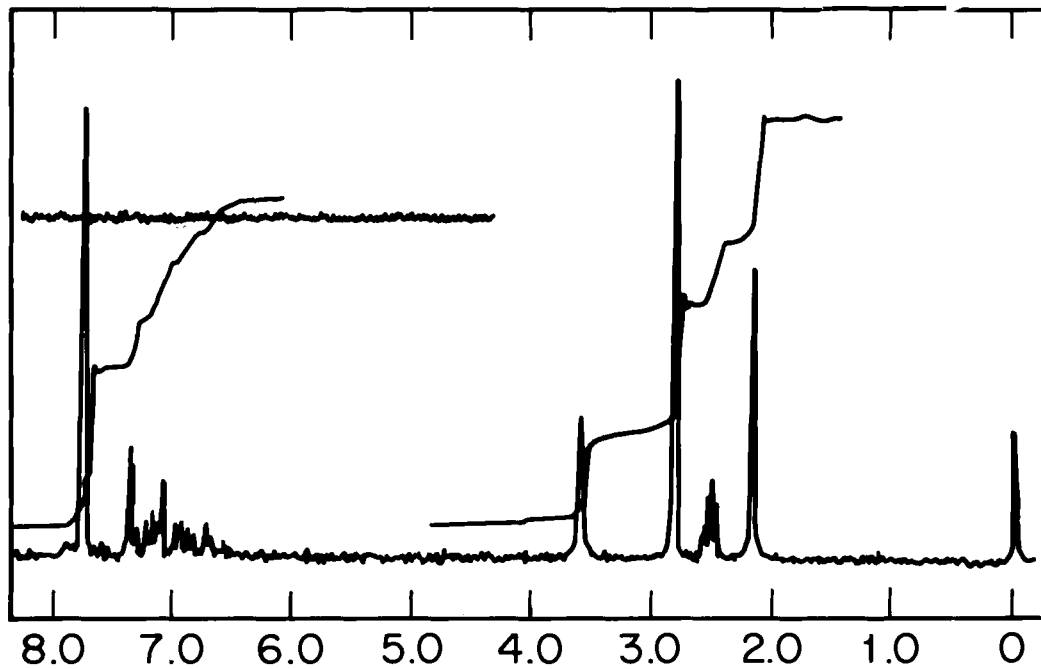


Figure 2. Mass Spectrum of Sulindac



Chemical Shift (ppm)

Figure 3. Proton NMR of Sulindac

TABLE II<sup>1</sup>H NMR Sulindac Assignments

<u>Chemical Shift</u> ppm ( $\delta$ )	<u>Pattern</u>	<u>Proton Assignment</u>
2.17	singlet	
2.83	singlet	
3.60	singlet	
$\sim$ 6.4 to 7.2	complex	
7.36	singlet	
7.74	singlet with weak satelites	

4.3.2 Carbon 13 Spectrum

The <sup>13</sup>C magnetic resonance spectrum is given in Figure 4 and was obtained using a Varian CFT-20 (FT mode) spectrometer with CDCl<sub>3</sub>/CK<sub>3</sub>OD (9:1) as the solvent at a concentration of 0.5M. The spectrum is consistent with the structure and the assignments are as follows: (8)

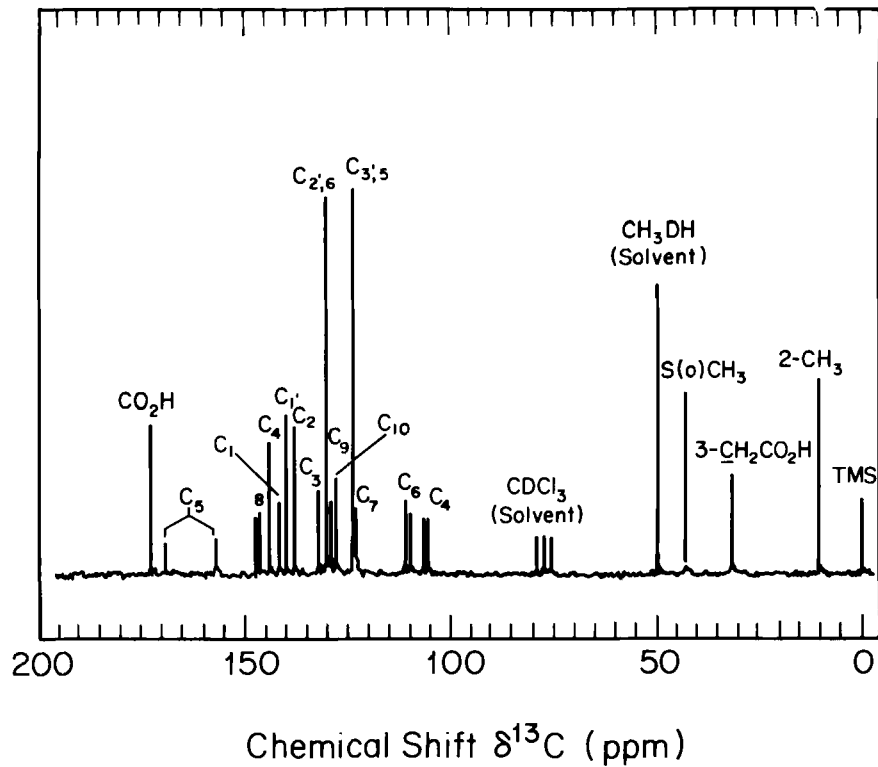


Figure 4.  $^{13}\text{C}$  NMR of Sulindac

TABLE IIICarbon-13 Chemical Shifts for Sulindac

Shift, $\delta^{13}\text{C}$ ppm)	Assignment
141.9	C
138.2	C
132.4	C
106.2	C
163.4	C
110.7	C
123.7	C
147.1	C
129.7	C
128.0	C (Benzal)
140.1	C1'
130.5	C2', C6'
124.1	C3', C5'
144.2	C4'
10.4	2-CH <sub>3</sub>
31.7	3-CH <sub>2</sub>
172.9	Carboxyl
43.2	SCH <sub>3</sub>

4.4 Ultraviolet Absorption Spectrum

The ultraviolet absorption spectrum of sulindac in methanolic 0.1N hydrochloric acid is found in Figure 5 and is characterized by maxima at 327, 284, 258 and 228 nm with  $E_{1\%}^{1\text{cm}}$  values of approximately 370, 420, 405 and 540 respectively.

4.5 Solubility

Sulindac is sparingly soluble in methanol and 95% ethyl alcohol, slightly soluble in ethyl acetate, and practically insoluble in water. The water solubility increases with increasing pH as indicated in the following table. (9)

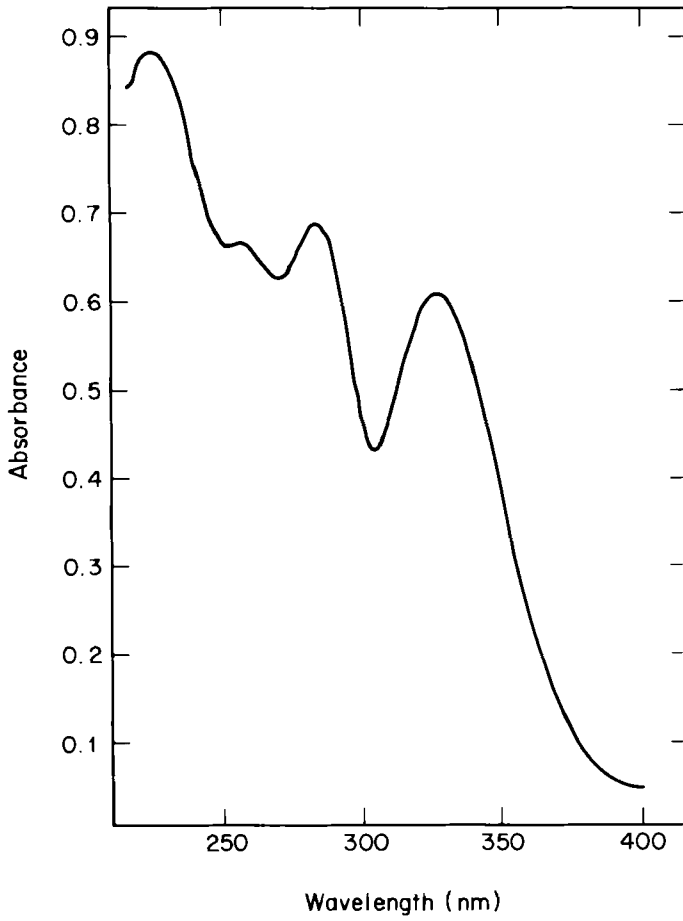


Figure 5. Ultraviolet Absorption Spectrum of Sulindac in Methanolic 0.1N HCl 1.625 mg/100 ml

TABLE IV

## Solubility of Sulindac

Solvent	Temperature (°C)	Solubility (mg/ml)
0.1N HCl	25	0.003
Water	25	0.003
pH 7.0 (Phosphate)	25	2.7
pH 7.4 (Phosphate)	25	3.4
pH 3.9 (Citrate)	37	0.01
pH 4.8 (Citrate)	37	0.05
pH 5.9 (Citrate)	37	0.35
pH 6.4 (Citrate)	37	1.0
Acetone	25	18
Chloroform	25	25
Ethanol	25	15
Ethyl Acetate	25	4
Hexane	25	1
Isopropanol	25	7
Methanol	25	30

4.6 Dissociation Constant

The pKa of sulindac is 4.7 at 25°C.

4.7 Partition Behavior

The distribution coefficient for sulindac between phosphate buffer pH 7.0 and octanol at 25°C is 0.28. Sulindac can be extracted quantitatively from aqueous acidic media into the following organic solvents: methylene chloride, chloroform, ethyl acetate and 3 volume % isoamyl alcohol in heptane. Sulindac can be back extracted into dilute alkali from these solvents.

4.8 Thermal Behavior

Sulindac melts with decomposition and therefore the observed temperature is dependent upon the conditions of the observation. Under USP Class la conditions, the temperature ranges from 182-185°C.<sup>(10)</sup> The DTA curve (Fig. 6) for sulindac at 20°C/min is characterized by a single endotherm with

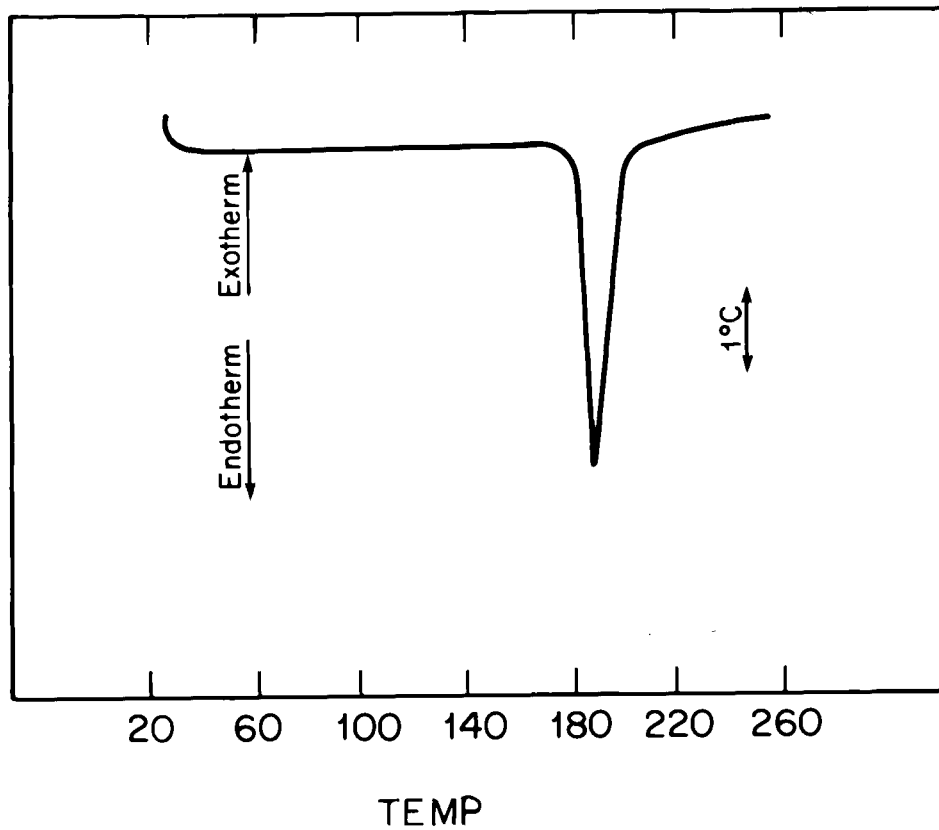


Figure 6. DTA Curve of Sulindac at 20°C/min.



peak temperature at approximately  $\sim 186$  C.

#### 4.9 Polarographic Behavior

In 0.1M tetraethylammonium perchlorate in acetonitrile, half-wave potentials of -1.38 and -1.52 were observed for sulindac. The reductions involve the C=C bonds and not the sulfur functionalities by analogy to the work of Hawson et al (11 and 12).

#### 4.10 Polymorphism

Sulindac is a polymorphic substance and can exist in two nonsolvated enantiotropic crystal forms designated Forms I (191°C) and II (186°C). Form II is the more stable polymorph at room temperature and temperatures below the transition point which is estimated to be 157°C from heats of fusion and melting points of the polymorphs. The transition temperature has also been estimated to be 165°C by extrapolations of solubility measurements of the two forms over the temperature range of approximately 20 to 80°C. The average of the two determinations yields a value for 161  $\pm$  7°C for the transition point.

Form I is the more stable form above the transition point up to its melting point of 191°C which is about five degrees above the metastable melting point of Form II. Although Form I is metastable with respect to Form II at room temperature, the rate of conversion of Form I to Form II is dependent upon the conditions. With solid Form I alone, the rate is so slow that it cannot be conveniently measured. In contact with water or aqueous buffered systems, the conversion requires weeks or months. In contact with ethanol Form I converts to Form II within 16 hours or less.

Form II, the more stable form at room temperature, is the preferred form. X-ray powder diffraction (Figs. 7 and 8), infrared absorption (Figs. 1 and 9) and differential thermal analysis (Figs. 6 and 10) are capable of distinguishing the two forms.

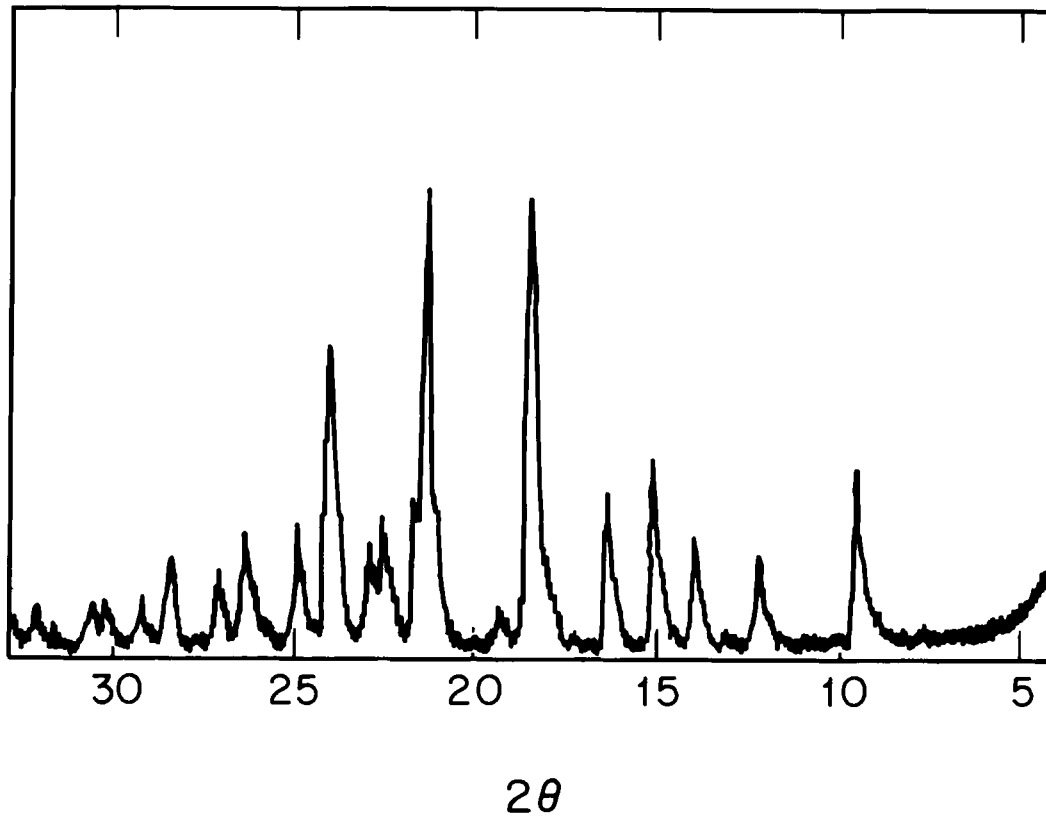


Figure 7. X-Ray Powder Diffraction Pattern Sulindac Form II (CuK $\alpha$  Radiation)

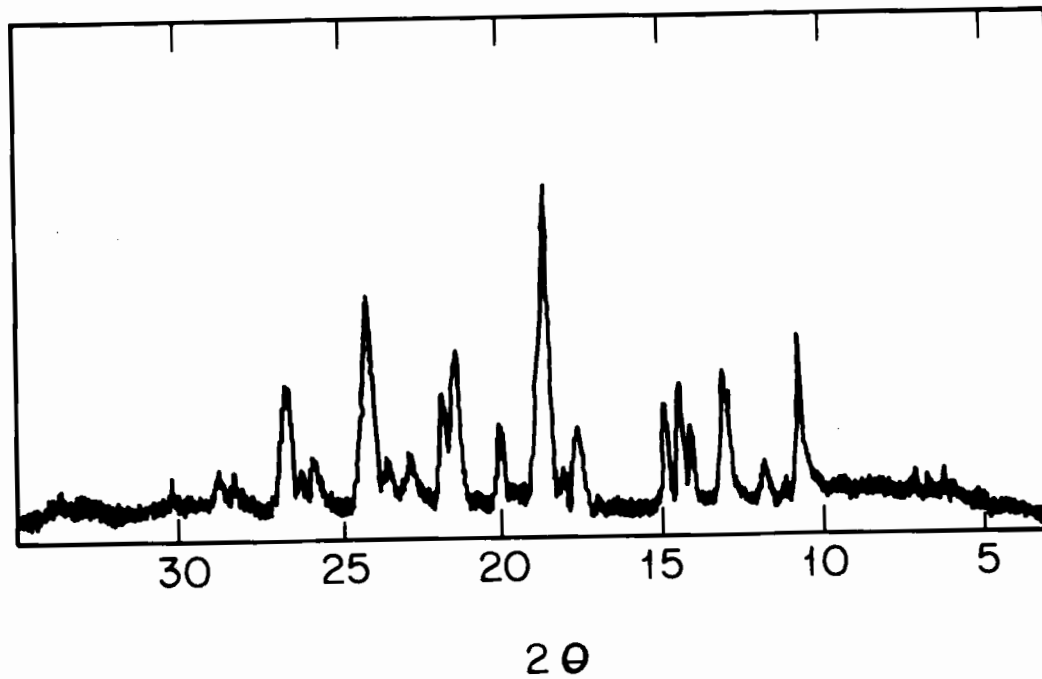


Figure 8. X-Ray Powder Diffraction Pattern Sulindac Form I (CuK $\alpha$  Radiation)

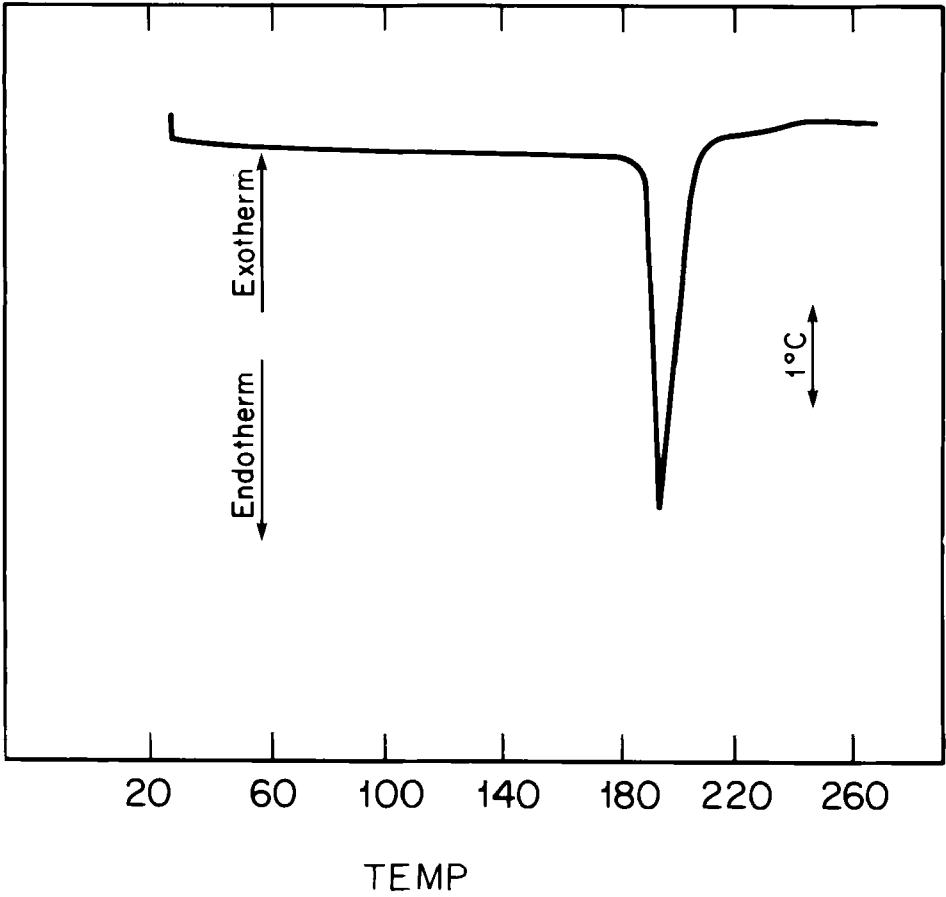


Figure 9. DTA Curve of Sulindac Form I at 20°C/min.

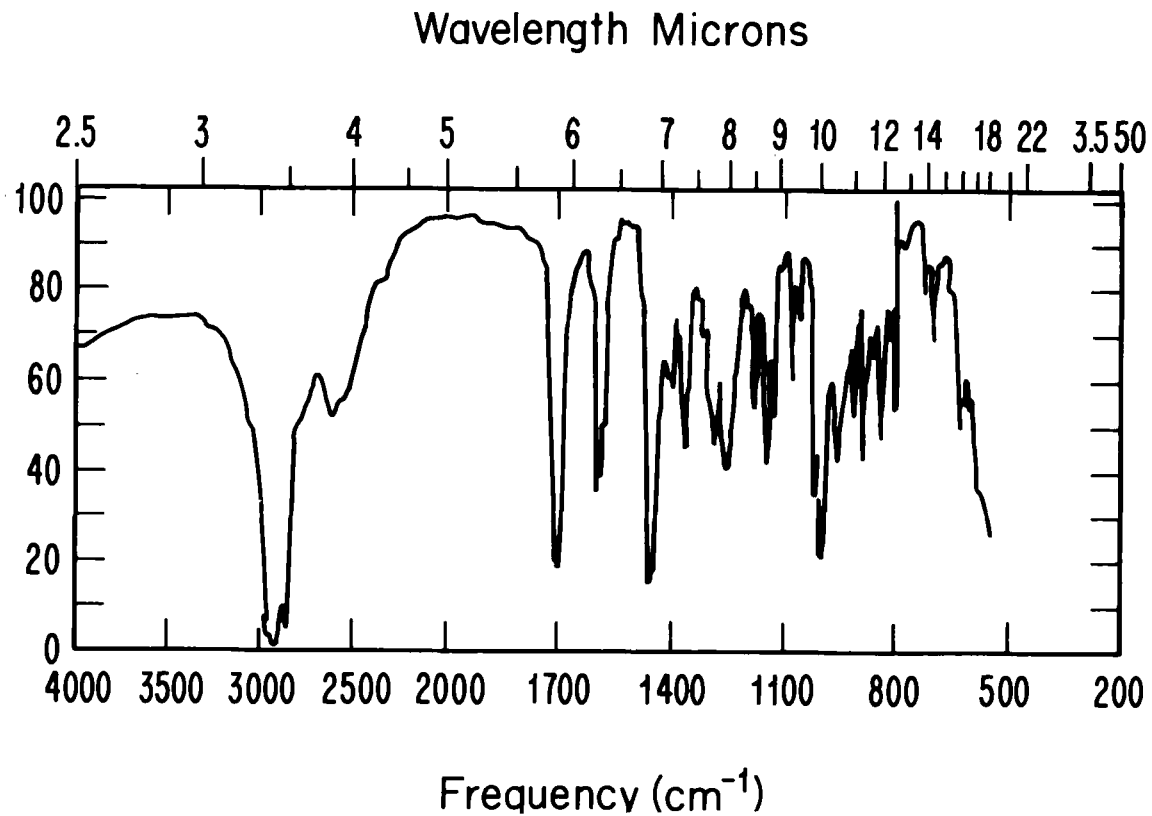


Figure 10. Infrared Absorption Spectrum of Sulindac Form I in Nujol Mull

## 5.0 Methods of Analysis

### 5.1 Identification

Comparison of infrared and absorption spectra with those of reference standard are methods of identifications specified by the USP-NF<sup>(13)</sup>.

### 5.2 Elemental Analysis (10)

The following elemental composition was observed for sulindac when approximately 2 mg of sample was analyzed with a Perkin-Elmer, Model 240 CHN Analyzer.

<u>Element</u>	<u>Theory %</u>	<u>Funds %</u>
C	67.40	67.63
H	4.81	4.72

### 5.3 Chromatographic Analysis

#### 5.3.1 Thin Layer Chromatography

Several TLC systems have been used for sulindac. Table V lists the solvents and the sulindac RF's.

TABLE V

TLC Solvents and Rf for Sulindac

<u>Solvent</u>	<u>Rf</u>
1. Chloroform/Ethyl Acetate/Acetic Acid 16:5:1	~0.4
2. Ethyl Acetate/Acetic Acid 97:3 and 94:6	~0.4
3. Chloroform/Acetic Acid 95:5	~0.3
4. Ethyl Acetate/Chloroform 1:1	~0.1
5. Carbon Tetrachloride/Acetic Acid 9:1	~0.2
6. Chloroform/Dioxane/Acetic Acid 25:1:1	~0.7
7. Benzene/Methanol/Ammonia 50:20:1	~0.2

Silica gel GF absorbents are used with all the solvent systems and detection is with short wave UV light (~254 nm) or with exposure to iodine vapor.

Solvent system #1 has been used for quantitative determination of impurities in sulindac. Quantum Q-7G plates are used and the plate is

scanned at 280 nm with a suitable densitometer<sup>(13)</sup>.

Approximate Rf's for sulindac related compounds for solvent systems #1 and #2 are listed below.

Compound	Rf	
	#1	#2
Sulfone analog	~0.6	~0.7
Sulfide analog	~0.7	~0.8
Ethyl ester sulindac	~0.5	~0.5
Trans isomer	~0.3	~0.3

### 5.3.2 Gas Chromatography

Sulindac has been chromatographed as the methyl ester (diazomethane) on a 6' x 10.25" 1% SE-30 on Supelcon 80/100 column under isothermal at 262°C with nitrogen as a carrier gas at ~100 ml/min. Detection was with electron capture and sample sizes ranges from ~50 to ~100 ng. The retention time of sulindac was approximately 7 minutes. The sulfide and sulfone analogs have retention times of 1.5 and 5 minutes respectively under the same condition<sup>(11)</sup>.

### 5.3.3 Liquid Chromatography

Several HPLC systems have been used for sulindac including one employing chloroform/ethyl acetate/acetic acid (800:200:2) as the mobile phase with a  $\mu$  Porasel, 30 cm, 10  $\mu$  particle size column; UV detection at 280 nm and a flow rate of 2.0 ml/min (500-3000 psi). Under these conditions sulindac chromatographed with a retention time of approximately 6.3 minutes. The sulfide and sulfone analogs chromatographed with retention times of ~1.8 2.5 minutes respectively<sup>(12)</sup>.

Other HPLC systems use Zorbax (silica gel) column 0.5 m x 8 mm or 0.25 m x 2.1 mm with mobile phases of chloroform/ethyl acetate/acetic acid 38:5:1<sup>(8)</sup> or methylene chloride/hexane/isobutanol/acetic acid/water 50:50:10:4:.046.

### 5.4 Titrimetry

Sulindac can be quantitatively assayed by potentiometric titration. Methanol is used as a

solvent for sulindac and the solution is titrated with standardized sodium hydroxide (0.1N) to a potentiometric end point using a glass-calomel electrode system(15).

#### 6. Drug Metabolic Products, Pharmacokinetics, Bio-availability

The major urinary metabolites are sulindac and its glucuronide, sulfone and its glucuronide and in trace amounts the insoluble sulfide metabolite and its glucuronide. The major biotransformation involves irreversible oxidation of the sulfoxide group of sulindac to sulfone and a reversible reduction to the sulfide. Studies in healthy subjects revealed that after oral administration of sulindac at least 88% is absorbed. After a single 200 mg oral dose the peak plasma concentrations of sulindac and its sulfone and sulfide metabolites were 4, 2, and 3 mg/ml respectively, and were attained after 1 hour for the parent drug and after 2 hours for each of the metabolites.

The sulfone and its conjugates are the major products excreted in urine, and account for  $27.6 \pm 2.8\%$  of the administered dose. Sulindac and its glucuronide accounted for  $20.2 \pm 0.2\%$  of the dose excreted in the urine. Mean renal clearance of sulindac and its sulfone metabolite was  $45.1 \pm 16.2$  and  $33.2 \pm 12.2$  ml/min respectively(16). The effective half-life for accumulation is about 7 hours for sulindac(17). The long half-life is due to extensive enterohepatic recirculation(18).

There are no reported bioavailability problems with sulindac tablets. No significant difference in plasma concentrations or urinary excretion of sulindac was found following administration of tablets or solution(17).



## 7. Identification and determination in body fluids and tissues.

The following methods have been used for the assay of sulindac in body fluids and tissues.

<u>Methods</u>	<u>References</u>
Radioactivity	(17)
Isotope dilution-radioimmunoassay	(19)
Ultraviolet	(17)
Thin-layer chromatography	(16)
Gas-liquid chromatography	(16)
Mass spectrometry	(16)
HPLC	(20)

## References

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# TETRACYCLINE HYDROCHLORIDE

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## Tetracycline Hydrochloride

Tetracycline and tetracycline hydrochloride have been mostly used synonymously in this monography. Literature cites quite often tetracycline but it is presumed to be valid also for tetracycline hydrochloride. Generally solutions of tetracycline are made in dilute acids. Physical properties, spectra etc are given for tetracycline hydrochloride.

### 1. History

During the course of experiments for the elucidation of the structure of the two earlier discovered compounds chlortetracycline (CTC) and oxytetracycline (OTC) it was found that hydrogenation of chlortetracycline resulted in halogenolysis and the product tetracycline (TC) retained the useful activity spectrum of the first two members of the family. TC appears to represent the first clinically successful antibiotic produced by sheer chemical manipulation of preexisting antibiotic. TC was found to be present in fermentations of both cultures streptomyces aureofaciens and streptomyces rimosus as well as in streptomyces viridofaciens (1).

### 2. Nomenclature

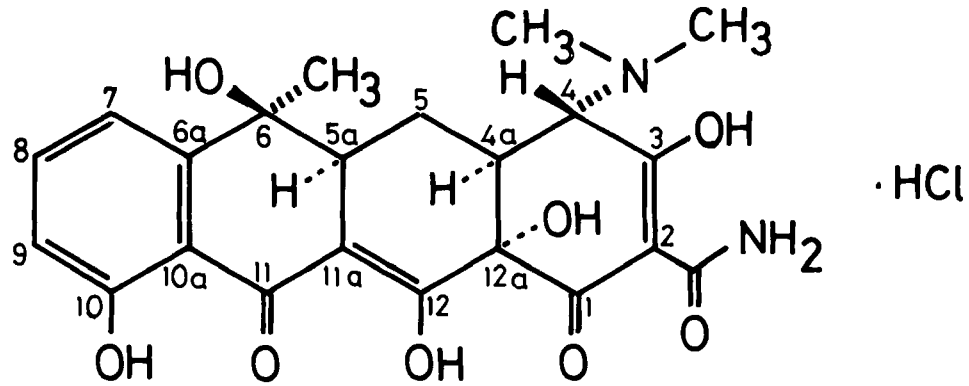
The name tetracycline derives from the naphthacene nucleus that it possesses. The rings are lettered A through D from right to left and the numbers start at the bottom of ring A. The enolized form as illustrated on next page has been selected arbitrarily and is used most exclusively in the literature. It is further defined as hydrochloride of 4 $\beta$ -dimethylamino-1,4,4a,5,5a,6,11,12a-octahydro-3,6 $\alpha$ ,10,12,12a $\beta$ -pentahydroxy-6-methyl-1,11-dioxonaphthacene-2-carboxamide.

### 3. Description

#### 3.1. Name, Formula, Molecular Weight

Tetracycline hydrochloride	
C <sub>22</sub> H <sub>25</sub> ClN <sub>2</sub> O <sub>8</sub>	480.90

## Tetracycline-Hydrochloride



Structural Formula

### 3.2. Appearance, Colour, Odour

A yellow crystalline powder, odourless, with a bitter taste.

### 4. Synthesis

The chemical total synthesis of tetracycline hydrochloride is economically not feasible. The original process involved catalytic hydrogenolysis of CTC to TC and then a directed biosynthesis using chlorine-free-culture-mediums (2,3). In fact the removal of chlorine from the medium or decreasing chlorine incorporation with a wide variety of chlorination inhibitors is technically quite important in order to facilitate processing without the need of expensive chromatographic steps as otherwise undesirable quantities of CTC are coproduced along with TC (4).

Fermentation and Isolation (5): Various tetracyclines are produced by submerged fermentation of different streptomyces species isolated in soil screening programmes and then nutritionally and genetically manipulated to optimize yields. The fermentations are done on a very large scale of about 20,000 to 50,000 gallons. The organism and strain chosen not only determine maximal yields but also the identity of the antibiotic formed. Chlorotetracycline fermentations frequently have up to 10% of tetracycline present. A wide variety of media have been employed for TC production. Fermentation starts with a tube of lyophilized culture which must be opened aseptically and transferred to agar slants for incubation. The cell population is then built up in stages. The pH of the fermentation is generally closely controlled between 6 and 7 by the periodic addition of the base, usually solid  $\text{CaCO}_3$  and the temperature is maintained between  $25^\circ$  and  $30^\circ$   $^\circ\text{C}$ . Many energy and carbon sources such as starch, dextrin, maltose, glucose and nitrogen sources including amino acids, casein, meat extracts, nitrates and ammonium salts have been employed. A variety of inorganic ions must be present and calcium

carbonate is specially noteworthy as it stabilizes the tetracyclines formed and may help increase yields by precipitating the antibiotics as they are formed and thus decreases their autotoxic effect on the producing organism. Several patents dealing with the fermentative production of tetracyclines are reported in literature. The yields of tetracyclines are reported to lie about in the range of 10 g/liter (6). There are a variety of procedures which have been employed for isolating the various tetracyclines from high-yielding fermentations. The processes in use are governed by the physical and chemical properties of the antibiotics and by economic factors. Tetracycline is an amphoteric substance and has an isoelectric point at 4.8 and its water solubility near neutral pH is about 1 mg/ml. The solubility in water is about ten times greater at pH 1 - 3 than at pH 5 - 6. The isolation of TC can be affected through the use of a liquid ion exchange process, extraction from filtered beer at pH 8.5 with n-butanol or MIBK and precipitation with a non-polar solvent as the free base (7), fractional precipitation of the less soluble TC-HCl from CTC-HCl (coproduced in the fermentation) (8) and precipitation as the calcium-complex (9). The tendency of TC fermentations to produce sufficient quantities of CTC also renders refining difficult on a commercial scale (10). TC-oxalate is less soluble than CTC-oxalate so that fractional crystallization is quite effective (11). Precipitation at about pH 4 in the presence of citrate (12), urea (13) and phosphate (14) has also been reported. Various cultures known to produce major fermentation-derived TC are also mentioned in literature (5).

## 5. Physical Properties

- 5.1. Solubility: It is freely soluble in water giving a clear solution which becomes turbid on standing, soluble in aqueous solutions of alkali hydroxides and carbonates, slightly soluble in alcohol and practically insoluble in various organic solvents (15,16).



Following solubility parameters are given in Table 1.

Table 1  
Solubility of TC-HCl and TC in various solvents in mg/ml at 28 °C (17).

	TC-HCl	TC
Water	10.9	1.7
Methanol	20+	20+
Ethanol	7.9	20+
Benzene	0.3	1.0
Petroleum ether	0.0	0.005
Carbon tetrachloride	0.1	0.3
Ethyl acetate	0.8	17.3
Acetone	0.8	17.4
Ether	0.6	3.7
Ethylene chloride	0.8	11.3
Dioxane	7.7	14.6
Chloroform	2.9	13.8
Pyridine	20+	20+
Benzyl alcohol	10.8	14.4

#### 5.2. Loss on Drying (15)

Not more than 2.0%, determined with 1.00 g by drying for 3 hours at 60 ° over phosphorus pentoxide at a pressure not exceeding 5 Torr.

#### 5.3. Dissociation Constant

The macroscopic  $PK_a$  values of tetracycline hydrochloride in water at 25 °C are reported to be 3.30, 7.68 and 9.69 (18, 19). The most acidic value is attributable to the unusual  $\beta$ -tricarbonyl system in ring A, the middle  $PK_a$  is associated with the vinylogous acidic  $\beta$ -dicarbonyl system of rings B and C and highest  $PK_a$  is then assigned to the protonated dimethylamino function at C<sub>4</sub>. A fourth  $PK_a$  value of 10.7 has also been reported and is also attributed to the phenolic OH group at C 10 (20).

#### 5.4. Specific Optical Rotation (15)

- 239 ° to - 258 °, determined in a 1.00% w/v solution in 0.01N HCl and calculated with reference to the dried substance.

#### 5.5. pH Value (15)

The pH of a 1.0% w/v solution is between 1.8 and 2.8.

### 5.6. Ultraviolet Spectrum

The quaternary carbon at C 12a separates the chromophores of the tetracyclines into two relatively distinct regions. The  $\beta$ -tricarboonyl system of ring A contributes a strong absorption band at about 260 nm while the aromatic ring D conjugates with the  $\beta$ -diketone moiety in rings B and C to produce a visible band at about 360 nm (21). The molecular extinction coefficients and  $A_{1\%}^{1\text{cm}}$  of TC-HCl in different solvents are reported to be (22):

	Methanol	0.1 N HCl	0.1N NaOH
Absorption maximum	363 nm	356 nm	380 nm
$A_{1\%}^{1\text{cm}}$	270 nm	269 nm	268 nm
$\epsilon$	331	301	366
	345	382	299
	15940	14480	17580
	16590	18380	14360

The UV spectra are given in Fig. 1.

### 5.7. Infrared Spectrum

The infrared spectrum of TC-HCl is given in Fig. 2. The spectrum was obtained with a Perkin-Elmer Spectrophotometer 257 from a KBr pellet. The several carbonyl groups of the TC are either amide in character or conjugated to double bond system or strongly hydrogen bonded. Thus the amide band at about 1670  $\text{cm}^{-1}$  and the large carbonyl envelope near 1620  $\text{cm}^{-1}$  are usually identical for almost all the tetracyclines whose spectra then differ mostly in the finger print region (21). The structural assignments may be correlated with the following band frequencies:

<u>Frequency(<math>\text{cm}^{-1}</math>)</u>	<u>Assignment</u>
3350	stretching vibrations of $\text{NH}_2$
3100	stretching vibrations of OH
2600-2800	Amine halide salt bands
1670	stretching vibrations of amide group
1620	stretching vibrations of carbonyl group
1450	characteristic vibrations of the aromatic ring

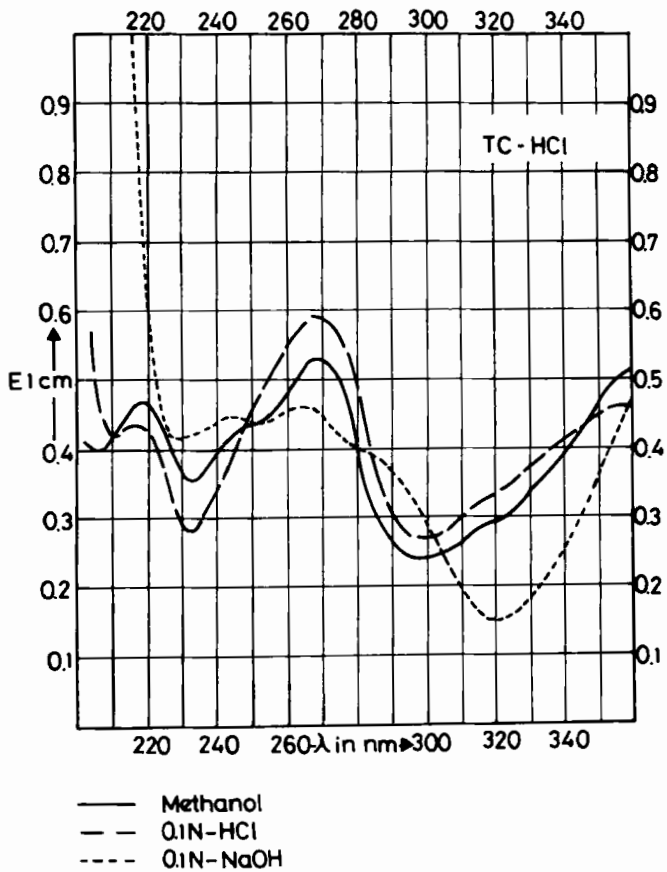


Fig. 1

UV Spectrum Of Tetracycline Hydrochloride  
In Different Solvents

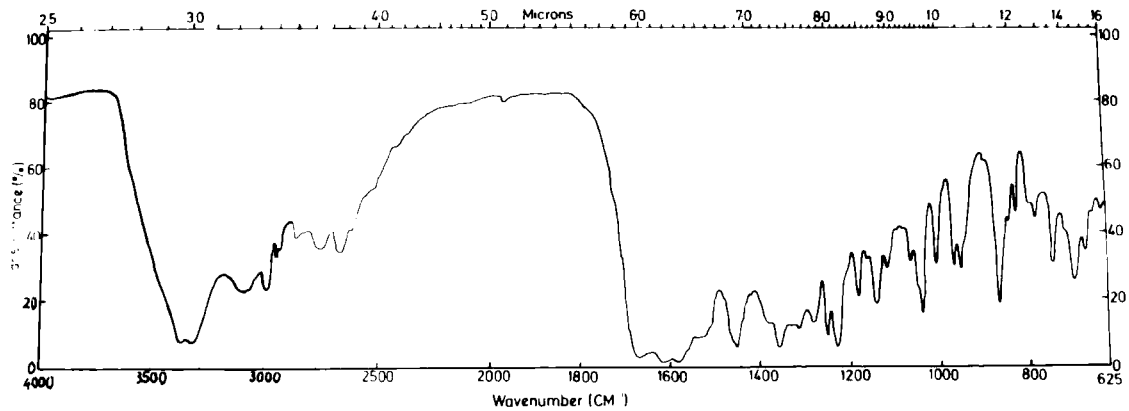


Fig. 2

IR Spectrum Of Tetracycline Hydrochloride, KBr Pellet,  
Perkin-Elmer 257 Spectrophotometer

### Nuclear Magnetic Resonance Spectrum

The nuclear magnetic resonance spectrum of TC-HCl as shown in Fig. 3 was obtained on a Varian T-60 NMR Spectrometer in deuterated methanol containing 1% tetramethyl silane as the internal standard. The following spectral assignments are made for Fig. 3.

<u>Chemical Shift</u>	<u>Assignment</u>
1.72 singlet	CH <sub>3</sub> at C 6
3.10 singlet	N(CH <sub>3</sub> ) <sub>2</sub> at C 4
7.20 doublet	H at C 7
7.58 doublet	H at C 8
6.95 doublet	H at C 9

They are approximately in agreement with values reported in literature, but the spectrum there was obtained with a HA-100 (23). The nmr shifts appear to be general regardless of solvent. Similar shifts are obtained in pyridine, trifluoroacetic acid and dimethyl sulfoxide. Particularly notable is the substantial downfield shift in the resonance due to the C 4 proton when the tetracyclines epimerize at C 4. This is especially convenient as both isomers are usually available and can be distinguished easily in this way. NMR was used to monitor the epimerization of TC since the dimethylamino resonance of TC and its C 4 epimer differ by 0.1 ppm (24). Formation of anhydrotetracycline could also be readily detected through NMR (23).

#### 5.9. <sup>13</sup>C Nuclear Magnetic Resonance Spectrum

carbon magnetic resonance measurements have been performed using the natural <sup>13</sup>C abundance. Many more resonances are present in these spectra compared with the proton nmr. The spectrum of TC-HCl, dissolved in D<sub>2</sub>O, is presented in Table 2. The signals are reported to be slightly different in DMSO (25).

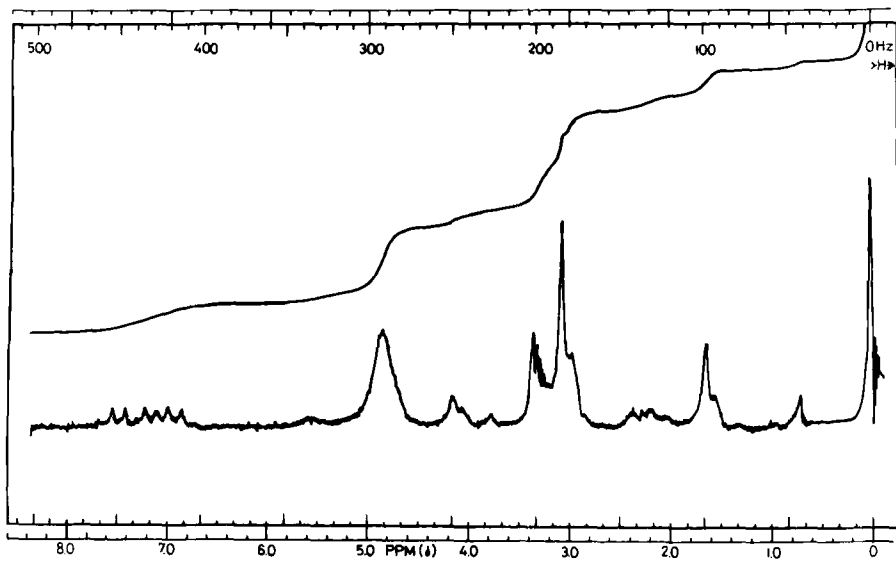


Fig. 3  
NMR Spectrum Of Tetracycline Hydrochloride,  
Varian T 60 Spectrometer

Table 2  
 $^{13}\text{C}$  NMR Spectrum of TC-HCl in  $\text{D}_2\text{O}$  (25)

Carbon Assignment	Chemical Shift
C 1	193.9
C 2	97.1
CONH <sub>2</sub>	173.0
C 3	187.3
C 4	70.7
HN <sup>+</sup> (CH <sub>3</sub> ) <sub>2</sub>	43.1 (quartet)
C 4a	35.0 (doublet)
C 5	27.3 (triplet)
C 5a	42.3 (doublet)
C 6	70.4
C - CH <sub>3</sub>	22.1
C 6a	146.7
C 7	118.6
C 8	138.4
C 9	116.8
C 10	161.9
C 10a	115.1
C 11	194.3
C 11a	107.3
C 12	172.3
C 12a	74.3

### 5.10. Mass Spectrometry

Due to its non-volatility and thermal instability the electron-impact mass spectra of tetracyclines is rich in fragment ions and weak in molecular ion intensity (26). In the highest mass region one sees  $m/e$  426 for dehydration followed by ions 409 and 391 resulting from loss of  $\text{NH}_3$  and water from the carboxamide function as well as 381 for loss of the whole group as  $\text{CONH}_3$ . Most of the fragments seen involve rings A and B because rings C and D become so quickly naphthalenoid. The mass spectrum of TC-HCl is given in Fig. 4.

Instrument: Varian Mat 44

Sample temperature: (direct inlet)

50°C - 250°C in 5 minutes

Source temperature: 200 °C

Electron energy: 80 eV

The prominent ions of this spectrum can be correlated to the structure as following:

M-HCl  $\text{H}_2\text{O}$ =426; M-HCl  $\text{H}_2\text{O}$   $\text{NH}_3$ =409;

M-HCl  $2\text{H}_2\text{O}$   $\text{NH}_3$ =391; M-HCl  $\text{H}_2\text{O}$   $\text{CONH}_3$  = 381;

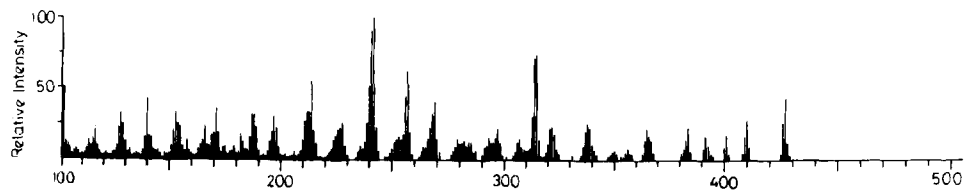


Fig. 4  
Mass Spectrum Of Tetracycline Hydrochloride



### 5.11. Circular Dichroism Spectra

TC-HCl gives useful ORD-CD spectra. The intensity of the A ring band and its relative absence in 4-epitetracycline provides a sensitive means of assay for epimeric mixtures. Mixtures of tetracycline and 4-epitetracycline were assayed utilizing the large difference in their circular dichroism spectra at 262 nm. The method is very rapid and requires only the experimental measurements of ellipticity at 262 nm and absorbance at 356 nm (27). The disadvantages of the assay are its inability to determine the content of anhydrotetracycline if it is present in large amounts. In Fig 5-6 molar ellipticity is illustrated graphically (27). The ability of this technique to reflect subtle conformational shifts in dilute hydroxylic solvents under a wide variety of conditions is a particular strong point. Interactions with complexing ions gave dramatic changes in the spectral bands from both chromophoric regions providing evidence for chelation in both areas. Quantitative studies of ion-binding led to the conclusion that little if any binding takes place below pH 3, that the binding of B C D rings takes place at pH 5 and the A ring binds at physiological pH's (28). The study of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  complexes of tetracycline in buffered solution was undertaken to determine their stoichiometry and the chelation sites. Circular dichroism was used to follow complex formation. Circular dichroism spectra of calcium and magnesium complexes of tetracycline in aqueous and methanol-water (90:10) systems are reported. Fig. 7-9 show these spectra as published by Newman and Frank (29). Calcium formed a 2:1 metal ion to ligand complex at C 10 and C 12 sites, while magnesium formed a 1:1 ratio complex, the chelating site being at C 11 (29). Circular dichroism spectra of TC-calcium complexes in different organic solvents and the effect of barbital sodium and L-tryptophan on it in alkaline and other buffer solutions are also known (30).

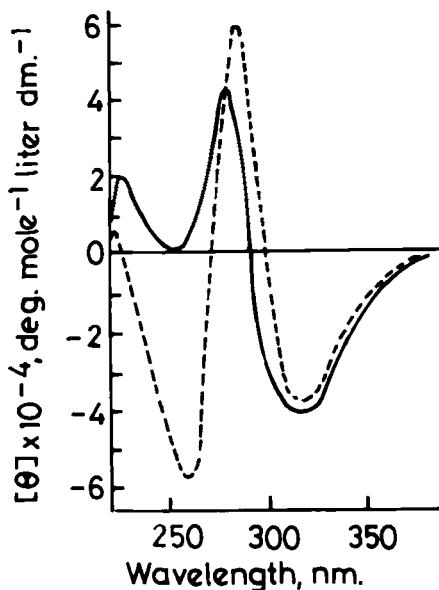


Fig. 5 Molar ellipticity ( $\Theta$ ) of tetracycline hydrochloride (—) & 4-epitetracycline ammonium salt (---) as a function of wavelength in 0.03 N HCl

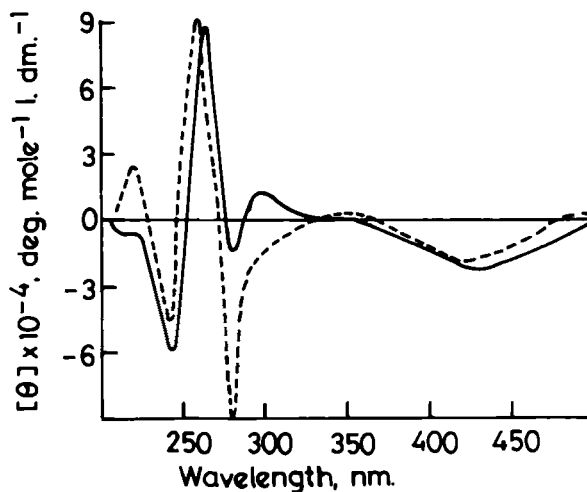


Fig. 6 Molar ellipticity ( $\Theta$ ) of anhydrotetracycline hydrochloride (---) and 4-epianhydrotetracycline (—) as a function of wavelength in 0.03 N HCl

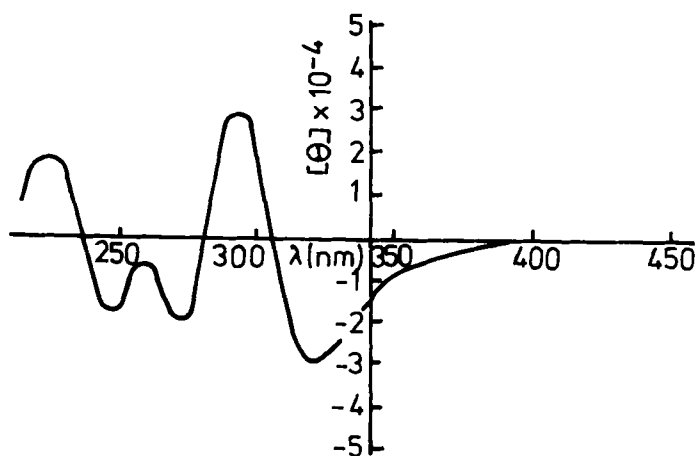


Fig. 7 Circular dichroism spectrum of tetracycline in methanol-water (90:10)

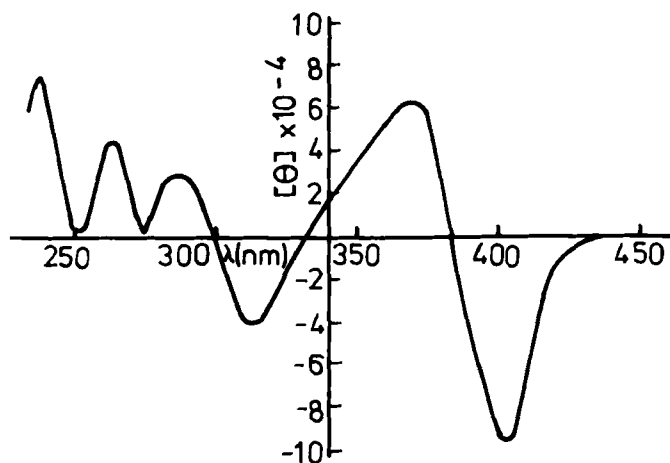


Fig. 8 Circular dichroism spectrum of the calcium complex of tetracycline in methanol-water (50:50)

### 5.12. Differential Thermal Analysis

Stability studies of TC have been done with differential thermal analysis (DTA) (31, 32). DTA was employed to study the effect of certain additives, magnesium stearate, talc and lactose on the stability of tetracycline. The incorporation of magnesium stearate has a profound influence upon the characteristic thermogram features of TC (Fig 10). The analysis was performed with a thermal analyzer with a heating rate of 5°/min (33). DTA was further used to identify some vitamins as the inactivating components in formulations containing TC-HCl and lactose with uncoated and coated vitamins' granules. The thermal analysis was done employing a Mettler T.A. 2000 differential thermal analyser with a constant heating rate of 10°/min (34).

### 6. Colour Reactions

To about 0.5 mg TC-HCl when conc. sulfuric acid is added a purplish-red colour is produced. On addition of 1 ml water the colour changes to deep yellow (15). When a solution of 2 mg TC-HCl in 2 ml water is reacted with 5 drops of iodine solution, a brown voluminous precipitate of periodide is obtained. 2 mg TC-HCl dissolved in 1 ml dilute sodium hydroxide are treated with 1 ml sulfanilic acid and few drops of sodium nitrite solution. A brown-orange coloured solution is obtained (35).

### 7. Degradation and Stability

The dimethyl amino function at C 4 of TC-HCl stands 1,3-diaxially eclipsed with respect to C 12a OH group in acidic solutions. Epimerization takes place and the product 4-epitetraacycline has no significant antibacterial activity. The reaction takes place most rapidly between pH 2-6 in aqueous solutions and is a reversible first-order reaction (36-42). In alkaline solutions using precise conditions epitetraacyclines are converted almost completely back to the bioactive isomer in the presence of chelating metals (43). This is due to the complexing metals tying together the C 4 -

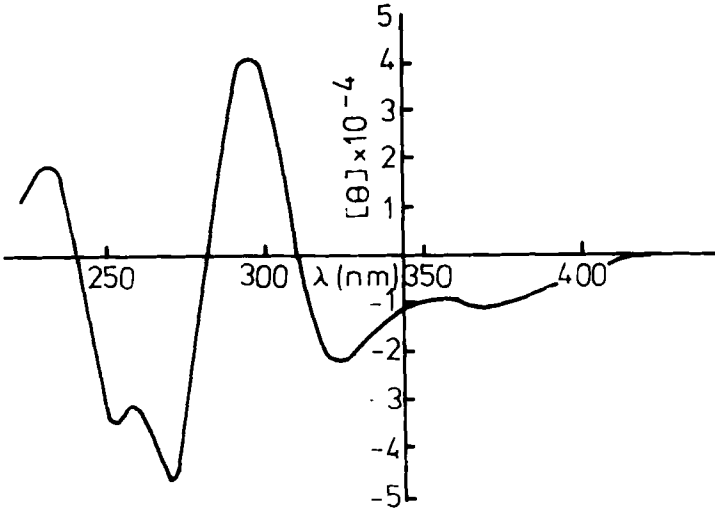


Fig. 9 Circular dichroism spectrum of the magnesium complex of tetracycline in aqueous solution

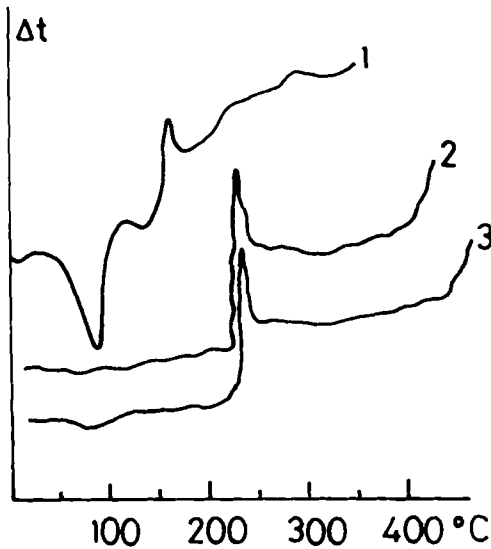


Fig. 10 Thermograms of tetracycline & mixtures with magnesium stearate; 1. tetracycline, 2. tetracycline + Mg. stearate (freshly prepared), 3. tetracycline + Mg. stearate (stored for 1 year)

dimethyl amino and C 12a - hydroxy functions thus overcoming their steric repulsion (44). Other groups which influence the epimerization include phosphate (41), citrate (41), urea (45) and some pharmaceutical ingredients (46). Additionally the reaction is catalysed by light. The equilibrium is reached, depending on the reaction-medium, at the level of about 40-50 % 4-epitetracycline following the 1st order reaction kinetics. Further epimerization is strongly dependent on pH, temperature and the buffer system used (47). In a solution of 0.2 mg TC-HCl in 10 ml water at pH 3.5 about 7% epitetracycline is formed in 48 hours (48). The C 6 - OH group is axial and antiperiplanar trans to the H at C 5a so that it is sterically ideal for an elimination reaction of second order. The anhydrotetracycline is produced due to dehydration at C 5a, 6. It is progressively formed at pH's lower than 2, specially upon heating. Anhydrotetracycline possesses in vitro bioactivity but has not found clinical applications (49-55). The degradation of tetracycline itself is superimposed by the concurring degradation reactions of the primary tetracycline degradation products. Epimerization of anhydrotetracycline (ATC) is faster and the dehydration of 4-epitetracycline (ETC) is slower than the corresponding degradation reactions of the intact tetracycline. Kinetics of dehydration of epitetracycline in solution to form epianhydrotetracycline (EATC) were studied using UV and visible spectrophotometry. The reaction was found to be first order with respect to epitetracycline and hydronium-ion concentrations (56). Degradation pathway of TC to ETC, ATC and EATC is presented in Fig. 11 as reported by Leeson (77). In alkaline medium tetracycline decomposes at the C 6 - OH site and isoderivatives with phthalide structure are obtained. The tetracyclines are stable in neutral or mildly acidic solutions. At pH extremes, specially at elevated temperatures bioactivity is progressively lost (57). Table 3 gives relevant stability data for

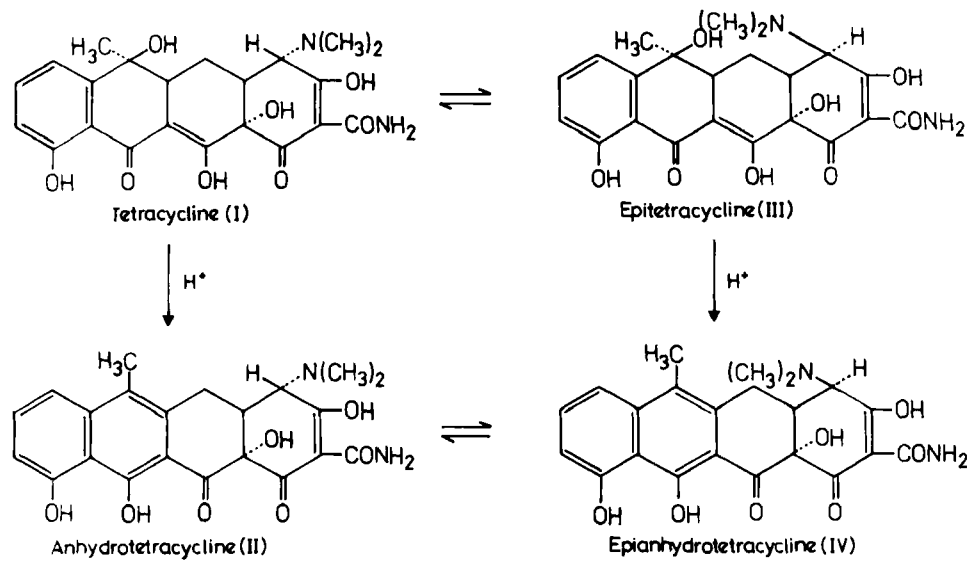


Fig. 11

Degradation Pathway Of Tetracycline Hydrochloride

the fermentation derived tetracycline.

Table 3  
Stability Data For the Fermentation-derived  
Tetracycline in Solution

Half-life	Conditions	Reference
2 min	0.2 N H <sub>2</sub> SO <sub>4</sub> /100°	58
1 min	1.0 N H <sub>2</sub> SO <sub>4</sub> /100°	59
15.5 hr	1.0 N H <sub>2</sub> SO <sub>4</sub> /25°	40
6.8 min	0.1 N NaOH/100°	59
11 hr	pH 10 buffer/22°	58
101 min	0.1 N NaOH/60°	40
12 hr	pH 8.8 buffer/24°	60
12 hr	pH 8.85 phosphate buffer	61

The stability of TC-HCl injection solutions can be enhanced through the addition of stabilising agents such as magnesium gluconate (62). Generally bivalent and trivalent cations such as magnesium, calcium, manganese, copper, nickel, iron and aluminium form chelates with TC which are mostly microbiologically inactive. This sort of complex-formation can be eliminated through the addition of citric acid, polyphosphates and glucoseamine hydrochloride (48). TC does not decompose in glucose solutions in the pH range 2-7.5 during a period of 6 hours. Propylene glycol, pyridin derivatives, pantothenic, lactic and tartaric acids may be used as stabilising agents and the TC solutions should be stored cool (47). Differential thermal analysis was employed to study the effect of certain additives such as magnesium stearate. It was found that magnesium stearate affects the stability of TC even in freshly prepared formulations (33). Photodecomposition of TC at oil-water interfaces has been studied. When the oils were irradiated in presence of TC, much larger amounts of peroxide were formed. This increase in the peroxide content was presumably due to the formation of the oil-soluble anhydrotetracycline derivatives (63). The stability of TC and some TC-complexes in suspensions has been tested.



Maximum stability of TC and its complexes with  $\text{CaCl}_2$ , urea and sodium hexameta-phosphate has been reported in weakly acidic medium (pH 4-5). Ascorbate anion influences the stability negatively. In non-aqueous vehicles the TC-urea-complex shows better stability than TC itself. Sodium metabisulfite and sodium hydrogensulfite influence the stability positively. Further it can be increased by treating the TC-suspensions with an inert gas (64). Studies of TC stability in aqueous solutions and the influence of pyridin derivatives, magnesium salts and aliphatic and aromatic acids has been extensively investigated by Kassem and coworkers (62, 65). Commercial tetracycline samples and formulations have been the subject of stability studies by various authors (66, 67, 68). It was found that newly manufactured TC preparations contain only small amounts of anhydrotetracycline and 4-epitetracycline. Storage under adverse conditions markedly increase the amount of degradation products, but storage under normal conditions results in only a slow increase in anhydrotetracycline and 4-epitetracycline. In syrups this can be correlated with loss in tetracycline potency. Citric acid was found to increase greatly the tendency of tetracycline degradation. TC-HCl is more stable than TC-phosphate (68). Epianhydrotetracycline could not be found over 1% in any of the commercial formulations tested (66). Problems of TC stability in eye-drops, injections and ways and means to increase this has been reported (69, 70, 71, 72). Powder or crystalline TC and TC-HCl samples darken in strong sunlight in a moist atmosphere. In aqueous solutions TC and TC-HCl degrade by epimerisation which leads to further decomposition products. It is rapidly inactivated at a pH less than 2 and is slowly destroyed at pH 7 and above. The rate of degradation of TC to epianhydrotetracycline is increased in the presence of citric acid (16). Influence of temperature on the stability of solid TC-HCl

was investigated by Dihuidi and coworkers (73). At 37° and 70 °C the stability was studied over a period of 27 months and at 50 °C over a period of 16 months. At 37 °C and 50 °C no decomposition is observed for TC nor for its related substances. At 70 °C a distinct decrease in TC is observed as well as a small increase in anhydrotetracycline. It is inferred from these values that at 20 °C and under conditions of low humidity TC-HCl is a very stable product having a  $t_{90}$  of at least 50 years but probably of about 350 years (73). The stability of solid samples of TC-HCl, TC and TC-phosphate aged at room temperature for up to 8 years has been followed. No appreciable decomposition was observed except for TC-phosphate which showed a significant increase of 4-epitetracycline and anhydrotetracycline after a period of 4 years (74, 75). Dony-Crotteux (76) reported that aqueous TC solutions containing riboflavin degrade in the presence of air and light. In addition suppression of degradation by the antioxidant ascorbic acid was observed. Some of the potency loss encountered results from epimerization catalysed by the buffer system. Parenteral TC-formulations containing ascorbic acid may be mixed with riboflavine for intravenous administration without concern for antibiotic stability. Although ascorbic acid itself appears to induce another degradation pathway, the loss is adequately covered by normal product overage (76, 77). The dehydration of TC at the C 5a-6 position as a function of acidity was investigated at various temperatures (78). TC epimerization kinetics has also been investigated using NMR spectrometry (23). The concentration of TC, ETC (epitetracycline), ATC (anhydrotetracycline) and EATC (epianhydrotetracycline) were studied in pH 1.5 phosphate solution at four temperatures in a degradation-pattern investigation. The study showed that epimerization of TC and ATC can take place at a low pH (79). Stability of TC and TC-HCl in methanol at 26 °C has also been performed (130).

Siewert and coworkers made a thorough study of TC stability in large number of formulations such as ointments, creams and hydrogels. All formulation showed a relatively quick TC decomposition at temperatures above 50 °C. Preparations containing non-aqueous ointment bases demonstrated a higher degree of stability than aqueous ones like creams and hydrogels. TC-HCl decomposed quicker than TC-base in aqueous systems (79a). In another study of the same authors 14 suspensions and 8 solutions were subjected to an accelerated stability test. 12 out of 22 preparations were calculated to be stable for a period of over three years under normal storage conditions. Two formulations decomposed to an extent of more than 10% at 61 °C within one day. Suspensions did not show a better stability than TC-containing solutions (79b).

#### 8. Related Impurities

Impurities of 4-epitetracycline, anhydrotetracycline and 4-epianhydrotetracycline have been limited in tetracycline hydrochloride by european pharmacopoeia (15), british pharmacopoeia (80) to 4% (ETC) and 0.5% each for ATC and EATC. USP XX (81) allows the content of EATC up to 2%. The permissible content of EATC in various TC formulations such as capsules and injections has been even increased to 3.0% by USP XX.

#### 9. Toxicity

The tetracyclines have a safety margin which is quite satisfactory under most circumstances. Acute toxicity data for mice is given for TC as following:

<u>Route of Administration</u>	<u>Acute Toxicity</u> <u>LD<sub>50</sub> (mg/kg)</u>
oral	6300
IV	170
IP	280

Overaged TC preparations degrading to EATC show a definite toxicity. Fatal reactions have been reported, although withdrawal of

the drug usually causes a slow reversal of the damage. Expiration dates should then be respected and TC should not be stored under conditions of excess heat and humidity. The LD<sub>50</sub> of EATC is 4.8 times higher than that of unchanged TC. The immunosuppressive effect is even reported to be 40-100 times higher (83). A comparative toxicity of ATC has not been reported. But the further degradation of ATC to EATC takes place much faster than the epimerisation of tetracycline.

10. Dissolution

The dissolution of TC was investigated using in vitro dissolution system of Stricker. Simulated gastric and intestinal juices were used as dissolution media separately. A good in vitro - in vivo correlation for TC was obtained (84). The aminomethylation of TC, a known process to make tetracyclines water-soluble and useful for parenteral administration has no significant influence on the bioavailability of the orally administered antibiotic. The in vitro adsorption of TC-HCl was studied on various antacids, namely magnesium trisilicate, magnesium oxide, calcium carbonate, bismuth oxycarbonate, aluminium hydroxide and kaolin. The reversibility of the adsorption process was studied in different media and at pH values similar to those of the gastrointestinal tract. In general 0.0143 N NaHCO<sub>3</sub> was found to possess higher eluting properties than 0.01 N HCl (85). The effect of various additives, PEG 6000, PVP, CMC on the adsorption of TC-HCl on magnesium trisilicate and milk was also studied. Conclusions were made that the incorporation of some of these additives may possibly reduce TC-antacid interactions and consequently improve the antibiotic availability when coadministered with antacids (86). The influence of additives on the in vitro release of drugs from hard gelatin capsules has been studied. A factorially designed experiment was used to assess the total percentage of TC-HCl released from hard gelatine capsules as the function of a) diluent - the diluents being

lactose, primojel and dry-flow starch - b) the quantity of diluent c) the presence and absence of magnesium stearate and d) the presence and absence of sodium lauryl sulphate (87). In a further study in vitro release of TC-HCl from hard-gelatine, soft-gelatine capsules and TC-HCl tablets in a number of commercial formulations was investigated. Majority of the commercial preparations conformed to the FDA regulations. The paddle and the flow-through-cell systems were used for the in vitro release of the drug (88). BP 80 prescribes a dissolution test for TC-tablets using a basket method and 0.1 M HCl as medium (88a). According to FDA regulations (89) tablets and hard-gelatine capsules containing 250 and 500 mg TC-HCl should release in first 30 minutes more than 60 and 50% of the drug respectively.

#### 11. Microbiological Methods

In vitro antimicrobial spectrum of TC as minimum inhibitory concentration in  $\mu\text{g/ml}$  can be given as following (89a):

<u>Ps.aeruginosa</u> 10	<u>A.aerogenes</u> 1.66	<u>Shigella Sonnei</u> 2.5
<u>S.typhosa</u> 1.56	<u>Vibrio comma</u> 0.58	<u>Kl.pneumoniae</u> 1.56
<u>S.aureus 5</u> 0.21	<u>S.aureus TC resist.</u> 60	<u>Ps.vulgaris</u> 100 <sup>+</sup>
<u>S.pyogenes</u> 0.06	<u>Ps.Morganii</u> 2	<u>E.coli</u> 0.73

The antibacterial activity of TC expressed in MIC-values (minimum inhibitory concentration) for a large number of gram-positive and gram-negative cocci, gram-negative bacteria and other microorganisms have been given in detail (89b). Microbiological assay methods are used due to their sensitivity but they are correspondingly nonspecific. The method is based upon a comparison of the inhibition of the growth of bacteria by

measured concentrations of the antibiotic to be examined with that produced by known concentrations of a reference standard of known activity. The assay for TC-HCl may be carried either by diffusion method or by turbidimetric method.

#### 11.1. Agar-Diffusion Method

The micro-organism used for TC is *Bacillus pumilus* NCTC 8241 and *Bacillus cereus* ATCC 11778. Buffer solutions of pH 4.5 in both cases and the incubation temperatures of 37 - 39° and 35 - 37 °C were used respectively (90).

#### 11.2. Turbidimetric Method

The growth of the test organism is measured by means of a suitable optical apparatus. The microorganism used for tetracycline are *Staphylococcus aureus* NCTC 6751 and *Staphylococcus aureus* ATCC 6538 P. The buffer solution with a pH of 4.5 and the incubation temperature between 35 - 37 °C are used (90, 91). Additives in TC formulations can influence the results obtained by microbiological methods (92). Comparison of the microbiological results has been made with those obtained through other methods (93, 94). Effects of certain tablet-formulation-additives such as starch, bentonite, veegum F, talc, liquid paraffin, stearic acid etc. on the antimicrobial activity of TC-HCl has been investigated (95).

### 12. Methods of Analysis

#### 12.1. Titrimetry

Titration of TC-HCl in non-aqueous medium are known. TC-HCl is dissolved in glacial acetic acid, 6% mercuric acetate solution added and then titrated potentiometrically with 0.1 N perchloric acid in dioxane (96, 97, 98). Indirect volumetric determination of TC-HCl after formation of a tetracycline-thiocyanochrom (III)-complex through oxidation with  $\text{KMnO}_4$ ,  $\text{KBrO}_3$  and  $\text{KIO}_3$  has also been reported (99).

#### 12.2. Visible and UV-Spectrophotometry

TC-HCl can be determined after dissolving it in 0.25 N NaOH. The yellow colour of the solution has an absorption maximum at

380 nm. Another method is the addition of ferric chloride solution to a dilute HCl solution of TC-HCl which gives an orange-brown colour with an absorption maximum at 490 nm (100, 101). Another procedure, originally developed for chlortetracycline, in which TC is dehydrated by heating in acid and the resulting solution is examined at 440 nm. This is preferred to direct spectrophotometric determination at 360 nm because there are fewer interferences at longer wavelengths (102, 103). The simplest method of all is to measure absorbance directly at 355 nm in dilute acid solutions (0.01N HCl) when relatively "clean" pharmaceutical formulations are involved. If dimethylformamide is used as solvent, the method can also be applied for TC determination in dragees (104). A spectrophotometric method for determining the amount of 4-epitetracycline is also known (40). The content of TC in presence of ATC can be determined through the application of absorbancy ratios, but the results are higher compared to those obtained in other studies (105). TC forms a rose colour with diazobenzene sulfonic acid in acid solutions which can be measured spectrophotometrically without interference from CTC and OTC (106). Another dye reaction used for analysis is the butanol soluble blue colour developed by TC with p-aminodimethyl aniline and sodium hypochlorite in neutral solutions (107). A spectrophotometrically screening method was used to screen powder, tablet and capsule samples for the contents of ATC and EATC in TC samples (68, 108). TC and its degradation products ATC and EATC have been determined spectrophotometrically as the metal complexes of titanium (III), zirconium (IV) and thorium (IV) salts at different wavelengths (109). The method has some advantages in respect to sensitivity and rapidity of measurements. Colorimetric determinations of TC after chemical reactions such as treatment with boric acid in sulfuric acid (110) or heating it with a mixture of sodium tungstate,

sodium carbonate and hydrogen peroxide (111) or reacting it with zirconium oxychloride in methanol and diazobenzoic sulphonic acid (106) are known. Other colorimetric methods for the determination of TC-HCl involve the use of ferric thiocyanate reagent, nitrosation method and an iodobismuthate reagent. TC-HCl was determined in various formulations such as powder, capsules and tablets (112), utilising these reagents. Spectrophotometric determination of TC and its degradation products ETC, ATC and EATC in synthetic mixtures and some solid-dosage forms on account of the differences in molar absorptivities at the wavelengths 254, 260, 267, 357 and 390 nm has been reported (113). A sensitive colorimetric method of analysis for TC-HCl and other antibiotics involves the formation of a coloured complex between TC and thorium (IV). The influence of pH and time upon the stability of the complexes was investigated. The method was applied to 29 different pharmaceutical dosage forms (114). Complex formation of uranyl acetate with TC has been utilized for its microdetermination. The mean stability constant of the 1:1 complex with absorption maxima at 404, 350 and 270 nm, as determined spectrophotometrically, amounted to  $1.2 \times 10^5$ , thus permitting the use of this procedure for TC microdetermination (115).

### 12.3 Fluorimetry

A quantum-corrected fluorescence spectrum of TC HCl (11 mg/50 ml) in 0.05 N NaOH taken with a Perkin-Elmer spectrofluorometer LS 5 is given in Fig. 12. Various fluorimetric methods of determination of tetracycline antibiotics are reported in literature (116, 117, 118, 119, 120). TC can be dehydrated to ATC by heating in acid solutions and taking the advantage of the greater lipophilicity of ATC it is extracted at about pH 4 - 5 with chloroform and then the fluorescence of aluminium-chelate is measured (116, 121). The content of TC has been determined fluorimetrically in partially decomposed aqueous solutions of different pH values. Calcium and diethyl barbituric acid complexes of TC were extracted into an



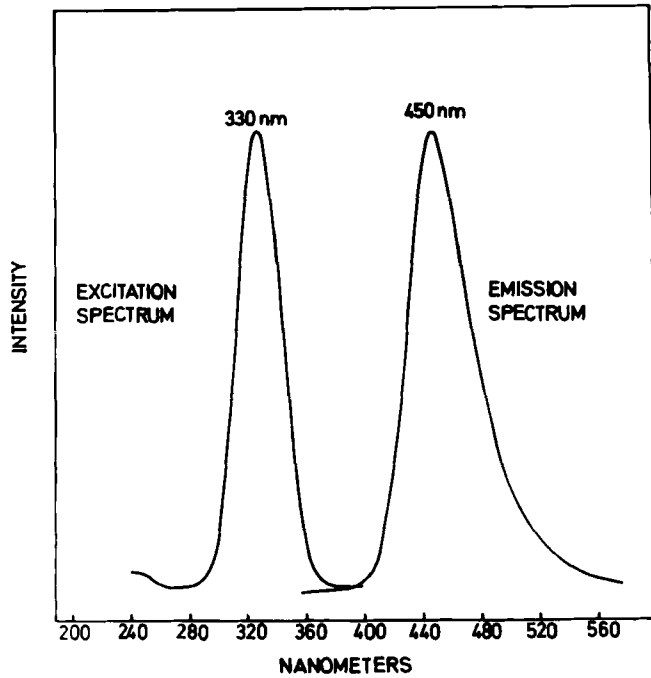


Fig. 12

Fluorescence Spectrum Of Tetracycline Hydrochloride in 0.05 N NaOH, Perkin-Elmer Spectrofluorometer LS 5

organic solvent and the fluorescence intensity was determined by using an excitation wave length of 365 nm mercury line and an emission wave length of 530-550 nm (glass filter). Strongly fluorescent extractable complexes of calcium and barbituric acid are formed only with undecomposed biologically active TC. Decomposition products of TC do not form such complexes or they are not extractable with an organic solvent (122). Spectrofluorimetric determination of TC in serum and urine is performed after isolation through adsorption on  $Al_2O_3$  at an excitation wavelength of 335 nm and emission wavelength of 414 nm. The assay is based on the strong fluorescence of a degradation product of TC formed on heating in alkaline solution. 0.2 - 5  $\mu$ g TC/ml in serum and 5 - 50  $\mu$ g/ml in urine can be determined (123). Fluorimetric assay of TC in mixtures of antibiotics is performed by formation of strongly fluorescent aluminium-TC complex without prior extraction or separation of individual antibiotics. Estimation was done by measuring fluorescence at 465 nm excitation and 555 nm emission wavelengths (124). TC-HCl is determined fluorimetrically after addition of perchloric acid to its solution in acetic anhydride as well as after extraction of ATC at pH 4.5 with chloroform. The excitation wavelength of 365 nm and emission wavelength 530-550 nm (glass filter) were used for measurements (125). TC formed ternary calcium complexes with barbital sodium and tryptophan in aqueous solutions. The fluorescence of TC and its complexes was measured at the emission maximum of 512 nm with the excitation wavelength of 390 nm (30). Fluorimetric determination of TC in biological materials is based on solvent extraction with ethyl acetate of mixed tetracycline-calcium-trichloroacetate-ion-pairs from aqueous solution. The excitation and emission maxima are reported to be 400 nm and 500 nm respectively. Detection limits of 0.05 and 0.125  $\mu$ g/ml were obtained (126, 127).

12.4 Chromatographic MethodsPaper Chromatography

Paper partition chromatographic methods have been widely applied to the analysis of tetracyclines (128, 129). Pharmaceutical aqueous suspensions for oral use are acidified with HCl and diluted with methanol. Crystalline formulations are dissolved only in methanol. A paper chromatographic method for TC determination in pharmaceutical preparations is based on the complexation of the antibiotic with a mixture of urea and disodium edetate on paper at pH 7.4. Urea helped in the separation of degradation products and led to the formation of well defined spots (130). Samples from fermentations must be acidified with oxalic acid to liberate TC from the mycelium. TC in filtrates may be precipitated in saturated solution of sodium tetraphenyl borate, precipitate dissolved in ethyl or butyl acetate and applied for paper chromatography. Various solvent systems and  $R_F$  values for paper chromatography are given in Table 4.

Table 4

Solvent System	$R_F$		Reference
n-butanol-acetic acid-water (4:1:5)	68	TC	131
n-butanol-conc. $NH_4OH$ -water (4:1:5)	24	TC	132
chloroform-nitromethane-pyridine (10:20:3) on paper impregnated with McIlvaine's citrate-phosphate buffer pH 3.5	28	TC	133
chloroform-n-butanol (4:1) saturated with McIlvaine's buffer pH 4.5	49	TC	134
n-butanol-acetic acid water (4:1:5) on paper impregnated with EDTA and dried	65 65 87 87	TC ETC ATC EATC	135

(continued)

Table 4 (continued)

n-butanol-ammonia-water	39	TC	135
(4:1:5) on paper impreg	15	ETC	
nated with EDTA and dried	62	ATC	
	40	EATC	
n-butanol-chloroform (9:1)			
saturated with 0.15M	34 - 42	TC	136
buffer pH 3.0, Whatman			
no. 4 paper impregnated			
with a 3.7% solution of			
chelaton III and wetted			
with aqueous phase			
ethyl acetate-acetone (4:1)			
saturated with disodium			
edetate and urea; What	37	TC	
man no. 1 paper impreg-			
nated with a mixture	45	TC-HCl	130
(1:3) of 10% urea in			
Mcllvaine's buffer pH	98	ATC	
7.4 and 5% disodium ede-			
tate in the same buffer	2	EATC	

Detection is made in UV light. The yellow fluorescence of tetracyclines and their epimers in UV light is greatly enhanced by exposing the paper to ammonia vapours. They may also be detected by Ehrlich's reagent or by starch-iodine following N-chlorination (137).

#### Column Chromatography

A partition chromatographic procedure was developed for the assay of tetracycline mixtures. Washed diatomaceous earth impregnated with buffered stationary phases containing EDTA have been used to separate and quantify TC mixtures in presence of degradation products. The compounds are eluted from a column of Celite 545 coated with a pH 7 buffer containing EDTA, glycerine and PEG 400 and then determined spectrophotometrically (138, 139, 140). ATC and ETC were determined in commercial and aged TC formulations through column chromatography using Celite 545 or Kieselgur moistened with EDTA in buffer solution as stationary phase and chloroform, methanol and n-butanol etc as eluents (68, 141, 142, 143).

Sephadex G-25 buffered at alkaline pH's has been found to possess excellent resolving power in some cases (144, 145, 146). A column liquid chromatographic method for determining TC in various dosage forms was automated. Each chromatogram consisted of a continuous flow separation of components followed by a spectral determination of the column eluate at a rate of 12 samples per hour (147). Gel chromatography has also been applied to the analysis of TC antibiotics using a Bio-Gel P-2 column with 0.1M acetic acid as an eluent (148).

#### Thin Layer Chromatography

TLC has been applied in european pharmacopoeia for the identification of TC-HCl as well as for detection of impurities and related products. TLC plates were prepared with a slurry of kieselguhr and sodium edetate solution adjusted to pH 7 or 7.5 and mobile phases of chloroform + ethyl acetate + acetone (4:4:2), previously saturated with 0.1M sodium edetate, and water + ethyl acetate + acetone (1:3:23) were used for identification and detection of impurities respectively (15). TLC has been widely used for the analysis of TC and related products (ETC, ATC, EATC) with various stationary phases and solvent systems. Stationary phases impregnated with EDTA solutions have been chosen often to avoid the complexation of TC with metal cations. Lloyd and Cornford (149) described an improved TLC limit test for the detection of ATC and EATC in TC using cellulose layer as stationary phase, buffered before development by spraying with a buffer solution (0.1 M disodium EDTA - 0.1 NH<sub>4</sub>Cl), and chloroform as mobile phase, previously saturated with the same buffer solution. The R<sub>f</sub>-values for TC, ATC, EATC were found to be 0.25, 0.93 and 0.48 respectively (149). A method for quantitative analysis of TC-HCl on microcrystalline cellulose thin layer plates is described (150). Determination of ATC and EATC in microgram range in degraded TC tablets through TLC is also reported (151).

Table 5 gives a number of stationary phases, solvent systems and where available  $R_f$ -values for TC and related products for TLC.

TLC has been used for qualitative and quantitative assay of degradation products of TC in TC-HCl, commercial formulations of various dosage forms such as powder, tablets and capsules etc. Determination has been performed after scratching the zone from TLC plates, elution of the drug and spectrophotometric measurement at 430 nm (155). Direct fluorimetry of the spots on TLC plates applying a 360 nm excitation and 540 nm emission wavelengths has been also performed. A linear relationship was found between the concentration of TC and related compounds in the range of 0.05-1  $\mu$ g and fluorescence intensity (157, 159, 160, 161, 162, 163). Chromatograms may be detected by bioautography with *Bacillus subtilis* using microbiological techniques (137, 169, 170, 171). Spraying with methanolic solution of ferric chloride (dark greyish spots), with antimony trichloride solution,  $MgCl_2$  in ethanol or methanolic triethanolamine solutions have been reported (137). Aqueous fast blue solution, diazotized p-nitroaniline, modified Sakaguchi reagent (boric acid in conc. sulfuric acid) and diphenyl picrylhydrazyl spray reagents have also been used for detection of TC and degradation products on TLC plates. Coloured spots on white background or yellow spots on a bluish-white background were obtained (156). The tetracyclines have been most commonly detected by their fluorescence under long wavelength UV light at 365 nm either with or without exposure of the chromatograms to ammonia vapours (80, 162, 164, 165, 168). TC-HCl was allowed to epimerize in phosphate and acetate solutions at room temperature and the reaction products were studied at suitable time intervals by TLC (156).

#### High Performance Liquid Chromatography

HPLC can separate and resolve TC-HCl from its degradation products ETC, ATC, EATC sensitively and reproducibly. The technique

Stationary phase	Table 5 Mobile Phase hR <sub>F</sub> -values	References
Kieselguhr, impregnated with citrate-phosphate buffer pH 5.5 containing 10% glycerine	dichloromethane - ethanol 95 % (9:1); TC:55-65; ETC:10; ATC:100; EATC:85;	152
Silica Gel G	n-butanol-oxalic acid-water (100:6:100); TC:38;	153
Silica Gel G	n-butanol-tartaric acid-water (100:6:100); TC:26;	153
Silica Gel impregnated with EDTA-water and activated	n-butanol saturated with water; TC:23	153
Kieselguhr G, impregnated with glycerol-phosphate-citrate buffer, pH 3.7	Chloroform-acetone(1:1) saturated with the same buffer	154
Kieselguhr impregnated with 5% aqueous EDTA solution adjusted to pH 9 with NaOH	Acetone-water (10:1) TC:69; ETC:29 ATC:84; EATC:55;	155
The same sorbent as above, but adjusted to pH 7.5	acetone-ethyl acetate-water (20:10:3); TC:71 ETC:38; ATC:88; EATC:46;	155

Kieselguhr G impregnated with EDTA+PEG 400+glycerine	methyl ethyl ketone saturated with McIlvaine's buffer pH 4.7 TC:53; ETC:20; ATC:93; EATC:47;	156
Same as above	dichloromethane-ethyl formate- ethanol (9:9:2) saturated with buffer pH 4.7; TC:36; ETC:12 ATC:83; EATC:50;	156
Kieselguhr Impregnated with mobile phase	ethylene glycol-water-acetone- ethyl acetate (2:2:15:15) TC:50; ETC:28; ATC:95; EATC:30;	157
Cellulose TLC plates impreg- nated with EDTA, pH adjusted to 9.0	n-butanol saturated with water TC:18; ETC:10; ATC:23; EATC:23;	158
Kieselguhr G impregnated with 0.1M EDTA	MIBK-ether-water-methanol- ethylene glycol (50:60:3:7.5:2.5) TC:16; ETC:4; ATC:83; EATC:22;	159
Microcrystalline cellulose plates, Merck	aqueous 0.25M MgCl <sub>2</sub> TC:72; ETC:68; ATC:29; EATC:16;	160
Same as above	aqueous 0.20M CaCl <sub>2</sub> TC:67; ETC:63; ATC:25; EATC:25;	160

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Table 5 continued

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Same as above	aqueous 0.25M BaCl <sub>2</sub> TC:63; ETC:60; ATC:24; EATC:17;	160
Same as above	aqueous 0.15M ZnCl <sub>2</sub> TC:65; ETC:65; ATC:22; EATC:18;	160
Microcrystalline cellulose impregnated with phosphate- citrate buffer and subsequen- tly submerged in methanolic ethylene glycol solution	twice in ethyl acetate saturated with water TC:35	161
Powdered cellulose m TLC plates impregnated with 0.1M EDTA	chloroform saturated with 0.1M EDTA, after develop- ment exposed to ammonia TC:6-23(band); ETC:3.5; ATC:92; EATC:52;	162

Kieselguhr plates impregnated with EDTA, adjusted to pH 7.5	acetone-ethyl acetate-water (20:10:3); TC:10; ETC:40; ATC:95; EATC:50;	163
Kieselguhr G impregnated with 0.1M EDTA	ethyl methyl ketone saturated with McIlvaine buffer pH 4.7 or alcohol; later plates exposed to ammonia; TC:56; ETC:30;	164, 165
Microcrystalline cellulose on TLC plates	0.1M EDTA with 0.1% NH <sub>4</sub> Cl; chloroform saturated with EDTA-NH <sub>4</sub> Cl solution; TC:72; ATC:36; EATC:52;	166, 167
Kieselguhr G treated with a buffer containing 0.1M EDTA at pH 7.0, glycerine and PEG 400	ethyl acetate saturated with 0.1M EDTA at pH 7.0	168

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has been successfully applied for the quantitative assay of TC and related compounds in all sorts of commercial solid and liquid dosage forms, stability studies, in body fluids, pharmacokinetics and drug metabolism investigations. Quite often tests for identity, potency and assay determination are performed with this method. Micro to sub-microgram amounts could be determined with ease. Different modes of HPLC, adsorption, reversed phase, ion-exchange and paired-ion chromatography have been applied. Butterfield separated various tetracyclines using an ion-exchange system (172). Later workers included EDTA in the system to prevent binding with the metal columns (75). Advantages of reversed phase HPLC over ion-exchange techniques have been given (173). Serum levels of as little as  $0.3 \mu\text{g/ml}$  TC have been determined through HPLC (174). A preliminary study of the effectiveness of ion-exchange, adsorption, liquid-liquid partition and reversed phase ion-pair chromatography indicated that only the last method showed promise. A  $18 \mu\text{m}$  partisil column gave plate heights in the range of  $0.15 - 0.3 \text{ mm}$ . The method was used to quantify the impurities in TC at round the 1% level (175). TCs may be separated with high efficiency (plate heights around  $0.05\text{mm}$ ) using SASand ODS-Hypersils in the pH range 3-5. Within this pH range the presence of EDTA at a concentration of about  $10^{-3} \text{ M}$  is essential for satisfactory chromatography. EDTA is thought to act as a zwitterion pairing agent with the zwitterionic form of the TCs (176). Table 6 gives various columns, mobile phases etc. reported in literature for the analysis of TC-HCl and TC.

Concentrations as low as  $4 \mu\text{g/mg}$  TC in urine were quantitated accurately. TC was extracted from urine as calcium complex (183). Calcium complex of TC was extracted from urine and plasma with ethyl acetate and then re-extracted into HCl. Concentrations of less than  $1 \mu\text{g/ml}$  in urine and  $1.5 \mu\text{g/ml}$  in plasma were determined with a relative standard deviation of less than 5% (184).

The addition of phenylbutazone to urine or plasma enhances the extraction of these compounds by ethyl acetate. The formation of phenylbutazone-tetracycline ion pairs and their role in extraction process is discussed (185). C 18 reversed phase column was used with the mobile phase water-acetonitrile-perchloric acid in two steps with varying amounts of acetonitrile. Six different TC preparations analysed contained ETC, ATC and EATC from undetectable to 7.78% (186). Limits of detection for ATC and EATC in TC were found to be 12 and 7 ng respectively through HPLC analysis (187). TCs can be extracted from serum or organ homogenates by means of acetonitrile containing buffers. The lowest concentrations of the intact molecule detectable are 0.2 µg/ml serum or blood and 0.4 µg/g organ. HPLC analysis yielded the same results as microbiological method (191). Reversed phase ion-pair chromatography with the use of tertiary amines as counter ions has been applied for the separation of TC analogs and their potential impurities (192). Retention of TC can be regulated by the addition of organic ammonium compounds to the mobile phase in reversed phase ion-pair chromatography (194). The addition of inorganic anions  $\text{Cl}^-$ ,  $\text{Br}^-$ ,  $\text{ClO}_4^-$  as their sodium salts to the mobile phase and their influence on the retention of TC has been studied (194). TC has been extracted from plasma as an ion-pair with tetrabutyl ammonium hydrogen phosphate into chloroform-1-heptanol and after reextraction into an acidic aqueous phase the separation was performed through reversed phase HPLC. Analysis of urine was done by direct injection (193).

Table 6

Column	Mobile Phase	Detection	Application	References
Pellicular cation-exchange resin	0.003M EDTA in water-ethanol (60:40)	254 nm	TC in formulations	177
$\mu$ Bondapak C 18 10 $\mu$ m	0.02M phosphate buffers, pH 2-5 in water-acetonitrile gradient	280 nm	TC in pharm. preparations	178
TMS bonded WLCU Si gel	0.1M HClO <sub>4</sub> in water-acetonitrile gradient 85:15 to 70:30	280 nm	separation of TC, ETC, ATC, EATC	179
Micropak CH	water-methanol-0.02M phosphate buffer (pH 7.6) and 1mM EDTA, gradient 25-50% v/v methanol	267 nm	TC from penicimocycline	180
Vydac RP 18 and Lichrosorb RP 8	0,15M (NH <sub>4</sub> ) <sub>2</sub> CO <sub>3</sub> -acetonitrile (96:4) pH adjusted to 8.4 with ammonia; citrate-phosphate buffer (pH 2.20)-acetonitrile (65:35)	350 nm	separation of TC from related compounds	181

Vydac RP 18 10 $\mu$ m	isopropanol-diethanolamine-phosphate ammonium EDTA-water	254,280,365 405 nm	TC from degradation products	182
TMS-bonded partisil 18 $\mu$ m	0.1M HClO <sub>4</sub> or 0.1M H <sub>2</sub> SO <sub>4</sub> or 0.05M HNO <sub>3</sub> - acetonitrile (85:15 to 70:30)	280 nm	separation of TC, ETC ATC, EATC	175
Zipax HCP polymer	0.001M EDTA, 0.01M H <sub>3</sub> PO <sub>4</sub> in water-methanol (87:13, v/v)	280 nm	separation of TC, ETC, ATC, EATC	75
639 ODS-Sil-X-1	0.005M EDTA, 0.05M (NH <sub>4</sub> ) <sub>2</sub> CO <sub>3</sub> in water-methanol (92:8)	280 nm	TC	173
Pellionex CP 172	0.1M Na <sup>+</sup> , 0.002M EDTA in water-ethanol (70:30)		TC, ETC, ATC, EATC	
ODS-Hypersil, C 18 5 $\mu$ m;	water-acetonitrile-dimethyl-formamide-0.001M	272 nm 280 nm	TC, ETC, ATC, EATC	176
SAS-Hypersil C 1,5 $\mu$ m	Na <sub>2</sub> EDTA-citric acid and acetic acid, pH adjusted to 3.0 or 5.0			

continued

Table 6 continued

Ion-X SA, conditioned with EDTA	0.005M EDTA, 0.05M NaCl in water-methanol, 95:5 and 70:30	375 nm	TC in urine extracts as calcium com- plex	183
$\mu$ Bondapak C 18, 10 $\mu$ m	0.01M NaH <sub>2</sub> PO <sub>4</sub> in water-acetonitrile 60:40 pH 2.4	355 nm	TC in urine plasma, tissues as calcium com- plex	184+185
640 ODS-Sil-X-1 10 $\mu$ m	Water-acetonitrile-per- chloric acid 76:22:1.8; 61:37:1.8	254 nm	TC, ETC, ATC, EATC	186
Zipax SCX 10 $\mu$ m	0.3M EDTA buffer, pH ad- justed to 7.0	429 nm	ATC, EATC in TC	187
Lichrosorb NH <sub>2</sub> , RP 8, 5 and 10 $\mu$ m	10% acetonitrile in 0.1M phosphoric acid with 10 <sup>-3</sup> M HIBS	357 nm	TC in mix- tures	188
Lichrosorb RP 8, 5 $\mu$ m	acetonitrile-0.1M citric acid 25:75	350 nm	Retention mechanism of TCs	189

$\mu$ Bondapak phenyl C 18,	a step gradient of 12-22% acetonitrile in 0.2M phosphate buffer at pH 2.2		ETC, ATC, EATC in TC powder and capsules	190
Nucleosil C 8, 10 $\mu$ m	25% acetonitrile-0.01M NaH <sub>2</sub> PO <sub>4</sub> , pH adjusted to 2.4	270, 357 nm	TC in blood and organs of animals	191
Lichrosorb RP 8, 5 $\mu$ m	phosphate buffer, tri- propylamine or N,N-di- methyloctylamine, pH 8.0, with 20-35% acetonitrile	280 nm	separation of TC and analogs and their impu- rities	192
Lichrosorb RP 2, 8, 18, 5 $\mu$ m	0.01M phosphoric acid in water-35% acetonitrile organic ammonium compounds in mobile phase	357 nm	ion-pair LC of TC, TC in plasma and urine	193,194
$\mu$ Bondapak phenyl C 18	methanol-water-1 M phos- phoric acid, gradient elution	280 nm	influence of temperature on solid TC	195

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### Gas Chromatography

The GLC determination of TC, ETC, ATC and EATC in aged TC samples has been reported. Trimethylsilyl derivatives of TCs were formed and gas chromatographed on six different columns, 3% OV-1, 3% JXR, 3% SE 52, 3% OV-17, 10% OV-25 and 10% OV-210. A 6 feet 3% JXR column at 260 °C isotherm separated most of the compounds. Different TC-HCl powder samples were analysed through GLC for their degradation products and the results compared with those obtained from microbiological and UV assay methods (74).

#### 12.5 Polarography

Tetracycline antibiotics have been determined qualitatively and quantitatively through polarographic methods (196, 197). Polarographic behaviour of tetracycline and some of its degradation products has been investigated in phosphate-acetate buffer solutions. The first reduction wave is attributed to the carbonyl group at C 1 and the second reduction step corresponds to the conjugated system at C 11 - C 12 (198). Oscillopolarographic methods have been also applied for the analysis of tetracycline and its degradation products (199, 200). Optimum conditions for the dc polarographic reduction of TC were studied. A boric acid-sodium borate buffer provided the best conditions for the electro-reduction of TC. Quantitative determination in various dosage forms were carried on (201). TC can be analysed through polarography and differential-cathode-ray polarography in a supporting electrolyte of 0.1M diethyl barbituric acid in 40% methanol and 0.1M lithium chloride at pH 4.7 (202). TC-HCl gives a polarographic step in a phosphate buffer solution, pH 6.2, with a half-wave potential of -1.45 to -1.51 V (203). A method involving ac polarography is also reported in literature (204). Electrochemical methods for the determination of TC have been reviewed by Unterman and Hughes (205, 206).

#### 13. Drug Metabolism and Pharmacokinetics

TC-HCl is incompletely and irregularly absorbed after oral administration. The

presence of metal ions such as calcium, magnesium, aluminium, zinc and iron reduce its absorption due to formation of chelates and coordination compounds. Alkalis also lower the serum concentration. Rectal absorption from suppositories is unsatisfactory. Absorption from stomach is minimal. Absorption from the intestines is incomplete and subject to considerable variation between individuals and is affected substantially by the contents of GI tract. Milk and food reduce TC absorption, phosphates may enhance it. Some practical improvement in absorption appears to have resulted from the exclusion of "inert" magnesium stearate or calcium carbonate from TC capsules (207, 208, 209, 210). Therapeutic blood concentrations are achieved more readily by increasing the frequency of administration than by increasing the dose. Therapeutic serum concentrations lie in the range of 2-5  $\mu\text{g}/\text{ml}$  whilst the toxic concentration is about 12  $\mu\text{g}/\text{ml}$  or over. Serum concentrations of 1-4  $\mu\text{g}/\text{ml}$  are maintained when oral doses of 250-500 mg are administered daily. An intramuscular dose of 100-250 mg brings about serum concentration of 2-4  $\mu\text{g}/\text{ml}$  during 1-2 hours. A serum concentration of 5-30  $\mu\text{g}/\text{ml}$  is reached 30 minutes after an intravenous dose of 250-300 mg TC. Serum half-life is about 9 hours when given orally in a single dose (16). A mean serum half-life is about 11 hours when administered orally in divided doses (211). TC is secreted in milk and saliva and crosses the placentas. It is readily taken up by newly formed bones and teeth in which it forms a TC-calcium orthophosphate complex. It remains bound to malignant cells for a longer time than to non-malignant cells. TC is excreted both in urine and bile. The concentration in bile may reach 10 to 20 times that of blood (212, 213). About 20% is excreted in urine in 24 hours, mainly as unchanged drug, after oral administration and 50% after intravenous doses. The average concentration of TC in urine after oral and im + iv administration is in the range of 100-300  $\mu\text{g}/\text{ml}$ . Urinary excretion is increased when

urine is alkaline (16). TC appears to be excreted in the urine by glomerular filtration as its half-life is strongly dependent upon the extent of protein-binding. TC bounds to plasma proteins in the range of 20-70% (16). It is also bound reversibly to serum proteins. In serum 53% TC is in the form of complexes with protein. Among the bound TC, 54% is with albumin, 13 and 19% with low and high density lipoproteins respectively, 6% with very high density lipoprotein and 8% is bound with other serum proteins. TC appears to dissolve in the lipophilic portion of the lipoprotein molecule, rather than to be associated with specific binding sites (214).

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# VITAMIN D<sub>3</sub> (CHOLECALCIFEROL)

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Kalamazoo, Michigan*

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## 1. Description

- 1.1 Name: Vitamin D<sub>3</sub>  
Generic Name: Cholecalciferol  
Chemical Name: 9,10-Secocholesta-5,7,10(19)-trien-3 $\beta$ -ol,(3 $\beta$ ,5Z,7E)-,

### 1.2 Formula and Molecular Weight

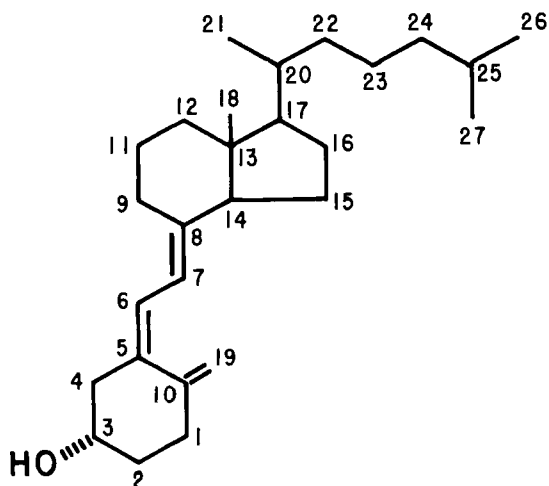
The chemical structure of vitamin D<sub>3</sub> is closely related to its precursor, 7-dehydrocholesterol, from which it is produced by a photochemical reaction. Therefore, vitamin D<sub>3</sub> is closely related structurally to the four-ring nucleus of steroids derived from the cyclopentanoperhydrophenanthrene ring system. No vitamin D activity is noticed until the B ring of 7-dehydrocholesterol is opened between C-9 and C-10. Thus, vitamin D<sub>3</sub> is a 9,10-seco steroid and its carbon skeleton is numbered accordingly (Scheme I). The important aspects of its chemistry center about the 5,6,7-cis-triene structure. The formula for vitamin D<sub>3</sub> is C<sub>27</sub>H<sub>44</sub>O and its formula weight is 384.64.

### 1.3 Storage and Handling

Crystalline vitamin D<sub>3</sub> is stored in hermetically sealed containers under nitrogen in a cool place and protected from light. Under such conditions, degradation is negligible for one year. Vitamin D<sub>3</sub> exists in thermal equilibrium with previtamin D<sub>3</sub> and the rate of equilibration markedly increases with temperature and time (1,2).

### 1.4 Brief History

The symptoms of vitamin D deficiency disease has been documented in 16th century literature. A clear picture of the basis for the disease and methods of treatment were unclear until the experiments by Sir Edward Mellanby (3,4). In the early 1920's, cod liver oil was known to cure rickets and xerophthalmia. The name vitamin D was given to



SCHEME I: Vitamin D<sub>3</sub> structure with hydrocarbon number assignments.

the antirachitic substance in cod liver oil (5-7) and differentiated from vitamin A by the fact that vitamin A activity in the oil was lost by heating while that of D was not. Hess and Steenbock discovered, independently (8-11), that the antirachitic activity of this vitamin could be induced by UV irradiation of rachitic animals or their food. Steenbock's discovery that antirachitic activity could be induced by irradiation of foods, particularly of the sterol fraction, ultimately led to the identification of the structure of vitamin D and to the eradication of rickets as a major medical problem (9,10,12).



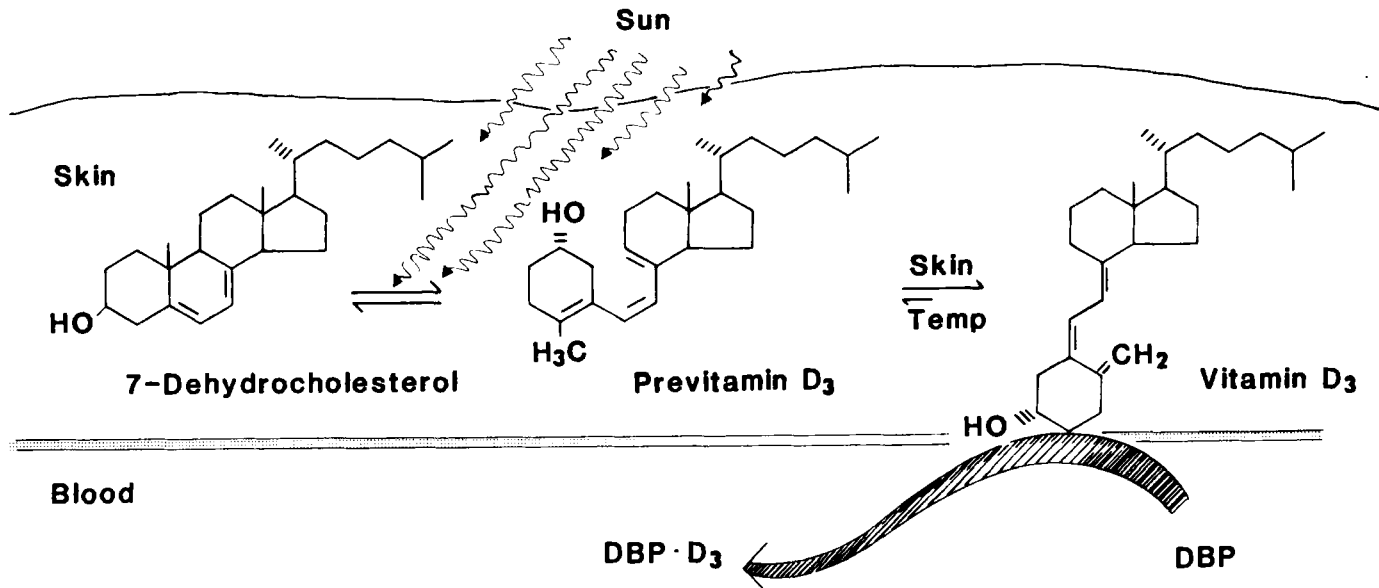
It was later discovered that vitamin D occurs in two active forms, ergocalciferol and cholecalciferol. Ergocalciferol is the synthetic form derived by the irradiation of ergosterol and is designated as vitamin D<sub>2</sub> (13,14). Cholecalciferol, the natural form, was identified (15,16) and designated as vitamin D<sub>3</sub>. Vitamins D<sub>2</sub> and D<sub>3</sub> are equally active in humans and other mammals, but D<sub>2</sub> is virtually inactive in poultry.

### 1.5 Biosynthesis

It has been known for a long time that vitamin D<sub>3</sub> is synthesized in the skin when exposed to sunlight from 7-dehydrocholesterol (7-DHC, Provitamin D<sub>3</sub>). The sequence of steps leading to the cutaneous photosynthesis of vitamin D<sub>3</sub> from 7-DHC was recently demonstrated (17). In Caucasian skin, all of the epidermal strata contain 7-DHC. When irradiated, 7-DHC is photochemically converted by a fast reaction to previtamin D<sub>3</sub> throughout the epidermis and the dermis. Once formed in the skin, previtamin D<sub>3</sub> is isomerized to D<sub>3</sub> by a slow nonphotochemical rearrangement at a rate dictated by skin temperature. Vitamin D<sub>3</sub> is then bound to a specific binding protein (DBP) and transported from the skin into the circulating blood. Thus the skin serves as the source of 7-DHC, a reservoir for the storage of the primary photoproduct, previtamin D<sub>3</sub>, and the organ where the slow thermal conversion of previtamin D<sub>3</sub> to D<sub>3</sub> occurs. The third process permits the skin to continuously synthesize D<sub>3</sub> and release it into the circulation for up to 3 days after a single exposure to sunlight. Scheme II (17) illustrates this sequence of events.

### 1.6 Potency Definition

The original reference standard of vitamin D was a solution of irradiated ergosterol in olive oil. The present international standard is a solution of pure crystalline cholecalciferol in olive oil containing 0.025 µg/mg of solution. One international unit (IU) is equivalent to 0.025 µg of crystalline D<sub>3</sub> or 1 µg of D<sub>3</sub> is equivalent to 40 IU. The USP reference standards are crystalline ergocalciferol or cholecalciferol packaged in sealed ampules. The USP standards and the international standards are equivalent in that 1 µg of each is equivalent to 40 IU.



SCHEME II: Diagram of the formation of previtamin D<sub>3</sub> in the skin, its thermal conversion to vitamin D<sub>3</sub>, and transport by binding proteins (DBP) in plasma into the general circulation (Reference 17). From ref. 17, copyright 1980 by the AAAS.

## 2. Physical Properties

### 2.1 Spectra

#### 2.11 Ultraviolet Spectrum

Figure 1 is the ultraviolet spectrum of a 10 mcg/ml solution of vitamin D<sub>3</sub> in methanol. The spectrum was obtained using a Cary Model 219 recording spectrophotometer (Varian Instrument Co., Palo Alto, CA). Vitamin D<sub>3</sub> and related compounds have a characteristic UV absorption maximum at 265 nm and a minimum at 228 nm. The extinction coefficient at 265 nm is about 17,500 and 15,000 at 254 nm. An index of purity of vitamin D<sub>3</sub> is a value of 1.8 for the ratio of the absorbance at 265 to that at 228 nm. The high absorbance at 254 nm enables one to use the most common and sensitive spectrophotometric detector used in high performance liquid chromatography (HPLC) for the analysis of vitamin D<sub>3</sub> in multivitamin preparations, fortified milk, other food products, animal feed additives etc.

#### 2.12 Infrared Spectrum

The infrared spectrum of a mineral oil mull of vitamin D<sub>3</sub> recorded on a Model FTS 15E instrument (Digilab, Cambridge, Mass.) is shown in Figure 2 (18). Table I shows assignments for the significant infrared absorption bands (18).

#### 2.13 Nuclear Magnetic Resonance Spectrum

Proton and C-13 NMR spectra of vitamin D<sub>3</sub> respectively are shown in Figures 3 and 4 (19). Spectra were obtained with a Model XL-200 NMR spectrophotometer (Varian, Palo Alto, CA). Tables II and III are the corresponding chemical shifts in the proton and C-13 NMR spectra which are based on the assignments made by Wing et al. (20) and Williams et al. (21), respectively.

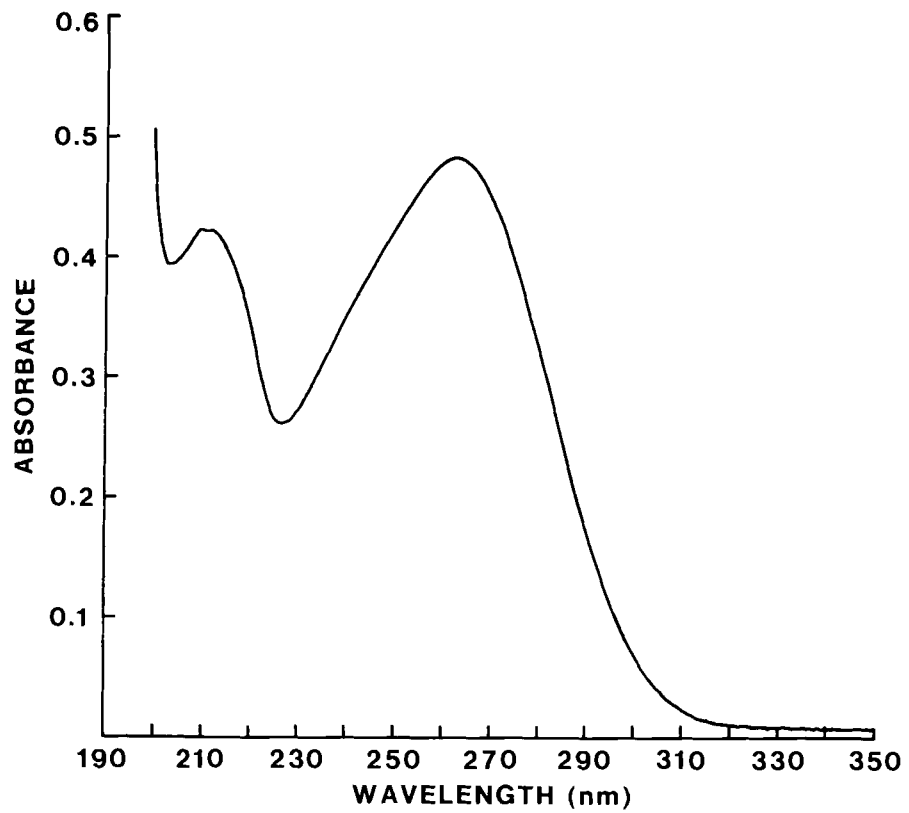


FIGURE 1: The ultraviolet spectrum of a  $2.60 \times 10^{-5}$  M solution of vitamin D<sub>3</sub> in methanol (1 cm cell).

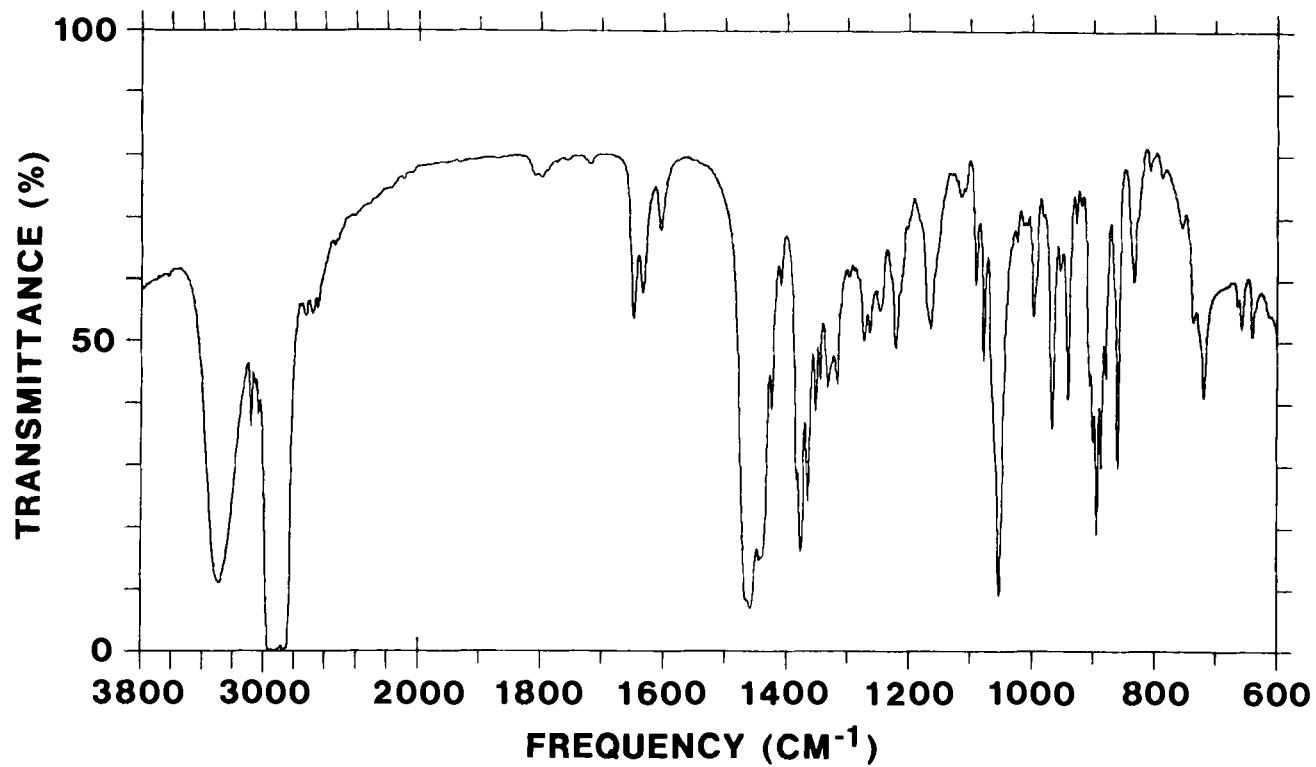


FIGURE 2: Infrared spectrum of a mineral oil mull of vitamin D<sub>3</sub>.

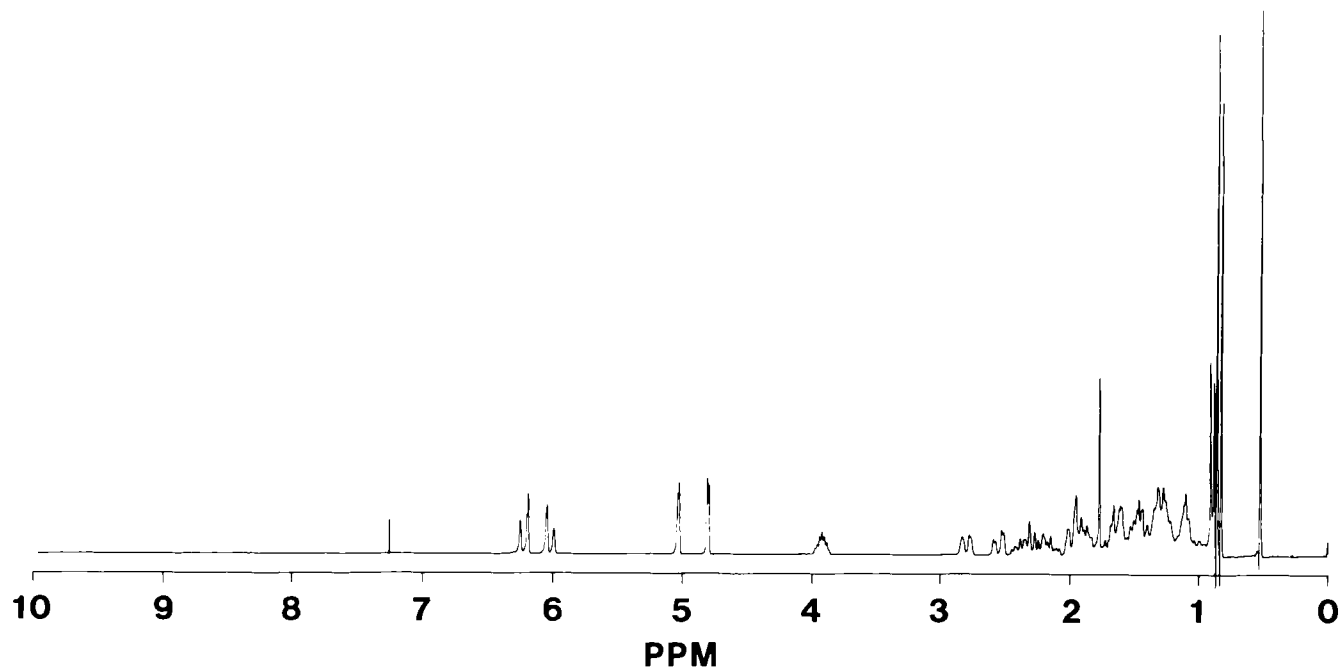


FIGURE 3: Proton NMR spectrum of vitamin D<sub>3</sub> in CDCl<sub>3</sub>.

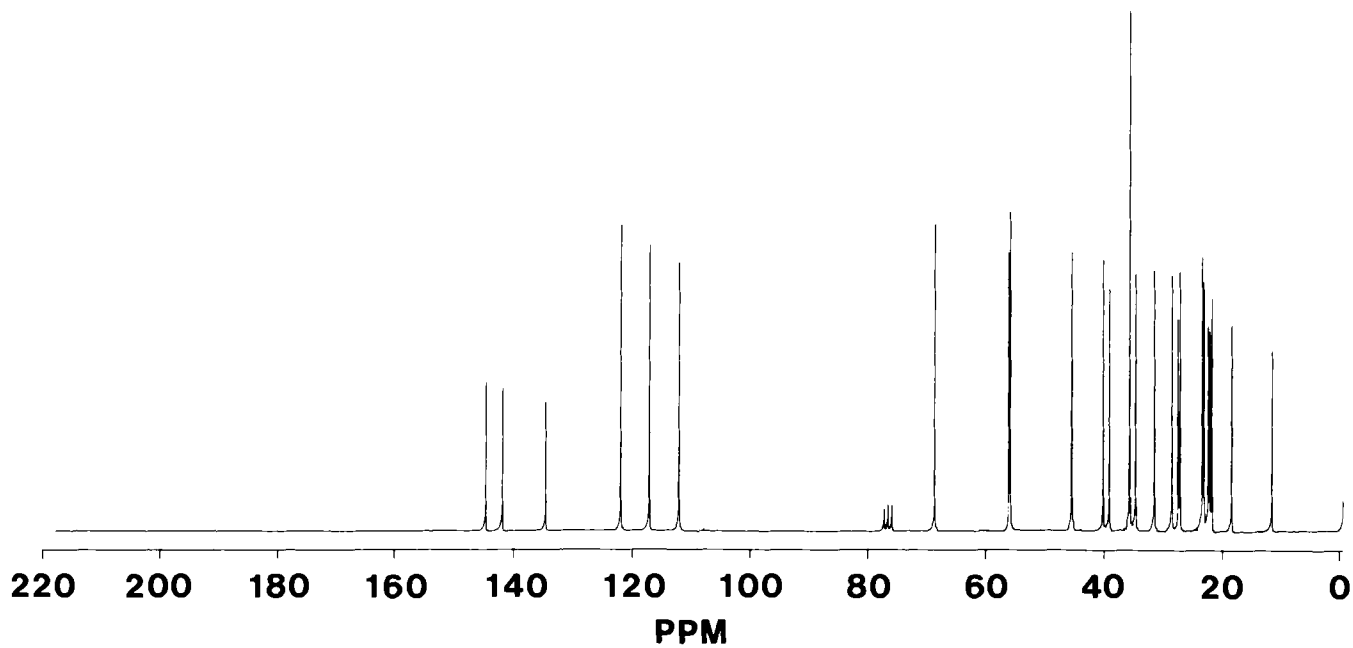


FIGURE 4:  $^{13}\text{C}$  NMR spectrum of vitamin  $\text{D}_3$  in  $\text{CDCl}_3$ .

TABLE I

Infrared Band Assignments for Vitamin D<sub>3</sub> (Ref. 18)

Wave Numbers $\text{cm}^{-1}$	Structural Feature	Assignment
3289	alcohol	O-H stretch
3077, 3055, 3031	alkenes	C-H stretch
1648, 1634, 1604	Conjugated alkenes	C=C stretch
1053	secondary alcohol (equatorial)	C-O stretch
895	alkene $R_1R_2$ C=CH <sub>2</sub> type	C-H out of plane bending
968, 942, 888 and/or 860	alkene	C-H out of plane bending



TABLE II

Proton NMR Spectral Assignment for Vitamin D<sub>3</sub> (Ref. 20)

---

Assignment	Chemical Shift	Shape
18 methyl	0.54	Singlet
27 methyl	0.85	Doublet
26 methyl	0.88	Doublet
21 methyl	0.92	Doublet
4 $\alpha$ H	2.55	Doublet Doublet
9 $\beta$ H	2.80	Doublet Doublet
3 $\alpha$ H	3.92	Multiplet
19 E H	4.83	Doublet
19 Z H	5.05	Doublet of Triplet
7 H	6.03	Doublet
6 H	6.24	Doublet

---

TABLE III

<sup>13</sup>C Chemical Shifts of Vitamin D<sub>3</sub> at 200 MHz in D<sub>2</sub>O  
(Ref. 21)

Carbon No.	Chemical Shifts	Carbon No.	Chemical Shifts
1	31.95	14	56.33
2	35.15	15	23.58
3	69.17	16	27.66
4	45.90	17	56.56
5	145.05	18	12.00
6	122.41	19	112.41
7	117.47	20	36.14
8	142.26	21	18.84
9	29.02	22	36.14
10	135.04	23	23.86
11	22.25	24	39.49
12	40.52	25	28.01
13	45.83	26	22.83
		27	22.56

## 2.14 Mass Spectrum

The electron impact mass spectrum of vitamin D<sub>3</sub> obtained with a CEC Model 21-110B instrument (Consolidated Electrodynamics Corporation, Pasadena, CA) by direct probe sample introduction is shown in Figure 5 (22). It shows a strong molecular ion at m/z 384 and a fragmentation pattern characteristic of vitamins D<sub>2</sub> and D<sub>3</sub>, their primary metabolites and other chemically related compounds. The fragment ion assignment is shown in Scheme III (22).

## 2.2 Crystal Properties

### 2.21 Melting Range

Crystalline vitamin D<sub>3</sub> obtained as fine needles from dilute acetone melts in the range of 84-88°.

### 2.22 Crystal Conformation

Trinh-Toan et al. (23) have shown by x-ray diffraction analysis that vitamin D<sub>3</sub> crystallizes in an equimolar ratio of the  $\alpha$  chair form in which the 19-CH<sub>2</sub> group is situated below the mean A ring plane and the  $\beta$  chair form in which the 19-CH<sub>2</sub> group is situated above the mean A ring plane. The 3-OH substituent occupies an equatorial position in the  $\alpha$  form and an axial position in the  $\beta$  form. Figure 6 shows the two independent molecules of vitamin D<sub>3</sub> displaying different solid-state conformations of the A ring. These solid-state conformations in the A ring correspond to those inferred from the <sup>1</sup>H NMR investigations on the solution conformations of vitamins D<sub>2</sub> and D<sub>3</sub> (24) and several metabolites including 1 $\alpha$ ,25-(OH)<sub>2</sub>-vitaminD<sub>3</sub> (25-27). The observation that both the mean torsional angles of 53.8 and 50.1 in ring A of molecules  $\alpha$  and  $\beta$ , respectively, are smaller than the corresponding experimental value of 55.9° found in cyclohexane is attributed to the flattening effect caused by the two exocyclic double bonds (23). Trinh-Toan et al. (23) have also reported that there is strong hydrogen bonding between the  $\alpha$  and  $\beta$  conformational forms. The molecules are well separated in the unit cell with all intermolecular nonhydrogen contacts being greater than 3.4 Å except for the relatively short O-H...O distances of 2.71 to 2.73 Å which indicate reasonably strong OH...O bonds. The authors conclude that the crystal packing of vitamin D<sub>3</sub> including the presence of both conformers is mainly dictated by steric constraints imposed by the infinite helical hydrogen-bonded oxygen chain.

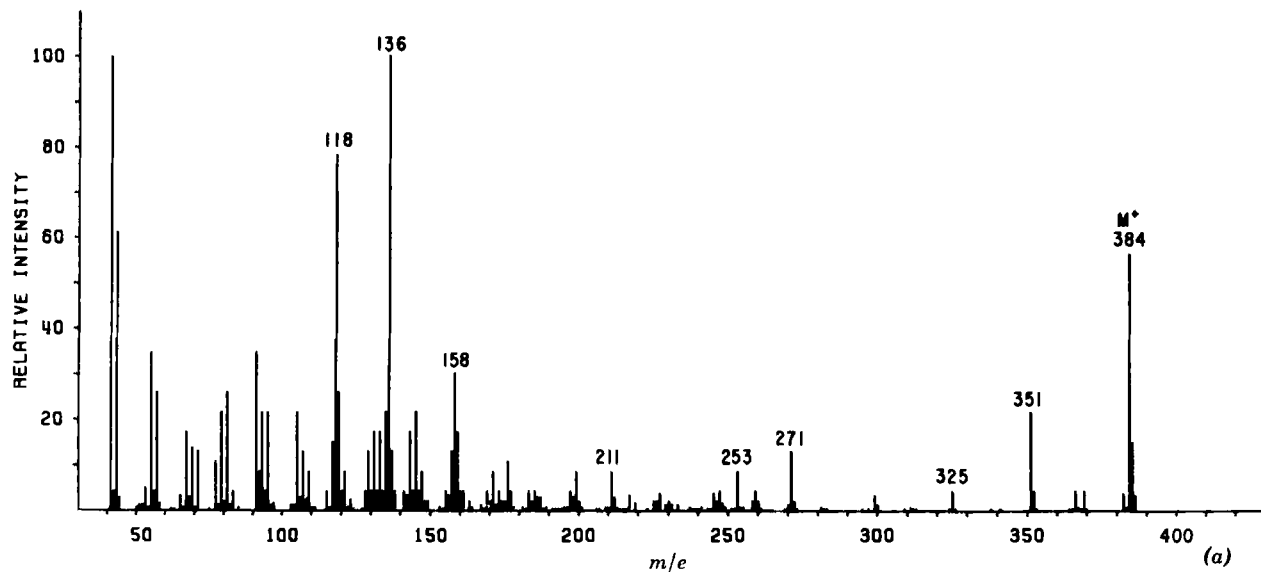
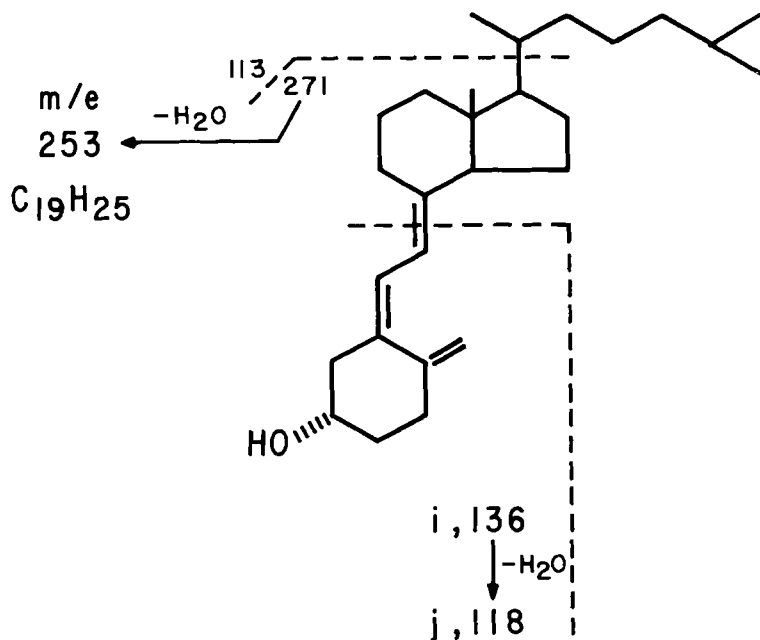


Figure 5. Electron impact mass spectrum of vitamin D<sub>3</sub>; 70 ev, 150° ion source, direct inlet (from ref. 22. Copyright © 1972. Reprinted by permission of John Wiley & Sons, Inc.)



Scheme III: Mass spectral fragmentation pattern of vitamin  $D_3$  (from reference 22. Copyright © 1972. Reprinted by permission of John Wiley & Sons, Inc.)

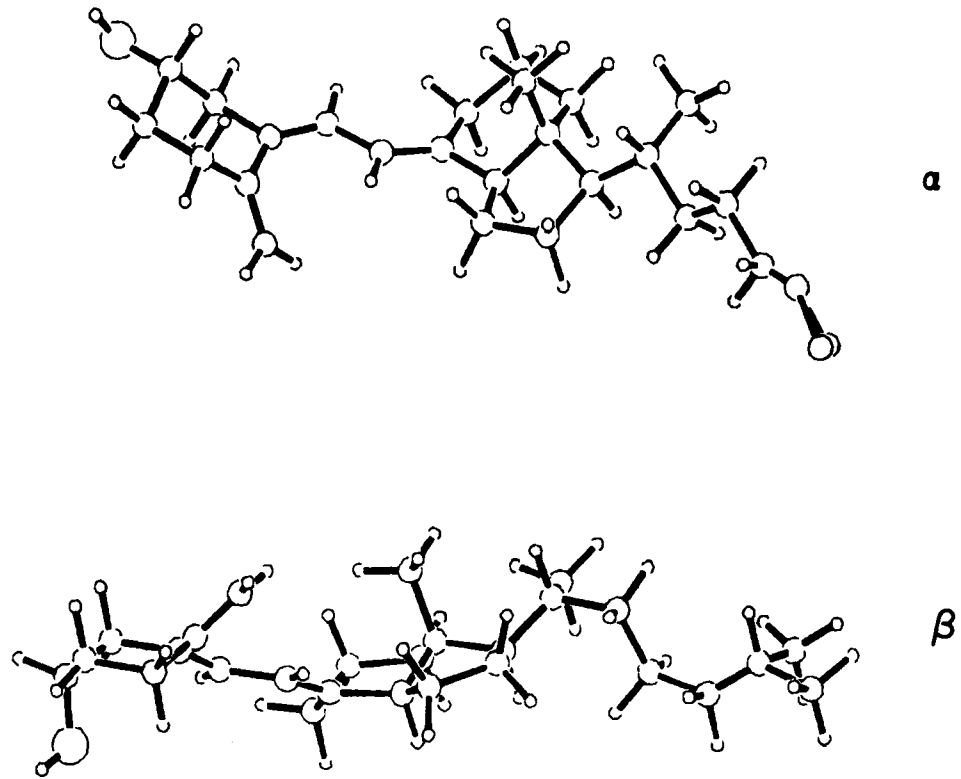


Figure 6. Crystal forms of vitamin D<sub>3</sub> as shown by x-ray diffraction. (Reprinted with permission from ref. 23.<sup>3</sup> Copyright 1976 American Chemical Society.)

### 2.23 Thermal Analysis

Differential scanning calorimetric (DSC) and the thermogravimetric (TGA) curves for vitamin D<sub>3</sub> are shown in Figures 7 and 8 (28). The curves were generated on a DuPont thermal analyzer (Model No. 1090, DuPont DeNemours and Co., Wilmington, Del.). The sample was contained in aluminum pans and the analysis was conducted under N<sub>2</sub> atmosphere. The heating rate for both was 5°/min. There is a precise melting endotherm in the DSC curve which peaks at 86.3°. The exotherm at 206° may be due to decomposition. The TGA curve shows a little weight gain in the melting range of 84-88° when the sample volume is reduced and the buoyancy effect of N<sub>2</sub> is altered. The sharp drop in weight at 128° may be due to boiling and possible splattering of the sample.

### 2.24 X-Ray Diffraction

Figure 9 is the x-ray powder diffraction pattern of crystalline vitamin D<sub>3</sub> (USP reference standard) (29). The values for the 2  $\theta$  angles and the corresponding "d" spacings are shown in Table IV.

### 2.3 Solubility

The solubility of crystalline vitamin D<sub>3</sub> (99 + % pure, Aldrich Chemical Company, Inc., Milwaukee, Wisconsin) was determined in our laboratories in commonly used laboratory solvents. It had over 10% solubility in methanol, ethanol, acetone, ethylacetate, hexane, chloroform, methylene chloride, dimethylformamide, cyclohexane, tetrahydrofuran, methylethylketone, isopropanol and cyclohexanone. The only solvent with low solubility was acetonitrile in which the solubility was about 0.18%.

### 2.4 Optical Rotation

The specific rotation of a freshly prepared 5 mg/ml ethanol solution of vitamin D<sub>3</sub> is between +100.5° and +112° (30).

## 3. Chemical Properties

### 3.1 Chemical Synthesis

Vitamin D<sub>3</sub> is chemically synthesized by the irradiation of 7-DHC and subsequent controlled heating of the

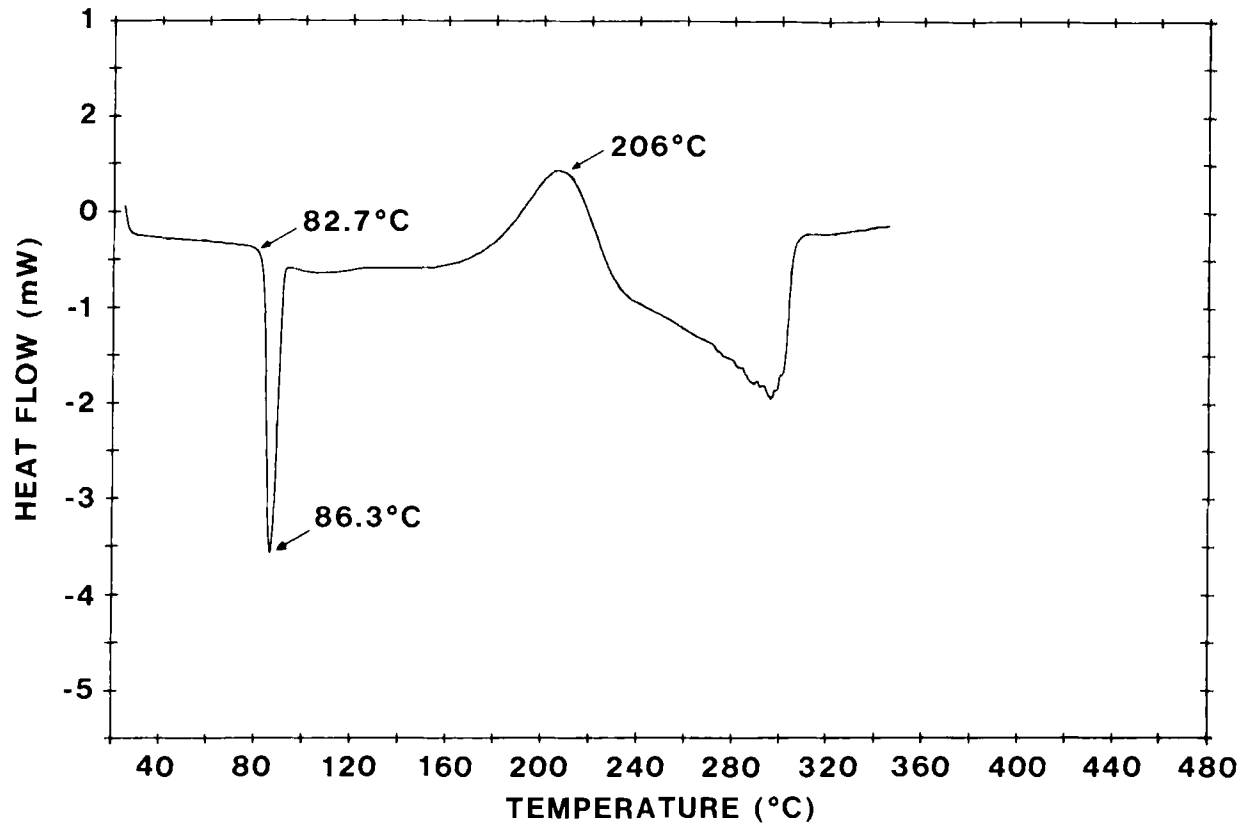


FIGURE 7: Differential scanning calorimetric curve for vitamin D<sub>3</sub>.



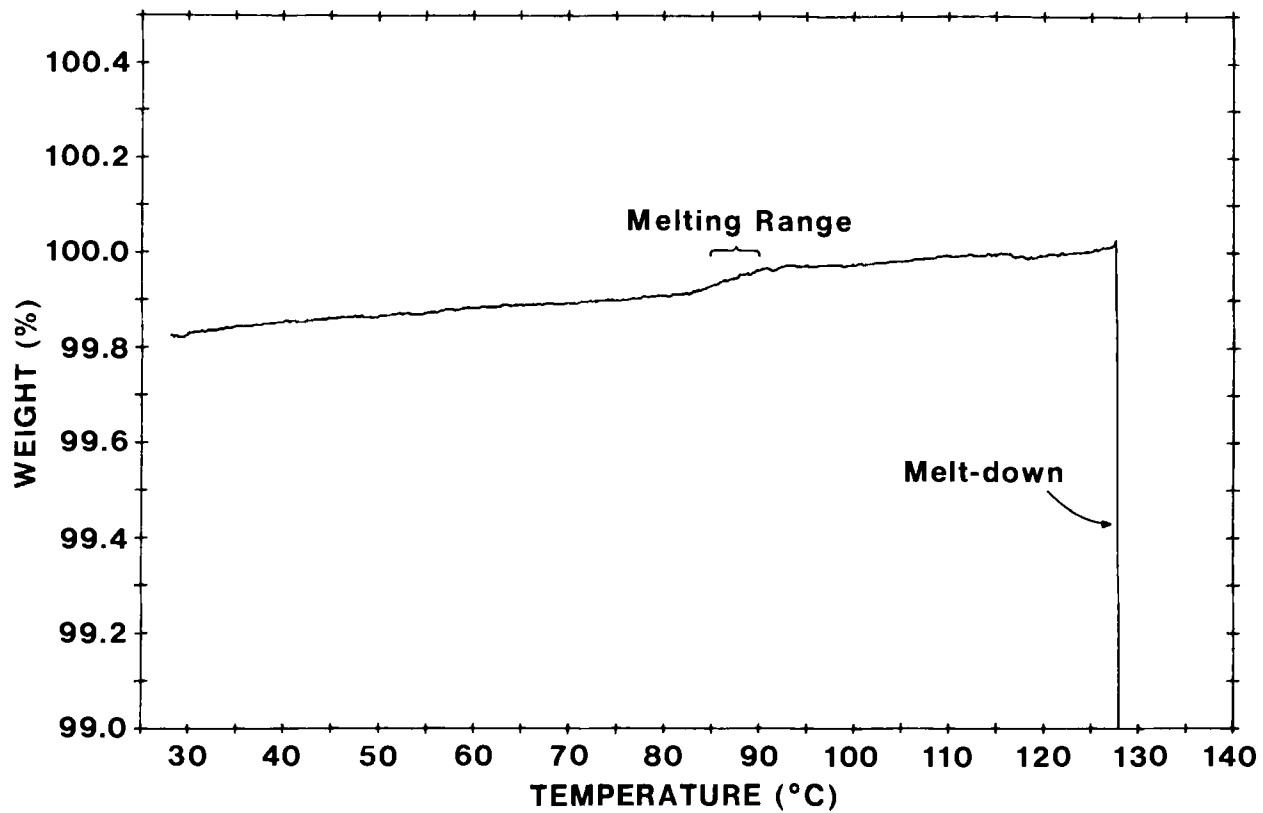


FIGURE 8: Thermogravimetric curve for vitamin D<sub>3</sub>.

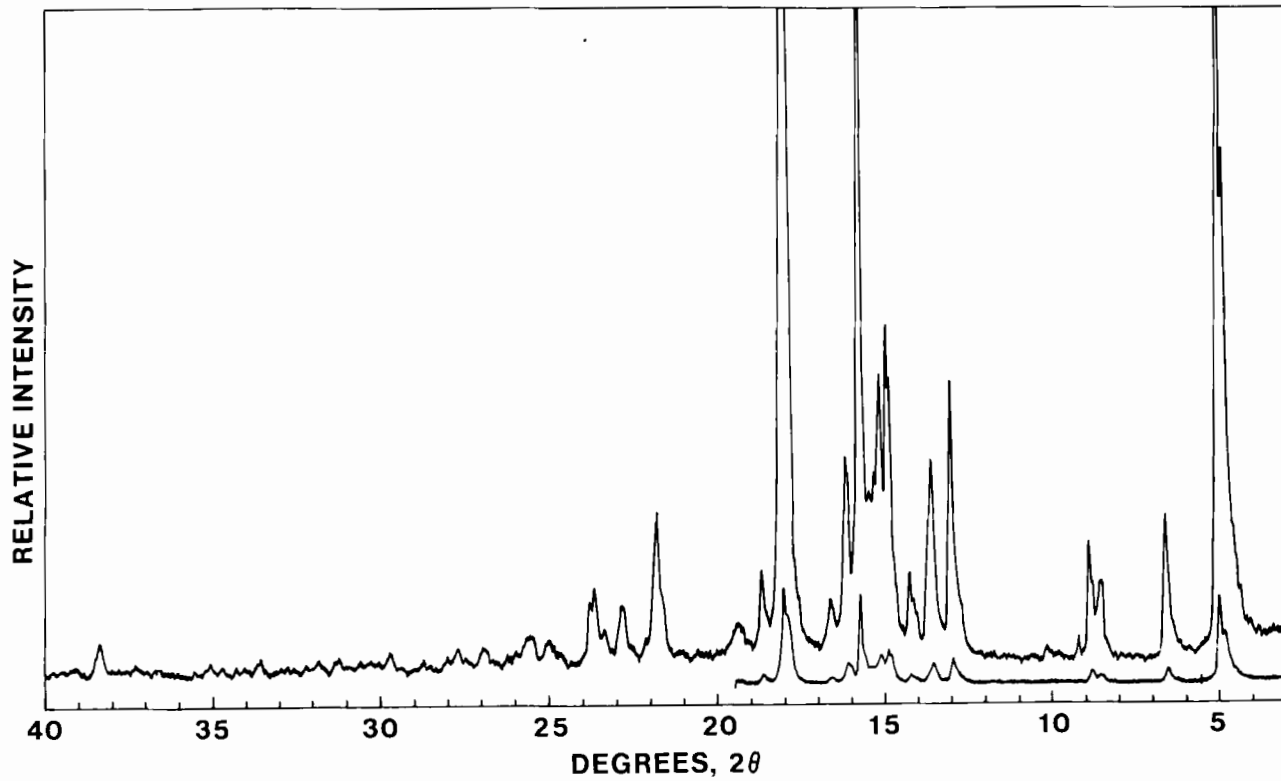


FIGURE 9: X-ray powder diffraction pattern for vitamin D<sub>3</sub>.

TABLE IV

X-Ray Powder Diffraction Pattern of Vitamin D<sub>3</sub> (Ref. 29)

---

$2\theta$	d-spacing (Å)	Relative Intensity
4.89	18.06	4
5.05	17.48	3
6.60	13.38	
8.60	10.27	
8.85	9.98	
9.20	9.60	
10.15	8.71	
13.00	6.80	
13.59	6.51	
14.25	6.21	5
14.95	5.92	
15.15	5.84	
15.75	5.62	2
16.15	5.48	
16.65	5.32	
18.05	4.91	1
18.65	4.75	
19.35	4.58	
21.79	4.08	
22.85	3.89	
23.35	3.81	
23.65	3.76	
25.10	3.54	
25.60	3.48	
27.05	3.29	
27.75	3.21	
29.80	3.00	
31.30	2.86	
31.90		
33.65		
38.45		

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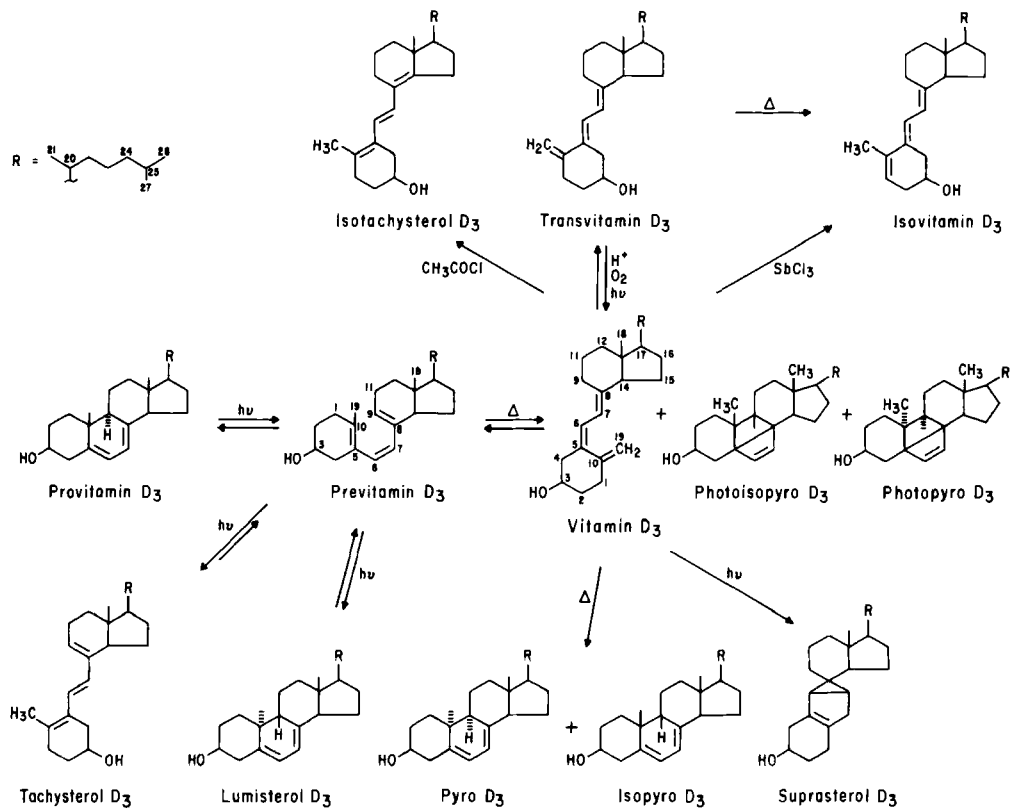
previtamin. A number of by-products are formed, most of which are removed during the clean-up steps. Scheme IV shows the structures of the known impurities and degradation products (31-33) that may be present in the synthetic vitamin D<sub>3</sub> concentrates. Some of the by-products have biological activity but others such as lumisterol, the suprasterols, and the pyro- and isopyrocalciferols have no antirachitic activity. Tachysterol and trans-vitamin D<sub>3</sub> have only slight antirachitic activity. Thus, the biological activity is primarily due to vitamin D<sub>3</sub> and previtamin D. The biological activity of previtamin D<sub>3</sub> is attributed to its *in vivo* conversion to vitamin D<sub>3</sub>. It may be mentioned that analogous compounds are formed during the synthesis of vitamin D<sub>2</sub> from ergosterol. The two forms differ only in the side chain. Vitamin D<sub>2</sub> has an extra methyl group on C-24 and a double bond between C-22 and 23.

### 3.2 Previtamin D<sub>3</sub> - Vitamin D<sub>3</sub> Equilibrium

The thermodynamics and kinetics of the thermal equilibrium between previtamin D<sub>3</sub> and vitamin D<sub>3</sub> have been studied (34,35). The isomerization of previtamin D<sub>3</sub> to vitamin D<sub>3</sub> is an exothermic first order reaction. The vitamin D<sub>3</sub>/previtamin D<sub>3</sub> equilibrium ratio depends on the temperature and can be calculated from the appropriate equilibrium and kinetic constants reported by Hanewald et al. (36). The rate constants for the equilibrium have been shown to be independent of the nature of the solvent, of acidic or basic catalysis and of factors known to affect free radical process (37,38). The percentages of vitamin D<sub>3</sub> in equilibrium with previtamin D<sub>3</sub> ranges from 98% at -20° to 78% at 80°. Thus, when vitamin D<sub>3</sub> is stored in the cold, the equilibrium constant hinders the conversion to previtamin D<sub>3</sub>.

### 3.3 Cis-Trans Equilibrium

The 5,6,7-cis-triene configuration of vitamin D<sub>3</sub> is important for its biological activity as the 5,6-trans form has very low activity. Exposure to iodine in non-polar solvents under diffuse light (39,40) or to mild acidic conditions (41) affords formation of the 5,6-trans isomer. The reverse transformation occurs photochemically (42). The 5,6-trans isomer can undergo further isomerizations upon exposure to heat (43) or acids or treatment with antimony trichloride. The cis and the trans forms of vitamin D<sub>3</sub> display characteristic ultraviolet properties. The cis form as shown earlier has a UV maxima at 265 nm. The trans form has a UV maxima at 273 nm.



Scheme IV. Photochemical, thermal, and chemical reaction pathways in the synthesis of vitamin D<sub>3</sub> (references 31,33). From *J. Pharm. Sci.* 71, 137 (1982). Reproduced with permission of the copyright owner.

### 3.4 Other Chemical Reactions

Vitamin D<sub>3</sub> can undergo very complex chemical reactions upon exposure to heat, light and chemical reagents. It is beyond the scope of this article to review these reactions. The reader is referred to recent reviews on the subject (99,100).

## 4. Methods of Analysis

The analysis of vitamin D<sub>3</sub> is difficult for the following reasons:

a. As shown in Scheme IV a number of by-products are formed during the chemical synthesis of vitamin D<sub>3</sub>, some of which may be present in the finished bulk form.

b. Vitamin D<sub>3</sub> concentrates are commercially available in a resin form in peanut oil and as a dry gelatin bead-let. The vitamin D<sub>3</sub> concentrates usually contain other oil soluble vitamins, especially vitamin A. The vitamin D<sub>3</sub>, therefore, has to be released from its matrix which is accomplished by saponification and extraction. It then has to be analyzed by a method that would distinguish it from all interfering isomers and other ingredients.

c. Vitamin D<sub>3</sub> exists in thermal equilibrium with previtamin D<sub>3</sub> (See Section 3.2). There is always uncertainty of the extent of this reaction even when a vitamin D<sub>3</sub> standard is treated under the same conditions as the sample.

d. The concentrations of vitamin D<sub>3</sub> is low in food products such as fortified milk, milk formulae, breakfast cereals, animal feeds etc. These samples, therefore, need extensive clean-up prior to analysis.

### 4.1 Identification Tests

The USP XX (30) describes the following identification tests for vitamin D<sub>3</sub>, which are reproduced verbatim:

a. The infrared absorption spectrum of a potassium bromide dispersion of it, in the range of 2  $\mu\text{m}$  to 12  $\mu\text{m}$ , exhibits maxima only at the same wavelengths as that of a similar preparation of USP Cholecalciferol RS.

b. The ultraviolet absorption spectrum of a 1 in 100,000 solution in alcohol exhibits maxima and minima at the same wavelengths as that of a similar solution of USP Cholecalciferol RS, concomitantly measured, and the respective absorptivities at the wavelength of maximum absorbance at about 265 nm do not differ by more than 3.0%.

c. To a solution of about 0.5 mg in 5 ml of chloroform add 0.3 ml of acetic anhydride and 0.1 ml of sulfuric acid, and shake vigorously; a bright red color is produced, and it rapidly changes through violet and blue to green.

d. Prepare without heating, and handle without delay, a 1 in 100 solution of squalane in chloroform containing 50 mg of cholecalciferol per ml, and prepare a Standard solution of USP Cholecalciferol RS in the same solvent and having the same concentration. Spot 10  $\mu$ l of the test solution and 10  $\mu$ l of the Standard solution on a line parallel to and about 2.5 cm from the bottom edge of a thin-layer chromatographic plate (See <621>) coated with a 0.25 mm layer of chromatographic silica gel mixture. Place the plate in a developing chamber containing and equilibrated with a mixture of equal volumes of cyclohexane and diethyl ether. Develop the chromatogram until the solvent front has moved about 15 cm above the line of application. Perform the development and subsequent operations in the dark. Remove the plate, allow the solvent to evaporate, and spray with a 1 in 50 solution of acetyl chloride in antimony trichloride TS: the chromatogram obtained with the test solution shows a yellowish orange area (cholecalciferol) having the same  $R_f$  value as the area of the Standard solution, and may show below the cholecalciferol area a violet area attributed to 7-dehydrocholesterol.

## 4.2 Quantitative Methods

Since vitamin D<sub>3</sub> is incorporated into a number of food additives for human and animal consumption, there is no one method that may be best suitable for all such products. Therefore, several analytical systems are described here to fit the need for a specific situation.

### 4.2.1 Biological Methods

Biological methods are the oldest for the analysis of vitamin D<sub>3</sub> and the one that is applicable for all types of samples. The biological methods can be divided

into three groups: curative, prophylactic, and those based on calcium absorption into the blood stream. All are based on the administration of measured doses of a standard vitamin D<sub>3</sub> preparation to a group of test animals and comparison of the biological responses with a similar group given the substance under test. A third group of test animals is used as controls.

The biological methods are popular for three reasons: 1) in most biological samples the concentration of vitamin D<sub>3</sub> is very low and is not suitable for more specific methods because of interfering materials even after extensive clean-up; 2) vitamin D<sub>3</sub> is effective biologically in trace amounts making it more sensitive than the best chemical methods; and 3) the biological methods are specific for vitamin D<sub>3</sub> and its biologically active metabolites. The chief disadvantages of the biological methods are in the high individual variations in the responses of test animals, the high cost, the time factor and that they do not differentiate vitamin D<sub>3</sub> from its biologically active impurities or metabolites.

The USP XX (30) and the Official Methods of Analysis of the Association of Official Analytical Chemists (AOAC) (44) describe in detail the curative method more commonly known as the rat line method. This method is not applicable to products offered for poultry feeding. For poultry feeds, and fish liver oils and their extracts, the AOAC (44) also describes in detail the chick bone ash method. In addition to the above two methods, there is a method based on the comparative measurement of serum calcium in rats referred to as Intestinal Calcium Transport Assay and another method based on the comparative amounts of calcium absorbed by control and test chicken referred to as Calcium Absorption Test. These methods are described by DeLuca and Blunt (45).

#### 4.2.2 Chemical Methods

The most widely used chemical method is the antimony trichloride colorimetric method. The method is applicable to vitamins D<sub>2</sub> and D<sub>3</sub> for their analysis in pharmacopeial preparations. The reaction product of vitamin D<sub>3</sub> with antimony trichloride is believed to be isovitamin D<sub>3</sub> (46, See Scheme IV). Antimony trichloride reacts with vitamin A also. Vitamin A occurs along with D<sub>3</sub> in many biological samples and is also an ingredient in many commercial products. Therefore, it is necessary to remove it and other interfering substances prior to reaction with



the reagent. This is generally achieved by saponification with alcoholic potassium hydroxide, extraction of the unsaponifiable fraction with petroleum ether, and clean-up on chromatographic columns. The procedure for the determination of vitamin D in pharmaceutical preparations is detailed in the USP (30).

This method is applicable with suitable modifications to biological samples like fish liver oils, fortified milk, etc., but the colorimetric procedures are being replaced when possible by more specific instrumental methods, usually HPLC.

#### 4.2.3 Paper Chromatography

Paper chromatographic separation of some sterols, provitamins and vitamins D<sub>2</sub> and D<sub>3</sub> has been reported by Peereboom et al. (47). The best chromatographic separation was achieved by reverse phase chromatography on paper impregnated with reagents shown in Table V, which also gives the R<sub>f</sub> values obtained. Details regarding the preparation and development of the paper are given. A number of detection systems are described which included exposure to UV light and reactions with phosphomolybdic acid, antimony trichloride, bismuth chloride, phosphotungstic acid, silicotungstic acid, a mixture of dimethyl-p-phenylenediamine and m-toluenediamine, Million's reagent and sodium iodate. Paper chromatographic systems are also described (48) by manufacturers of radiolabeled vitamin D<sub>3</sub> for monitoring purity. The systems reported are reverse phase chromatography with 95% aqueous methanol on paper treated with 10% mineral oil in benzene and with 10:1 acetic acid-water on paraffin coated paper.

#### 4.2.4. Thin Layer Chromatography

Thin Layer Chromatography is used by major manufacturers of radiolabeled vitamin D<sub>3</sub> (48) for monitoring purity. The systems described are; 1) silica gel G with 10% acetone in hexane; 2) silica gel G with acetone-hexane 1:1 and acetone-chloroform 1:1; 3) silver nitrate-impregnated silica gel G with chloroform or chloroform-acetone 9:1; and 4) silica gel saturated with silicone oil using 4:1 acetone-water. It may be mentioned that these operations are carried out under very carefully controlled conditions to prevent or minimize decomposition. TLC is an excellent technique for separating vitamin D<sub>3</sub> from interfering materials, but it has to be used with due care in the quantita-

TABLE V

R<sub>f</sub> Values of Vitamin D<sub>3</sub> on Paper Chromatographic Systems  
(Ref. 47)

Stationary Phase	Mobile Phase	R <sub>f</sub> x 100
Quilon	MeOH-H <sub>2</sub> O-ethylene glycol monomethyl ether (65:20:20)	76
Quilon	MeOH-H <sub>2</sub> O (95:5)	91
Paraffin	Acetic Acid-H <sub>2</sub> O (84:16)	36
Paraffin	Ethylene glycol monoethyl ether-n-propanol-MeOH-H <sub>2</sub> O (35:10:30:25)	46
Paraffin	N-propanol-MeOH-H <sub>2</sub> O (15:82:3)	68
Paraffin	MeOH-H <sub>2</sub> O (85:15)	68

tive analysis of vitamin D<sub>3</sub>, especially in biological samples containing small quantities of the vitamin. The R<sub>f</sub> values for vitamin D<sub>3</sub> on Silica Gel GF<sub>254</sub> in common organic solvents as determined in the authors' laboratory are shown in Table VI (49). These values are reported to enable one to develop suitable systems in unknown matrices.

Hashmi et al. (50) have reported R<sub>f</sub> values for both water and oil soluble vitamins on silica gel and alumina plates by circular thin layer chromatography. These systems are fast (< 2 minutes development time) and convenient for detecting and estimating the amounts of vitamin D<sub>3</sub> in different matrices. The authors have also listed detection systems for all the vitamins. The chromatographic systems described in this report are shown in Tables VII and VIII.

#### 4.2.5. Gas Liquid Chromatography (GLC)

Although GLC procedures have been used extensively for the determination of numerous physiologically important steroids and hormones, only a few investigators have attempted to develop similar techniques for vitamin D<sub>3</sub>. The major problem with the GLC analysis of vitamin D<sub>3</sub> is due to its open ring structure incorporating three conjugated double bonds in a 5,6-cis configuration. The 5,6-cis double bond causes less controllable thermal cyclization into the pyro- and isopyrociferols at operating GLC temperatures, resulting in two peaks. Another reason for the lack of interest in GLC analysis is that vitamin D<sub>3</sub> concentration in biological systems is low and the samples generally contain structurally similar compounds requiring extensive sample clean-up.

Murray et al. (46) overcame the difficulty of the thermal isomerization of vitamin D<sub>3</sub> into two peaks by converting it into isovitamin D<sub>3</sub> by treatment with antimony trichloride which gave a single peak by GLC. They reported successful application of the method for the determination of vitamin D<sub>3</sub> in biological samples. Sheppard et al. (51) treated vitamin D<sub>3</sub> with acetyl chloride to convert it quantitatively to isotachysterol D<sub>3</sub> (See Scheme IV) which gave a single peak by GLC. Nair and deLeon (52) prepared the 5,6-trans vitamin D<sub>3</sub> by treating vitamin D<sub>3</sub> with iodine followed by exposure to UV light. They then prepared the pentafluoropropionyl or the heptafluorobutyryl esters of the trans-D<sub>3</sub> which chromatographed as the isotachysterol D<sub>3</sub> esters as a single peak. Using <sup>63</sup>Ni electron capture detec-

TABLE VI

R<sub>f</sub> Values of Vitamin D<sub>3</sub> on Silica Gel GF<sub>254</sub> TLC Plates<sup>1</sup>  
for Common Solvents

Solvent	R <sub>f</sub>
Hexane (1.9) <sup>2</sup>	0
1,4-Dioxane (2.2)	0.67
Benzene (2.3)	0.10
Toluene (2.4)	0.07
Ethyl Ether (4.3)	0.55
Chloroform (4.8)	0.23
Ethyl Acetate (6.0)	0.70
Methylene Chloride (9.1)	0.23
Cyclohexanone (18.3)	0.90
Acetone (21)	0.78
Ethanol, Absolute (24.3)	0.73
Methanol (32.6)	0.67
Acetonitrile (38.8)	0.78
Hexane-Acetone, 9:1	0.27
Hexane-Acetone, 1:1	0.75
Acetone-Chloroform, 1:1	0.70

<sup>1</sup>Analtech Inc., Newark, DE.

<sup>2</sup>The numbers in parentheses are the dielectric constants of the solvents at 20°C.

TABLE VII

Circular Thin Layer Chromatographic Systems for  
Vitamin D<sub>3</sub> on Silica Gel D-0, Camag Without Binder  
(Ref. 50)

Mobile Phase	R <sub>f</sub> x 100	Detecting Reagents and Colors		
		A	B	C
Benzene-Petroleum Ether (4:1)	33	Grey (0.7) <sup>1</sup>	Bluish Grey (0.7) <sup>1</sup>	Greyish Green (0.8) <sup>1</sup>
Cyclohexane-Methyl Ethyl Ketone (21:4)	35	"	"	"
Toluene-Methyl Ethyl Ketone (99:1)	40	"	"	"
Cyclohexane-Cyclopentanone (24:1)	48	"	"	"
Cyclohexane-Ethyl Ether (4:1)	35	"	"	"
Cyclohexane-Methyl Iso-propyl Ketone (9:1)	50	"	"	"
Toluene-Chloroform (9:1)	32	"	"	"
Cyclohexane-Acetic Acid (22:3)	66	"	"	"
Benzene-Cyclohexane (9:1)	34	"	"	"
Toluene-Cyclohexane (9:1)	23	"	"	"
Cyclohexane-Methanol (43:7)	32	"	"	"

(Continued)

TABLE VII - Continued

Mobile Phase	R <sub>f</sub> × 100	Detecting Reagents and Colors		
		A	B	C
Cyclohexane-Methyl Ethyl Ketone (21:4)	50	Grey (0.7) <sup>1</sup>	Bluish Grey (0.7) <sup>1</sup>	Greyish Green (0.8) <sup>1</sup>
Cyclohexane-Ethyl Ether (4:1)	52	"	"	"
A 70% Perchloric Acid				
B Concentrated Sulfuric Acid				
C Saturated Solution of antimony pentachloride in carbon tetrachloride.				

<sup>1</sup> The numbers in parentheses represent the minimum amounts (μg) of vitamin D<sub>3</sub> that can be detected and identified on the plates.

TABLE VIII

Circular Thin Layer Chromatographic Systems for Vitamin  
D<sub>3</sub> on Aluminum Oxide G (E. Merck)  
(Ref. 50)

Mobile Phase	R <sub>f</sub> x 100	Detecting Reagents and Colors		
		A	B	C
Benzene-Petroleum Ether (4:1)	53	Brown (0.6) <sup>1</sup>	Grey (0.3) <sup>1</sup>	Light Brown (0.4) <sup>1</sup>
Cyclohexane-Methyl Ethyl Ketone (21:4)	53	"	"	"
Toluene-Methyl Ethyl Ketone (99:1)	52	"	"	"
Cyclohexane-Cyclopentanone (24:1)	47	"	"	"
Cyclohexane-Ethyl Ether (4:1)	30	"	"	"
Cyclohexane-Methyl Isopropyl Ketone (9:1)	31	"	"	"
Toluene-Chloroform (9:1)	45	"	"	"
Cyclohexane-Acetic Acid (22:3)	63	"	"	"
Benzene-Cyclohexane (9:1)	59	"	"	"
Toluene-Cyclohexane (9:1)	38	"	"	"
Cyclohexane-Methanol (43:7)	47	"	"	"

(Continued)

TABLE VIII - Continued

Mobile Phase	R <sub>f</sub> x 100	Detecting Reagents and Colors		
		A	B	C
Methanol-glacial Acetic Acid (49:1)	82	Brown (0.6) <sup>1</sup>	Grey (0.3) <sup>1</sup>	Light Brown (0.4) <sup>1</sup>

A Saturated Solution of antimony pentachloride in carbon tetrachloride.

B 70% perchloric acid.

C Concentrated sulfuric acid.

<sup>1</sup> The numbers in parentheses represent the minimum amounts (μg) of vitamin D<sub>3</sub> that can be detected and identified on the plates.



tor, they were able to detect nanogram quantities of vitamin D<sub>3</sub>.

In recent years GLC methods have been superceded by HPLC methods. There has not been much interest in capillary GLC analysis of vitamin D<sub>3</sub>. Capillary column technology, however, has improved so much in recent years that this technique may have applicability in the determination of trace amounts of vitamin D<sub>3</sub> in biological matrices especially in conjunction with an electron capture detector.

#### 4.2.6. High Performance Liquid Chromatography (HPLC)

In recent years HPLC procedures have evolved as the methods of choice for the analysis of vitamin D<sub>3</sub> in bulk drug (32,33,53-61), pharmaceutical preparations (62-73), fortified milk (74-82), animal feed supplements (83-89), margarine (79), infant formulae (79), cod liver oil (90-93), and chicken egg (96). It may be pointed out that these reports represent the latest in the state-of-the-art in the HPLC analyses of vitamins D<sub>2</sub> and D<sub>3</sub>. In spite of the intensive effort by a number of investigators and a few collaborative studies, there is no consensus on widely acceptable HPLC methods for vitamin D analysis in the bulk drug, pharmaceutical preparations, and fortified milk.

##### 4.2.6.1 HPLC Analysis of Bulk Vitamin D<sub>3</sub>

Bulk vitamin D<sub>3</sub> may contain some of synthetic by-products shown in Scheme IV. Tartivita et al. (33) have reported an excellent chromatographic system which showed resolution of most of the photochemical isomers and reaction by-products. The chromatograms obtained on a 30-cm x 4-mm i.d. commercial microparticulate silica column using a 70:30:1 mixture of chloroform (free from ethanol and water), n-hexane, and tetrahydrofuran at a flow rate of 1 ml/min is shown in Figure 10. The detection was by a 254 nm UV detector. Using this system, vitamin D<sub>3</sub> was quantitated in a resin sample containing 20 x 10<sup>6</sup> IU/g with a relative standard deviation of 1.37%. This procedure is essentially the basis for the USP XX (30) procedure for the analysis of bulk vitamin D<sub>3</sub> which is reproduced below, in its entirety.

Standard Preparation - Transfer about 30 mg of USP Cholecalciferol RS, accurately weighed, to a low-actinic, 25-ml volumetric flask, add isoctane to volume, mix, and sonicate for 5 minutes. Store at 0 ± 5°, and use this stock

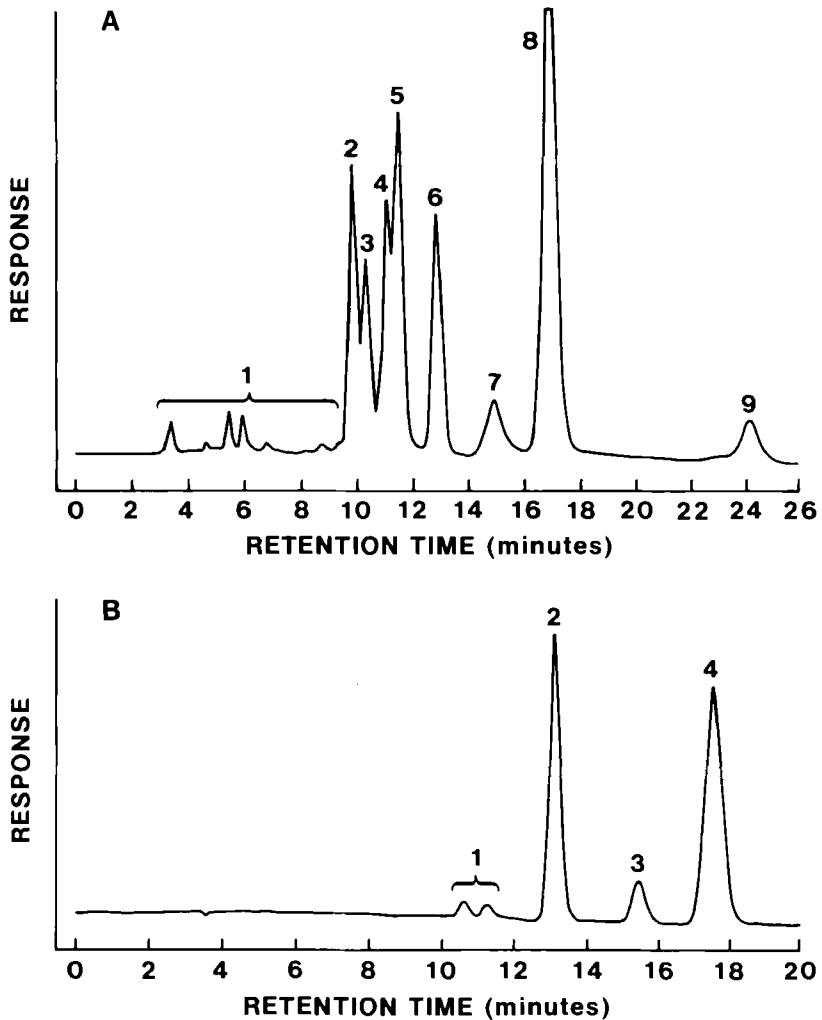


FIGURE 10: High Performance Liquid Chromatograms: (A) A synthetic mixture of photochemical isomers and reaction products of vitamin D<sub>3</sub>. Key: (1) unknowns; (2) trans-vitamin D<sub>3</sub>; (3) previtamin D<sub>3</sub>; (4) lumisterol<sub>3</sub>; (5) isotachysterol<sub>3</sub>; (6) p-dimethylaminobenzaldehyde (internal standard); (7) tachysterol<sub>3</sub>; (8) vitamin D<sub>3</sub>; (9) 7-dehydrocholesterol. (B) A typical vitamin D<sub>3</sub> resin. Key: (1) resin impurities; (2) p-dimethylaminobenzaldehyde (internal standard); (3) tachysterol<sub>3</sub>; (4) vitamin D<sub>3</sub>. (Both from Reference 33; reproduced with permission of the copyright owner.)

solution within 3 days. Pipet 5 ml of this stock solution into a low-actinic, 50-ml volumetric flask, dilute with isooctane to volume, and mix to obtain a solution having a known concentration of about 120  $\mu\text{g}$  per ml.

Assay Preparation - Transfer about 30 mg of Cholecalciferol, accurately weighed, to a low-actinic, 25-ml volumetric flask, and proceed as directed for Standard Preparation, beginning with "add isooctane to volume," to obtain a solution having a concentration of about 120  $\mu\text{g}$  per ml.

Alcohol-Free Chloroform - Prepare 2 Chromatographic columns by packing 2 chromatographic tubes with an amount of dry, activated, 80- to 200-mesh alumina sufficient to half-fill each tube (See Column Adsorption Chromatography under Chromatography <621>), and mount one column above the other. Extract 500 ml of chloroform with three 75-ml portions of water, and discard the aqueous extracts. Pass the chloroform layer through the Chromatographic columns, collect the eluate in a glass-stoppered flask, insert the stopper, and mix. Prepare this solvent on the day of use.

Mobile Phase - Prepare a filtered and de-gassed mixture of alcohol-free chloroform, n-hexane, and tetrahydrofuran (about 70:30:1 by volume). The ratio of components and the flow rate may be varied to meet system suitability requirements.

Chromatographic System - Typically, a high-pressure liquid chromatograph, operated at room temperature, is fitted with a 30-cm x 4-mm stainless steel column packed with chromatographic column packing L3\*. An ultraviolet detector that monitors absorption at the 254-nm wavelength is used.

System Suitability Preparation - Pipet 5 ml of the stock solution, prepared as directed under Standard preparation, into a 25-ml volumetric flask, dilute with isooctane to volume, and mix. Heat this solution at reflux for 2 hours, cool, and irradiate for 1 hour under long-wavelength and short-wavelength ultraviolet light. This solution contains cholecalciferol, pre-cholecalciferol, and tachysterol.

System Suitability Test - Chromatograph five injections of the heat-equilibrated Standard Preparation, and measure

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\*Porous silica microparticles, 5 to 10  $\mu\text{m}$  in diameter.

the peak response as directed under Procedure. The relative standard deviation for the peak response does not exceed 2.0%. The retention times observed for the System Suitability Preparation, chromatographed as directed for Procedure, are between 10 and 11 minutes for pre-cholecalciferol, between 14 and 16 minutes for tachysterol, and between 16 and 18 minutes for cholecalciferol. The resolution between tachysterol and cholecalciferol is not less than 1.0.

Procedure - Equilibrate the Standard Preparation and the Assay Preparation in the dark at 80° for 2.5 hours, accurately timed. Cool, and introduce equal volumes (5 to 10  $\mu$ l) of the heat-equilibrated Standard Preparation and Assay Preparation into the high-pressure liquid chromatograph (See chromatography <621>) by means of a suitable sampling valve. Measure the peak responses obtained for the Assay Preparation and the Standard Preparation, and calculate the quantity, in mg, of C<sub>27</sub>H<sub>44</sub>O in the portion of Cholecalciferol taken by the formula  $0.25C(A_U/A_S)$ , in which C is the concentration, in  $\mu$ g per ml, of USP Cholecalciferol RS in the Standard Preparation, and A<sub>U</sub> and A<sub>S</sub> are the peak responses for cholecalciferol obtained for the Assay Preparation and the Standard Preparation, respectively.

#### 4.2.6.2. HPLC Analysis of vitamin D<sub>3</sub> in Pharmaceutical Preparations

HPLC methodology permits rapid and generally quite simple analysis of vitamin D<sub>3</sub> in pharmaceutical bulk drugs, occurring as oil solutions, beadlets, or resins. However, for many multivitamin products, excipient components with differing degrees of purity, other vitamins and their degradation products, and whether vitamin D<sub>3</sub> is present as beadlets, oil, or resin, can make HPLC methodology a challenging procedure. Since the extinction coefficient for D<sub>3</sub> at 254 nm is very high (~15,000) standard, single wavelength detectors at 254 nm are generally used.

Reverse-phase HPLC procedures for vitamin D<sub>3</sub> in bulk drug were reported in the early 70's by William et al. (54) using a DuPont Permaphase® ODS column (DuPont, Wilmington, Del.) and 78% methanol in water as the mobile phase. A similar column and mobile phase (DuPont's Zorbax® ODS Column and 95% methanol in water) were reported to give increased resolution of the two forms of vitamin D (62) in multivitamin formulations. Complete separation of vitamins D<sub>2</sub> and D<sub>3</sub> by reverse-phase chromatography in model multivitamin preparations were reported by Osadca and Araujo (63). In this

analysis they used a Vydac column (Separations Group, Hespera, CA.) and a mobile phase of 90% methanol in water. Tscherne and Capitano (64) also reported complete separation of  $D_2$  and  $D_3$  with a  $\mu$  Bondapak<sup>®</sup> C<sub>18</sub> column (Waters, Milford, Mass.) and a mobile phase of 86.5% methanol in water with added silver nitrate. Separation of the two vitamins was satisfactory, however, the presence of silver nitrate in itself can give rise to a multitude of problems.

A reverse-phase HPLC assay, as part of the Association of Official Analytical Chemists report on analysis of fat-soluble vitamins, was described by DeVries et al. (65). Analysis were made with a Merck LiChrosorb RP-18 column (Manufacturing Chemists, Inc., Cincinnati, OH) and a acetonitrile:propionitrile:water (79:15:6) mobile phase. Although adequate chromatography was realized, the authors were concerned that problems arose concerning influence of temperature, dissolution of sample and purification of solvents in the mobile phase. For these reasons they recommended normal-phase chromatography. Separation of vitamins  $D_2$  and  $D_3$  with their systems was not discussed.

Normal-phase HPLC on silica columns are also used extensively in  $D_3$  analysis of vitamin products with non-polar mobile phase containing polar modifiers. Krol et al. (66) separated  $D_3$  from pre- $D_3$  and from a mixture of other vitamins using an adsorptive silica support introduced in 1972 (Vydac<sup>®</sup>, supplied at that time by Applied Science Laboratories, Inc. State College, Penn.). The hand-packed column was used in conjunction with a mobile phase of pentane:tetrahydrofuran (97.5:2.5). Sterule (32) used aluminum oxide as column support with chloroform as the mobile phase. Separation of  $D_3$  and its isomers and from vitamin A acetate was achieved.

DeVries et al. (67) reported the summary of studies by a number of collaborating laboratories for the HPLC assay of vitamin D in multivitamin preparations. Saponification and a reverse-phase Merck LiChrosorb RP-8 column were used for sample cleanup. The analytical column was a Partisil<sup>®</sup>, 5  $\mu$ m column (Whatman, Clifton, N.J.) with hexane-amyl alcohol (99.65:0.35%) as the mobile phase. The cleanup procedure although a departure from the usual analytical methods, was incorporated to ensure predictable, interference-free vitamin D assays ( $D_2$  and  $D_3$  co-elute).

Normal-phase columns, either Water's  $\mu$  Porasil<sup>®</sup> or DuPont's Zorbax-sil<sup>®</sup>, were used for the data of Sheridan's

(68) final report of the Pharmaceutical Manufacturer's Association-Quality Control Section (PMA-QC). The data covered collaborative studies on vitamin D using HPLC. A mobile phase of chloroform:hexane:tetrahydrofuran (70:30:1) was used with detection at 254 nm. The HPLC method separated vitamin D from interfering compounds that could be present, namely 7-dehydrocholesterol, tachysterol, isotachysterol pre-vitamin D, trans-vitamin D, lumisterol, and ergosterol, from other vitamins (A acetate, A palmitate, tocopherol, tocopheryl acetate, and phytonadione), and from antioxidants. Samples and standards were treated identically as to time and temperature so that only vitamin D need be measured, since for practical purposes previtamin D would be the same for sample and standard. Vitamins D<sub>3</sub> in resin and vitamin D<sub>2</sub> in beadlets were treated differently for sample preparations. The resin was dispersed in isooctane, treated with ultrasonication, and diluted with isooctane. Beadlets were added to a mixture of DMSO:water (3:1), shaken vigorously, and heated at 50°C for 20 minutes. After cooling to room temperature, hexane was added, the mixture was then shaken and centrifuged. The hexane layer was removed and the extraction procedure with hexane was carried out three more times. The combined hexane extracts were then dried with anhydrous sodium sulfate and reduced to dryness with a roto-evaporator. The dry residue was then dissolved in isooctane for injection on to the column. The HPLC procedure was considered advantageous because micro-particulate columns were reproducible and available commercially, ambient temperature requirements reduced thermal breakdown of D, and time-consuming saponification was not necessary. The method was considered superior to methods of the USP (30) and AOAC (44).

Based on the report of Sheridan (68), Walker et al. (69) were able to assay a variety of pharmaceutical products for D<sub>2</sub> (and reportedly D<sub>3</sub>, although no data were given). A Si-5A silica column (Brownlee Labs., Santa Clara, Calif.) was used in conjunction with a mobile phase comprised of a mixture of chloroform:water-saturated hexane:hexane:tetrahydrofuran:acetic acid (60:15:25:1.5:0.4). As with the HPLC assay of the PMA-QC report (68), D<sub>2</sub> and D<sub>3</sub> co-eluted. Comments were made concerning the addition of polar modifiers or changes in the ratio of mobile phase to enhance chromatography.

Building on published literature and based on empirical trials, an efficient reverse-phase HPLC system and sample preparation scheme has been developed for vitamin D<sub>3</sub> in

multivitamin products in the author's laboratory for in-house use (70). Although the method does not appear in open literature, it has been used extensively in our laboratories and will be described in some detail. It is felt that the sample preparation and treatment scheme, the use of two internal standards, and the capability to switch mobile phases to rid the column of very slow eluters are helpful in routine quality control assays and for stability evaluation of vitamin D<sub>3</sub> in a variety of pharmaceutical products.

In this procedure, the HPLC analysis is carried out on a DuPont Zorbax ODS® column using methanol, acetonitrile and water 10:2:1 at the mobile phase. This system resolves vitamins D<sub>3</sub> from D<sub>2</sub> and from degradation products and other fat soluble vitamins. Didecyl and dinonyl phthalate were used as the internal standards. The latter was included for use in the event that extraneous peaks interfered with the didecyl phthalate peak. Figure 11 shows a chromatogram of an extract of a multivitamin formulation with the two added internal standards.

Multivitamin liquid suspensions were prepared for assay using a hexane extraction of the sample from dimethyl sulfoxide:10% glacial acetic acid (9:1). A small volume of hexane containing the internal standards was added quantitatively to the extracting solution. Provisions were made to automatically rinse the column between injections with methanol:tetrahydrofuran:dimethyl sulfoxide (25:25:1) to remove any slow eluting materials. Column rinsing was initiated and controlled (Digital Valve Sequence Programmer, Valco, Houston, Texas), by an automatic sampler. Mobile phase and column rinse switching was made with a valve (Model 5302 Rheodyne, Cotati, Calif.), fitted with an air activator (Model 5300, Rheodyne). Air to the valve was controlled by a solenoid valve (No. 062E1-3-10-20-35, Humphrey Products, Kalamazoo, Mich.). With this chromatographic system dinonyl phthalate eluted in about 15 minutes, vitamin D<sub>3</sub> in 25 minutes, and didecyl phthalate in 30 minutes. Column rinse was complete in approximately 10 minutes, permitting an analysis time of about 40 minutes.

Oil formulations of multivitamin products were diluted with hexane, passed through a SEP-PAK® silica cartridge (Waters, Milford, Mass.) where the oil was retained. The cartridge was then flushed with methanol to elute D<sub>3</sub> into a container having the appropriate amount of internal standard, previously evaporated to dryness. The cartridge use was based on the reports from Water's (71) and from R.A.

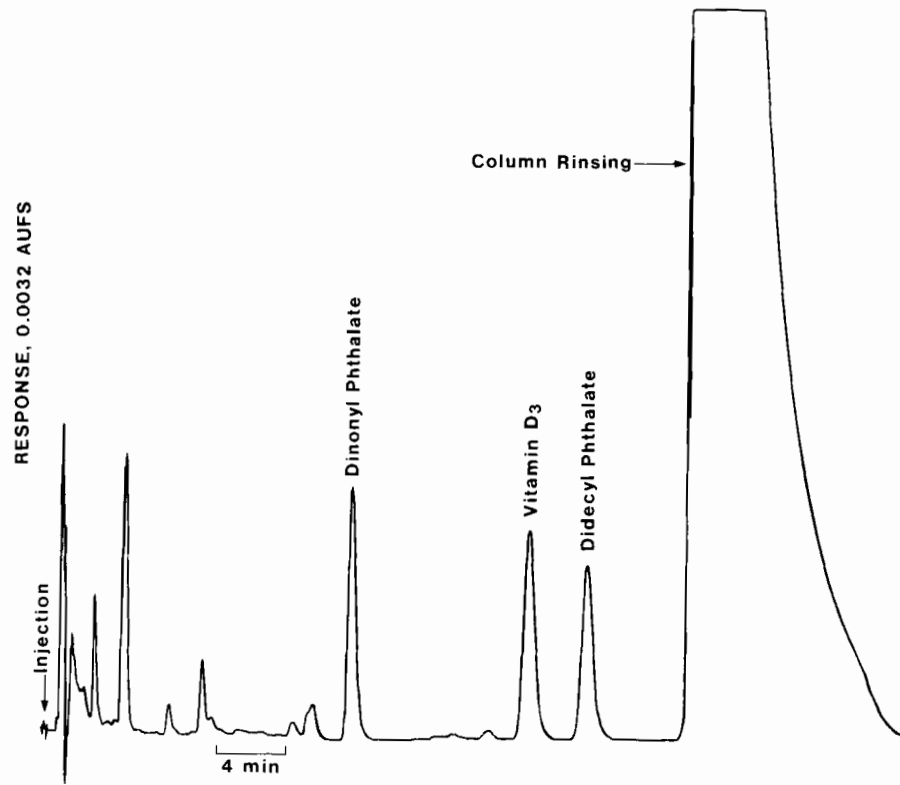


FIGURE 11: High Performance Liquid Chromatogram of a commercial multivitamin liquid suspension.



Pask-Hughs and D.H.Calam (72).

HPLC procedures for vitamin D in most instances have solved problems of interference from the matrix and degradation products, presence of isomers and homologues, other fat-soluble vitamins and their companion compounds. Stable and highly efficient columns, an easily obtainable and stable detector operated at 254 nm (near absorbance maximum of 265 nm), and reliable sample preparative techniques and apparatuses have made HPLC a most appealing technique. A choice of techniques with or without internal standards is available, depending on the amount of development time that can be committed and the presence or absence of suitable areas on the chromatogram for the insertion of an internal standard peak. The addition of an internal standard at the earliest time possible in the sample preparation scheme can add considerably to the accuracy of the HPLC method. It can aid in various ways, namely; correct for recovery, negate bias from losses in isolation steps, improve precision associated with aliquot removal and dilution, and importantly, compensate for changes in instrumental performance, particularly, long chromatographic runs. Valve switching for columns and mobile phases can decrease chromatographic time by diverting unwanted peaks or flushing the column after desired peaks have been obtained. In essence, HPLC has been utilized as one of the most powerful analytical tools available for vitamin D analysis in pharmaceutical products. Even greater utility of HPLC can be expected as higher efficiency columns and extraction procedures, better column hardware, and increasingly efficient instrumentation are developed and made available.

#### 4.2.6.3 Analysis Vitamin D<sub>3</sub> in Milk and Milk Powder

Milk sold in the USA and Canada is required by law to be fortified with either vitamin D<sub>2</sub> or D<sub>3</sub> at the level of 400 IU/quart which is equivalent to 10 ng/ml or 10 ppb. It is important for nutritional reasons that compliance with the regulation be checked by analyzing the fortified milk. Moreover, it is quite possible that a harmful excess could be added by mistake, and therefore it is imperative that errors in fortification be detectable. Of all the methods available HPLC appears to be the most reliable one even though it requires careful handling of the sample. A number of methods have been reported for the analysis of vitamin D<sub>3</sub> in whole milk (74-79), fortified

dried milks (76,77,80), skim and chocolate milk (81), instant nonfat dried milk (82) and infant formula (79). There is still no widely accepted method but the general approach (76) involves saponification and extraction of the unsaponifiable residue followed by an HPLC cleanup on a nitrile bonded silica column to separate vitamin D<sub>3</sub> and its isomers from interfering substances. The fraction corresponding to vitamin D<sub>3</sub> is collected and quantitated on a microparticulate silica column using 0.35-1% amyl alcohol in n-hexane as the mobile phase. The HPLC method is suitable for the determination of either vitamin D<sub>2</sub> or D<sub>3</sub> in milk. It is remarkable that it is possible to determine fairly accurately the amounts of vitamin D present in whole milk and in different forms of reconstituted milk.

#### 4.2.6.4 Determination of Vitamin D<sub>3</sub> in Animal Feeds

The bulk use of commercially produced vitamins D<sub>2</sub> and D<sub>3</sub> are for the supplementation of poultry, cattle, swine and pet feeds. The concentration of vitamin D<sub>3</sub> in animal feed premixes ranges from 30,000 to 150,000 IU/kg. It is in the range of 500 to 4000 IU/kg in the final feed. The animal feed composition varies depending on the availability and prices of the feed ingredients. Therefore it is a very challenging problem to develop an acceptable method for the analysis of vitamin D<sub>3</sub> in animal feeds. However, there are already reports (83-89) of partially successful efforts in this regard. A collaborative study (88) was conducted under the auspices of Association of Official Analytical Chemists, Washington, D.C. The procedure based on the one developed by Knapstein et al. (89) involves saponification of 25 g of the powdered sample, extraction of the unsaponifiables, cleanup on an alumina column using ether-hexane mixtures, additional cleanup on a C<sub>18</sub> bonded microparticulate silica column using CH<sub>3</sub>CN-MeOH-H<sub>2</sub>O (50:50:5) as the mobile phase and final quantitation on a microparticulate silica column (250 x 4.6 (id) mm) using 0.35% amyl alcohol in n-hexane as the mobile phase. Results are very encouraging and it can be expected that practical methods will be available in the near future.

#### 4.2.6.5 Determination of Vitamin D<sub>3</sub> in Cod Liver Oil

Prior to the availability of synthetic vitamins D<sub>2</sub> and D<sub>3</sub>, fish liver oils were the primary sources of vitamin D<sub>3</sub>. The commonly used oil is that from

the cod liver which contains about 100-150 IU of vitamin D<sub>3</sub> per gram. Fish liver oils also contains large amounts of vitamin A and cholesterol. In cod liver oil the weight ratio may be 1:100:3000 for vitamin D<sub>3</sub>, vitamin A and cholesterol. The main difficulty in analyzing cod liver oil for vitamin D<sub>3</sub> is in separating it from the large excess of cholesterol. Even though precipitation of cholesterol with digitonin or by freezing are standard procedures, these methods are physically cumbersome and also result in loss of vitamin D<sub>3</sub> by occlusion. Therefore, the analysis of vitamin D<sub>3</sub> in cod liver oil is a difficult problem. Ali (90) has reported a method based on a column chromatographic clean-up followed by a thin layer chromatography and spectrophotometry. HPLC procedures have been reported by Ali (91), Egaas and Lambersten (92) and Stancher and Sonta (93). Of these the procedure by Stancher and Zonta (93) appears to be the simplest. The procedure is reportedly applicable for the simultaneous determination of vitamins D<sub>3</sub> and E, but the recoveries of the preliminary extraction step using either hexane or ethyl ether is in the range 60-75% only. The authors do not report the overall recovery for the procedure.

From the above discussion it is clear that there is scope for more work in developing a better method for vitamin D<sub>3</sub> in cod liver oil.

#### 4.2.6.6. Determination of Vitamin D<sub>3</sub> in Chicken Egg Yolk

The chicken egg yolk, as deduced from its antirachitic activity, contains 1-2 µg of cholecalciferol and this can be raised further by increasing the intake of vitamin D<sub>3</sub> of the laying hen (94). The concentration in yolk is 5-10 times higher than that of total vitamin D<sub>3</sub> plus its metabolites in blood plasma of normal chickens and 50-100 times higher than the concentration in any other tissue (95). Consequently, the chicken egg yolk is a potent source of vitamin D<sub>3</sub>.

Because several of the metabolites of vitamin D<sub>3</sub> are biologically active, the molecular species of vitamin D<sub>3</sub> which passes into the yolk cannot be determined just from measurement of antirachitic activity. Consequently, a reliable and sensitive method for determining the amount of the unchanged form of vitamin D<sub>3</sub> would be extremely beneficial to those interested in the metabolism and other factors that influence the chicken to deposit vitamin D<sub>3</sub> in

the egg yolk.

Packson et al. (96) have recently reported an HPLC method for the analysis of vitamin D<sub>3</sub> in egg yolk. They use vitamin D<sub>2</sub> as an internal standard which is added to 50 g of the freeze-dried egg at the beginning of the analysis to compensate for losses during the several manipulative steps in the procedure. The sample is saponified for 30 minutes in a N<sub>2</sub> atmosphere. The unsaponifiables are extracted with 1:1 mixture of petroleum ether and diethyl ether. The extract after washing free of base is evaporated. The excess steroids are removed by precipitation by chilling from 90:10 methanol-water solution and removed by filtration. The extract is evaporated to dryness, dissolved in a small volume of light petroleum ether and subjected to TLC on silica gel plates. The band representing vitamin D is scraped, eluted with HPLC grade methanol, concentrated and subjected to HPLC on a 250 x 4 mm i.d. stainless steel column packed with a C<sub>22</sub> reversed-phase packing (Magnusil C<sub>22</sub> 5x) using either 95:5 or 90:10 methanol-water as the mobile phase. Under the HPLC conditions vitamin D<sub>2</sub> eluted before vitamin D<sub>3</sub> in about 14-17 minutes. The two peaks were not completely resolved. Replicate analysis of 5 samples showed vitamin D<sub>3</sub> content in the range 1.3-1.9 µg/100 g (mean 1.6 ± 0.2 µg/100 g) of whole freeze-dried sample. This is equivalent to 0.32 µg/20 g of whole egg. This value is considerably lower than the currently accepted value of 1-2 µg per egg yolk weighing 15-20 g (94).

## 5. Metabolites of Vitamin D<sub>3</sub>

### 5.1 Primary Metabolites

For nearly 30 years following the discovery of vitamin D<sub>3</sub>, relatively little was known about its metabolic fate in man. It was thought that vitamin D<sub>3</sub> acted directly on the target tissues of intestine and bone. However, the time lag reported by Carlsson (97) between the administration of vitamin D<sub>3</sub> and its physiological response led investigators to suspect that vitamin D<sub>3</sub> was metabolically altered before it became biologically active.

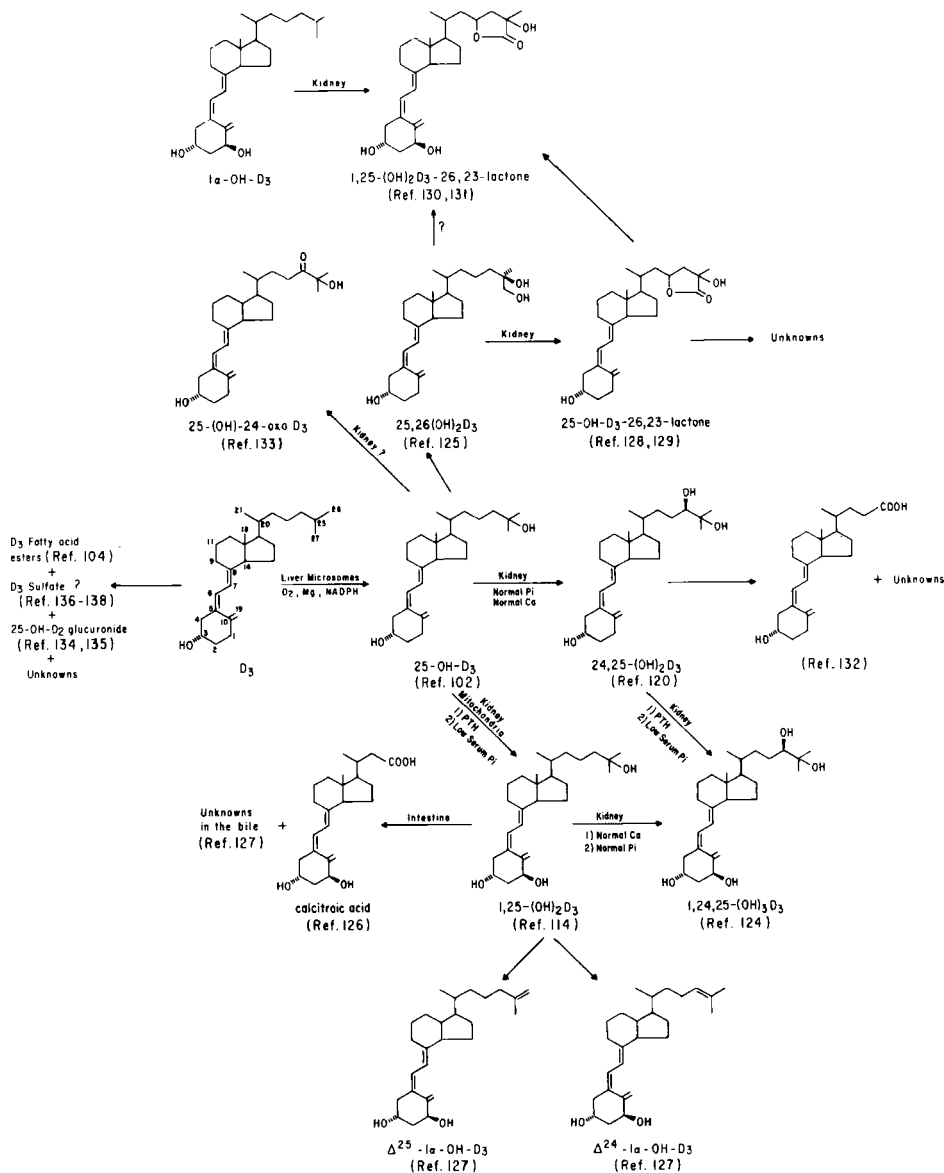
Major developments in the metabolic study came after selective <sup>3</sup>H and <sup>14</sup>C labeled vitamin D<sub>3</sub> with high specific activities were synthesized in the mid-1960's. Since then, massive efforts by a number of investigators have led to the discovery of a large number of metabolites, the hormonal nature of the primary metabolites in the maintenance of

calcium homeostasis, the nature of the specific proteins that carry the metabolites to target tissues, the enzymes involved in the transformations and the intricate role played by other endocrine organs in the control of the metabolism of vitamin D<sub>3</sub>. These findings have been reviewed extensively (98-100). The currently known metabolic pathway of vitamin D<sub>3</sub> is shown in Scheme V. Vitamin D<sub>3</sub> from the diet or that generated in the skin is absorbed into the blood. The concentration of vitamin D<sub>3</sub> in the blood is very low, but excess vitamin is stored in the fat depots. Depletion of vitamin from fatty tissues depends on the turnover of fat which is generally a slow process.

Lund and DeLuca (101) administered [<sup>3</sup>H] vitamin D<sub>3</sub> to rats and found biologically active metabolites in the bone, liver and serum. The aqueous-soluble metabolites from the tissues and the feces did not have vitamin D activity. At least three biologically active metabolites were isolated from the chloroform-soluble portion of the extract. One of these was found in large amounts in the liver, blood and bone. In 1968, Blunt et. al. (102) proved convincingly that this major metabolite is 25-hydroxyvitamin D<sub>3</sub> (25-OH-D<sub>3</sub>). Two other groups of investigators (103,104) independently found clues to the metabolic hydroxylation of vitamin D<sub>3</sub>. It was soon established that 25-hydroxylation of vitamin D<sub>3</sub> takes place primarily in the liver (105,106) and that 25-OH-D<sub>3</sub> is the major form of circulating vitamin D<sub>3</sub> in human plasma (107).

Initially, 25-OH-D<sub>3</sub> was considered to be the main biologically active metabolite of vitamin D. But soon it was discovered that physiological concentrations of 25-OH-D<sub>3</sub>, like vitamin D<sub>3</sub>, are incapable of stimulating either intestinal calcium transport or bone calcium mobilization (108-110). Earlier work (111,112) with [ $1\alpha$ -<sup>3</sup>H] vitamin D<sub>3</sub> had shown that one of the unknown metabolites had lost its tritium from the C-1 position. Fraser and Kodicek (113) established that this active metabolite is 1-oxygenated 25-OH-D<sub>3</sub> and that it was produced in the kidney. A short time later Lawson et al. (114) identified this metabolite to be  $1\alpha$ , 25-dihydroxyvitamin D<sub>3</sub> (1,25-(OH)<sub>2</sub>D<sub>3</sub>) which was confirmed by other investigators (115,116).

The endogenous level of 25-OH-D<sub>3</sub>, the primary metabolite of vitamin D<sub>3</sub>, has been determined by a number of investigators and found to be in the 15-30 ng/ml in normal Caucasian plasma. The concentration of 25-OH-D<sub>3</sub> increases with increased vitamin D<sub>3</sub> intake and to extended exposure to



Scheme V. Metabolic pathway for vitamin D<sub>3</sub>. From *J. Pharm. Sci.* 71, 137 (1982). Reproduced with permission of the copyright owner.

sunlight. As pointed out earlier, it is the major circulating and readily available form of vitamin  $D_3$ . The endogenous level of  $1,25-(OH)_2D_3$ , on the other hand, is very low, between 20 and 40 pg/ml of plasma. The formation of  $1,25-(OH)_2D_3$  is feedback regulated depending on the need for calcium in the blood. At the present time,  $1,25-(OH)_2D_3$  is considered to be the most potent of all the known metabolites of vitamin  $D_3$  for controlling calcium and phosphorus absorption from the intestine and mobilization of these elements from the bone when necessary.

## 5.2 Secondary Metabolites

It became known during the work on the identification of  $25-OH-D_3$  and  $1,25-(OH)_2D_3$  that there were other metabolites some of which were identified in quick succession. Of these the important ones from a biological standpoint are  $24R,25$ -dihydroxyvitamin  $D_3$  [ $24,25-(OH)_3D_3$ ] and  $1,24,25$ -trihydroxyvitamin  $D_3$  [ $1,24,25-(OH)_3D_3$ ]. Omdahl and DeLuca reported (117) that when the synthesis of  $1,25-(OH)_2D_3$  was suppressed by a block of the kidney hydroxylase which produces  $1,25-(OH)_2D_3$  from  $25-OH-D_3$ , a new metabolite appeared. This metabolite is made exclusively in the kidney (118,119) and was identified as  $24,25-(OH)_2D_3$  (120). The concentration of this metabolite in human is low but much higher than that of  $1,25-(OH)_2D_3$ . Taylor et al. (121) reported a value of  $1.68 \pm 0.82$  ng/ml among several normal volunteers. It was demonstrated (122,123) that under normal or hypercalcemic conditions,  $24,25-(OH)_2D_3$  is the major circulating metabolite of  $25-OH-D_3$ . Like  $25-OH-D_3$  and  $1,25-(OH)_2D_3$ , it is capable of elevating serum calcium and supporting bone growth on a normal calcium, normal phosphorus diet. However, unlike  $1,25-(OH)_2D_3$ , it has little ability to mobilize calcium from the bone. Boyle et al. (122,123) had also shown that  $24,25-(OH)_2D_3$  has to be metabolized to a more polar metabolite in the kidney before it became biologically active. This metabolite was isolated by Holick et al. (124) in pure form from chicken kidney homogenates and identified as  $1,24,25-(OH)_3D_3$ . The biological activity of  $1,24,25-(OH)_3D_3$  parallels the biological activity of  $24,25-(OH)_2D_3$  (123). There is no conclusive data on the concentration of  $1,24,25-(OH)_3D_3$ . It is known to be lower than that of  $1,25-(OH)_2D_3$  and, therefore, is in the very low picogram per milliliter range in the plasma.

### 5.3 Metabolites of Very Low or Unknown Biological Activity

A number of metabolites of vitamin D<sub>3</sub> with extremely low or of unknown biological activity have been discovered. Scheme V shows the structures of these metabolites, their origin and also appropriate literature citations of their discovery. The metabolites are 25,26-dihydroxyvitamin D<sub>3</sub> discovered by Suda et al. (125), 1 $\alpha$ -OH-24,25,26,27-tetranor-D<sub>3</sub>-23-carboxylic acid (calcitric acid) (126),  $\Delta^{24}$ -1 $\alpha$ -hydroxyvitamin D<sub>3</sub> (127),  $\Delta^{25}$ -1 $\alpha$ -hydroxyvitamin D<sub>3</sub> (127), 25-hydroxyvitamin D<sub>3</sub>-26,23-lactone (128,129), 1 $\alpha$ -25-dihydroxyvitamin D<sub>3</sub>-26,23-lactone (130,131), 25,26,27-tris-nor-vitamin D<sub>3</sub>-24-carboxylic acid (132), 25-hydroxy-24-oxocholecalciferol (133), and possibly 25-hydroxyvitamin D<sub>3</sub>-25-glucuronide (134,135). Initial experiments with radiolabeled vitamin D<sub>3</sub> showed very little radioactivity in the aqueous portions after the tissues were extracted with a mixture of chloroform and methanol. However, the presence of water soluble metabolites has always been suspected. The presence of vitamin D<sub>3</sub> sulfate in human milk was reported by a few investigators (136-138). Recently Hollis et al. (139) used a more specific HPLC method for vitamin D sulfate in human milk whey and did not find any (detection limit 1 ng/ml). However, it is established that human milk has antirachitic activity. Lakdawala and Widdowson (137) have reported that even in winter in the United Kingdom, breast milk protects infants from rickets. Therefore, the metabolite that protects infants from rickets while on breast milk remains to be clearly identified.

Leading investigators are certain that there are many unidentified metabolites of vitamin D<sub>3</sub>. For example, according to Onisko et al. (127), there are substantial amounts of unknown metabolites of 1,25-(OH)<sub>2</sub>D<sub>3</sub> in bile. These are water-soluble, negatively charged compounds which are rendered chloroform-soluble after methylation suggesting that these are unknown carboxylic acid metabolites. Administration of radioactive vitamin D<sub>3</sub> daily for 1-2 weeks (140) have led to the discovery of a few unknown metabolites. It is not known if any of these have important biological activity.

## 6. Vitamin D<sub>3</sub> As A Prohormone

Vitamin D<sub>3</sub> is unique in that under normal circumstances it is formed in the skin in sufficient quantities when humans and animals are exposed to sunshine, and extra sup-



plementation is unnecessary. The previtamin D<sub>3</sub> that is formed in the skin is slowly released into the body as vitamin D<sub>3</sub>. Vitamin D<sub>3</sub> is also unique in that it has to be metabolized in sequence to 25-OH-D<sub>3</sub> and to 1,25-(OH)<sub>2</sub>D<sub>3</sub> or to some as yet unknown metabolite before it becomes physiologically active. Since 1,25-(OH)<sub>2</sub>D<sub>3</sub> acts on tissues remote from its production site, it meets the criteria and definition of a hormone. In true hormonal form, its biogenesis is regulated by hypocalcemia or hypophosphatemia. Then vitamin D<sub>3</sub> can be considered a prohormone and 25-OH-D<sub>3</sub> as a prehormone.

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## **PROFILE SUPPLEMENTS**

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# TOLBUTAMIDE

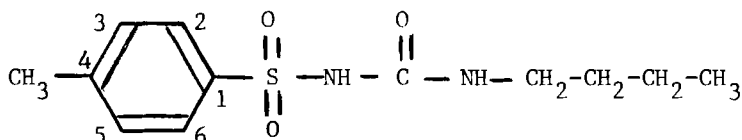
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TOLBUTAMIDE

Tolbutamide, N-[(butylamino)carbonyl]-4-methylbenzenesulphonamide; N-(butylamino) carbonyl-p-toluene sulphonamide is a sulphonylurea that is orally active as a hypoglycemic agent. The drug stimulates the pancreatic islet beta cells to release extra insulin. It also inhibits phosphodiesterase, which preserves cyclic AMP and thus favors glycogenolysis in a number of tissues.



Tolbutamide

1. Physical Properties1.1 Crystal Shape

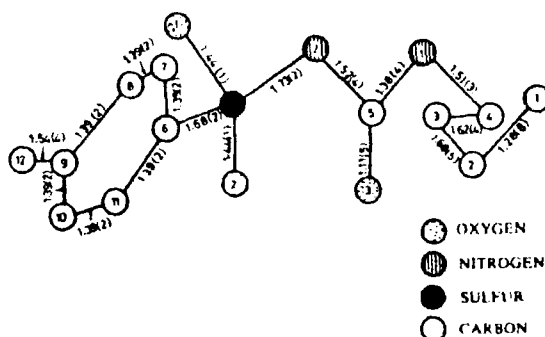
Tolbutamide exists in two polymorphs, one was obtained either by crystallization of tolbutamide from benzene solution after addition of hexane, or by precipitation from solution in aqueous ammonia by addition of acetic acid, and the other, metastable form was obtained from ethanolic solution after addition of water. The two forms were characterized by infrared spectroscopy, x-ray diffraction and d.t.a.(1)

1.2 Crystal Structure

Nirmala and Gowda (2) have reported that tolbutamide crystallizes in the orthorhombic space group  $Pn2_1a$ , with  $a=20.16$  (2),  $b=9.05$  (1),  $c=7.85$  (1)  $\text{\AA}$ ,  $Z=4$ ,  $D_c=1.25$ ,  $D_m=1.24$   $\text{Mgm}^{-3}$  and  $\mu$  ( $\text{CuK}\alpha$ )= $1.968$   $\text{mm}^{-1}$ . The structure was solved by the Patterson search method and refined to an R factor of 0.083 for 615 visually measured reflections. The structure is stabilized by hydrogen bonding between the polar group and Van der Waals interactions between the non polar groups. The

hydrogen-bond network and the dynamics of the protons are of great importance in explaining the properties of the crystals and their phase transitions.

The following figure represents a projection of tolbutamide molecule showing the atom numbering and bond lengths ( $\text{\AA}$ ). The authors excluded the hydrogen atoms for clarity (2).



### 1.3 Mass Spectrum

The electron impact (EI) mass spectrum at 70 eV recorded on Varian Mat 311 mass spectrometer and the methane derived chemical ionization (CI) mass spectrum obtained with Finnigan 4000 mass spectrometer are shown in Figures 1 and 2 respectively. The (EI) spectrum Fig.

1 shows a base peak at  $m/e$  91. Other major fragment at  $m/e$  (relative abundance) 108(71), 155(48), 65(27), and 197(15). The (CI) spectrum Fig. 2 is surprisingly simple, the base peak  $m/e$  271 corresponds to  $M^+$  ion. Other major fragments are at  $m/e$  117, 172, 200, 61 and  $m/e$  74.

### 1.4 Carbon-13 Nuclear Magnetic Resonance Spectrum

The carbon-13 NMR completely decoupled and off-resonance spectra are shown in Fig. 3 and 4 respectively. Both were recorded over 5000 Hz range in deuterated dimethylsulphoxide on FT-80 A-80 MHz NMR spectrometer using tetramethylsilane as reference standard. The carbon chemical shift value are assigned on the basis of signal multiplicity, chemical shifts and the comparison with the chemical shifts of model compounds. Table 1 summarizes the carbon chemical shifts of tolbutamide.



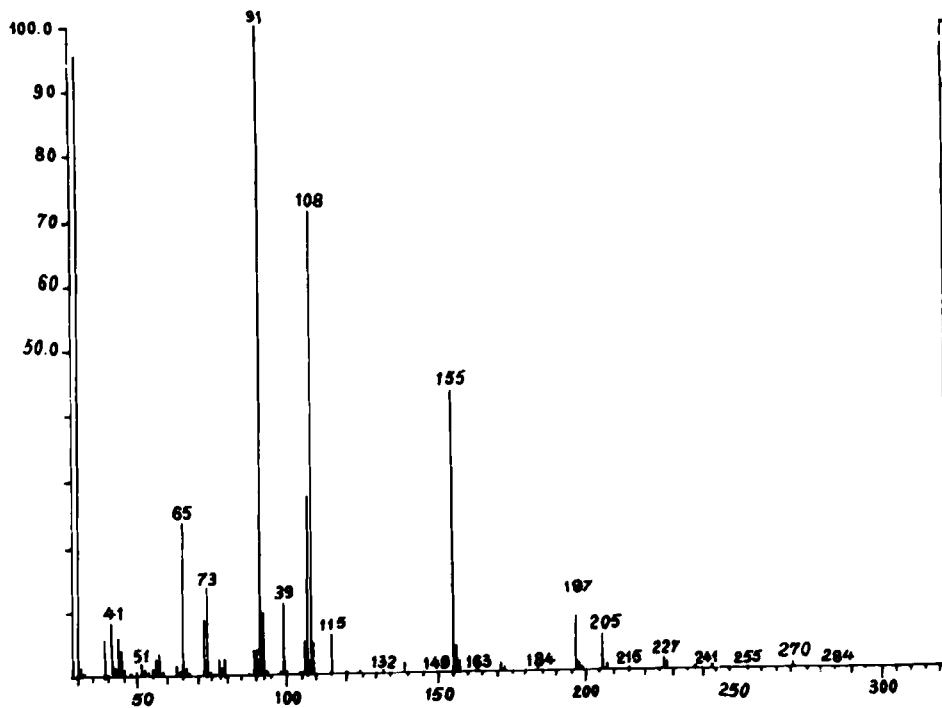


Fig. 1. Mass spectrum of tolbutamide (EI) determined by direct probe insertion.

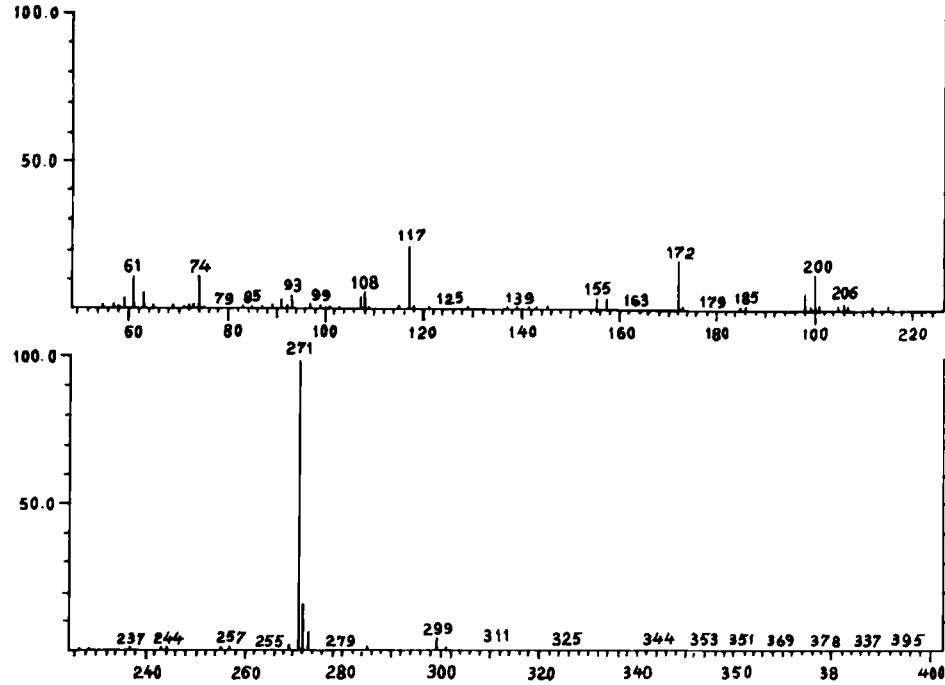


Fig. 2. Mass spectrum of tolbutamide (Cl) determined by direct probe insertion.

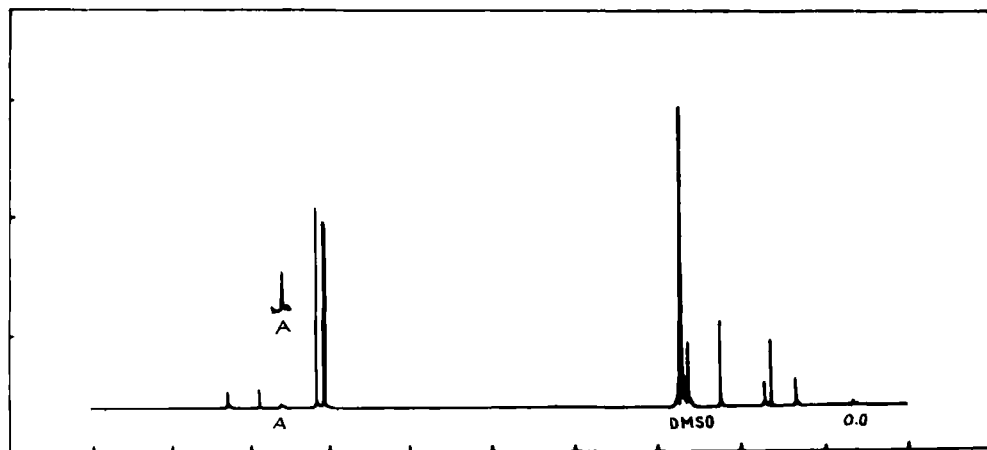


Fig. 3. C-13 nuclear magnetic resonance spectrum (proton-noise decoupled) of tolbutamide.

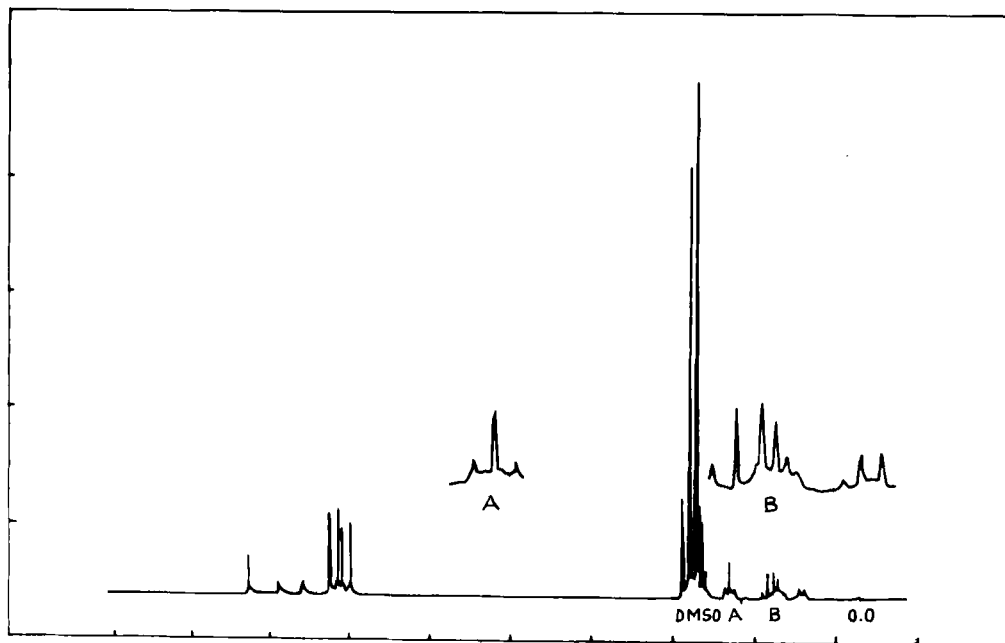
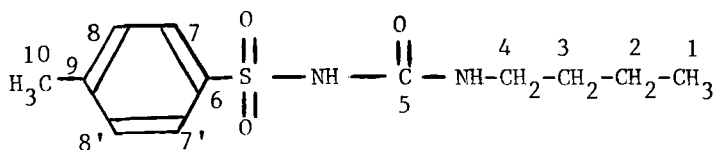


Fig. 4. <sup>13</sup>C nuclear magnetic resonance spectrum (off-resonance) of Tolbutamide.

Table 1. Carbon Chemical Shifts of Tolbutamide.



Carbon	Multiplicity	Chemical Shift (ppm)
C <sub>1</sub>	(q)	13.26
C <sub>2</sub>	(t)	19.30
C <sub>3</sub>	(t)	20.90
C <sub>4</sub>	(t)	38.98
C <sub>5</sub>	(s)	151.45
C <sub>6</sub>	(s)	148.36
C <sub>7</sub>	(d)	127.09
C <sub>7'</sub>	(d)	127.09
C <sub>8</sub>	(d)	129.23
C <sub>8'</sub>	(d)	129.23
C <sub>9</sub>	(s)	137.62
C <sub>10</sub>	(q)	31.28

## 2. Methods of Analysis

### 2.1 Titrimetric Methods

#### 2.1.1 Aqueous Titration

The methods of El-Fatary *et al* (3) involves measurements of the pKa values of tolbutamide in different solvents and titration of the drug in a suitable medium with standard alkali to the

pH value corresponding to the pKa of it in the medium. Accurate and precise results were obtained in the concentration range 1 to 10 mg ml<sup>-1</sup> in aqueous 60% acetone.

### 2.1.2 Non-Aqueous Titration

Tolbutamide in tablets and pure forms was dissolved in tetramethylurea and titrated with 0.1 N lithium methoxide in benzene-methanol medium, the end point was determined visually with 0.2% azo-violet in toluene as indicator (4).

## 2.2 Spectrophotometric Methods

### 2.2.1 Ultraviolet

- a. Abdel Hady et al (5) reported two spectrophotometric methods for the quantitation of tolbutamide without interference from the tablet excipients. In the first (Glenn's) method, the absorbance of tolbutamide in 95% ethanol was measured at 250-270 nm at 4 nm intervals and the p2 coefficient calculated. The coefficient was linearly related to concentration within a range of 0.1-0.4 mg/ml. The second method is based on the formation of a complex of a ratio 1:1 with basic dye brilliant cresyl blue or safranin T, the complex was easily extracted with chloroform and the absorbance of the chloroform extract was measured against either a blank or reference experiment at 615 or 510 nm for brilliant cresyl blue or safranin T methods respectively. The results of these methods are more accurate than those of the traditional ultraviolet spectrophotometric methods.
- b. Tolbutamide was determined in presence of thiamine hydrochloride and pyridoxine hydrochloride, by measuring the difference in absorbance at 274 and 276 nm in 95% ethanol medium, the interference by thiamine hydrochloride or pyridoxine hydrochloride is negligible. Mean recoveries (10 determinations) were 100.0 to 100.8 and the standard deviation  $\approx$  0.5% (6).

- c. Tolbutamide was determined in citrated blood contaminated with its metabolites 1-butyl-3-(4-carboxybenzenesulphonyl) urea and 1-butyl-3-(hydroxymethylbenzenesulphonyl) urea (7). The haemolyzed blood in aqueous phosphate buffer of pH 5 was shaken with heptane-chloroform (4:1) for 20 minutes, the organic phase was shaken with 0.1 M sodium hydroxide solution for 20 minutes then the aqueous alkali phase was mixed (20:1) with 3M hydrochloric acid solution and the extinction was measured at 230 nm. Blank solution containing uncontaminated drug were used in all instance.

### 2.2.2 Spectrofluorimetry

Among other antidiabetic drugs, tolbutamide was determined in formulations and in biological fluids by acid hydrolysis, reaction of the resulting amine with acetylacetone and formaldehyde, and fluorimetric determination of the resulting substituted dihydropyridine at 600 nm, with excitation at 480 nm (8).

### 2.2.3 Proton Magnetic Resonance Spectrometry

Al-Badr and Ibrahim (9) reported an accurate, rapid and precise method for the determination of tolbutamide and some other hypoglycemic agents in pure and tablet forms using proton magnetic resonance spectroscopy. The method involves comparing the integration of the aromatic doublets of tolbutamide at 7.33-7.8 ppm with that of the singlet of hexamethylenetetramine (internal standard) at 4.61 ppm using dimethylsulfoxide as solvent. Recovery is  $100\% \pm 1.5$  and  $99.6\% \pm 1.4$  for pure tolbutamide and its tablet dosage form respectively.

### 2.2.4 Mass Spectrometry

- a. Tolbutamide and its metabolites in plasma and urine were determined using chemical ionization mass spectrometry. The drugs and its metabolites were extracted with ethyl ether from samples of plasma and urine to which  $^2\text{H}$ -labeled internal standards have been added. The extract was then treated with excess

diazomethane. Quantitative measurements were made on protonated molecular ion (which is free from interference), with use of isobutane as reagent gas, by single ion monitoring for determination of single component or in scan mode for multi-component mixtures. Levels down to 0.5  $\mu\text{g ml}$  in plasma can be determined by this method (10).

- b. A high resolution mass spectroscopic analysis of methylated tolbutamide derivatives was reported by Sabih (11). The methyl derivative of tolbutamide obtained by treatment with dimethyl sulphate was subjected to g.l.c. on a stainless steel column (6 ft. X 0.125 in) packed with 1% OV-1 on Gas-Chrom Q, temperature programmed from 150 to 190 $^{\circ}$  at 2 $^{\circ}$  per minute and operated with helium as carrier gas (30 ml per minute). A mass spectrum of the column effluent was obtained at 70 eV with a double-focussing instrument linked to a digital data system.
- c. Braselton et al (12) determined tolbutamide and its metabolites in human serum by the formation of thermally stable derivatives (N-methyl-N $^{-}$ -trifluoroacetyl) and analyzing this derivatives by g.l.c.-m.s. method. The g.l.c. was performed on a glass column (1.5 m X 2 mm) operated at 170 $^{\circ}$  with helium as carrier gas. The g.l.c.-m.s. system was coupled with PDP-8 computer for data processing, a detection ion limit of 1 p mol is claimed.

## 2.3 Chromatographic Methods

### 2.3.1 Gas Chromatographic

Gas chromatography has frequently been used for the analysis to tolbutamide. The type of column, carrier gas and other experimental conditions are listed in Table II.

### 2.3.2 Liquid Chromatography

Tolbutamide, among other sulphonylureas, was determined in pharmaceutical products by high-speed liquid chromatography. The column used



Table II

## Gas Liquid Chromatography of Tolbutamide.

Drug Source	Phase and Column	Carrier gas	Temperature (°C)	Derivative used	Detector	Internal Standard	Ref.
Serum	1% SP-2100 on supelcoport (100-120 mesh). Glass Column (1.8 m X 2 mm).	nitrogen (30 ml min <sup>-1</sup> )	165	N-Methyl-N-trifluoroacetyl.	FID*	Chlorpropamide.	(12)
Plasma	3% QV-17 on Gas-Chrom O (100-120 mesh). Glass column 2.1 m X 0.2 mm.	nitrogen	220	methyl	EC**	Hexadocosane	(13)
Serum	3% OV-17 on Chromosorb W-HP (100-120 mesh), shaped glass column (6 ft. X 0.25 in).	nitrogen (50 ml/min <sup>-1</sup> )	190	methyl	FID	4-chloro-N-propylbenzene sulphonamide.	(14)
Plasma & urine	10% W-98 on Chromosorb W-HP (80-100 mesh), stainless steel column (6 ft. X 0.125 in).	helium (30 ml/min <sup>-1</sup> )	150	methyl	FID	Chlorpropamide.	(15)
Plasma	3.8% UC W-98 on diatoport S (80-100 mesh) glass column (4 ft. X 0.25 in).	nitrogen (25 ml/min <sup>-1</sup> )	220	methyl	FID	Chlorpropamide.	(16)

\* Flame Ionization Detector

\*\* Electron Capture

(100 cm X 2.1 mm) was packed with 1% ethylene-propene copolymer on Zipax with a mobile phase of 0.01 M - disodium hydrogen citrate containing 15% of methanol (pH 4.4). Detection at 254 nm was used and peak area were integrated. The procedure was applied to compressed tablets. (17).

### 2.3.3 High-Performance Liquid Chromatography

Several high-performance liquid chromatographic methods for the quantitation of tolbutamide with or without its metabolites in body fluids have been reported. The chromatographic systems investigated for the analysis of this drug are presented in Table III.

## 3. Pharmacokinetics

Pond et al (23) reported that the rate of metabolism of tolbutamide was decreased by chronic administration of certain drugs, they claimed that the tolbutamide half-life was increased by chronic administration of sulphaphenazole (9.5 hrs to 28.6 hrs), phenylbutazone (7.9 hrs to 23.1 hrs), and oxyphenbutazone (8.1 hrs to 30.2 hrs). The rate of elimination of tolbutamide was decreased within one to two hours after a single dose of sulphaphenazole and the half-life was increased from 9.2 hrs to 25.7 hrs. In contrast, phenylbutazone and oxyphenbutazone, administered as a single dose 800 mg have no immediate effect on tolbutamide elimination. It is suggested that phenylbutazone and oxyphenbutazone act by inducing a form of a cytochrome P-450 with low activity for tolbutamide hydroxylation, whereare, sulphaphenazole acts by direct inhibition of the microsomal mixed function oxidase system.

Another group of investigators (24) showed that tolbutamide was eliminated faster in patients with hepatitis than in those without liver disease.

During cholestasis, the metabolism of tolbutamide is greatly altered, thus, tolbutamide (100 mg/kg) injected i.v. into rats with experimental cholestasis was eliminated more rapidly than in control animals, this increase was owing to the increase in liver weight and microsomal protein (25). In patients with recurrent intrahepatic cholestasis no differences in tolbutamide metabolism were noted as compared to the control group. In cholestatic hepatitis the plasma half-life of tolbutamide appeared unchanged. The

Table III

## HPLC of Tolbutamide.

Drug Source	Column	Mobile Phase	Internal Standard	Detector	Remarks	Ref.
Plasma	$\mu$ Bondapack C <sub>18</sub>	Acetonitril: 0.05 M H <sub>3</sub> PO <sub>4</sub> buffer pH 3.9 (7:13) 2.5 ml min <sup>-1</sup> .	Chlorpropa- mide	254 nm	Calibration curve was rectilinear for 2-80 $\mu\text{g ml}^{-1}$	(18)
Serum and plasma	Silica beads coated with $\mu$ bondapack C <sub>18</sub> .	1% acetic acid (adjusted to pH 5.5) : acetonitril (18:7) 2.2 ml min <sup>-1</sup>	3-isopentyl 1-(toluene p-sulpho- nyl) urea.	254 nm	The limit of detection is is 6 mg l <sup>-1</sup> and recovery is 95%.	(19)
Plasma	$\mu$ bondapack C <sub>18</sub>	Acetonitril: 0.05% H <sub>3</sub> PO <sub>4</sub> (9:11). 1.5 ml min <sup>-1</sup> .	-	200 nm	The calibration graphs cover the pages of 5-300 $\mu\text{g ml}^{-1}$	(20)
Plasma	ODS-Sil-X-1	22% aqueous aceto- nitril.	Chlorpropa- mide.	223 nm	The carlibration graphs rectilinear for 25-500 mg l <sup>-1</sup> .	(21)
Tablets	Li Chrosorb Si 60	0.06% acetic acid in ethanol: tetrahydro- furan: hexane 1:2:22	Prednisone	254 nm	The results of undegraded tablets agreed within 0.3% with results by U.S.P.	(22)

most striking variations were observed in the patients with extra hepatic biliary obstruction, in such cases, the metabolism of tolbutamide was accelerated as evidence by a significant decrease of plasma half-life (165 minutes versus 384 of the control. However, the metabolism of tolbutamide in vitro did not show any difference between normal and cholestatic liver. Preliminary results in vitro suggested that the bile salt could displace tolbutamide from albumin binding thus increasing the amount of the free drug available for biotransformation by the liver. (26)

Darby et al (27) have reported that there is no significant correlations were found between the hepatic microsomal kinetic constants for  $^{14}\text{C}$ -tolbutamide metabolism in vitro and the plasma half-lives and plasma clearance of the drug administered orally.

#### 4. Identification

The B.P. (1980) (28) describes the following identification test for tolbutamide:

- A. Dissolve 25 mg in sufficient methanol to produce 100 ml. The light absorption, in the range 220 to 350 nm, exhibits a band at about 228 nm and maxima at 258 nm, 263 nm and 275 nm. The diluted solution of tolbutamide in methanol (0.001% w/v) exhibits a maximum only at 228 nm; A (1%, 1 cm) at 228 nm, about 490.

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# RESERPINE

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1. Description	738
1.1 Nomenclature	738
1.2 Formulae	738
1.3 Molecular Weight	740
1.4 Elemental Composition	740
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## 1. Description

### 1.1 Nomenclature

#### 1.1.1 Chemical Names

- (a) 11, 17  $\alpha$ -Dimethoxy-18  $\beta$ -[(3,4,5 - trimethoxybenzoyl) oxy]-3  $\beta$ , 20  $\alpha$ -yohimban-16  $\beta$ -carboxylic acid methyl ester.
- (b) Yohimban-16-carboxylic acid, 11, 17-dimethoxy-18-[(3,4,5-trimethoxybenzoyl) oxy]-, methylester, (3 $\beta$ ,16 $\beta$ ,17 $\alpha$ ,18 $\beta$ ,20 $\alpha$ ).
- (c) Methyl 18  $\beta$ -hydroxy-11,17  $\alpha$ -dimethoxy-3 $\beta$ , 20 $\alpha$ -yohimban-16  $\beta$ -carboxylate 3,4,5-trimethoxybenzoate (ester).
- (d) 3,4,5-trimethoxybenzoyl methyl reserpate.
- (e) Methyl 18-O-(3,4,5-trimethoxybenzoyl) reserpate.

#### 1.1.2 Generic Names

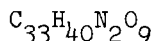
Reserpine

#### 1.1.3 Trade Names

Austrapine; Bioserpine; Crystoserpine; Eskaserp; Hiserpia; Orticalm; Quiescin; Rau-sed; Reserpex; Reserpoid; Rivasin; Roxinoid; Sandril; Sedaraupin; Serfin; Serolfia; Serpanary; Serpasil, Serpasol; Serpate; Serpen; Serpine; Serpiloid.

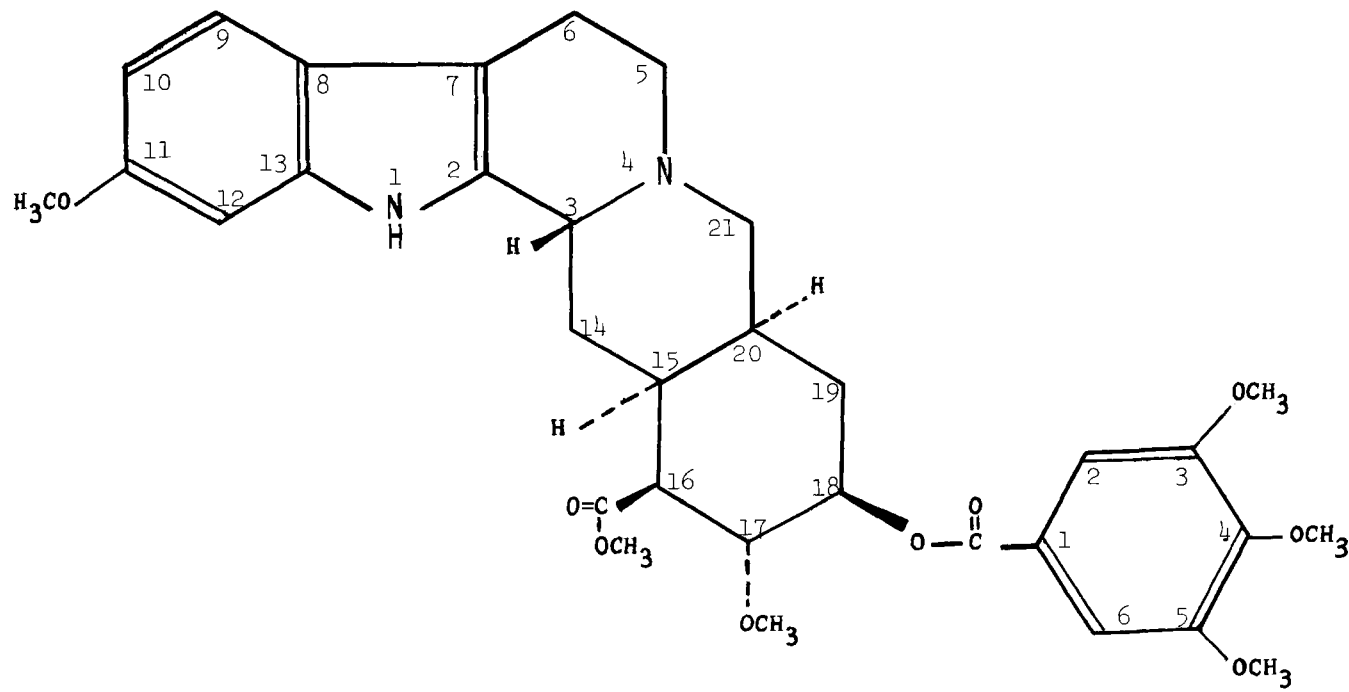
## 1.2 Formulae

### 1.2.1 Empirical



### 1.2.2 Structural

The structure (facing page) was confirmed by the total synthesis of Reserpine (1-3).



Reserpine

1.2.3 CAS Registry No.

[50-55-5]

1.2.4 Wiswesser Line Notation

T F6 D5 C666 E M ON  
 && TTTJ\_HO1 SOVR C  
 O1 DO1 EO1 &\_TO1 UV01 (4)

1.3 Molecular Weight

608.70

1.4 Elemental Composition

C, 65.12%; H, 6.62%; N, 4.60%; O, 23.66%

1.5 Appearance, Color, Odor and Taste

Long prisms from dilute acetone.  
 White to pale fawn small crystals or crystalline powder, darkening slowly on exposure to light.  
 Odorless and has a bitter taste.

2. Physical Properties2.1 Eutectic Temperature

Acetaminosalol	183°	] hot stage method (5)
Dicyandiamide	208°	
Phenolphthalein	225°	

2.2 Solubility

Very sparingly soluble in water, slightly soluble in ethyl alcohol and methyl alcohol.  
 Soluble at 20° in 6 parts of chloroform, in 90 parts of acetone and in 2000 parts of ethanol.

2.3 Loss on Drying

When dried for 3 hours at 60° at a pressure not exceeding 0.7 kPa (about 5 torr), loses not more than 0.5 per cent of its weight; use 0.5g. (6)

## 2.4 Spectral Properties

### 2.4.1 Infrared Spectrum

The IR spectrum of reserpine as KBr-disc was recorded on a Perkin Elmer 580B Infrared Spectrophotometer to which Infrared Data station is attached (Fig. 1).

The structural assignments have been correlated with the following frequencies (Table 1).

Table 1. IR Characteristics of Reserpine

Frequency $\text{cm}^{-1}$	Assignment
3430	N-H stretch of indole ring
2940	C-H stretch
2840	$\text{CH}_3$ , $\text{CH}_2$ -stretch
1732	$\text{O}=\overset{\text{O}}{\text{C}}-\text{CH}_3$
1710	$\text{O}=\overset{\text{O}}{\text{C}}$ -aromatic
1625	C-N stretch, C=C alkene
1588	C=C aromatic
1500	
1455	
1332	$\text{CH}_2$ -bending
1275	C-O-C
1250	
1225	

Other characteristic absorption bands are: 1410, 1188, 1120, 1060, 1030, 1005, 975, 940, 872, 820, 800, 763, 740, 710, 615  $\text{cm}^{-1}$ .

The IR data of reserpine have been also reported by several authors (7-11).

### 2.4.2 $^{13}\text{C}$ -NMR Spectrum

The  $^{13}\text{C}$ -NMR noise decoupled and off resonance spectra are presented in Fig. 2 and Fig. 3 respectively. Both were recorded over 4000 Hz range in deuterated chloroform on a Varian FT80 A-80 MHz spectrometer, using 10 mm. sample tube and tetramethyl silane as a reference standard at 22°.

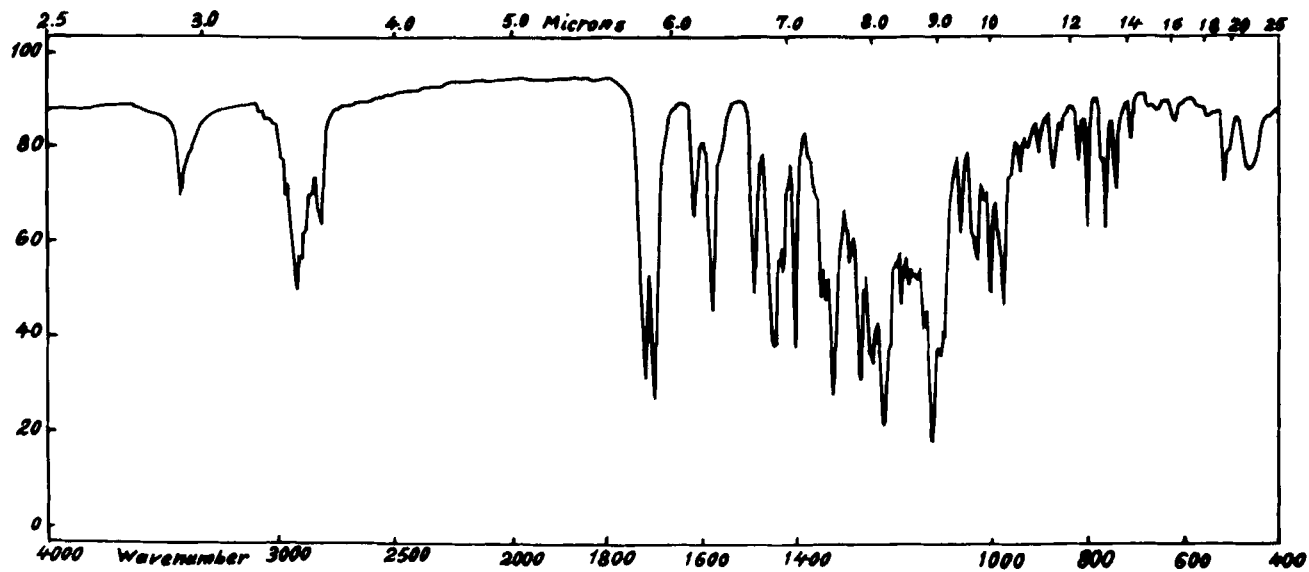


Fig. 1. The IR spectrum of reserpine as  $\text{KBr}_R$ - disc.

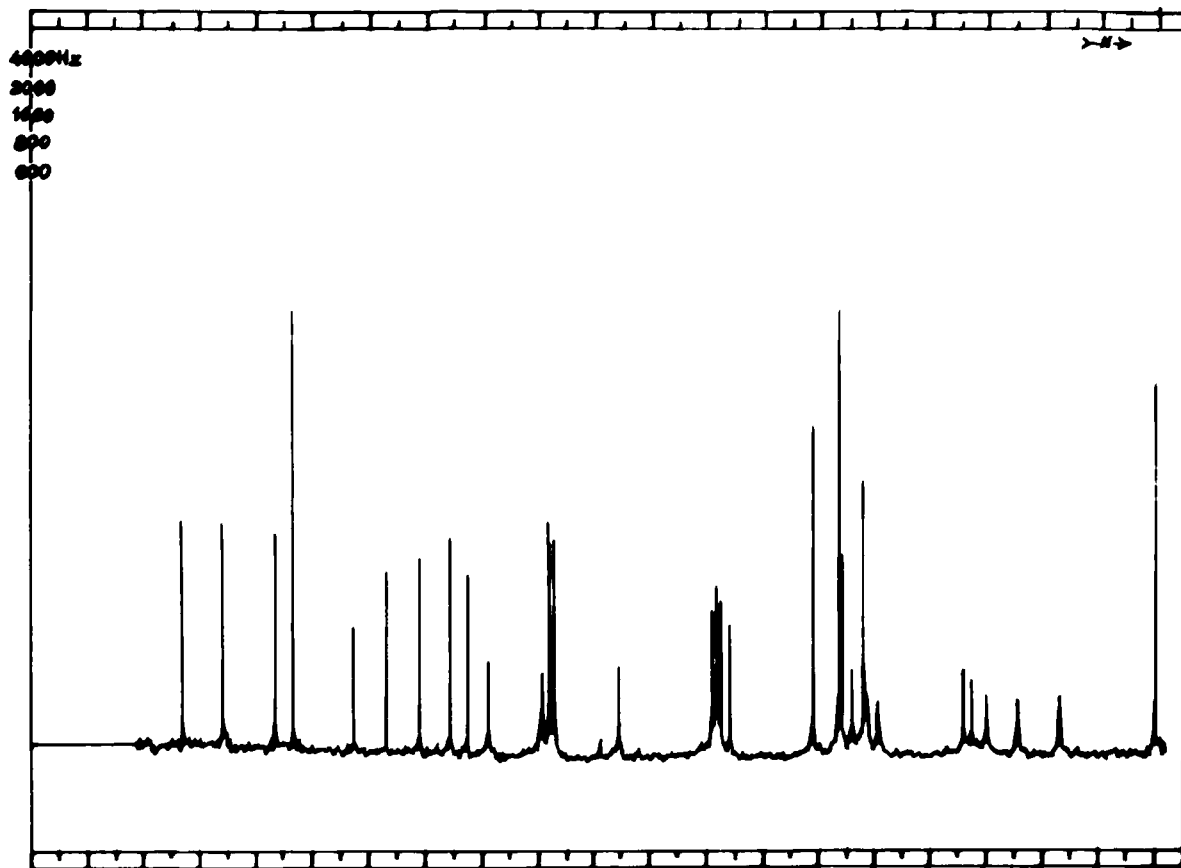


Fig. 2. The  $^{13}\text{C}$ -NMR Noise Decoupled Spectrum of Reserpine

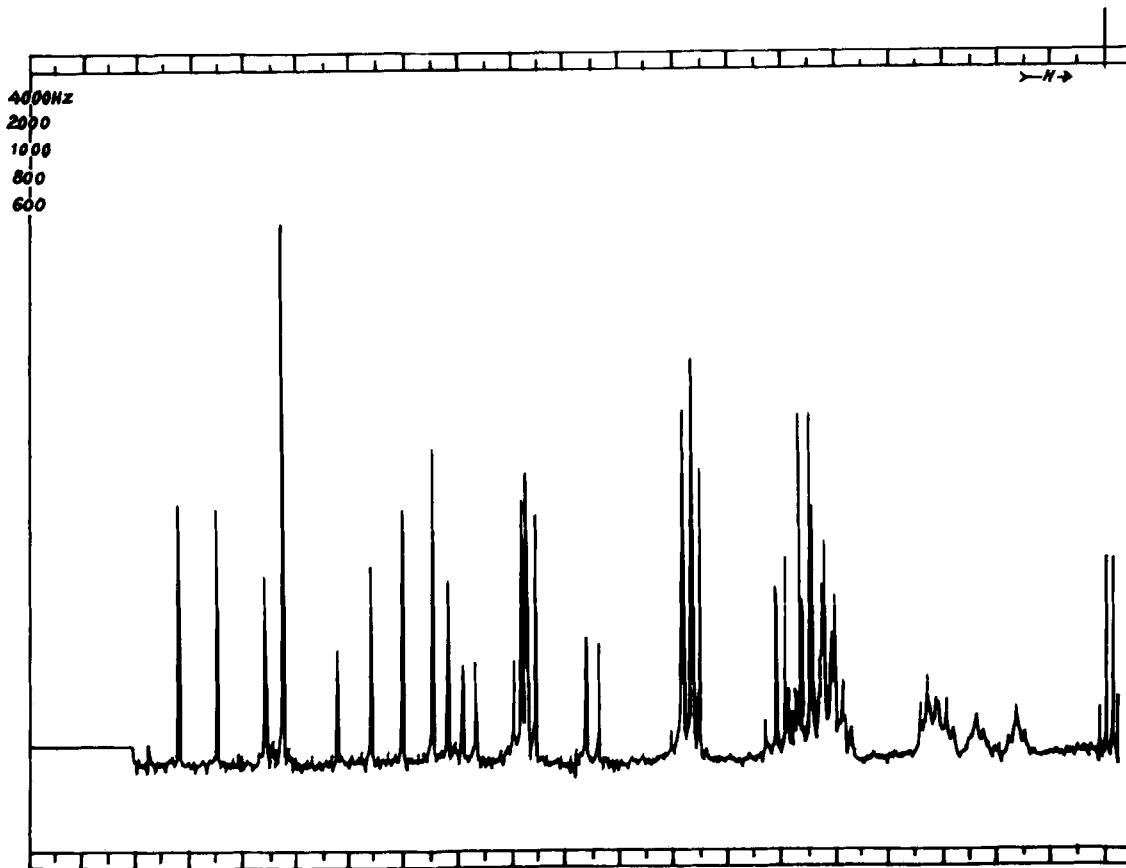


Fig. 3.  $^{13}\text{C}$ -NMR off resonance spectrum of reserpine.

The carbon chemical shifts are assigned on the bases of the additivity principals and off resonance splitting pattern (Table 2).

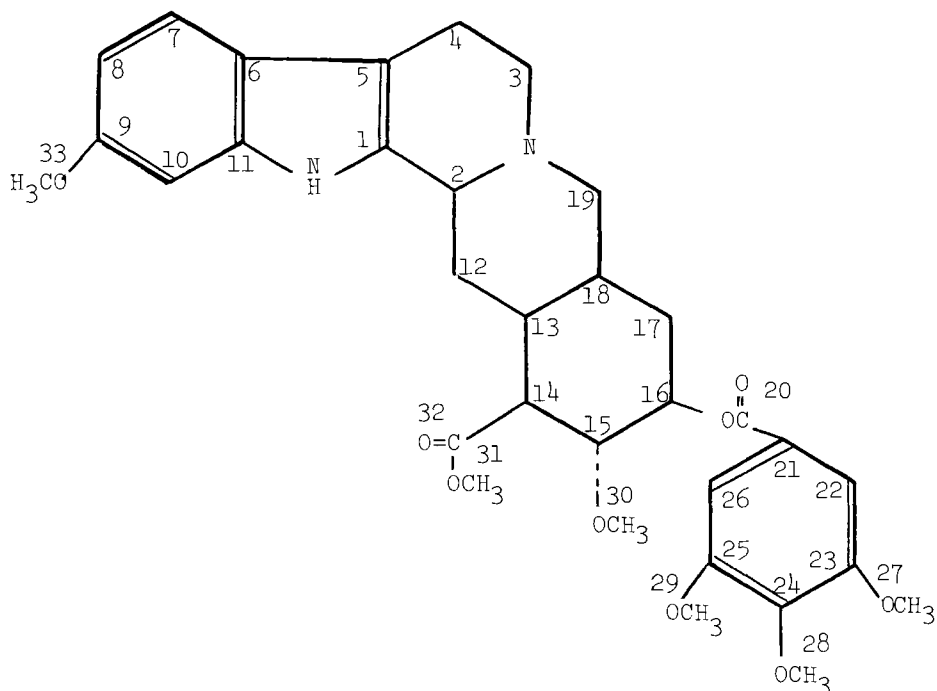


Table 2. Carbon Chemical Shifts of Reserpine

Carbon no.	Chemical shift [ppm]	Carbon no.	Chemical shift [ppm]
C <sub>32</sub>	172.84(s)	C <sub>30</sub>	60.86(q)
C <sub>20</sub>	165.56(s)	C <sub>27</sub> ,C <sub>29</sub>	56.24(q)
C <sub>9</sub>	156.09(s)	C <sub>28</sub> ,C <sub>33</sub>	55.67(q)
C <sub>23</sub> ,C <sub>25</sub>	153.02(s)	C <sub>2</sub>	53.87(d)
C <sub>24</sub>	142.36(s)	C <sub>14</sub>	51.80(d)
C <sub>11</sub>	136.50(s)	C <sub>31</sub>	51.80(q)
C <sub>1</sub>	130.61(s)	C <sub>3</sub>	51.39(t)

(Continued)



Table 2 (continued)

C <sub>21</sub>	125.38(s)	C <sub>19</sub>	49.01(t)
C <sub>6</sub>	122.22(s)	C <sub>18</sub>	33.99(d)
C <sub>7</sub>	118.49(d)	C <sub>13</sub>	32.36(d)
C <sub>8</sub>	108.89(d)	C <sub>17</sub>	29.85(t)
C <sub>5</sub>	107.76(s)	C <sub>12</sub>	24.31(t)
C <sub>22</sub> , C <sub>26</sub>	106.89(d)	C <sub>4</sub>	16.84(t)
C <sub>10</sub>	95.33(d)		
C <sub>15</sub>	] 78.88(d)		
C <sub>16</sub>		78.06(d)	

s = singlet; d = doublet ; t = triplet;  
q = quartet.

Other <sup>13</sup>C-NMR data for reserpine have been also reported (12-14).

#### 2.4.3 Mass Spectrum

The mass spectrum of reserpine is presented in Fig. 4. This was obtained by electron impact ionization and run on a Varian MAT 311 by direct inlet probe at 180°C. The spectrum was recorded with the aid of the Incos data system. The electron energy was 70 eV and the accelerating voltage was 2.4 KV.

The spectrum (Fig. 4) shows a molecular ion peak M<sup>+</sup> at m/e 608 with a relative intensity 100%.

The most prominent fragments, their relative intensities and some proposed ion fragments are given in table 3.

Table 3. Mass fragments of reserpine

m/e	relative intensity %	Ions
608	100	M <sup>+</sup>
609	36.0	[M+H] <sup>+</sup>
607	45.3	[M-H] <sup>+</sup>

(Continued)

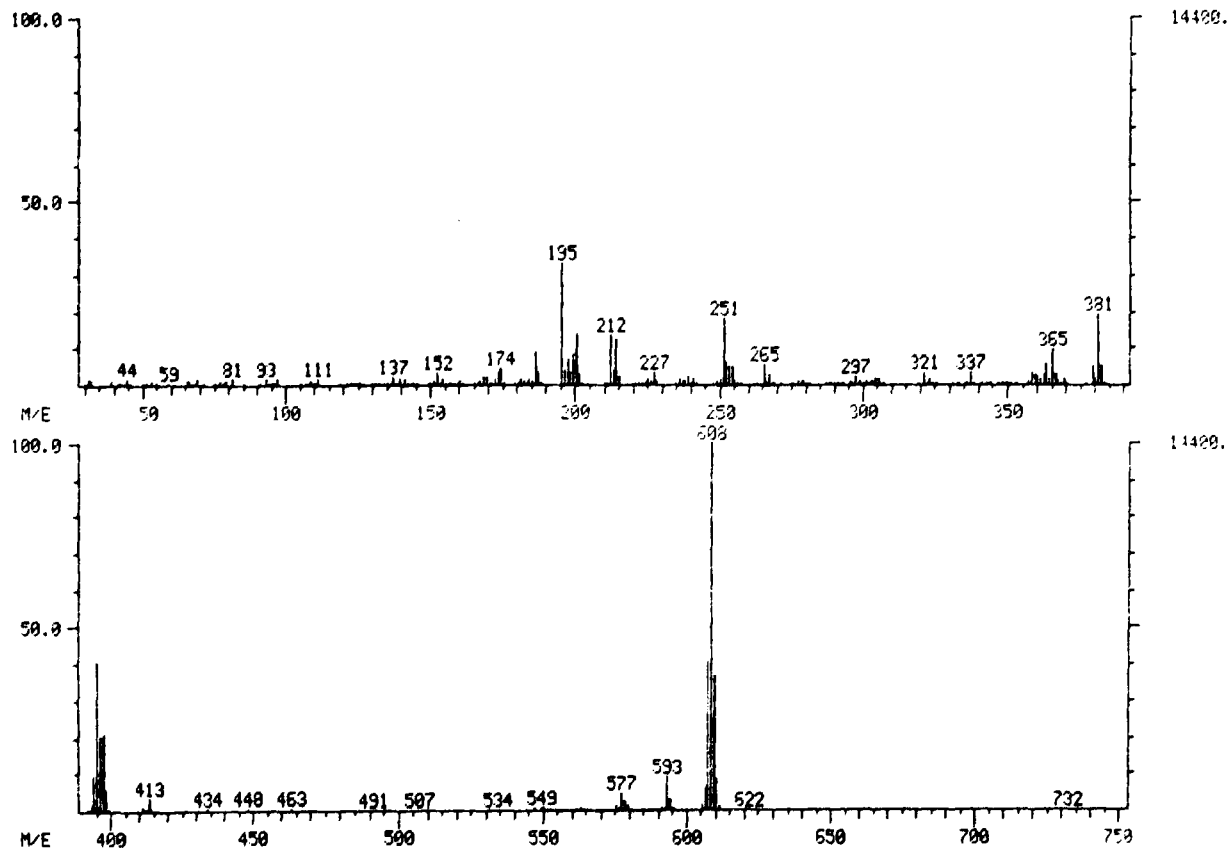
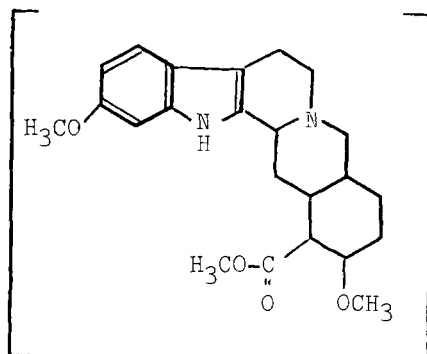


Fig. 4. The mass spectrum of reserpine.

398

21.3



397

20.0

-

396

41.3

-

381

20.0

-

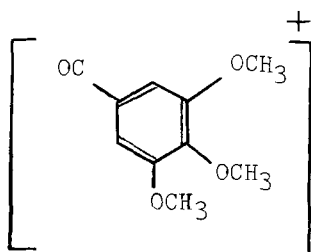
251

18.7

-

195

33.3



Other mass spectral data for reserpine have been also reported (4,15,16).

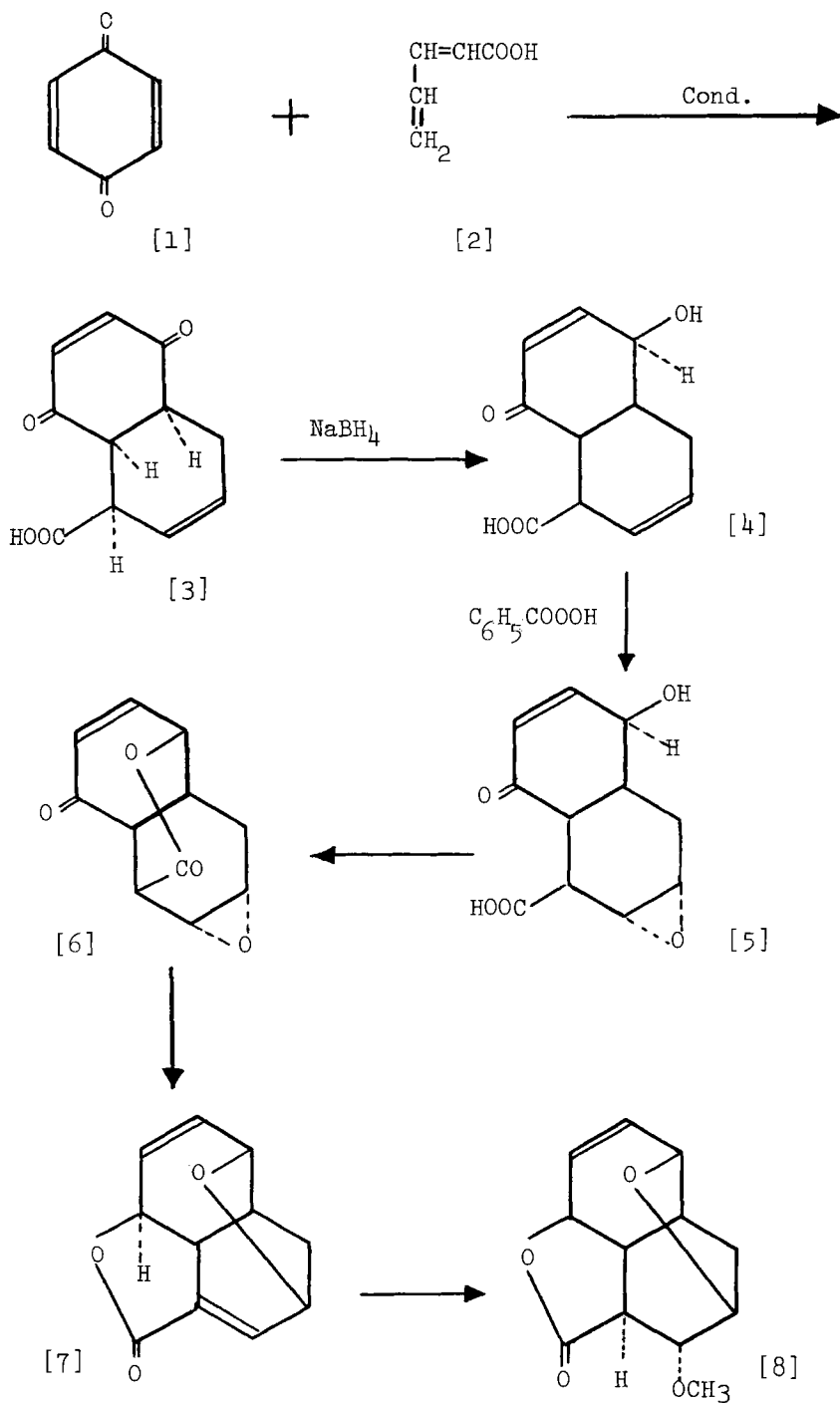
### 3. The Total Synthesis of Reserpine

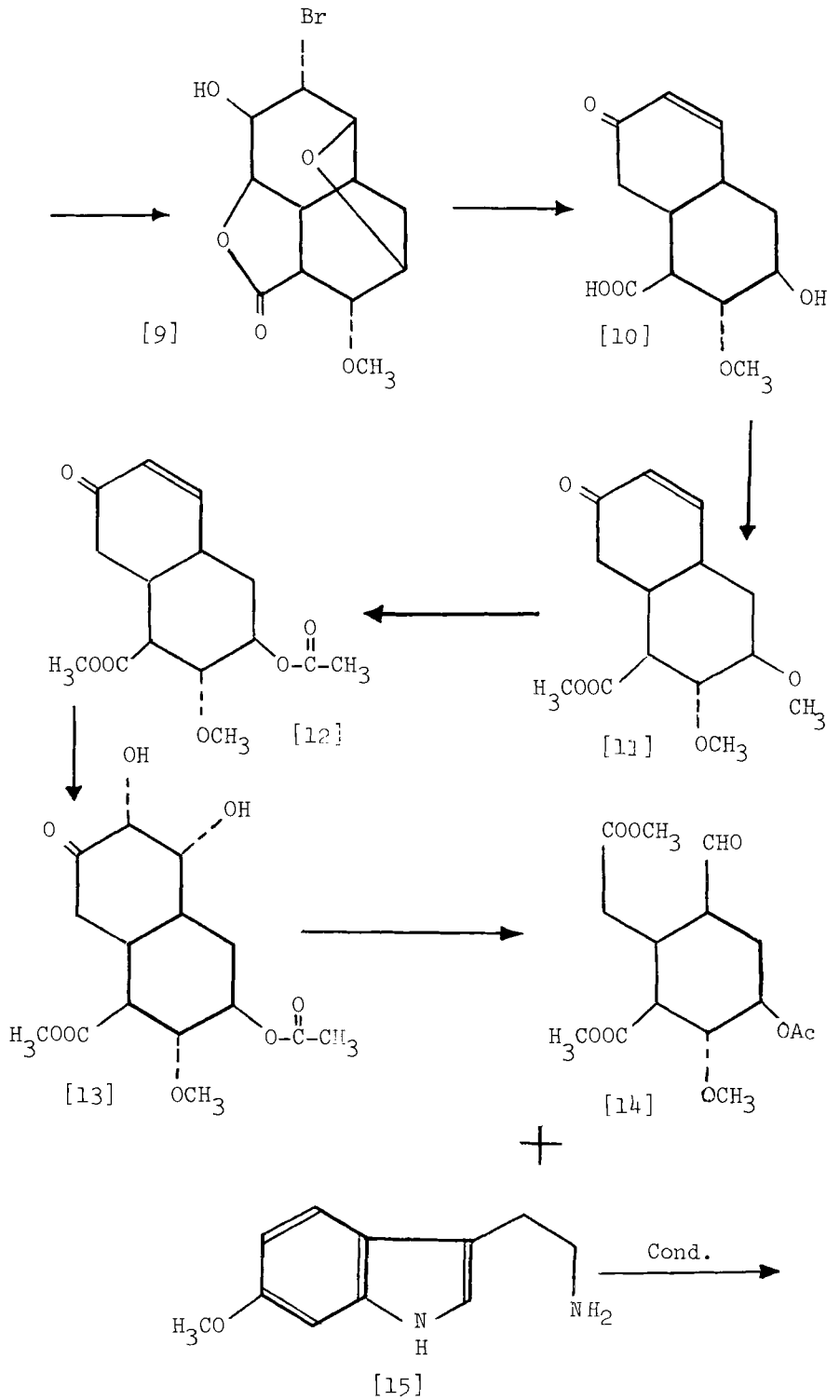
The total synthesis of reserpine was achieved by Woodward et al. (1-3) in 1956. The remarkable Woodward's synthesis of reserpine is summarised as follows:-

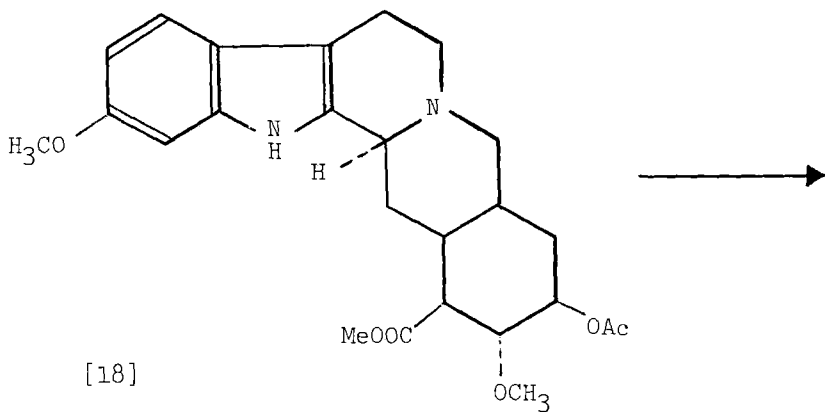
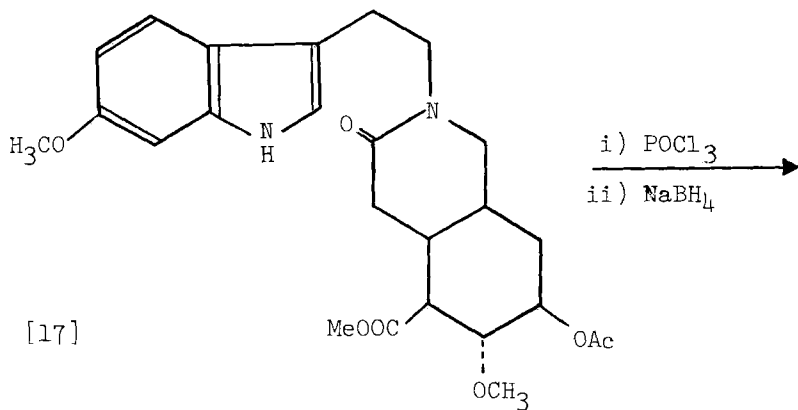
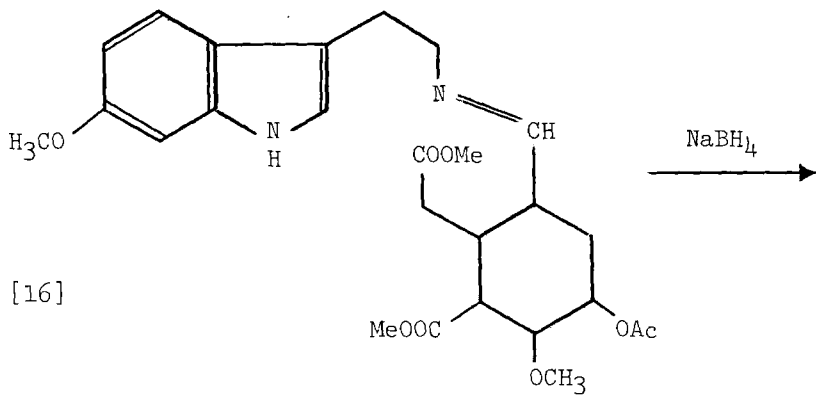
p-Benzoquinone [1] is condensed with vinylacrylic acid [2] to give the adduct [3] which is reduced by sodium borohydride to the alcohol [4]. This is oxidised by perbenzoic acid in benzene-dioxane to the oxide [5]. The corresponding lactone [6] obtained from [5] by the action of acetic anhydride and sodium acetate in benzene. [6] is transformed by aluminium isopropoxide in hot isopropyl alcohol into the ether [7]. This by the action of sodium methoxide in methanol gives the methoxy-ether [8]. [8] is treated with N-bromosuccinimide in warm aqueous solution in the presence of sulfuric acid to give the bromohydrine [9] which is oxidized by chromium trioxide in acetic acid to the corresponding ketone, and this is transformed by short treatment with zinc in cold glacial acetic acid to the hydroxy-acid [10]. The latter is treated with diazomethane in dioxane to give the methyl ester [11]. This is converted to the acetate [12] by acetic anhydride in pyridine and then to the diol [13] upon treatment with aqueous osmium tetroxide, followed by potassium chlorate. The diol [13] is esterified with diazomethane to give the methylester [14]. Condensation of [14] with 6-methoxytryptamine [15] in benzene to give the condensate [16] which is reduced with sodium borohydride in methanol to give the lactam [17]. This is cyclized with phosphorous oxychloride and reduced with sodium borohydride to dl-methyl-O-acetyl-isoreserpate [18]. Upon saponification and treatment with hydrochloric acid and warmed with N-N-dicyclohexylcarbodiimide in pyridine [18] is transformed into isoreserpic acid lactone [19]. This is isomerized with pivalic acid in xylene to give the more stable reserpic acid lactone [20]. Metholysis is followed to give methyl reserpate [21] which is condensed with 3,4,5-trimethoxybenzoylchloride [22] in pyridine to give dl-reserpine [23]. This is readily resolved via the highly crystalline l-reserpine d-camphor-10-sulfonate to give l-reserpine, identical in respects with natural reserpine.

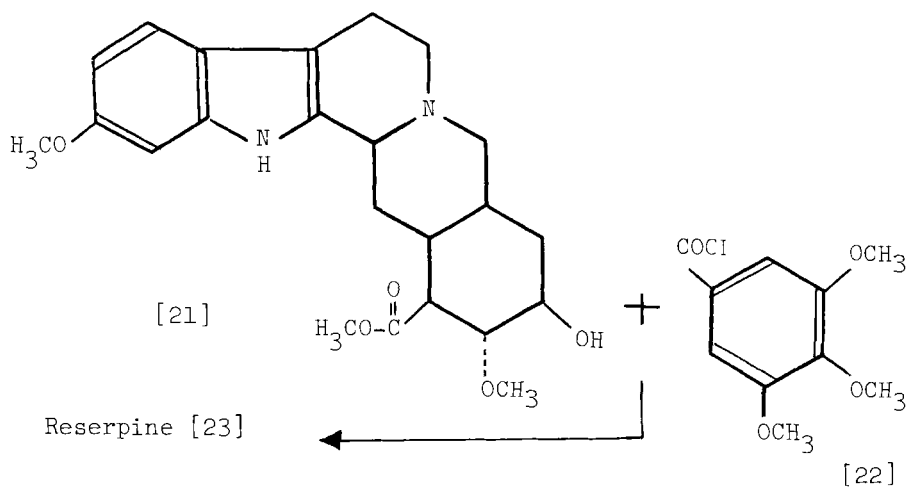
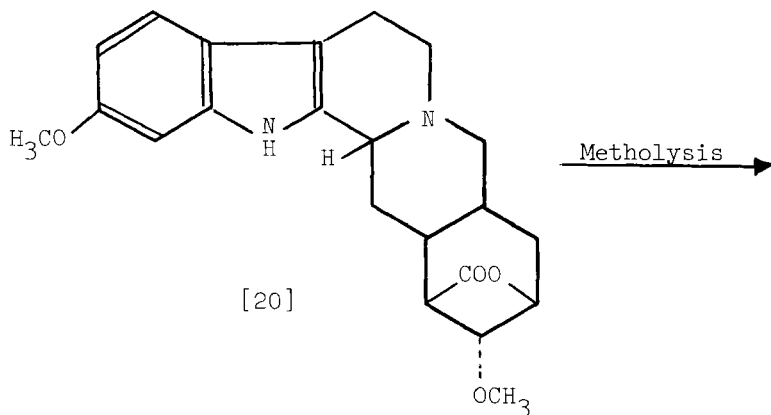
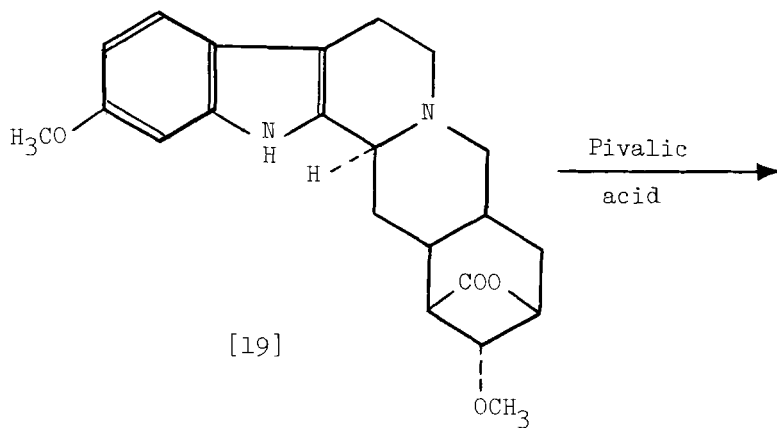
The total synthesis of reserpine is presented in scheme I.

A simplified method for the synthesis of reserpine has been patented (17).

Scheme I: Total Synthesis of Reserpine









#### 4. Biosynthesis of Reserpine

The biosynthetic pathways of reserpine and other alkaloids of *Rauwolfia* have been extensively studied by several authors. Leete (18, 19) fed DL-[2-<sup>14</sup>C]-tryptophan into *Rauwolfia serpentina* (3 years old plants) which led to the formation of radioactive ajmaline, serpentine and reserpine (18). Later, he found that radioactive serpentine was labeled solely at C-5 indicating that tryptophan was a direct precursor of the  $\beta$ -carboline moiety of this alkaloid (19). Other radioactive precursors have been administered to *Rauwolfia* plants such as [1-<sup>14</sup>C] acetate (20), [2-<sup>14</sup>C] acetate (21), [2-<sup>14</sup>C] alanine (20) and [2-<sup>14</sup>C] glycine (21, 22). All incorporated into ajmaline and reserpine.

Thomas (23) predicted that the non-tryptamine moiety of the indole alkaloids is derived from a cyclopentanoid monoterpene precursor. Wenkert (24) independently reached to the same conclusion.

Battersby and Co-workers (25-28), Money et al (29) and Leete et al (30) all reported the specific incorporation of [2-<sup>14</sup>C] mevalonic acid and [2-<sup>14</sup>C] geraniol into representative examples of the corynanthe, aspidosperma and iboga groups of alkaloids, and in each case the distribution of radioactivity was in full agreement with the monoterpene hypothesis developed by Thomas (23) and Wenkert (24), and according to the biogenetic isoprene rule that monoterpene are formed in nature by suitable modification of geranyl pyrophosphate (31).

The Biosynthetic pathway of reserpine is presented in scheme II.



## 5. Drug Metabolism and Pharmacokinetics

Reserpine is absorbed rapidly following oral administration. Maximum blood concentrations are reached in approximately 2 hours with reported values of 0.14 to 0.18 mcg/dl in whole blood and 0.13 to 0.15 mcg/dl in plasma(32).

Reserpine is metabolised by the liver(32-34)with more than 90% excreted as metabolites(33). The major urinary metabolites are methyl reserpate and 3,4,5-trimethoxybenzoic acid. Other metabolites are shown in Scheme 3 (35).

Reserpine has a half-life range of 50 to 100 hours (34). A biological half-life in whole blood of 386 hours and a biological half-life in plasma of 271 hours have been reported(32). Detectable levels of reserpine may be found after 11 days from administration of the drug. Reserpine is not removed either by hemodialysis or by peritoneal dialysis(36).

## 6. Methods of Analysis

### 6.1 Identification tests

The following identification tests are mentioned in the British Pharmacopoeia (6).

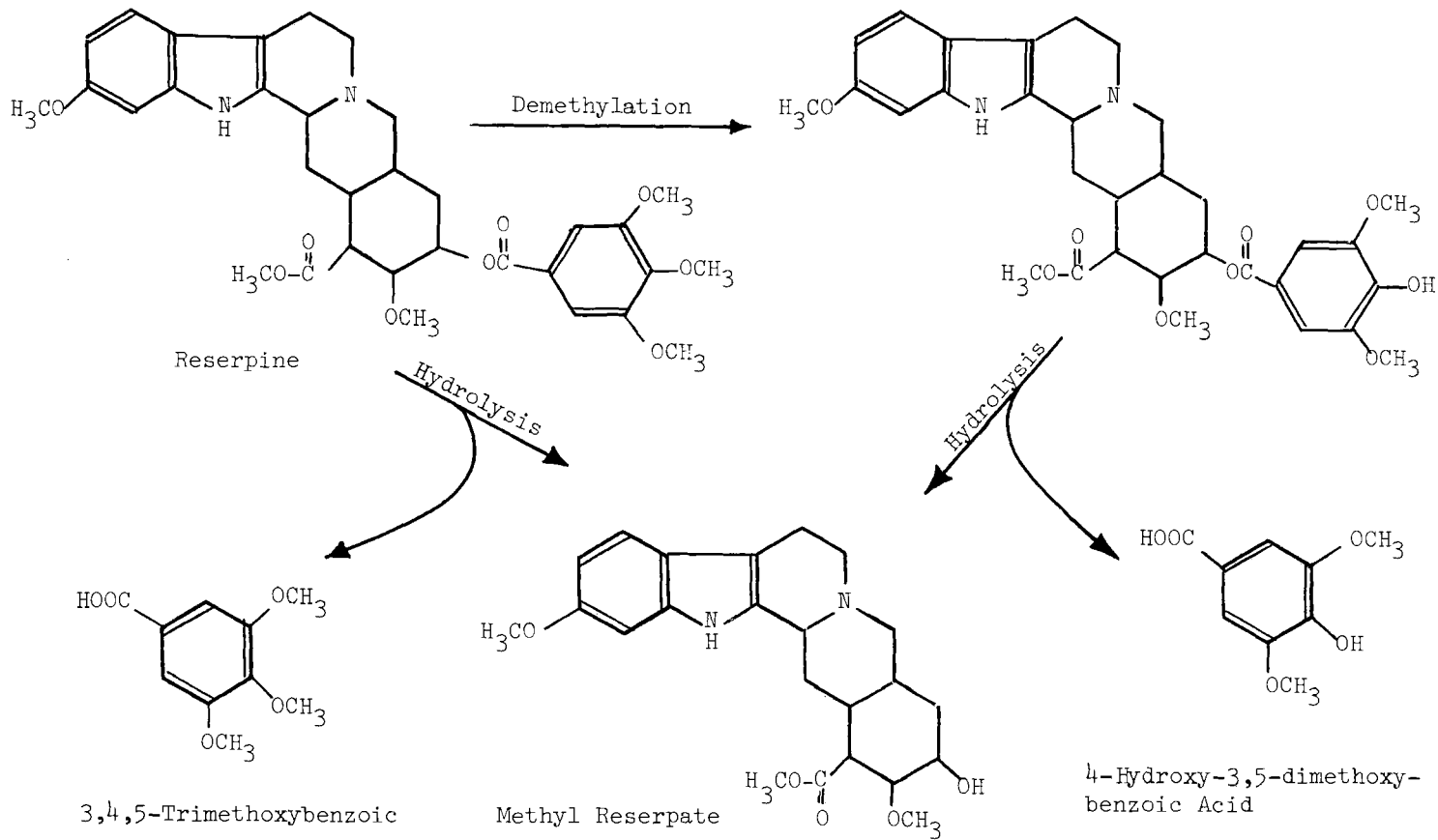
1. 20 Mg of reserpine to be dissolved in 10 ml of chloroform and 1 ml of this solution is diluted to 100 ml with ethanol (96%). The light absorption in the range 230 to 350 nm, exhibits a maximum at 268 nm; absorbance at 268 nm, is about 0.55.

Absorbance over the range 288 nm to 295 nm is about 0.34.

2. Upon the addition of 1 ml of a 0.1% w/v solution of sodium molybdate in sulfuric acid to 1 mg reserpine; a yellow color is produced immediately which changes to blue within two minutes.

3. Upon the addition of 0.2 ml of a freshly prepared 1% w/v solution of vanillin in hydrochloric acid; a rose pink color is produced within two minutes.

4. Upon mixing 0.5 mg of reserpine with 5 mg of 4-dimethylaminobenzaldehyde, 0.2 ml of glacial acetic acid and 0.2 ml of sulfuric acid; a green color is produced. Upon the addition of 1 ml glacial acetic acid; the color changes to red.



Scheme III: Metabolism of Reserpine

Other identification tests (7) are as follows:

1. Reserpine in ethanol exhibits in the ultra-violet region maxima at 267 nm (E 1%, 1 cm 239) and 294 nm (E 1%, 1 cm 150).
2. When drops of sulfuric acid-formaldehyde are added to reserpine; a grey-green color is produced which changes to brown.
3. When a solution of ammonium vanadate is added to reserpine; a green color is formed.
4. Vitali's test gives with reserpine purple flash color which changes to orange then to brown color.

## 6.2 Microcrystal tests

A solution of 0.1% w/v of reserpine in concentrated hydrochloric acid was used for the following microcrystal tests :-

1. A solution of 1% w/v ammonium thiocyanate was added to the above solution; a stellate like crystals were formed (Fig. 5) (7,37).
2. A solution of 0.5% w/v potassium cyanide was added to the above reserpine solution; small rosette crystals were formed (Fig. 7) (7).
3. Hager's reagent was added to the above reserpine solution to give irregular blade crystals (Fig. 8) (37).
4. To a solution of reserpine in acetone, a solution of 0.5% w/v potassium cyanide was added to give radiating rod crystals (Fig. 6) (37).

## 6.3 Titrimetric Method

A non-aqueous titration method is described for the determination of some alkaloids including reserpine and their dosage forms using 0.005M chloranilic acid solution in 1,4-dioxane as the titrant. The end point is determined by measuring the change in absorbance of the sample at 535 nm. Quantitative recoveries with good reproducibility are reported for reserpine and other alkaloids (38).

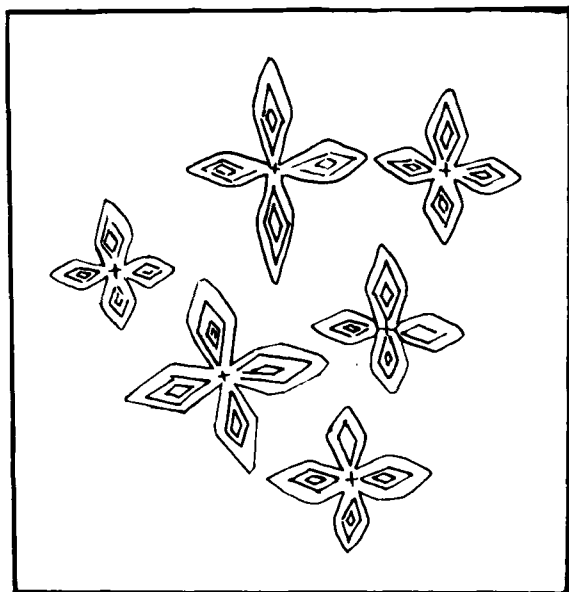


Fig. 5. Microcrystals of reserpine with ammonium thiocyanate.

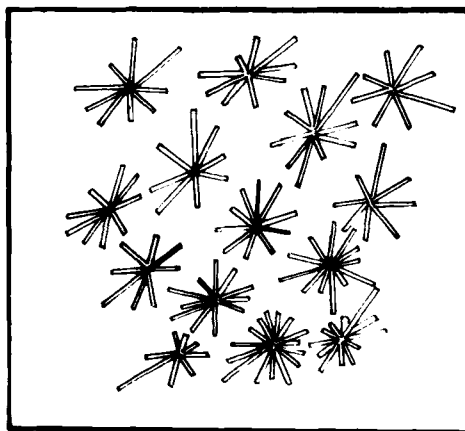


Fig. 6. Microcrystals of reserpine in acetone with potassium cyanide.

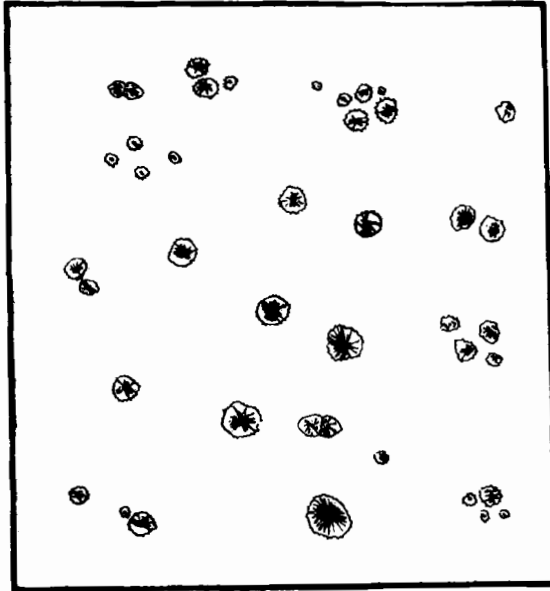


Fig. 7. Microcrystals of reserpine with potassium cyanide.

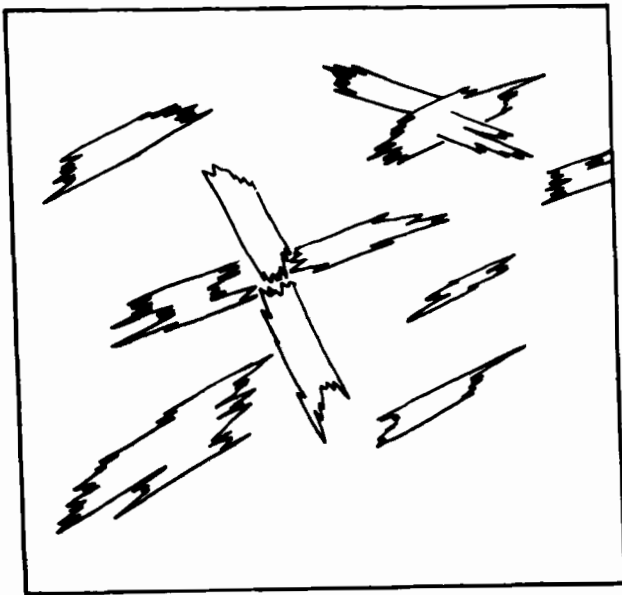


Fig. 8. Microcrystals of reserpine with Hager's reagent.

## 7. High Performance Liquid Chromatography (HPLC)

Reserpine can be determined in pharmaceutical preparations by HPLC(39) as follows:-

Reserpine is extracted from powdered tablets with water saturated with ethyl acetate, after addition of a solution of propiophenone in ethyl acetate saturated with water (as internal standard). After centrifugation, the ethyl acetate layer is analysed by HPLC on a 10  $\mu$ m Lichrosorb RP-8 column using methanol/0.05M sodium phosphate monobasic mixture (1:1) as the mobile phase. The flow rate is adjusted to 2 ml/minute. Detection is carried out under UV at 254 nm. (Fig. 9).

Another HPLC system to analyse reserpine and hydrochlorothiazide in two-component tablet formulations(40): 0.1-0.2 mg of reserpine and 25-50mg of hydrochlorothiazide in tablet form are shaken with 0.05% polythiazide solution in tetrahydrofuran (10 ml) for 20 minutes, centrifuged for five minutes at 2000 r.p.m. and 7 ml of the supernatant liquid is injected to HPLC using Lichrosorb Si 60 column and a mixture of 77% hexane, 18% isopropyl alcohol, 5% chloroform and 0.01% diethylamine as the mobile phase. The flow rate is adjusted to 1.5 ml minute. Detection is performed under UV at 254 nm.

A third method is described for the quantitative determination of chlorthalidone in pharmaceutical dosage forms containing reserpine(41).

Powdered tablets of chlorthalidone and reserpine are extracted with acetonitrile/water (9:1), centrifuged and the supernatant layer is injected to HPLC using a column of Pellamidon at 35°C and the solvent isopropanol/acetic acid/water/hexane (60:3:1:36) as the mobile phase. Detection is carried out under UV at 254 nm.

Reserpine and other antihypertensive drugs can be analysed by reverse phase HPLC(42) as follows:-

0.5% drug sample in the mobile phase is injected into HPLC fitted with either octadecyltrichlorosilane (C<sub>18</sub>) column or with diphenyldichlorosilane (phenyl) column. Several mobile phases are used. Acetonitrile or absolute methanol mixed with aqueous solutions of 0.1 or 1.0% ammonium acetate, 0.5 or 1% ammonium chloride and 0.2 or 1% ammonium carbonate.

A flow rate of 1.4 ml/min is maintained. Detection is carried using UV detector.



Reserpine in plasma can also be determined by HPLC(43) as follows:-

Equine plasma (2 ml) saturated with aqueous sodium borate (3 ml) and benzene (2 ml) are mixed and centrifuged until phases separated. The benzene layer is collected and evaporated to dryness at 50°C under nitrogen. The reserpine so isolated is oxidised with  $N_2O_5-H_3PO_4$  reagent for 10 minutes. 2 ml is injected to HPLC apparatus fitted with Bondapak  $C_{18}$  column using methanol/aqueous 0.01M sodium heptanesulfonate (13:7) as the mobile phase with flow rate of 2.5 ml/minute. Detection is carried out under fluorescence.

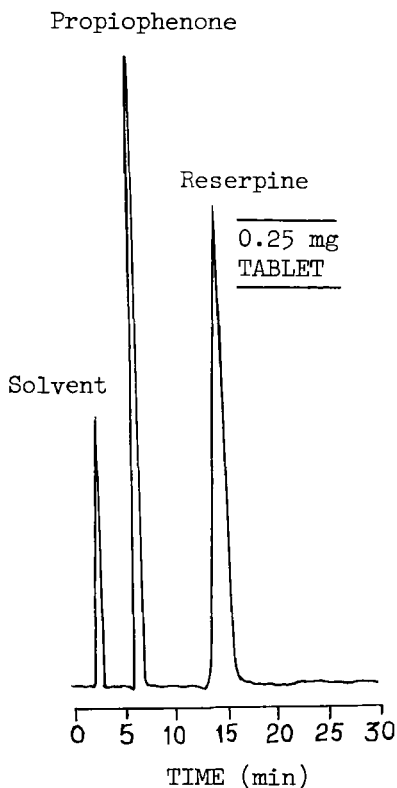


Fig. 9 HPLC of Reserpine in Tablets(39).

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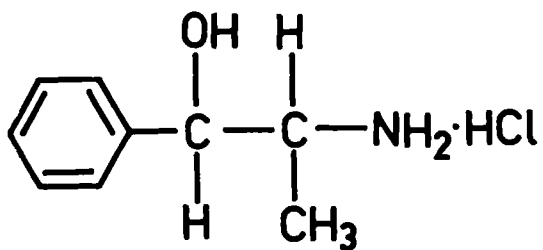
## ERRATUM

### PHENYLPROPANOLAMINE HYDROCHLORIDE

Volume 12, p. 358.

#### 2.1 Name, Formula, Molecular Mass

The structural formula should be replaced by the following:



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# CUMULATIVE INDEX

Bold numerals refer to volume numbers

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