Analytical Profiles

of

Drug Substances

Volume 8

Edited by

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PREFACE

Although the official compendia list tests and limits for drug substances related to identity, purity, and strength, they normally do not provide other physical or chemical data, nor do they list methods of synthesis or pathways of physical or biological degradation and metabolism. For drug substances important enough to be accorded monographs in the official compendia, such supplemental information should also be made readily available. To this end the Pharmaceutical Analysis and Control Section, Academy of Pharmaceutical Sciences, has undertaken a cooperative venture to compile and publish *Analytical Profiles of Drug Substances* in a series of volumes of which this is the seventh.

The concept of analytical profiles is taking hold not only for compendial drugs but, increasingly, in the industrial research laboratories. Analytical profiles are being prepared and periodically updated to provide physicochemical and analytical information of new drug substances during the consecutive stages of research and development. Hopefully, then, in the not too distant future, the publication of an analytical profile will require a minimum of effort whenever a new drug substance is selected for compendial status.

The cooperative spirit of our contributors has made this venture possible. All those who have found the profiles useful are requested to contribute a monograph of their own. The editors stand ready to receive such contributions.

Thanks to the dedicated efforts of Dr. Morton E. Goldberg, a long cherished dream has come to fruition with the publication of *Pharmacological and Biochemical Properties of Drug Substances,* M. E. Goldberg, editor, published by APhA Academy of Pharmaceutical Sciences. This new series supplements the comprehensive description of the physical, chemical, and analytical characteristics of drug substances covered in *Analytical Profiles of Drug Substances* with the equally important description of pharmacological and biochemical properties.

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Drug substances appearing in the new series will be cross-referenced in the cumulative index.

The goal to cover all drug substances with comprehensive monographs is still a distant one. It is up to our perseverance to make it a reality.

Klaus Florey

ASPIRIN

Klaus Florey

1.   Introduction
     1.1 Foreword
          The writing of an analytical profile of
aspirin, this drug of drugs, poses two major
dilemmas. The first is in the name itself. There
are many countries where Aspirin is still a trade-
name of the German firm Bayer AG, and Acetylsali-
cylic Acid is used as the generic. Yet, I decided
to use the former because it is the U.S.P. and B.P.,
the better known worldwide and the more elegant
name.
          Aspirin has now been available for close
to 80 years, and its usefulness and popularity are
undiminished. Consequently, the literature is
voluminous and also undiminished. A complete cover-
age would be beyond the scope of an analytical pro-
file. I have endeavored to cover the newer litera-
ture as comprehensibly as possible and have included
only those older references which I found of histor-
ical interest. To all those who have labored in
the vineyard of aspirin and who go unreferenced in
this profile, I tender my sincere apologies.

     1.2 History
          The documented facts of the discovery of
aspirin are quickly told. It was synthesized by the
German chemist Felix Hoffmann (1868-1946) in the
laboratories of Farbenfabriken Bayer, Elberfeld,
Germany in 1897 (Fig. 1). The compound was tested
pharmacologically by H. Dreser[1] and clinically
among others by Wohlgemuth [2] and Witthauer[3] who
documented the antirheumatic, antipyretic and
analgesic properties free of the undesirable side
effects of salicylic acid.

          Apparently, there was some initial reluc-
tance at Bayer to market the new compound since it
was thought that the field was already crowded with
new drugs. But opposition faded when the new drug
got the support of Carl Duisberg, then the general
manager of Bayer. Duisberg, of course, was the
great chemist and industrialist who built Bayer into
the chemical giant of world renown. After the
inspired trade name Aspirin, a contraction of acetyl
and "spirsäure" (salicylic acid), was coined in the
offices of Bayer - Euspirin was also considered and
fortunately discarded[4] - it was marketed in tablet
form in 1899 and conquered the world.

Figure 1.   Laboratory notebook entry of Felix
            Hoffmann, describing his first prepara-
            tion of aspirin.   The initials CD on the
            page are those of Carl Duisberg.
            (Courtesy of Bayer A.G., Leverkusen,
            Germany)

What motivated Hoffmann to undertake this momentous synthesis? Legend has it that he wanted to help his father who was suffering from rheumatism and who was no longer able to tolerate sodium salicylate, then widely used in rheumatic and arthritic diseases. Salicylic acid occurs naturally in several plants. The analgesic and antipyretic properties of willow bark were already known in antiquity to Hippocrates and the blossoms of spiraea ulmaria (meadow sweet) were used in the middle ages. Salicylic acid was crystallized from willow bark extracts in the early years of the last century, and Kolbe, in 1859, was able to synthesize it from sodium phenolate and carbon dioxide. His student von Heyden worked out a commercially feasible process and started a factory to produce salicylic acid which made possible its widespread use in rheumatic diseases. However, its bad taste, stomach irritation and other side effects were a strong incentive to search for derivatives which retained its efficacy without its disadvantages. Acetylation of the hydroxyl group was one of the logical modifications. Acetylated salicylic acid had already been described three times in the literature (see Section 3). Von Heyden and possibly also Merck, Darmstadt, are reputed to have experimented with aspirin without being able to produce the pure drug.

At the time when Felix Hoffmann prepared pure aspirin successfully in the Bayer laboratories, one of his colleagues was Arthur Eichengrün, who had been hired by Carl Duisberg in 1896, while Hoffmann had been hired in 1894. Eichengrün, as an old man, had to undergo the horrors of the infamous Nazi concentration camp in Theresienstadt which he survived. In 1949, Eichengrün published his memoirs relating to the invention of aspirin[5] which was then a half-century old. He claimed that it was he who told Hoffmann to prepare acetylsalicylic acid. Acetylation certainly was on Eichengrün's mind, since he had also experimented successfully with the acetylation of cellulose about the same time. He went on to fame as the inventor and developer of rayon and safety film. Eichengrün also claimed that another colleague of Bayer, the pharmacologist Dreser, opposed clinical trials. However, the memory of the 82 year-old Eichengrün must have been faulty when he

wrote these rather bitter reminiscences concerning
Hoffmann's, Dreser's and his own role in the discov-
ery of aspirin because, in 1913, Eichengrün wrote a
chapter on "The Pharmaceutical Research Laboratory"
in the book History and Development of Farben-
fabriken Bayer, Vorm. Friedr. Bayer & Co., Elber-
feld by F. Fischer, 1913[4], where he laid no paternity
claim to aspirin and described Dreser's role correct-
ly. The pertinent passage (p. 412) translates as
follows: "Acetylsalicylic acid, prepared by Felix
Hoffmann .... rested unnoticed for 1½ years
among the preparations rejected by the pharmacologi-
cal laboratory until in 1898, during unrelated in-
vestigations, Dreser's attention was again drawn to
it. On account of the observation that the acetyl
compound was increasing cardiac activity in contrast
to salicylic acid itself, he recommended a clinical
trial of the product...."

         Felix Hoffmann did not publish his version
of the discovery, nor did he obtain a German patent,
since the synthesis had been previously described.
Farbenfabriken Bayer did obtain a U.S. Patent[6] in
1900 which named him as the inventor. Chemical
Abstracts reveal no subsequent publications by him,
nor is there any record that he was publicly honored
for his contribution. However, in 1899, he was
appointed director of the pharmaceutical research
and marketing division of Bayer. He retired in
1928[7].

         In many ways, the story of the discovery
of aspirin is typical for the way in which new
drugs are invented and developed in pharmaceutical
research laboratories, where many individuals have
to make a contribution and where it is often diffi-
cult to fathom completely what thought processes,
suggestions and interactions lead to a successful
new drug.

2. Description
    2.1 Name, Formula, Molecular Weight
         Aspirin is acetylsalicylic acid, also
salicylic acid acetate and 2-(acetyloxy)-benzoic
acid (50-78-2). The last name is currently popular
in Chemical Abstracts.

$$\text{structure: benzene ring with COOH and OCOCH}_3\text{ substituents}$$
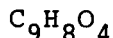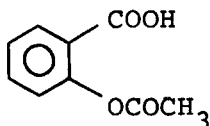
$C_9H_8O_4$ M.W. 180.16

2.2 Appearance, Color, Odor
Aspirin is a white, crystalline powder. It is odorless but might have a faint odor of acetic acid.

3. Synthesis
The first synthesis of aspirin is credited to Gerhardt[8] in 1853. Gerhardt was investigating mixed organic acid anhydrides and, among others, reacted acetylchloride with sodium salicylate. He obtained a solid product, undoubtedly impure acetylsalicylic acid, which immediately and without further characterization he hydrolyzed with aqueous sodium carbonate to salicylic and acetic acids. Next it was prepared by reaction of salicylic acid with acetylchloride by H. von Gilm[9] in 1859, who described a crystalline product. In 1869, K.Kraut[10] had a student, A. Prinzhorn, prepare acetylsalicylic acid by the methods of Gerhardt and von Gilm and obtained an identical product by both methods with a reported melting point of 118.5°. Kraut also correctly observed that the product is not an acid anhydride as assumed by Gerhardt but rather a phenolic ester. Felix Hoffmann[6] used acetic anhydride for its preparation.

$$\text{salicylic acid (benzene with COOH and OH)} \quad + \quad
\begin{array}{c} CH_3COCl \\ \text{or} \\ \dfrac{CH_3CO}{CH_3CO}\!\!>\!\!O \\ \text{or} \\ CH_2=CO \end{array}
\quad \xrightarrow{\text{catalyst}} \quad \text{aspirin (benzene with COOH and OCOCH}_3\text{)}$$
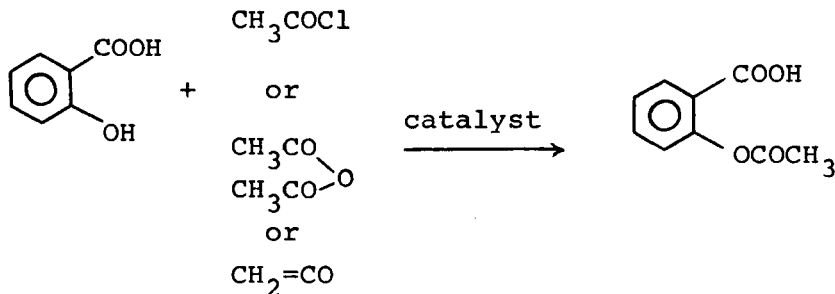
Essentially, all methods of synthesis are variations of the reaction of acetylchloride, acetic anhydride or ketene[11] with salicylic acid using a variety of catalysts such as pyridine[12] or sulfuric acid[13] and reaction conditions (c.f. 14). The preparation of aspirin labeled with a ^{14}C-labeled acetyl group has also been reported.[15] Efforts to improve the commercial processes continue to the present day.

4.   **Physical Properties**
  4.1   **Spectra**
    4.11   **Infrared**
          The assignment of the KBr infrared spectrum (Figure 2) of aspirin (U.S.P. reference standard #0675-F-4) is summarized in Table 1.[16] It agrees essentially with a spectrum published previously.[17] A reflection spectrum has also been presented.[18]

TABLE 1

Infrared Spectrum Interpretation

Wavelength (cm^{-1})	Assignment
2300-2500	carboxyl OH
1760	vinyl ester C=O
1690	aromatic acid C=O
1610 ⎤ 1580 ⎬ 1490 ⎦	aromatic C=C stretch
1220 ⎱ 1190 ⎰	=C-O (acid and ester)
760	ortho subst. phenyl C-H bending

    4.12   **Ultraviolet**
          Aspirin in 0.1N sulfuric acid[19] and in dilute trichloroacetic acid[20] exhibits maxima at 229 nm (E_1^1 484) and 276 nm (E_1^1 65.5). In chloroform a maximum was found at 277 nm (E_1^1 68).[21]

    4.13   **Fluorescence - Phosphorescence**
          The native fluorescence of aspirin, in contrast to salicylic acid, is a weak one and has been studied only recently.[22] Excitation wavelength maximum is at 280 nm and emission maximum is at 335 nm. Maxima for salicylic acid are at 308 and 450 nm respectively.
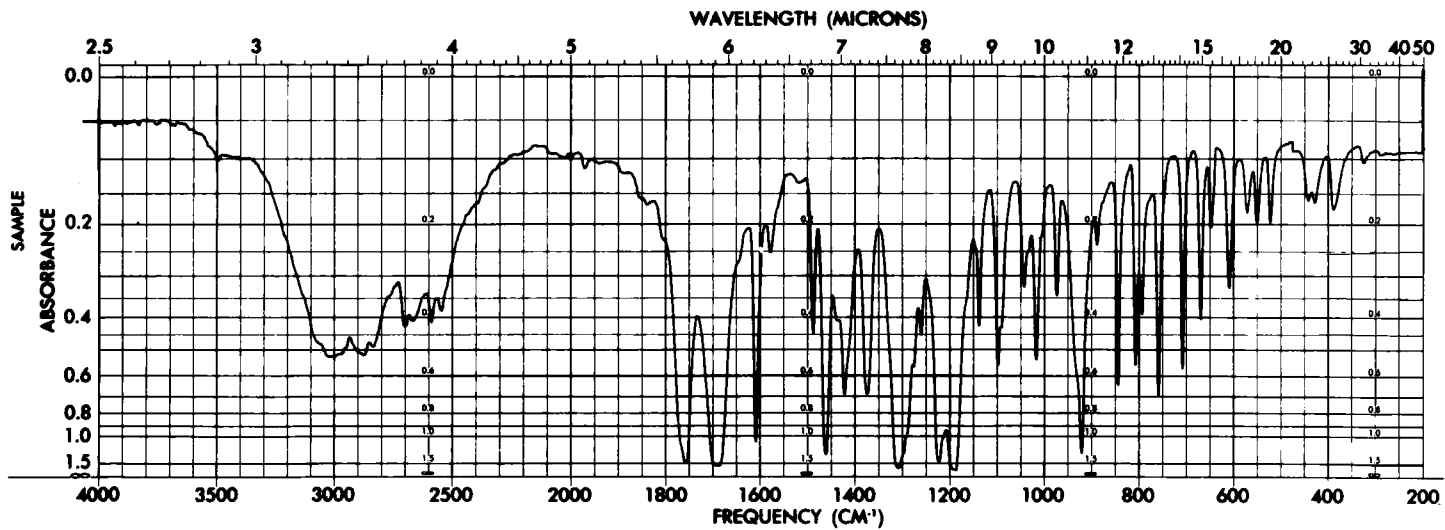
Figure 2. Infrared Spectrum of Aspirin (U.S.P. Reference Standard) KBr pellet. Instrument: Perkin-Elmer Model 621

The phosphorescence emission maximum was found at 410 nm.[23]
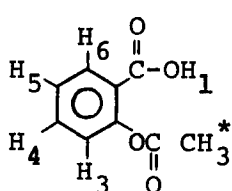
4.14 Raman·
Raman spectra are described and discussed in the following references: 24, 25

4.15 Nuclear Magnetic Resonance
The 60 and 100 MHz proton magnetic resonance spectra of aspirin have been published as part of analytical[26-29] and biochemical studies.[30,31]

The 100 MHz pmr spectrum of a deuterochloroform solution containing tetramethylsilane as an internal reference was obtained on a Varian Associates XL-100-15 spectrometer equipped with a Nicolet pulsed Fourier accessory.[32] (Figure 3.) The rms error for the experimental and calculated spectra shown in Figure 4 is 0.2. The proton assignment is shown below.

	δ	
H_1	12.04	$J_{3,4} = 8.05$Hz
H*	2.34(3H)	$J_{3,5} = 1.34$Hz
H_3	7.13	$J_{3,6} = 0.3$Hz
H_4	7.61	$J_{4,5} = 7.80$Hz
H_5	7.33	$J_{4,6} = 1.74$Hz
H_6	8.11	$J_{5,6} = 7.96$Hz

The differences in the pmr spectrum previously reported in aqueous media[30] are attributed to the solvent used. The proton-proton couplings of the aryl protons are virtually identical, however.

The fully decoupled ^{13}C-NMR spectrum of aspirin in CD_3OD (200 mg/ml) is shown in Figure 5. The spectrum was obtained on a Varian XL-100-15 NMR spectrometer equipped with a Transform Technology TT-100 FT data system. The data represent the transformation of a 400 pulse FID obtained using 4096 data points with a 15 second delay time between accumulations.[33] Peaks a-i (Figure 5) arise from the nine carbons of aspirin. The seven peak multiplet centered at $\delta=49.0$ ppm
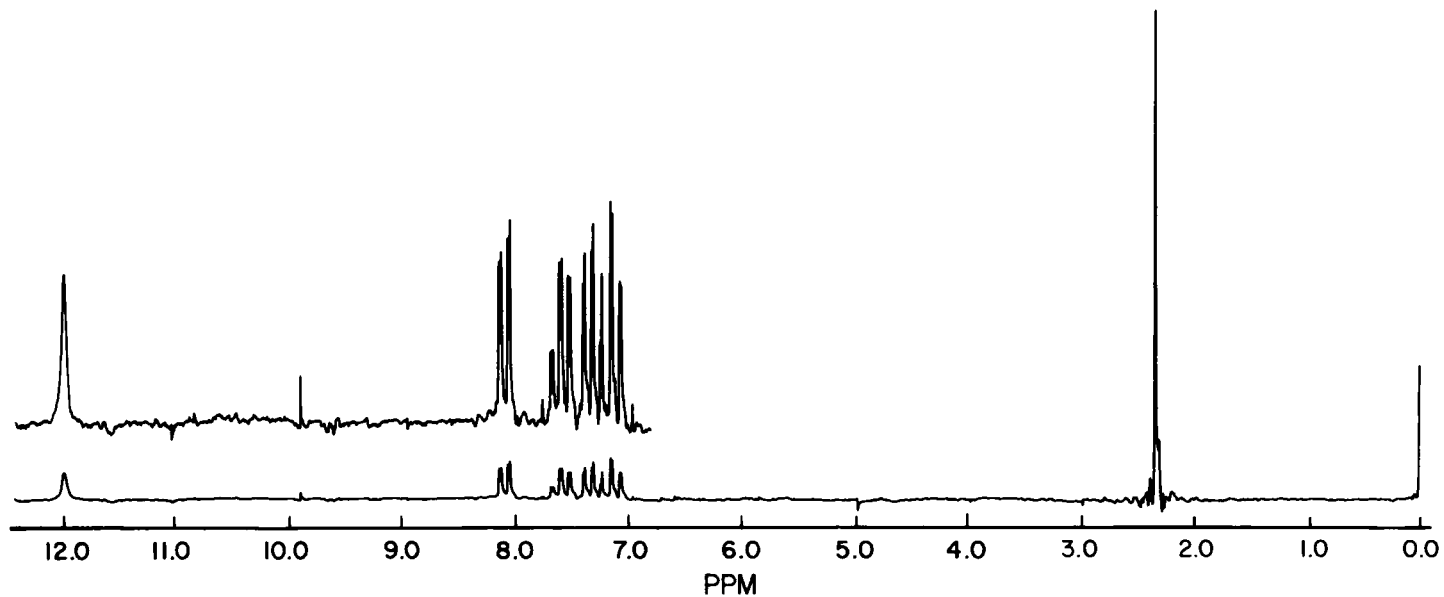
Figure 3. 100 MHz PMR Spectrum of Aspirin. Instrument: Varian XL-100-15

Simulated

8.25   8.00   7.50   7.00

PPM
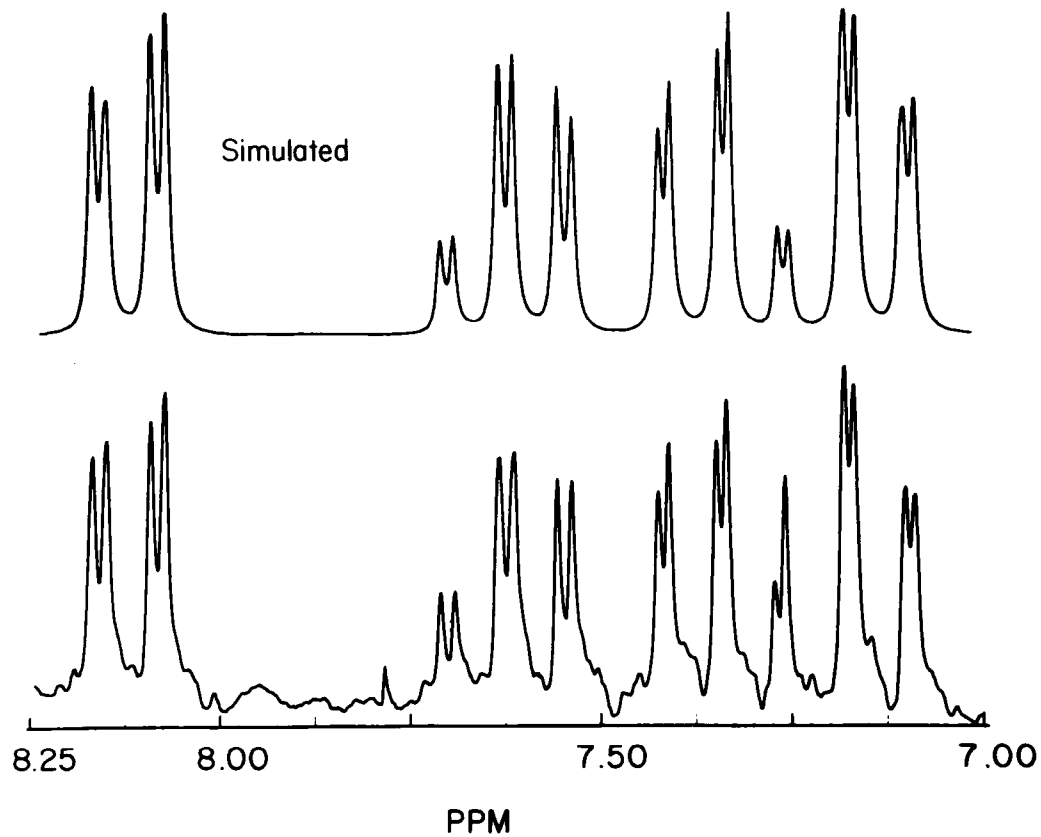
Figure 4. Simulated and calculated PMR Spectra of Aspirin.
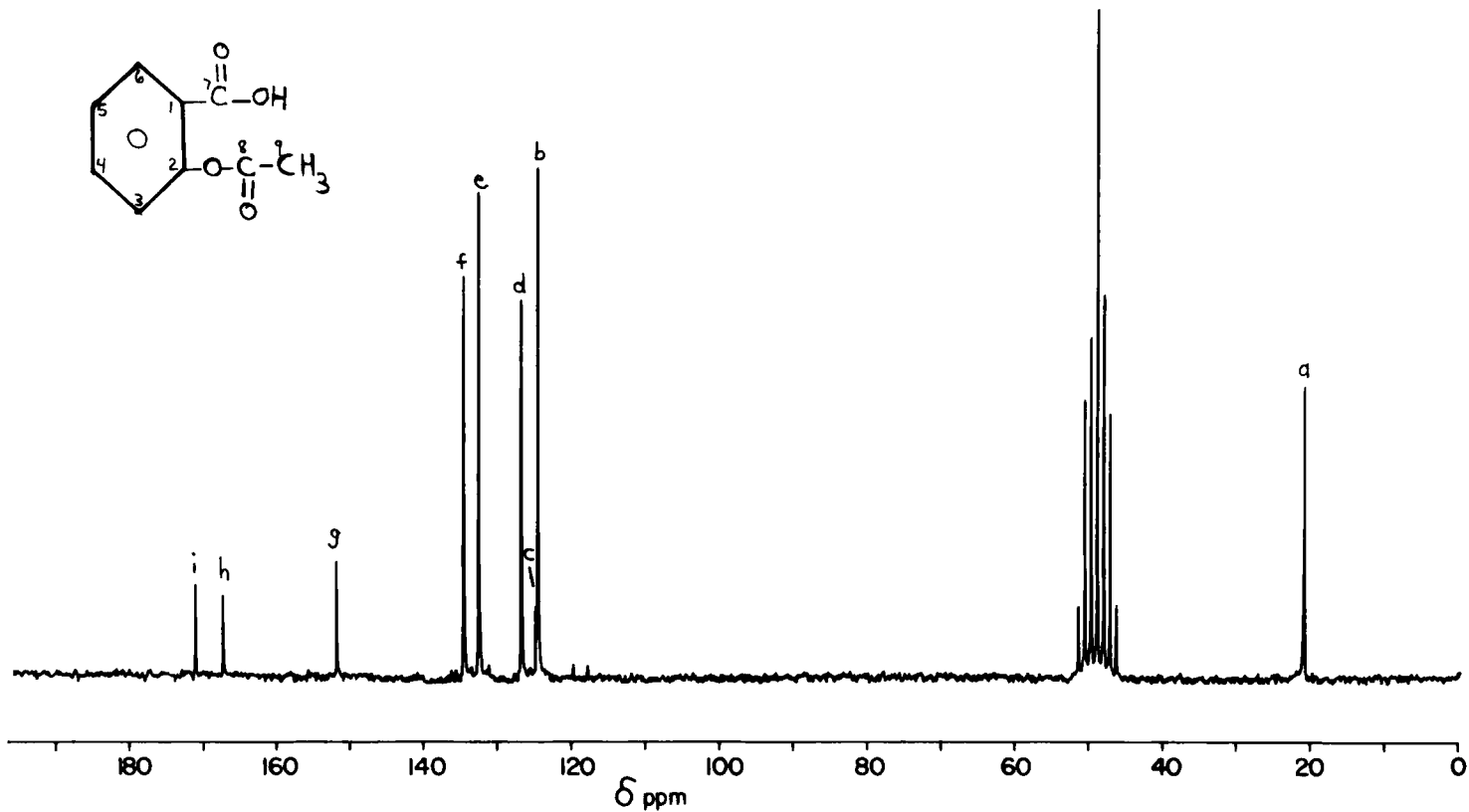
Figure 5. ^{13}C-NMR Spectrum of Aspirin in CD_3OD. Instrument: Varian XL-100-15

arises from the solvent. Assignment of each of the
peaks can be made on the basis of their chemical
shifts and the coupling information summarized in
Table 2.   Peak a is the sole peak in the aliphatic
region of the spectrum and is, therefore, assigned
to methyl carbon (C-9). As expected, the fully
coupled spectrum exhibits four peaks in this region
with a $^1J_{CH}$ of 130 + 2 hz. Peaks b,d,e and f can
be assigned to the four singly protonated aromatic
carbons. Each is strongly coupled to one proton
with a $^1J_{CH}$ of '65 + 2 hz. Weak long range coup-
ling can also be observed. Peak b,d,e and f can be
assigned to carbons 3,5,6 and 4, respectively by
comparison of their chemical shifts to values pre-
dicted on the basis of substituent effects observed
in model systems. Peaks c,g,h and i must arise
from non-protonated carbons since they do not
exhibit appreciable splitting in the fully coupled
spectrum. Chemical shift predictions based on sub-
stituent effects enable assignment of peaks c and g
to the C-1 and C-2 aromatic ring carbons, respec-
tively. The remaining peaks h and i must arise from
the carbonyl carbons. Comparison of their respec-
tive chemical shifts to those of model compounds
suggest their assignment to the carboxyl (C-7) and
acetoxy carbonyls (C-8), respectively. This assign-
ment is confirmed by the observation of a long range
coupling of peak i to the three protons of the
methyl group.

TABLE 2
^{13}C-NMR Data for Aspirin

Peak	δ (ppm)[a]	Assignment Carbon #	$^1J_{CH}$[b]	Multiplicity[b]
a	21.6	9	130	(4)
b	124.6	3	164	(2)
c	124.9	1	c	m
d	126.9	5	165	(2)
e	132.6	6	165	(2)
f	134.7	4	163	(2)
g	151.9	2	c	m
h	167.4	7	c	m
i	171.2	8	c	m (4)

a) ppm from TMS external via the relationship
   δ(CD_3OD)=49.0 ppm.
b) obtained from the fully coupled spectrum.

c) none observed.
m) weakly coupled multiplets due to long range effects.

4.16 Mass

The low resolution mass spectrum was obtained on an AEI MS-902 double-focussing mass spectrometer equipped with a frequency-modulated analog tape recorder at a source temperature of 100° C. above ambient (approximately 130° C.).[32] By adjusting the sample flow and the electron multiplier gain, the maximum sensitivity was obtained without clipping the most intense peak. The recorded analog spectrum was processed on a PDP-11.[34] Except for differences in the intensity of the molecular ion (M^+), the intensities shown in Figure 6 are virtually identical to the previously published spectrum.[35] Differences could be ascribed to instrument design, source temperatures or even source design.

The assignment of a number of fragment ions of the mass spectrum is shown in Figure 7.

Figure 7.

$$M^+ \xrightarrow{-C_2H_2O} m/z\ 138 \xrightarrow[-H_2O]{m^*\ 104.3} m/z\ 120$$

$$m/z\ 92 \xrightarrow[-HCO]{-CO} \begin{array}{l} m/z\ 64 \\ m/z\ 63 \end{array}$$
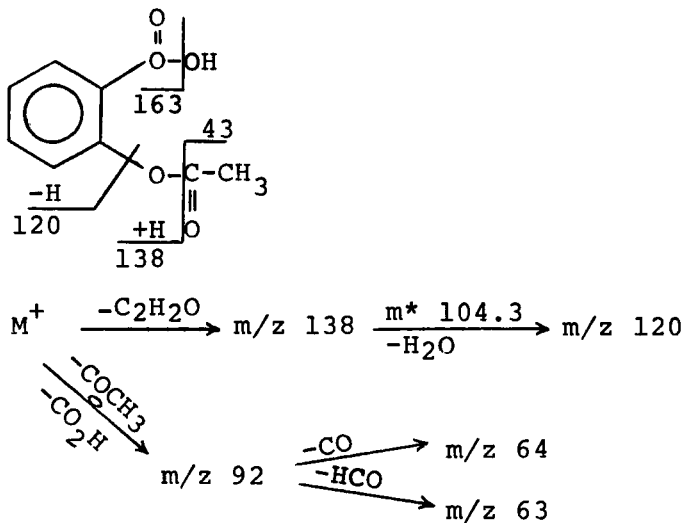
The metastable ion at m/z 104.3 supports the loss of the elements of water from the m/z 138 rearrangement ion.
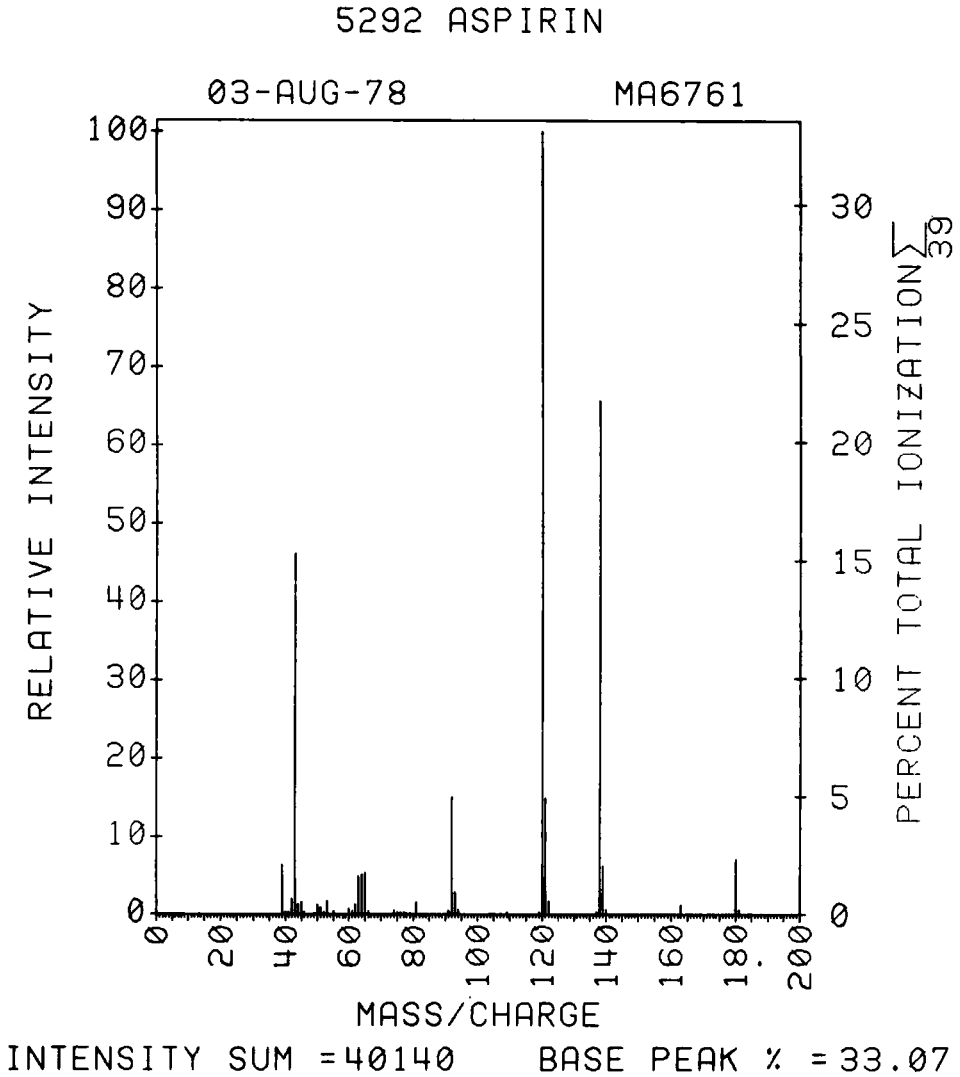
Figure 6.　Mass Spectrum of Aspirin.
　　　　　　Instrument:　AEI MS 902

The mass spectrum of aspirin has
been used as an aid in the rapid identification of
toxic materials isolated from urine, blood or
gastric aspirates of drug abuse patients.[36,37]

4.2   Solid Properties
4.21   Melting Range
The melting point of aspirin is
nothing very definitive having variously been given
between 118-144°[38] and a good deal of work has been
done to get the best methods for compendial
use.[39-43]   Early commercial preparation melted
around 135°.[44]   The European Pharmacopeia[45] gives a
melting point of 141° to 144° as determined by the
instantaneous method.   For further discussion, see
Polymorphism (Section 4.242).

4.22   Differential Thermal Analysis
When aspirin (USP reference
standard) was heated at a rate of 15°/min. in air,
a single endotherm was observed with a T onset = 134°
and T peak = 139°.[46]   DTA and TGA patterns of
aspirin have also been previously studied[47] and used
for forensic drug identification.[48]

4.23   Thermogravimetric Analysis
When aspirin (USP reference
standard) was heated at 20°/min. and a N_2 flow of
20 cc/min.,no weight loss was observed at less than
130°.[46]

4.24   Crystal Properties
4.241   Single Crystal X-Ray
Diffraction
The crystal structure of
aspirin was determined by Wheatley.[49] The monoclinic
crystals have a space group of $P2_1/C$.   The dimension
of the unit cell are: a=11.446Å; b=6.596A; c=11.388
Å; β=95° 33'; n=4.

4.242   Powder X-Ray Diffraction
The powder x-ray diffrac-
tion pattern of aspirin is presented in Table 3 and
Figure 8.[50]

4.243   Polymorphism
In 1968, Tawashi[51] claim-
ed that aspirin exists in several polymorphic forms.
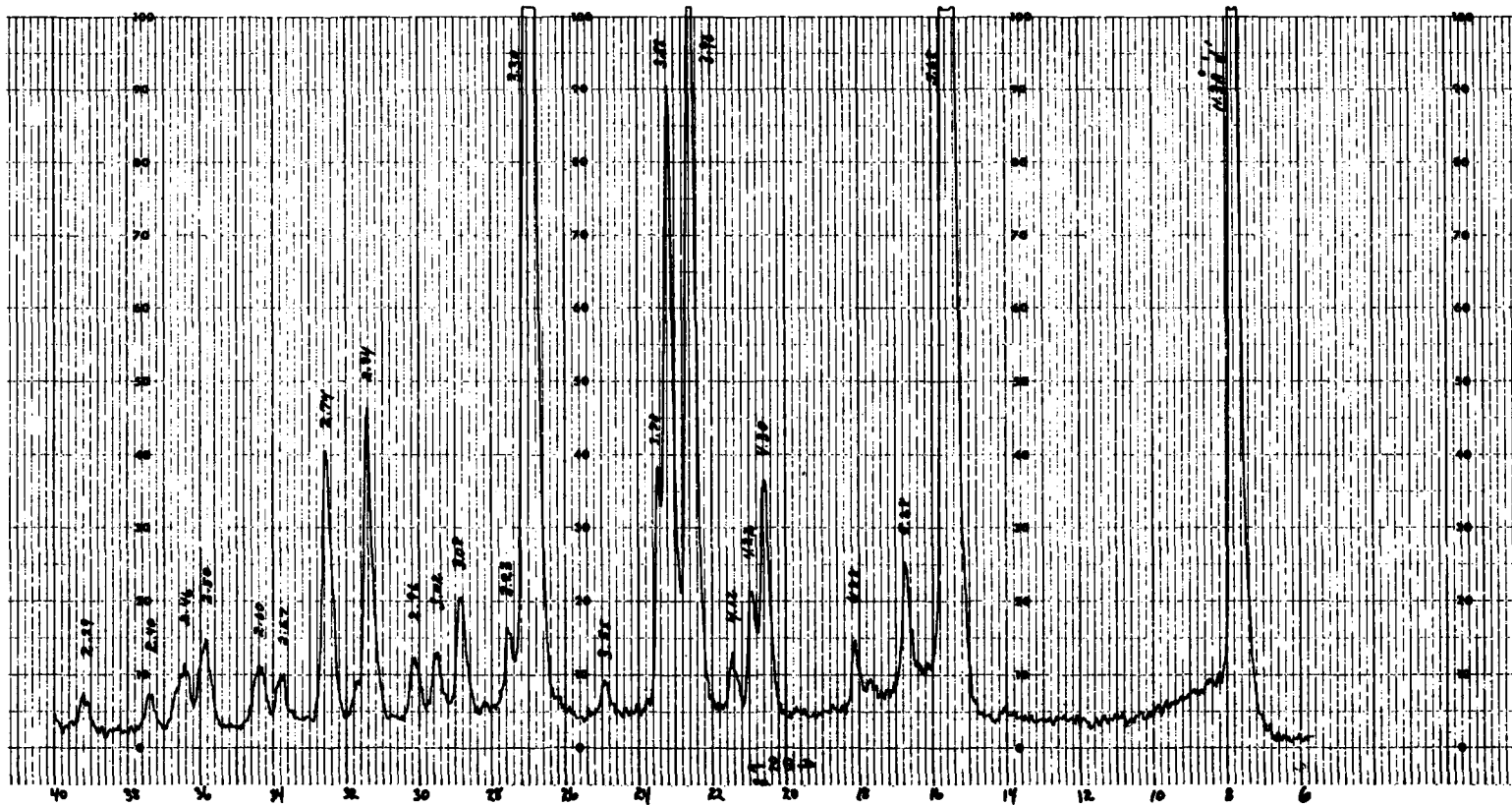
Figure 8. Powder X-Ray Diffraction Pattern of Aspirin (U.S.P. Reference
Standard). Instrument: Norelco

TABLE 3

Powder X-Ray Diffraction Pattern of Aspirin
(U.S.P. Reference Standard)

2θ (Deg.)	D (Å)	Relative Intensity	2θ (Deg.)	D (Å)	Relative Intensity
7.80	11.3	0.539	27.56	3.23	0.054
14.09	6.25	0.033	28.85	3.08	0.068
15.63	5.68	1.000	29.62	3.02	0.040
16.78	5.28	0.087	30.26	2.96	0.039
18.19	4.88	0.038	31.54	2.84	0.120
20.63	4.30	0.079	32.57	2.74	0.110
20.95	4.22	0.035	33.85	2.67	0.051
21.53	4.12	0.030	34.50	2.60	0.037
22.56	3.93	0.268	36.04	2.50	0.049
23.20	3.83	0.210	36.55	2.46	0.048
25.00	3.55	0.033	37.45	2.40	0.034
27.05	3.30	0.427	39.37	2.29	0.038

This started a flurry of activity. DeBisschop[43] claimed to have obtained three different crystal forms but stressed that the only stable one is the monoclinic one melting at 142° C.

However, the claim for true polymorphism of aspirin was questioned or refuted in several laboratories[52-56] and can be best summarized in the words of G. Schwartzman:[57]

"It is generally accepted that true polymorphism results in distinct optical and spectral properties. The data presented are entirely negative in these respects. The evidence accumulated agrees with the findings of Pfeiffer[55] and questions the formation of aspirin polymorphs. We believe that the different crystal habits were caused by the solvents used for crystallization. The dissimilar melting points are probably due to the poor transfer of heat caused by the larger crystal size or to possible crystal defects."

4.244 Optical Constants

Aspirin has been described as monoclinic, a:b:c = 1.7322:1:1.7322, β=95° 4.25'. Indices for 576 μμ are: d=1.5042; β=1.6424

and z=1.6554; 2V=15° 46'. The optical plane is
normal to (010) and lies in obtuse angle β.[58] Very
similar constants are presented in reference 41.

4.245 Polarized Crystal
                   Absorption Spectrum
                   The polarized absorption
spectrum of a single crystal of aspirin was measur-
ed which indicated that aspirin may be spectroscop-
ically treated as perturbed benzoic acid.[59]

4.25 Calorimetry
The heat of combustion at
constant volume was determined as 859.3 kcal/Mol.[60]
Aspirin tablets make good samples for use in oxygen
bomb calorimetry.[61]

4.3 Solution Properties
4.31 Solubility[62-64]

	g/ml
Water at 25°	0.0033
Water at 37°	0.01
Water at 100°	0.03
Ethanol	0.2 − 0.4
Chloroform	0.025 − 0.06
Carbon tetrachloride	0.0004
Ether	0.1 − 0.2
Abs. ether	sparingly soluble
Benzene	0.0033
Petroleum ether	insoluble

The solubility in polyethylene
glycol 400 and in aqueous solution of other poly-
ethylene glycols has been described.[65-66] The
effect of selected surfactants above and below the
critical micelle concentration (CMC) on aspirin
solubility[67] was studied.

4.32 Dissociation Constant (pKa)
In 1913, Springer and Jones[68]
determined the dissociation in aqueous solution at
various temperatures. At 25° they determined the
dissociation constant as 2.8 x 10^{-4} (pKa 3.55). The
Merck Index[62] gives a value of 3.27 x 10^{-4} (pKa
3.49) at 25°.

When the apparent pKa was de-

termined in DMF, using quaternary butyl ammonium
hydroxide as the titrant, the pKa observed depended
on the solvent of crystallization. From ethanol
(m.p. 140-142°), a value of 8.99; from hexane (m.p.
121-124°), a value of 9.19 was obtained. The latter
higher value was ascribed to internal hydrogen
bonding of the carbonyl to the hydroxy group.[69]
This apparent pKa should not be confused with the
true pKa of 3.5 (see above).

4.33   Partition Coefficients
When aspirin was partitioned
between buffers pH 1-7 and octyl alcohol, partition
coefficients ranging from k=17.7 (pH 1) to k=0.025
(pH 7) were obtained.[70] Earlier, coefficients of
0.32 in toluene:water and 1.81 in chloroform:water
were determined.[71]

4.34   Dielectric Constant, Dipole Moment

Dielectric constant:	Dipole Moment:	Ref.
$\sim$ 2.35	2.09	72
5 to 7	–	73
	5.65 calc.;	74
	4.36 observed	
	0.93 (acc. to	75
	Onsager by immersion)	

4.35   Radiation Absorption
The absorption coefficient of a
collimated beam of ^{60}Co z-radiation was determined
for aspirin. See reference 76 for details.

5.   Methods of Analysis
5.1   Historical Synopsis
As the following pages of this section
will show, there is hardly a new method of analysis
which is not immediately tried for the determina-
tion of aspirin as such, or in formulations and
biological fluids. The analysis of aspirin is
intricately interwoven with that of salicylic acid,
its precursor and degradation product. From the
very first, residual salicylic acid was determined by
the convenient reaction with ferric salts -- typical
for phenols -- which give a violet complex with
salicylic acid.

In spite of the plethora of methods, the

compendial approach to determine purity and strength
of aspirin has been very conservative. A monograph
for aspirin was introduced into U.S.P. not earlier
than Volume X (1926) and has not been changed in its
essentials for the last fifty years. Aside from
such niceties as ash, carbonizable substances, chlo-
ride, sulfate and heavy metals, the mainstays then
(U.S.P. X, 1926) and now (U.S.P. XIX, 1975) are:
a) identification by a color test with ferric chlo-
ride after heating, saponification to salicylic
acid, identified as a white precipitate and an odor
test (ethyl acetate), b) residual salicylates as
determined by a color matching test with ferric
ammonium sulfate with a 0.1% limit and c) an assay
involving saponification to salicylic and acetic
acids and back titration of excess alkali with a
purity specification of 99.5 to 100.5%.

The European Pharmacopeia[45] uses essential-
ly the same analytical methods as U.S.P.

In contrast to aspirin itself, the U.S.P.
monograph for aspirin tablets has undergone consid-
erable changes. For some reason, U.S.P. does not
use the ferric salt test for free salicylic acid, as
does the British Pharmacopeia of 1973. Apparently,
certain excipients such as citric and tartaric acid
interfere with this reaction.[77] Already in 1913, a
double titration method was developed[78] which was
made an official method in 1926.[79] This method was
used as the assay method when the aspirin tablets
monograph was introduced into U.S.P. XII in 1942.
For identification, the same two tests as for aspir-
in itself were prescribed then (U.S.P. XII) and now
(U.S.P. XIX); however, due to the pioneering work of
Higuchi, Banes, Smith and Levine in the '50's,[80] a
test for non-aspirin salicylates was introduced
using a siliceous earth column for separation from
excipients and aspirin, and spectrophotometric fin-
ish at 306 nm. A limit of 0.3% is specified.

The same column method with different elut-
ing solvents and a spectrophotometric finish at
280 nm is used for the assay with limits of 95 to
105 percent.

    5.2 Identity and Color Tests
Aspirin can be identified by the following
name tests:

Test	Color	Ref.
Trinder's reagent	Purple after hydrolysis	63
McNally's test	Red	63
Mandelin's reagent (Ammonium vanadate)	Green with blue tint; changes to red-violet	81
Feigl	Saponif. to salicylic acid. Reaction with KOH at 130°: Violet fluorescence	82
Kulberg	Saponif. to salicylic acid. Addition of $FeCl_3$ in HCl: red-violet coloration	83
Vitali Morin	Orange to red	84
Kofler	Microscopic Identification	85

For identification by infrared, see Section 4.11. Identification in combination products by mass spectrometry has been described.[86]

5.3   Quantitative Analysis

5.31   Elemental

The percent of carbon, hydrogen and oxygen is as follows:[62]

	% (theoretical)
C_9	60.00
H_8	4.48
O_4	35.53

5.32   Colorimetric

The use of basic organic dyes for ion pair extraction-photometric determination has been described.[87]   After ammonia treatment, an orange-red color with $CuSO_4$ and H_2O_2 (Denigès) can be quantitated.[88]   A water insoluble violet complex (λ max. 620 nm) with 2-picoline-Cu(II) has also been reported.[89]

5.33   Ultraviolet

The maximum at 277 nm has been used to determine aspirin in tablets after chromatography (see Section 5.43).   It also has been used to determine aspirin in mixtures with other drugs (cf. 21).   For simultaneous determination of

aspirin and salicylic acid, see Section 5.61.

5.34 Infrared
The infrared spectrum has been used
to determine aspirin in combination products.
Accuracy of 1-2% has been claimed (cf. 90,91). For
aspirin, the absorption maximum at 1765 cm^{-1} has
been used.[92]

5.35 Fluorescence - Phosphorescence
Although fluorescence of aspirin, as
contrasted to that of salicylic acid, is weak (see
Section 4.13), it has been used for the determina-
tion in tablets.[22] Phosphorimetry (see Section
4.13) has been described as useful in the determin-
ation of aspirin in blood serum and plasma.[23] Since
the phosphorescence of salicylic acid at the maxi-
mum of 410 nm is about 500 times weaker, it does
not interfere.

5.36 Titrimetric - Electrochemical
Although theoretically aspirin could
be titrated directly with alkali, this tends to
give inaccurate results due to its instability in
alkali and, therefore, the compendial methods back
titrate after saponification (cf. 79). However,
non-aqueous titration is possible and desirable,
particularly for determination in combination
products. Sodium methoxide in benzene-methanol is
used as the titrant and methylisobutyl ketone as
the solvent. The end point is determined potentio-
metrically.[93] Alternately, tetrabutyl ammonium
hydroxide and DMF as titration solvent have also
been used.[94,95] Titration in ethylene diamine has
also been described.[96] Potentiometric measurements
of ion-pair association and selective acid strength
in ethylene diamine and water has been reported.[97]
Potentiometric titration in aqueous medium has also
been described[98] as has colorimetric.[99]

The direct current and alternating
current polarographic response of aspirin in an a
aprotic organic solvent system (acetonitrile - 0.1M
tetrabutyl ammonium perchlorate) has been
studied.[100] The following values were obtained:

1. dc half-wave potential: $E_{\frac{1}{2}}$ = -1.64
2. ac fundamental harmonic peak
   potential: Ep = -1.76

3. ac second harmonic minimum
   potential: E min = 1.87

n values calculated from the
three modes are: 0.44; 0.45 and 0.40. Approximate
detection limits (moles/liter) for the three modes
are: 5 x 10^{-5}; 1 x 10^{-4}; 1 x 10^{-4}.

On a rotating disk electrode,
aspirin was reduced to the aldehyde.[101]

5.37 Miscellaneous (NMR)
Nuclear magnetic resonance spectro-
metry has been used to quantitate aspirin in a
combination product with a coefficient of variation
of 1.1.[102] For quantitation, the shift at 2.3 ppm
representing the ester methyl group was used.

5.4 Chromatographic Methods
5.41 Paper
Paper chromatographic systems have
been tabulated in Table 4.

TABLE 4

Solvent systems:	R_fA*	R_fS*	Detection	Ref.
Pet-ether, Methanol, Benzene and Water (25:20:20:0.05)	0.05	–	$FeCl_3$	103
Iso-propyl alcohol, Water, Ammonia (15:85:10)	–	–	$FeCl_3$	104
Methanol, Water, Ammonia (10:90:10)	–	–	$FeCl_3$	104
Butanol, 25% Ammonia (4:1)	0.45	–	U.V.	105
0.75% Nitric Acid	0.8	0.6	Fluorimetry	106

*R_fA = aspirin; R_fS = salicylic acid.

Cellulose anion-exchange paper
chromatography with 5% acetic acid-n-propanol (5:1)
gave good separation, detected by U.V. light, of
aspirin (Rf. 0.07) from salicylic acid
(Rf. 0.51).[107]

5.42   Thin Layer
TLC has been used to identify and quantify aspirin in pharmaceutical preparations and body fluids. Data have been summarized in Table 5. Readout by densitometers[108,109] and TLC separation as student experiments[110] have also been described.

5.43   Column
As mentioned in the historical synopsis (Section 5.1), Levine[121] perfected the compendial partition column procedure in which aspirin in chloroform is first trapped in an immobile phase of sodium bicarbonate on a column of siliceous earth (celite) then eluted with a solution of acetic acid in chloroform and measured spectrophotometrically. This has been also used for separation in combination products.[80] For the determination of salicylic acid in presence of aspirin by this method, see Section 5.61. Ion exchange columns filled with strongly or weakly basic anion exchange resin in the acetate or chloride cycle have also been used for separation of aspirin in combination products.[122,123,124] This has also been adapted for a student experiment.[125] A Sephadex-G25 column has been used for the separation of aspirin from salicylic acid.[126]

5.44   High Pressure Liquid
This newest of chromatographic techniques has already been used quite extensively for the determination of aspirin in pharmaceutical products. Separation on columns filled with anion-exchange resin[127] or cation-exchange resin with and without counter ions[128,129] were investigated in detail. Silica surface columns have also been used[130] as have been reverse phase (octadecyl) ones[131-134] U.V. detection was used throughout.

5.45   Gas-liquid
Determination of aspirin by gas-chromatography was first reported by Hoffman and Mitchell[135] in 1963 who separated it from other tablet ingredients by direct chromatography on tetrafluoroethylene polymer coated with Dow-Corning silicone, using a flame ionization detector and an integrator. A glass-bead column, coated with carbowax and isophthalic acid has also been used.[136] Most other investigators made the methyl- or trimethylsilyl-derivative prior to chromatography. As

TABLE 5

Thin Layer Chromatography of Aspirin

Support	Solvent System	Rf.Asp.	Rf.Sal.	Detection	Ref.
Silica	MeOH-HOAc-Et$_2$O-C$_6$H$_6$ (1:18:60/120)	$\sim$0.9	-	UV	111
Silica Gel G	Et$_2$O-AcOEt (1:4)	0.26	-	-	112
Silica	C$_6$H$_6$-HOAc-MeOH-CHCl$_3$-Pet-ether	0.42	0.44	UV	113
Polyamide	CH$_3$Cl$_3$-C$_6$H$_6$-90% HCO$_2$H (5:1:0.1)	0.44	0.24	UV	114
Polyamide	iso-PrOH-H$_2$O-90% HCO$_2$H· (1:5:6:01)	0.32	0.08	UV	114
Polyamide	CH$_3$Cl$_3$-90% HCO$_3$H (20:0.1)	0.47	0.24	UV	114
Polyamide	Cyclohexane-CHCl$_3$-HOAc (4:5:1)	0.61	0.41	UV	114
Silica Gel G	Cyclohexane-CHCl$_3$-HOAc (50:40:10)	0.36	-	K$_4$(Fe(CNa))$_3$	115
Polyamide	CHCl$_3$-Cyclohexane-HOAc-Dioxane (40:60:1:10)	0.48	0.14	UV	116
	CHCl$_3$-Cyclohexane-HOAc (40:60:1)	0.37	0.12	UV	116
Silica Gel G	EtOH-IsoProOH-Xylene-CHCl$_3$ (12.5:12.5:25:50)	0.04	-	Iodine	117

TABLE 5 (continued)

Support	Solvent System	Rf.Asp.	Rf.Sal.	Detection	Ref.
Silica	$CHCl_3-(CH_3)_2CO$ (9:1)	0.075	–	NH_4 Vanadate	118
	MeOH – NH_3 (100:1.5)	0.75	–	NH_4 Vanadate	118
Silica Gel GF	EtOAc-t-PrOH-NH_3 (40:30:3)	0.25	–	UV	119
Silica Gel GF	EtOH-HOAc-H_2O (60:30:10)	0.92	–	UV	119
Silica Gel GF	C_6H_6	0.10	–	UV	119
Silica Gel 60	C_6H_6-Dioxane-HOAc (60:20:2)	–	–	Fluorimetry	120

methylating agents, diazomethane in ether alco-
hol[137] or tetrahydrofurane,[138] methanol with boron
trifluoride[139] and methyl iodide with potassium
carbonate[140] were used. For trimethylsilylation
hexamethyl disilazane,[141-143] N-O-bis(trimethyl-
silyl) acetamide and other trimethylsilyl donors,[144]
BSTFA[145-146] and MSTFA[147] have been tried. It was
noted that derivatization with BSTFA or MSTFA re-
sulted in slight hydrolysis of aspirin.[147]

    5.5 Electrophoretic Methods
        Aspirin can be separated from salicylic
acid by ionophoresis at a pH of 4-5.[148] Separation
of aspirin in combination products has been achiev-
ed with paper strip electrophoresis, using buffers
at pH 2-8 and a 200 V. applied potential.[149]
Aspirin was separated from metabolites by paper
electrophoresis in a phthalate buffer of pH 3.2 and
an ionic strength of 0.0125-0.0500.[150]

    5.6 Determination of Impurities
        5.61 Salicylic Acid
                As has already been pointed out, the
determination of salicylic acid is intricately
interwoven with that of aspirin itself. There is
the convenient color reaction with ferric chloride
which was already used by Dreser[1] to determine free
salicylic acid in his own urine after the ingestion
of aspirin. However, this reaction is not too
specific and considerable work has gone in the de-
velopment of interference free methods.

                My task has been made easy since
there is an excellent review by C.A. Kelly[151] on
the determination of salicylic acid in aspirin and
aspirin products. A more recent review has been
compiled by S.L. Ali.[152]

                I, therefore, have restricted my
tabulation of methods to those references which
discuss determination of salicylic acid in bio-
logical specimens, which were not covered by Kelly,
to important references which have been published
since the Kelly review and references which are
pertinent to other sections of this profile.

References for the determination of salicylic acid:
    1. Colorimetric (iron complex): 77,78,153,154
            (Folin-Ciocalteu reagent): 218,219

2. Nonaqueous titration: 104
3. Iodometric: 131
4. Spectrophotometric: 20, 156-158
5. Fluorometric: 22,159,160
6. Infrared: 92
7. Column: 57,137,138,103
8. PC (for Rf values and solvent systems see
        Section 5.41): 106,107
9. TLC (for Rf values and solvent systems see
        Section 5.42): 114,116,119
10. VPC: 106,107,140,141,144,145,146,147
11. HPLC: 152,164
12. Automated: 165-167

5.62 Acetic Acid

It is easily forgotten that aspirin
degrades to acetic as well as salicylic acid. And,
indeed, any smell aspirin might have is due to ace-
tic acid. However, the volatility of acetic acid
does not make the determination of acetic acid a
reliable tool to measure stability or degradation.

A.N. Smith in 1920[168] devised a
method to determine acetic acid in aspirin by
bubbling dry air through a thin layer of powdered
aspirin, trapping the acetic acid in water and back-
titrating it with alkali. Gas chromatography[169] has
also been used.

5.63 Acetylsalicylic Anhydride and Acetyl-salicylsalicylic Acid

In addition to salicylic and acetic
acids, very small quantities of acetylsalicylanhy-
dride (0.0012 to 0.024%) and acetylsalicylsalicylic
acid (0.03 to 0.1%) have been found in aspirin prep-
arations. The former has been determined by gas
chromatography,[140] TLC[170] and spectrophotometry,[171]
the latter by gas chromatography.[140,144] A contro-
versy is still ongoing (cf. 152) whether the occa-
sionally observed hypersensitivity against aspirin is
caused by these two impurities and whether the basis
of the adverse reaction is immunological.

6. Stability - Degradation

That aspirin is sensitive to moisture is well-
known and most of us at one time or another have
observed the growth of salicylic acid whiskers on
aspirin tablets left for too long in a humid bath-

room wall cabinet. The hydrolysis of aspirin to
salicylic acid was already of concern to the pharma-
cologist, Dreser[1] (see Section 1.2), who in 1899
prepared what nowadays one would call a preformula-
tion profile. He tested the hydrolysis of aspirin
in acid and alkaline solutions and, by perusing text-
books of Ostwald and Nernst to devise the proper
equations, determined hydrolysis constants of
3.3×10^{-4} at "body temperature" in acid medium and
2.5×10^{-2} at room temperature in alkaline medium.
He also observed that aspirin was easily split in
weakly alkaline medium which had consequences for
the metabolism (see Section 7). One could say that
he established the first profile for the pH depend-
ence of hydrolysis of aspirin. Of course, the
definition of pH was unknown at the time.

The next systematic study of the hydrolysis of
aspirin in water, albeit at 100° C., was undertaken
by Rath[172] who determined a hydrolysis constant of
about 0.17 depending on experimental conditions.
Much work has been done since, and it is quite
evident that this seemingly simple hydrolysis to
acetic and salicylic acids is both complex and
controversial. I am fortunate that I can refer the
reader to the excellent and detailed review by Clark
A. Kelly[151] already mentioned in Section 5.61. The
most complete and thorough kinetic studies of the
factors involved in the hydrolysis of aspirin are
undoubtedly those by Edwards[173], which were further
elaborated by Garrett.[174]

Stability and decomposition kinetics of aspirin
both as a solid and in solution continue to be
studied. The topochemical decomposition pattern of
aspirin tablets has been explored.[175] The degrada-
tion of aspirin in the presence of sodium carbonate
and high humidity was studied by x-ray diffrac-
tion.[176] The activation energy of decomposition by
water vapor in the solid state was found to be
30 kcal/mol.[177] The effect of common tablet excip-
ients on aspirin in aqueous suspension was also
studied.[178]

An exhaustive study of the stability of aspirin
in polyethylene glycols (substituted, unsubstituted
and esterified), as well as other polyhydric alcohols,
was undertaken by Whithworth and collaborators.[179-183]

It was found that decomposition is in part due to
transesterification and that substitution increases
stability. The effect of gamma radiation on aspirin
has been described.[184] The pH stability profile of
aspirin according to Edwards has been made the sub-
ject of a student experiment.[185]

7. Pharmacokinetics - Drug Metabolic Products

When Dreser[1] investigated the pharmacological
properties of aspirin in 1897 (see Section 1.2), he
postulated that, based on the easy hydrolysis of
aspirin in weakly alkaline medium, aspirin also
should be converted to salicylic acid in vivo. And,
indeed, he found that only 22 minutes after in-
gestion of aspirin, his own urine gave a positive
reaction for salicylic acid with ferric chloride.
After 12 hours, he could no longer detect salicylic
acid in his urine. He found no evidence for aspirin
itself in urine, nor did he find a combination of
aspirin with glycine analogous to the formation of
hippuric acid already known at the time. He had
evidence for formation of another nitrogen contain-
ing derivative. This early investigation should
give food for thought to those who believe that
pro-drugs and pharmacokinetics are recent discover-
ies.

In 1911, Neuberg[186] detected small amounts of
gentisic acid (2,5-dihydroxybenzoic acid) in the
urine of dogs dosed with aspirin. The metabolism of
aspirin is intertwined with that of salicylic acid,
but I was unable to ascertain who first reported
the metabolic formation of salicyluric acid, the
major metabolite of both salicylic acid and aspirin,
specifically after administration of aspirin.

Reviews by Puetter[187] and by Levy,[188-190] taken
together present a comprehensive picture of the
pharmacokinetics and metabolism of aspirin.

Once absorbed, aspirin is rapidly converted
to salicylic acid. After i.v. administration, the
half life of aspirin in the human organism was found
to be only 15 minutes[191] by Rowland and Riegelman
who also estimated that only 20% of the in vivo
hydrolysis takes place in blood.[192]

Apparently, all tissues investigated possess

esterases which can split aspirin; however, most of
it seems to be hydrolyzed in the liver.[187] While
the major pathway of hydrolysis leads to free ace-
tic acid, it is noteworthy that a significant
portion of the acetic acid is not set free but used
for transacetylation of certain factors which play
a role in the inhibition of platelet aggregation[193]
only shown by aspirin but not by salicylic acid.
The kinetics of this important pathway still need
exploration.[187]

Aspirin serum esterase activity is species
dependent.[194] In man it is sex linked since it was
found to be higher in men than women.[195] It also
seems to be age dependent.[196]

Once aspirin is hydrolyzed to salicylic acid,
it follows the metabolic pathway of the latter.
The metabolic products of salicylic acid are pre-
sented in Figure 9. Over the last decade, the
pharmacokinetics of salicylates in the healthy and
diseased organism have been explored in great
detail by Levy.[188] Salicylic acid (II) is elimina-
ted by five parallel and competing pathways (Fig. 9)
leading to renal excretion. These are: excretion
unchanged (II), conjugation with glycine to form
salicyluric acid (III) -- the major pathway --
conjugation of the carboxyl or phenolic hydroxyl
group to form glucuronides IV and V and hydroxyla-
tion to gentisic acid (VI). To a minor degree,
salicyluric acid can be further conjugated with
glucuronic and sulfuric acids.[187] The major
metabolic pathways (salicyluric and salicyl phenol
glucuronide) are easily saturated in the usual
dosage range for treatment of inflammation.[197] For
the clinical implications of these non-linear
pharmacokinetic characteristics, I refer to the
reviews by Levy.[188-190]

To properly treat bovine "sufferers of head-
aches" (inflammation) the pharmacokinetics of
aspirin in cattle have also been explored.[198]

8. Bioavailability - Dissolution
    In the last two decades, the concept of bio-
availability has gained prominence and with it
dissolution as a possible in vitro model for drug
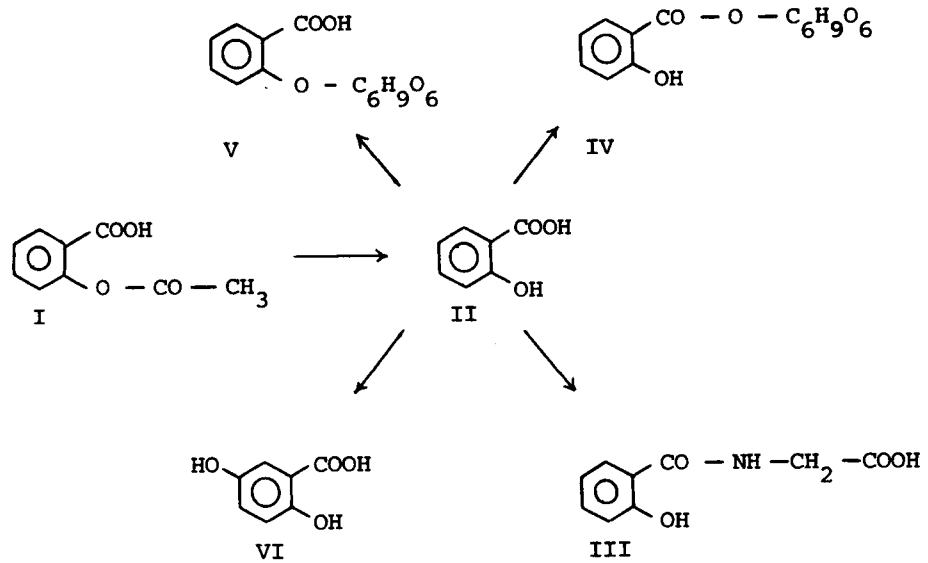absorption. In 1960, Swintosky and Blythe[199]

Figure 9. Drug Metabolic Products of Aspirin

compared the relative availability of aspirin from
enteric coated and compressed tablets by measuring
the excretion of salicylate. About the same time,
Levy[200],[201] started to compare dissolution and
absorption rates of different commercial aspirin
tablets, found good correlation and proposed that
the U.S.P. tablet disintegration test be replaced
by a dissolution test; a suggestion which as of
this writing has not been heeded. These studies
were extended and further perfected by Gibaldi.[163]
Many other papers on this subject have appeared.
Particularly, the influence of the crystal habitat
(see Section 4.243) on absorption has been studied
(cf. 202). Significant intraindividual, but not
interindividual, variations in blood plasma aspirin
levels were found in young male subjects after
administration of aspirin as tablets or solutions.
The plasma level curves obtained for tablets were
more variable than those obtained for solutions.[203]
Levy[204] found that using urinary excretion measure-
ments for evaluation of aspirin dosage forms, with
different absorption rates in man, requires
that such measurements be made during the first
hour after drug administration. The use of a pH-
stat for testing dissolution of various aspirin
dosage forms has been described.[205]

9.   Determination in Biological Fluids and Tissues
     All the advances in pharmacokinetics and drug
metabolism described in Sections 7 and 8 would not
have been possible without the availability of the
proper analytical methods. The following is a tab-
ulation of publications in this field, most of
which have already been discussed in Section 5. It
should be mentioned that a few publications talk
about aspirin blood levels, but really mean sali-
cylate levels. The following tabulation covers
only those papers where aspirin was differentiated
from other salicylates by chromatography or other
means. It seems that the "workhorse" for serum
salicylate levels is still the colorimetric (ferric-
nitrate) method of Brodie, Udenfriend and Coburn[153]
published in 1944, or modifications thereof.
Simplified versions (cf. 206) may lead to erroneous
results under certain conditions.[207] The method is
also applicable for urinary metabolites after
proper hydrolysis (cf. 208). For other methods
restricted to salicylic acid, see Section 5.61.
The first gas chromatographic separation of aspirin

and salicylic acid in plasma was described by
Rowland and Riegelman,[141] who presented a brief
review of earlier methods.

Methods for aspirin:
Colorimetric (with differential extraction):218,219
Spectrophotometric (differential): 20, 209
Fluorometric (as salicylic acid): 160, 210
Phosphorimetric: 23
VPC: 137,141,143,145,146
PC: 106,107,119
TLC: 118,211
Mass Spectrometric: 37
Radioisotopic: 212-214

10. Determination in Pharmaceutical Preparations
     The following tabulation of references high-
lights those methods (see Section 5) useful in
pharmaceutical analysis.

1. Determination in tablets:
        Differential U.V.:   157,158
        Automation: 166
        Fluorometric: 22
        Column: 121
        TLC: 120
        GC: 136,139,144

2. Determination in buffered tablets:
        Fluorometric: 159
        Column: 161,162

3. Determination in combination products:
    a. General Identity tests: 83,86,103,104,105,
                                 112
    b. Aspirin, caffeine, acetophenetidine
        (and more components)
        UV: 21,215
        IR: 91-93
        NMR: 102
        Electrophoresis: 149
        TLC: 110,111
        Column: 80
        Ion-exchange column: 123
        HPLC: 127,130,131
        VPC: 135,142
        Pot. titration: 216

     c.   Aspirin, acetaminophen, caffeine:
         Nonaqueous titration: 94,217
         Potentiometric: 102

     d.   Aspirin-barbiturate combinations:
         Nonaqueous titration: 93

     e.   Cough-cold mixtures:
         HPLC: 132

11. Acknowledgments

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The literature has been covered systematically
through Chemical Abstracts, Volume 88 (1978), with a
few stray references beyond.

BROMOCRIPTINE METHANESULPHONATE

Danielle A. Giron-Forest and W. Dieter Schönleber

1.   Introduction

1.1 History

The most valuable pharmacological properties of the pep-
tide type ergot alcaloids induced a variety of attempts of
chemical derivatisation of the parent compounds (1,2 + lit.
quoted therein). In one of these the bromination of α-ergo-
cryptine led to a product of highly interesting pharmacology,
namely bromocriptine.

It became known to suppress prolactine secretion, and it
is therefore a useful tool in the treatment of prolactine
dependent disorders, such as galactorrhea associated with
hyperprolactinemia and postpartum, as well as certain kinds of
sterility (3 - 9). In more elevated doses, the drug is a
potent antiparkinsonicum. In addition, there is recent evi-
dence of bromocriptine playing an important role in the trace
heavy metals balance of the brain (10).

1.2 Name, Formula, Molecular Weight

Bromocriptine mesilate is 2-bromo-α-ergocryptine methane-
sulphonate or 2-bromo-12'-hydroxy-2'-(1-methylethyl)-5'-(2-
methylpropyl-5'α-ergotaman-3',6',18-trione methanesulphonate
or Bromocriptinum (INN). It is the active ingredient in
Parlodel® dosage forms.
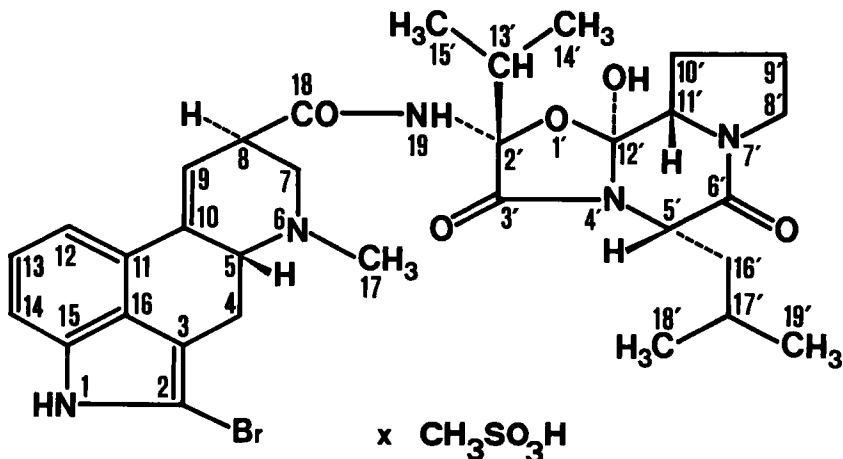
Chemical Abstracts Registry Numbers :

          25614-03-3
          (26409-15-4)
          (47830-26-2)

Average Molecular Weights :

     base :   654.61

     mesilate: 750.71

Average Formulas :

     base : $C_{32}H_{40}BrN_5O_5$

     mesilate: $C_{33}H_{44}BrN_5O_8S$

1.3. Appearance, Colour, Odour

Grey tinged white or light yellow, finely crystalline powder, odourless or of weak, characteristic odour.

2.   Physicochemical Properties

2.1 Elemental Analysis

The elemental analysis of bromocriptine mesilate yielded the following results (11):

element	% calculated	% found
C	52.8	53.2
H	5.9	6.0
Br	10.6	10.5
N	9.3	9.2
O	17.0	16.8
S	4.3	4.4

2.2 Spectra

2.21 Infrared

The infrared spectrum of bromocriptine mesilate in a KBr pellet is given in fig. 1. It was recorded on a Perkin Elmer 257 infrared spectrophotometer.
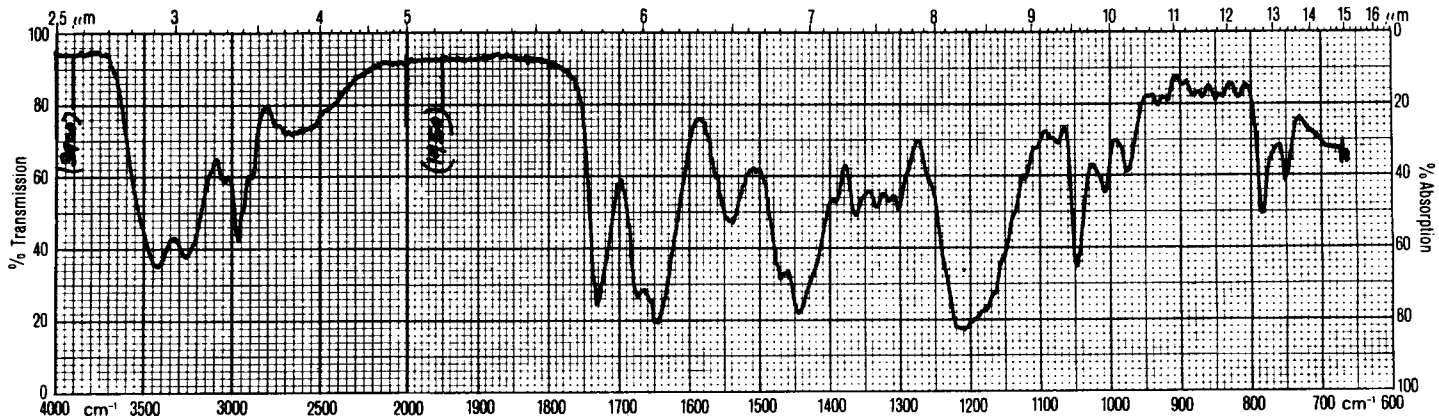The main characteristic bands are the following :

Figure 1.   Infrared Spectrum of Bromocriptine Mesilate
in a KBr Pellet.
Instrument: Perkin Elmer 257

wave number (cm^{-1})	assignment
3200 - 2900	C-H-stretching vibrations
1728	C=O-stretching of 5-membered ring lactam (cyclol)
1642, 1672	C=O-stretching of 6-membered ring lactam (amide I)
1560	amide-II band and C=C-stretching

The spectrum of the base has been reported by P.A. Stadler and co-workers (11).

2.22 Ultraviolet

The ultraviolet spectrum of bromocriptine mesilate was recorded on a Philips 1700 uv spectrophotometer in 0.1 M methanolic methanesulphonic acid solution. It is given in fig. 2. A maximum occurs at about 308 nm with a log molar absorptivity of 4.0. In 1:1 dichloromethane/methanol solution the absorption maximum was reported as 306 nm log = 3.988 (11).

2.23 Fluorescence

Bromocriptine mesilate exhibits fluorescence like the other lysergic and isolysergic acid type alcaloids. In 2 percent ethanolic tartaric acid solution, the emission maximum appears at 402 nm (excitation at 325 nm). See fig. 3.

2.24 Proton Nuclear Magnetic Resonance

The PMR spectrum of bromocriptine mesilate in deuterated dimethyl sulphoxide as obtained on a Bruker HX-90 NMR spectrometer is presented in fig. 4. TMS served as internal standard. The characteristics of the spectrum are given in the following table (see also 12, 13 and lit. quoted therein):

PMR-spectrum and Assignment

Chemical Shift downfield (ppm)	Intensity	Multiplicity	Assignment
11.85	1 H	Singlet	indole-N$\underline{H}$
10.45	1 H	Singlet	N$\underline{H}$ $^{+}$
9.55	1 H	Singlet	CON$\underline{H}$ (18-19)
7 - 7.3	4 H	Multiplet	$\underline{H}$-C$_{12}$, C$_{13}$, C$_{14}$ and O$\underline{H}$

Figure 2.   Ultraviolet Spectrum of Bromocriptine Mesilate
            in 0.1 M Methanolic Methanesulphonic Acid.
            C_A = 0.05 mg/ml, C_B = 0.012 mg/ml.
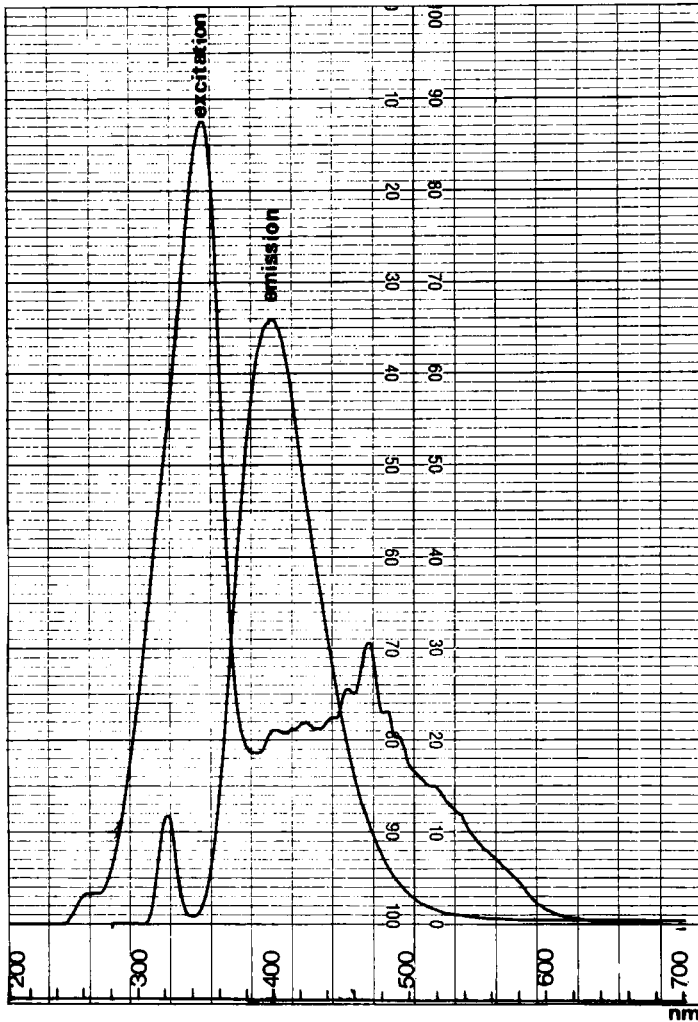   Instrument: Philips SP 1700

Figure 3.   Fluorescence Spectrum of Bromocriptine
             Mesilate in 2 % Ethanolic Tartaric Acid.
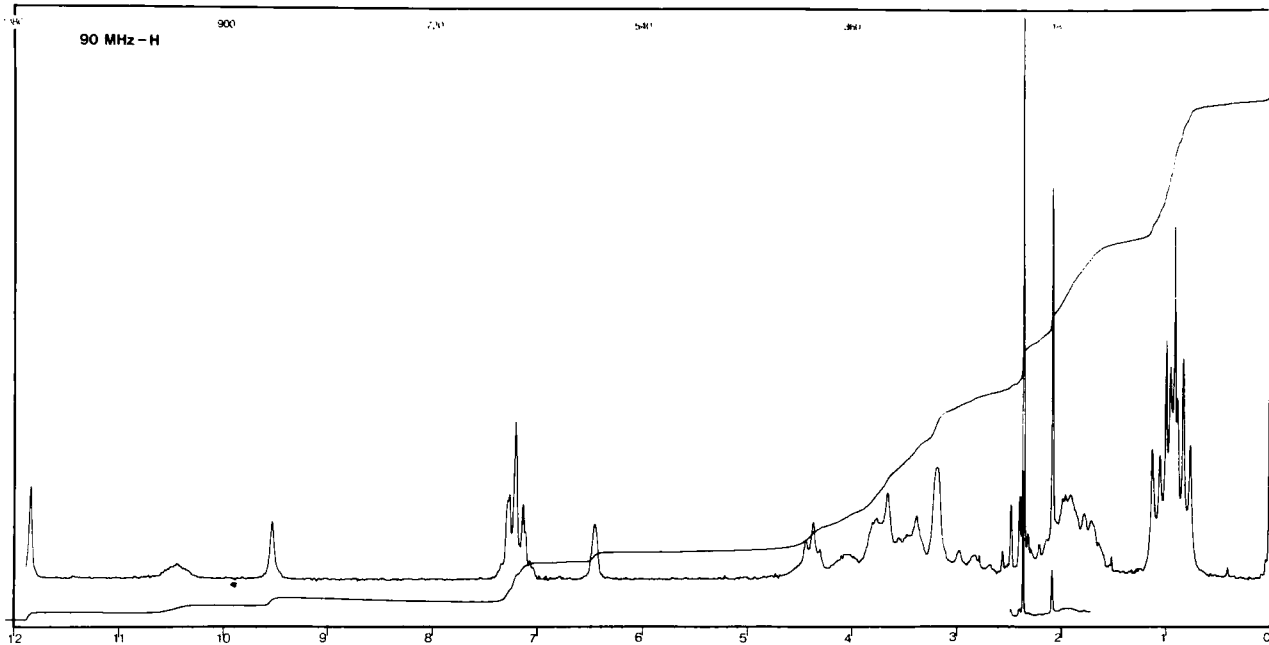   C = 65 μg/ml.
             Instrument: Perkin Elmer MPF-3

90 MHz−H

Figure 4. PMR Spectrum of Bromocriptine Mesilate in $(CD_3)_2SO$,
Instrument: Bruker HX-90 E

6.47	1 H	Singlet	$H-C_9$
4.3 - 4.5		Triplet	$H-C_{5'}$
3.2	16 H	Singlet	$6-CH_3$ (C-17)
3 - 4.2		Multiplet	$H-C_{8'}$; $H-C_{11'}$; $H-C_4$; $H-C_5$; $H-C_7$; $H-C_8$ $CH_3COCH_2CH_3$; H_2O (recryst. solvent)
2.37	3 H	Singlet	CH_3-SO_3H
2.1		Singlet	$CH_3COCH_2CH_3$
2.1 - 1.5	8-9 H	Multiplet	$5'-CH_2$; $5'-CH$; $2'-CH$; $H-C_{9'}$; $H-C_{10'}$
0.75-1.15	12 H	Multiplet	$2'CH_3$; $5'-CH_3$ and $CH_3COCH_2CH_3$

2.25 Carbon-13 Magnetic Resonance Spectrum

The spectrum of bromocriptine mesilate has been recorded
in dimethyl sulphoxide using a Bruker HX-90 NMR spectrophoto-
meter (fig. 5). The assignment of the individual signals is
given in fig. 6.

2.26 Mass Spectrum

The low resolution electron impact mass spectral pattern
of bromocriptine mesilate (fig. 7) corresponds quite nicely
to those of the other non- and dihydrogenated ergot alcaloids
(12, 13 and lit. quoted herein).

The molecular peak M of the base shows up at 653 and 655,
respectively, reflecting the natural abundance of the bromine
isotopes, and M-18 at 635/637 mass units. As in the parent
compounds the next smaller fragment appears at M-228, indica-
ting a substantial loss of the peptide section constituents
with the tentative fragment structure F1. From this species
the isopropyl group splits off easily to yield the line pair
at 382/384 mass units. Loss of the entire peptide moiety leads
to the formation of 2-bromo-lysergamide F2 at 345/347 m.u.,
the mass spectral behaviour of which corresponds to that of
the bromine-free compound. Thus, it gives rise to peak pairs
at 300/302 and 299/301, respectively, presumably by loss of
formamide or by the rupture of the tetra-hydro-pyridine ring,
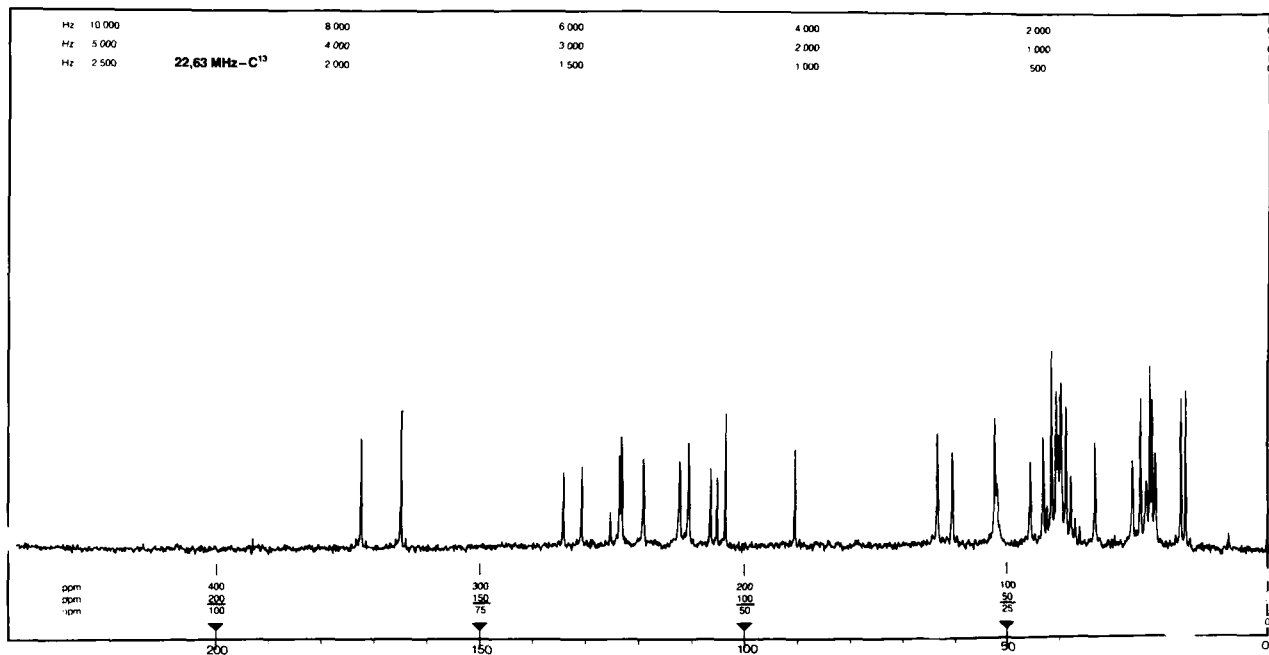
Figure 5. 13-C-NMR Spectrum of Bromocriptine Mesilate in $(CD_3)_2SO$, Instrument: Bruker HX-90 E
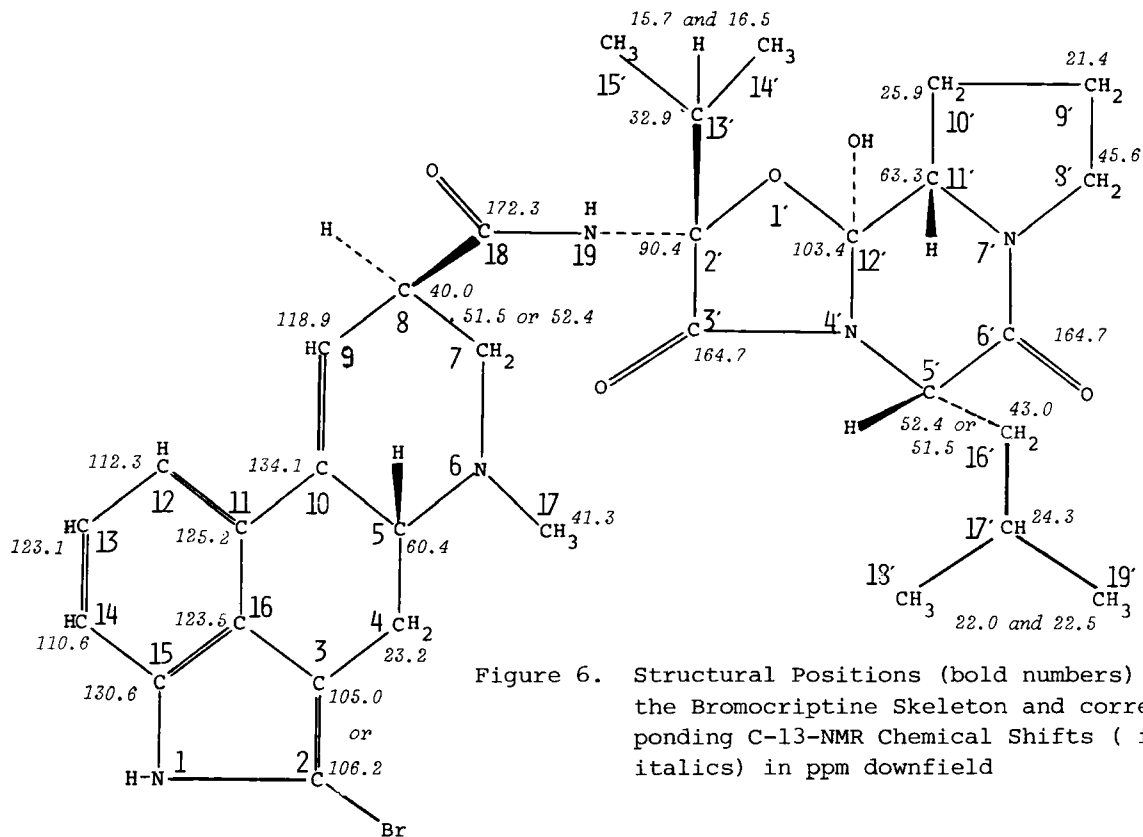
Figure 6. Structural Positions (bold numbers) in the Bromocriptine Skeleton and corresponding C-13-NMR Chemical Shifts (in italics) in ppm downfield
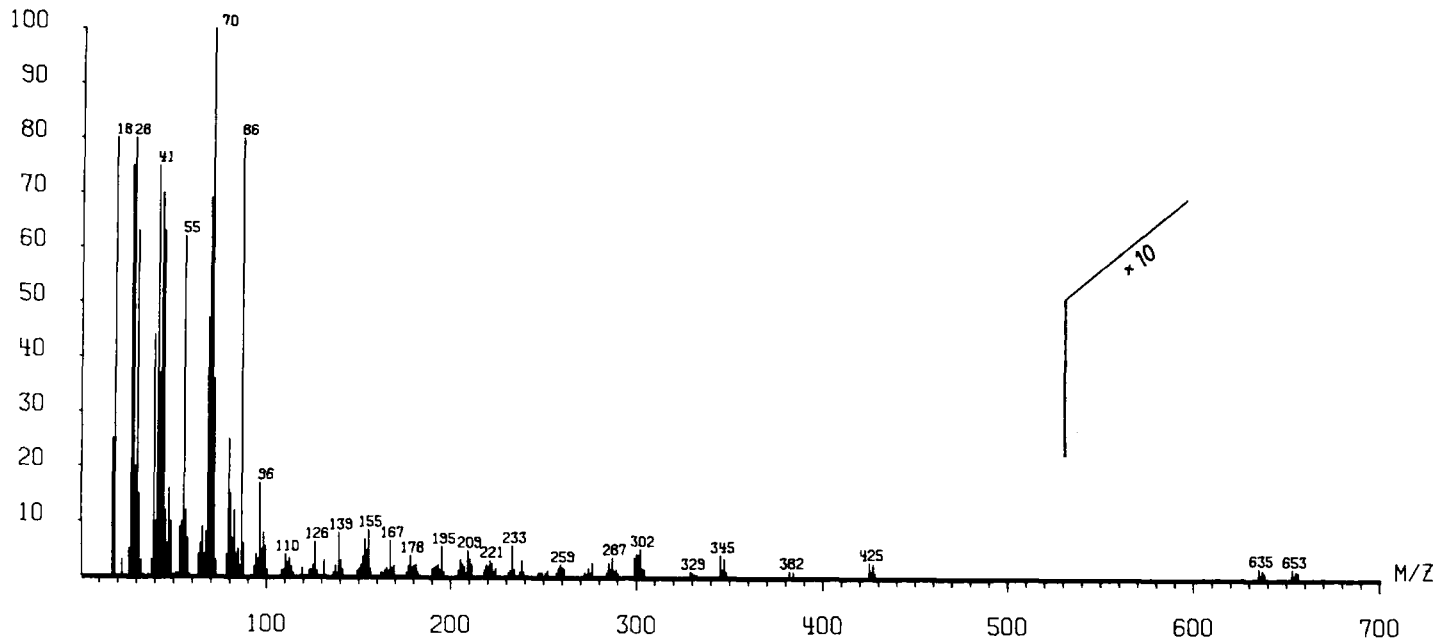
Figure 7. Low Resolution Electron Impact Mass Spectrum of
Bromocriptine Mesilate
Instrument: CEC 21-110B; Energy 70 eV, Ion Source
Temperature 160 - 200° C

a decay mode that was proposed earlier (14,15) for lysergic acid derivatives. Peak doublets at 285/287 and 274/276 m.u. indicate fragments of illicit structure still containing bromine.

F1

425/427 m.u.

F2
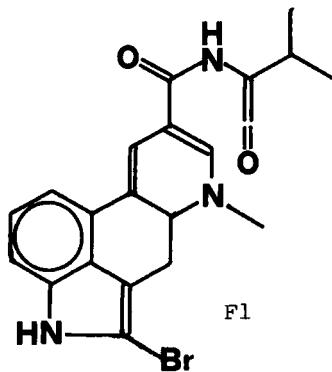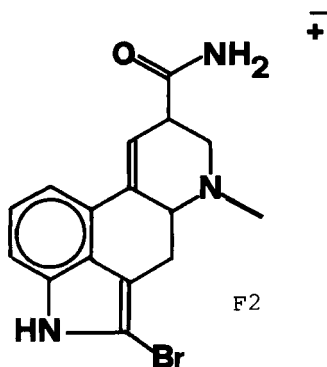
345/347 m.u.

The peptide moiety itself (308 m.u.) obviously does not appear in the spectrum, whereas its fragmentation pattern with peaks at m/e 195 (F3), 167 (F3-CO), 155 - 153 (F4), 125 (F5), 86 (F6), and base peak 70 (F7) mass units, is clearly under-stood (14,15). The mesilate shows a signal at m/e 96.

F3

195 m.u.

F4

154 m.u.

F5

F6

F7
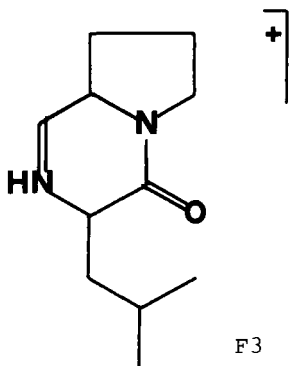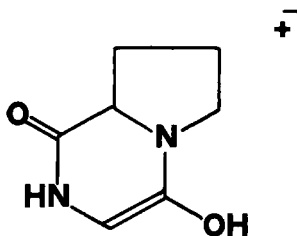
125 m.u.   86 m.u.   70 m.u.
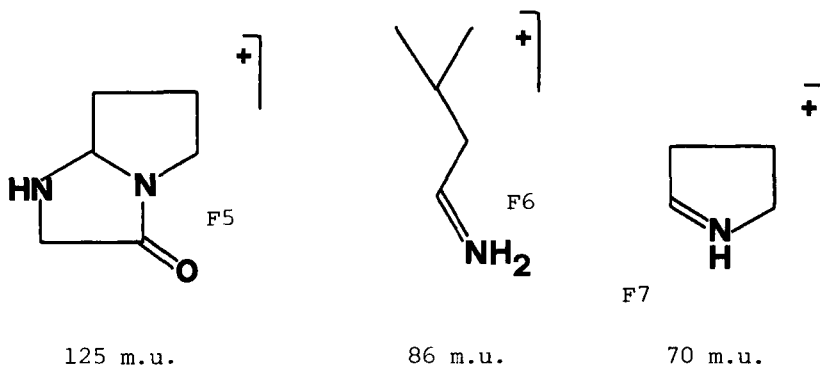
2.3 Crystal Properties

2.31 Melting Characteristics

Bromocriptine mesilate decomposes between 180 and 200 °C, thus a melting point or melting range cannot be given.

2.32 Polymorphism

Investigations for the occurrence of polymorphism have been undertaken by ir spectroscopy, differential scanning calorimetry and x-ray powder diffraction (Guinier-de Wolff). No polymorphism has been observed so far. An amorphous form may be prepared artificially by rapid evaporation of a methanolic solution of the drug substance.

2.33 X-Ray Diffraction

X-ray structural analysis of bromocriptine, crystallized as the base from dichloromethane/diethylether, has been carried out on a CAD 4-diffractometer with CuKα-radiation (16). 3371 reflexions were within sin $\Theta/\lambda < 0.62$ $Å^{-1}$. Assessment of the structure was achieved by computation to a refinement of R = 0.033 for the absolute configuration.
The crystal data were: Space group P 2_1, a = 10.681, b = 13.454, c = 11.049, β = 99.75°, z = 2.
In fig. 8 the resultant conformation of bromocriptine base is depicted.

2.34 Differential Scanning Calorimetry

The DSC thermogram of bromocriptine mesilate, obtained with a Perkin Elmer DSC-2 instrument at a heating rate of 20 °C/min. and in a nitrogen atmosphere, is shown in fig. 9.
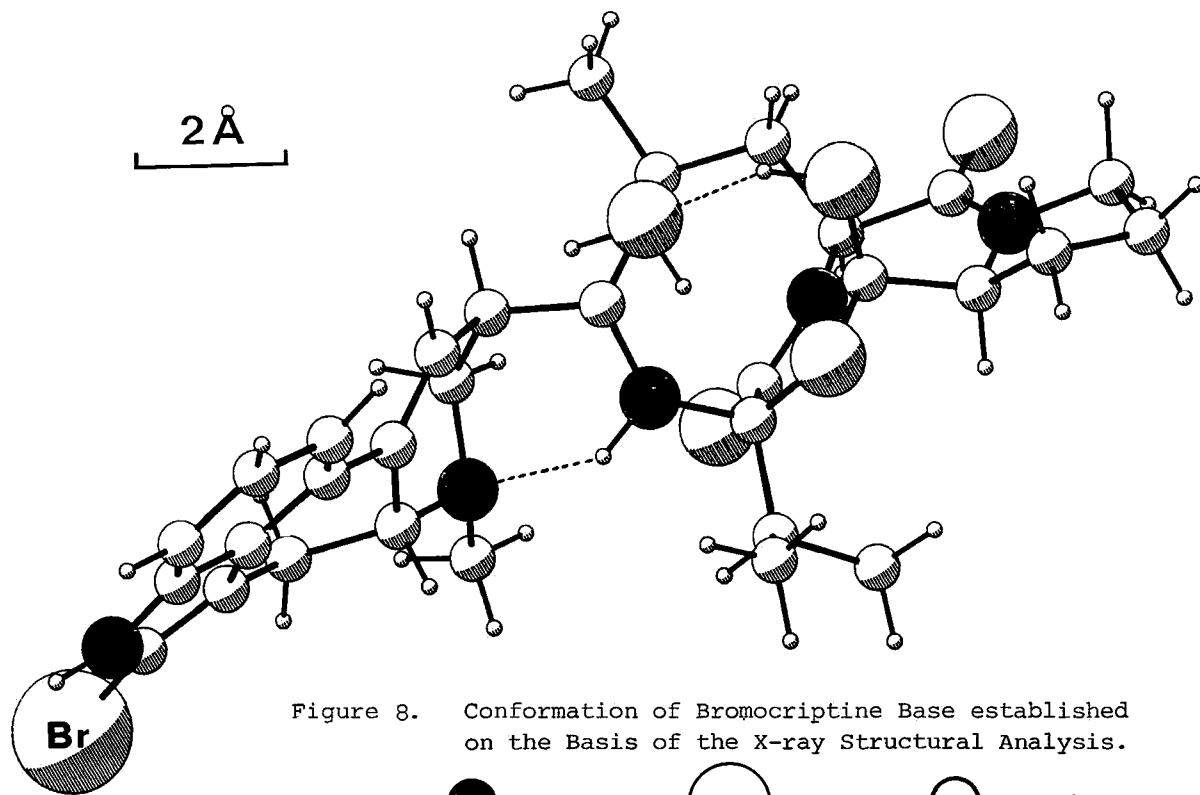
Figure 8.   Conformation of Bromocriptine Base established
on the Basis of the X-ray Structural Analysis.

● = Nitrogen, ◯ = Oxygen, ◯ = Carbon,

○ = Hydrogen
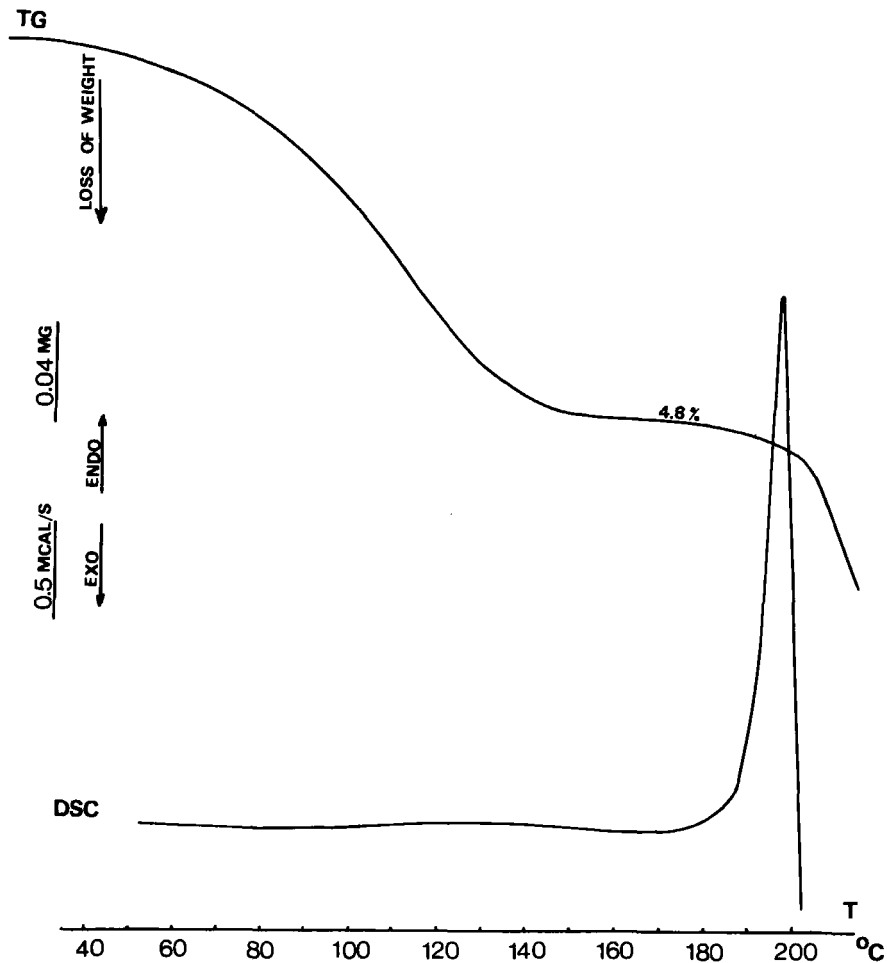
Figure 9.   Differential Scanning Calorimetry and.
             Thermogravimetry Curves of Bromocriptine
             Mesilate, each at a heating rate of 20°C/min.
             and a Nitrogen Flow of 15 ml/min.
             Instruments: Perkin Elmer DSC-2
                          Perkin Elmer TGS-1

The melting endotherm is followed immediately by a strong
exothermic degradation. Since bromocriptine mesilate decompo-
ses under melting, the transition temperature is strongly
dependent on the heating rate. A broad but weak endotherm
between 40 and 100 °C indicates the volatilization of sorbed
recrystallization solvent (usually butanone-2, see section 3).

2.35 Thermogravimetry

The thermogram of bromocriptine mesilate, carried out on
a Perkin Elmer TGS-1 thermobalance, is given in fig. 9.
The sample temperature was raised at a rate of 20°C/minute
maintaining a nitrogen atmosphere. A considerable loss of
weight, attributed to a loss of sorbed solvent (s. above), is
observed below 130 °C. Sample decomposition obviously starts
after melting.

2.4. Solubility

The solubility of bromocriptine mesilate was determined
in a variety of solvents equilibrated by vibration during
24 hours at 25 °C. They are as follows:

Solvent	Solubility in mg/g	Solubility in g/100 ml
water	0.8	0.08
methanol	910	72
ethanol	23.0	1.8
2-propanol	1.2	0.1
acetonitrile	1.6	0.12
acetone	0.2	0.015
ethyl acetate	0.2	0.015
chloroform	0.45	0.06
benzene	<0.1	<0.02
hexane	<<0.1	<<0.01

At 22 ± 2 °C bromocriptine mesilate dissolves readily
(>2 %) in propylene glycol, 50 % ethanol, 95 % ethanol and
n-octanol, whereas it is poorly soluble (<0.1 %) in simulated
gastric and intestinal fluids at 37 ± 2 °C.

2.5 Dissociation Constant

Due to the low solubility of bromocriptine mesilate in
water, the pK_a value had to be determined in methyl cello-
solve/water 8:2 (w/w). Titration at ambient temperature yield-
ed pK_a as 4.90 ± 0.05 for a 0.0078 M solution.

2.6 Partition Coefficients

The partition coefficients of bromocriptine mesilate between water of pH 1.2 and n-octanol on the one hand, and water of pH 7.5 and n-octanol on the other, have been determined at 37.0 ± 0.5 °C.

$$\text{water pH 1.2/n-octanol} \quad 1 : \quad 90$$
$$\text{water pH 7.5/n-octanol} \quad 1 : 235$$

2.7 Optical Rotation

Theoretically, with 6 chiral centers within the molecule (at 6, 9, 2', 5', 11' and 12' positions) 64 diastereomeric forms are possible. However, bromocriptine is sterically well defined at all of these positions, as it is derived from the naturally occurring α-ergocryptine.

The specific optical rotation of bromocriptine mesilate in different solvents is given below for 20° C corrected for loss on drying. A Perkin Elmer polarimeter 241 was used, the actual concentration being 10 mg/ml.

	wavelength (nm)				
solvent	589	578	546	436	365
	specific optical rotation in degrees				
dichloromethane/ methanol 1:1	101.0	107.5	130.1	327.8	329.4
ethanol	100.6	107.0	129.6	328.7	331.3
dimethyl formamide	127.4	135.2	162.0	388.0	392.7

For the specific rotation of 2-bromo-α-ergocryptine and -inine bases see (11).

3. Synthesis

Bromocriptine base is manufactured by bromination of α-ergocryptine with N-bromosuccinimide in dioxane solution. The mesilate is then formed by addition of methanesulphonic acid. The salt is recrystallized from butanone-2 (s. fig. 10) (2,3,11).

α-Ergocryptine Base

↓ N-Bromo-succinimide

Bromocriptine Base

↓ Methanesulphonic Acid

Bromocriptine Methanesulphonate
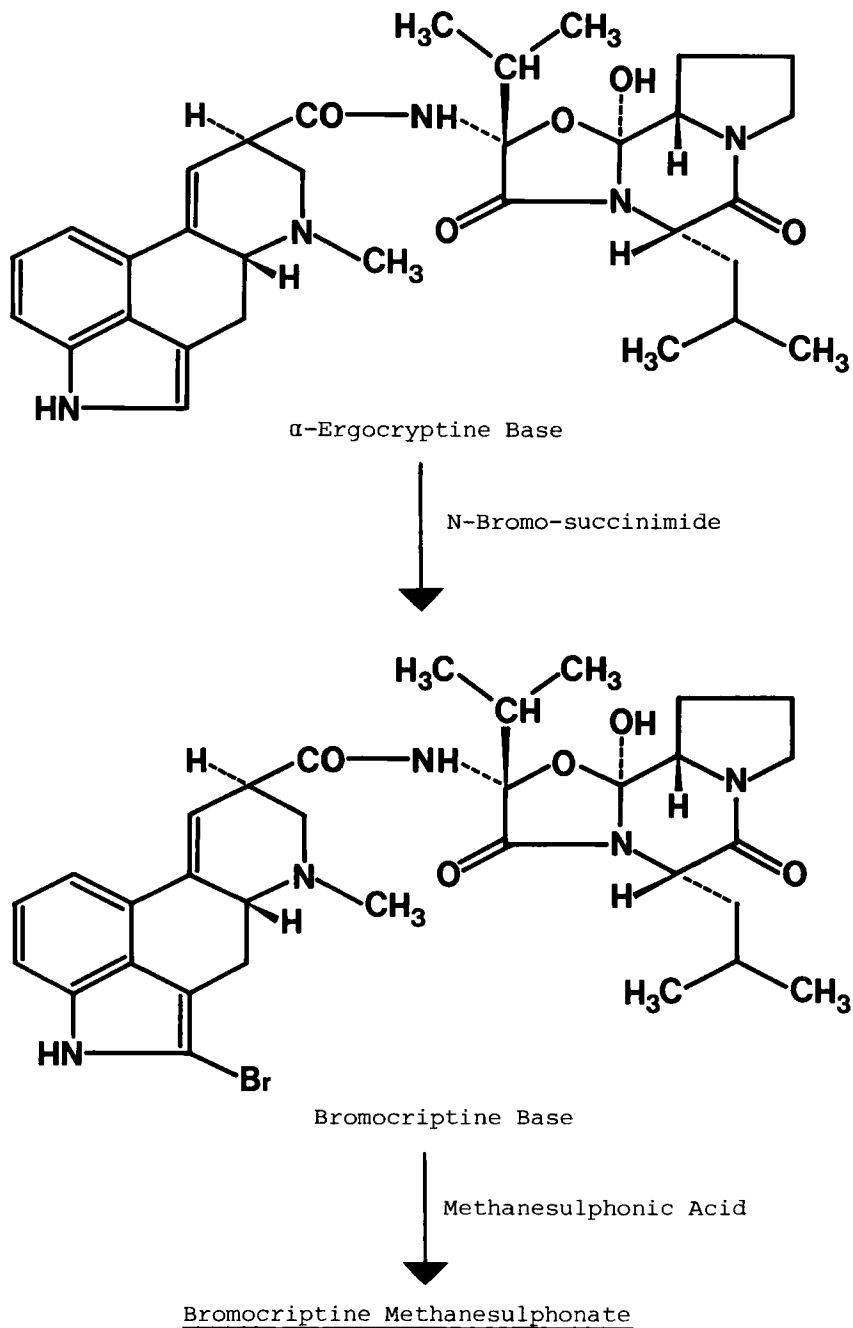
Figure 10.   Route of Synthesis

4.   Stability and Degradation Processes

Possible degradation pathways of ergot alcaloids at the example of ergotamine tartrate have been clearly summarized by H. Bethke et al. (17) and B. Kreilgaard (13).

As a nonhydrogenated ergot alcaloid, bromocriptine is relatively sensitive to autoxidation both in solid and in dissolved states, but degradation products have not yet been elucidated. Similarity to the oxidative transformation of the parent compounds is to be strongly anticipated (12).

In hydroxyl-containing solvents, nonhydrogenated ergot peptide alcaloids are readily epimerized at C-8 to an equilibrated mixture of the lysergic and iso-lysergic acid series, called the -ine/-inine forms (-ine = 8β, -inine = 8α)(12,18 and lit. quoted therein). The hydrolysis of the lysergic acid amide bond is of minor importance for the beginning degradation, but, of course, prevalent under more drastic hydrolysis conditions.

Under the same conditions, but at elevated temperatures, the C-2'-center is very likely to be inverted (aci-inversion) yielding a more acidic isomeric compound.

The light-induced addition of water to the 9,10-double bond of bromocriptine yielding the so-called lumi-products, is of high probability (12,18). They have, however, not yet been isolated or characterized. The corresponding 10α-methoxy-lumi-derivative could be prepared by the photo-catalyzed addition of methanol (19) under slightly acidic conditions.

4.1   in Bulk

Although bromocriptine mesilate is sensitive to heat and light, it is stable for up to 3 years at ambient temperature (20) when stored in sealed polyethylene bags contained in twist-off amber glass bottles. In warm and tropical climate (30 °C/75 percent relative humidity) and under identical package conditions, it is stable for 1 year, however only for 3 months at 50 °C.

If not properly protected, bromocriptine adsorbs up to 6 percent by weight of water in tropical climate.

4.2 in Solution

Bromocriptine follows the behaviour sketched above and is
thus rather labile in aqueous or aqueous/alcoholic solutions,
particularly in the presence of acid, yielding mainly the
equilibrated mixture with its 8-epimer (1) and to a smaller
extent, its hydrolysis products 2-bromo-lysergamide and
2-bromo-lysergic acid and their 8α-isomers, respectively.

4.3 in the Dosage Form

Bromocriptine mesilate is marketed as Parlodel® capsules
(10 mg for the treatment of Parkinson's disease) and tablets
(2.5 mg as lactation suppressor). Both forms have proved
stable at least for 4 years when stored at ambient temperature
in amber glass bottles (22).

5.   Biopharmaceutical Aspects (23)

5.1. Pharmacokinetics

The disposition of bromocriptine has been studied in
several animal species and man following single oral and
intravenous administration of the drug labelled with either
tritium or carbon-14.

The enteral absorption of bromocriptine from an aqueous
solution amounts to 30 - 40 % as determined from the sum of
the cumulative biliary and urinary excretion of radioactivity
(parent drug + metabolites) in bile duct cannulated animals.

The blood levels following oral and intravenous doses
are very low in all animal species. This, most likely, is due
to the marked affinity of the drug for various tissues and the
rapid hepatic extraction of the absorbed fraction. The main
route of excretion is the bile. Less than 5 % of the dose are
recovered in the urine of intact animals after oral or intra-
venous administration.

In man, the extent of enteral absorption is estimated to
be at least of the same order of magnitude than determined for
animals. Absorption is rapid with an approximate rate constant
of 1.4 h^{-1} ($t^{1}/2$ = 30 min).

Peak plasma levels are reached about 1.5 h after oral ingestion, the maximum concentrations being in the order of 2 - 3 ng equivalents/ml (parent drug + metabolites) for an oral 1 mg dose. The elimination from the plasma is biphasic and proceeds with mean half-lives of 6 h (α-phase) and 50 h (β-phase). Similar elimination half-lives are obtained from the urinary excretion. The cumulative renal excretion is practically the same after oral and intravenous administration and amounts to 6 - 7 % of the radioactivity dosed. The main portion of the dose, either oral or intravenous, is eliminated by the biliary route into the faeces. The kinetics of bromocriptine has been demonstrated to be linear in the oral dose range from 2.5 to 7.5 mg.

Parent drug is bound to bovine and human plasma proteins to an extent of 89 - 96 % (in vitro concentration range 0.2 - 80 µg/ml) and exerts a pronounced affinity to various tissues.

5.2 Metabolism

Bromocriptine is rapidly and completely metabolised in animals and man. The major components of the urinary metabolites have been identified as 2-bromo-lysergic acid and 2-bromo-isolysergic acid. Apart from the hydrolytic cleavage of the amine bond and the isomerization at position 8 of the lysergic acid moiety, a third principal biotransformation pathway consists in the oxidative attack of the molecule at the proline fragment of the peptide part, predominantly at position 8', giving rise to the formation of a number of hydroxylated and further oxidized derivatives of bromocriptine, and in addition of conjugated derivatives thereof.

6. Toxicology

The acute toxicity of bromocriptine mesilate has been determined in the mouse as 230 and 2620 mg/kg i.v. and p.o., respectively. In the rabbit, the corresponding values were 12 and >1000 mg/kg (2). Thus, bromocriptine proved less toxic than the nonhydrogenated ergot alcaloids by one order of magnitude, resembling the behaviour of the dihydrogenated derivatives.

Chronic toxicity studies were carried out with rats, dogs, and rhesus monkeys (2). In nearly all cases, the principal effect produced was ischemia of some part of the body. The well-known emetic effect of ergot alcaloids in dogs was particularly pronounced with bromocriptine, even with oral doses of as low as 0.1 mg/kg.

7.   Analytical Methods

7.1   Application of General Tests

Compared with the unsubstituted ergot alcaloids (1,18) the introduction of bromine into the 2-position of the indole nucleus diminishes the reactivity of the molecule in respect to general alcaloid tests. Nevertheless, they still remain applicable.

1. Keller's test. Bromocriptine mesilate is dissolved in glacial acetic acid to which have been added traces of ferric chloride. After carefully layering with concentrated sulfuric acid, a green colour is produced at the interface. Usually an intense blue-violet colour occurs with 2-H-substituted compounds.

2. Van Urk's reaction. (24) Bromocriptine mesilate is dissolved in methanol. After addition of van Urk's reagent and vigorous shaking a blue colour develops slowly, which is substantially weaker than with 2-H-ergot alcaloids.

3. Meyer's test. Bromocriptine mesilate is dissolved in methanol/water 3:10. After addition of one drop of diluted hydrochloric acid and one drop of Meyer's reagent (mercuric potassium iodide) and shaking, a white precipitate is produced.

7.2   Titration

Bromocriptine mesilate may be assayed in glacial acetic acid/acetic anhydride 1:7 by titration with 0.1 N perchloric acid. The endpoint may be determined potentiometrically using a glass/calomel electrode system.

The methanesulphonic acid content of bromocriptine mesilate is usually determined by titration with 0.1 N methanolic potassium hydroxide. The endpoint may be detected potentiometrically using a glass/calomel electrode system.

Residual N-bromosuccinimide from the manufacturing process may be identified and/or quantified by making use of its oxidation potential by titration of liberated iodine after addition of potassium iodide in acetic acid (25).

7.3 Spectroscopic Methods

7.31 Infrared

Infrared spectroscopy is utilized for identification purposes during the analysis of the drug substance. (see 2.21)

7.32 Ultraviolet

Spectrophotometric analysis of bromocriptine mesilate is carried out directly using the uv maximum at about 305 nm in methanolic methanesulphonic acid. However, the method is not very specific.

It was preferred to first separate the impurities from bromocriptine by thin layer chromatography and then to isolate the substance by elution from the silica gel of the plate with methanol. The intact active ingredient is measured in 0.01 M methanolic methanesulphonic acid (26).

7.33 Colorimetry

In moderately acidic solutions bromocriptine mesilate readily forms ion pairs with anionic dyes such as picric acid, bromothymol blue, methyl orange, which are extractable with an organic solvent. A procedure has been developed both for direct assay and for assay following chromatographic separation from the impurities. Therein bromocriptine mesilate is allowed to react with bromothymol blue at pH 2.5. The resulting ion pair is then extracted with benzene and its concentration determined at 410 nm (25).

7.34 Proton Magnetic Resonance

PMR spectroscopy may be used for identification of the drug substance. (see 2.24)

7.4 Chromatography

7.41 Paper

Paper chromatography was applied formerly to the determination of bromocriptine.

conditions: system 1: stationary phase: 25 % formamide
                      mobile phase   : carbon tetrachloride/
                                        diethyl ether 1:1

```
system 2: stationary phase: 10 % dimethylphthalate
          mobile phase    : dimethylformamide/
                            0.5 N hydrochloric
                            acid 15:85
```

The drug substance is visualized by reaction with iodine vapour.

Rf values of bromocriptine and precursor

	system 1	system 2
Bromocriptine	0.88	0.09
α-ergocryptine (precursor)	0.70	0.31

7.42 <u>Thin Layer</u>

The relative instability of bromocriptine makes it very difficult to avoid artifact formation in test solutions or during spotting on the plate. Therefore, bromocriptine mesilate, usually dissolved in chloroform/methanol 1:1, has to be spotted on the plate very rapidly and with the exclusion of light. The separation being terminated, the mobile phase is removed by means of high vacuum for 30 minutes.

A great number of tlc systems have been investigated for the separation of by-products, degradation products, metabolites, and excipients. Also a variety of spraying reagents have been tested (see table). The most advantageous one was Dragendorff's reagent with consecutive spraying by 30 % hydrogen peroxide.

```
methods: stationary phase: silica gel 60 F254,            (27)
                           Merck tlc plates, no.5715

         mobile phase 1  : dichloromethane/dioxane/96 percent
                           ethanol/conc.ammonia 180:15:5:0.1
                           (v/v/v/v)

         mobile phase 2  : chloroform/methanol/formic acid
                           78:20:2 (v/v/v)

         mobile phase 3  : chloroform/96 percent ethanol/conc.
                           ammonia 192:8:0.35 (v/v/v)
```

In the procedure with mobile phase 1 and 2, the visualization is accomplished by screening under uv light (254 and 366 nm) and in addition by spraying with Dragendorff's reagent, modified by Munier and Deboeuf, followed by 30 percent hydrogen peroxide.

| compound | R_{st} values | |
	mobile phase 1	mobile phase 2
bromocriptine	1.0 (Rf 0.5)	1.0 (Rf 0.7)
2-bromo-α-ergocryptinine	1.5	1.1
α-ergocryptinine (isomer of precursor)	1.4	1.0
α-ergocryptine (precursor)	0.6	0.5
2-bromo-lysergic acid	0.0	0.25
2-bromo-isolysergic acid	0.0	0.35
2-bromo-lysergamide	0.5	0.15
2-bromo-isolysergamide	0.5	0.35

Mobile phase 3 may be used for the detection and semi-quantitative determination of residual N-bromosuccinimide. Thereby, the plate is sprayed with water and then placed in a chlorine atmosphere for 10 minutes. Excess chlorine is removed by placing the plate in a stream of warm air for another 10 minutes. After spraying with o-toluidine reagent, the evaluation is made against a dilution of N-bromosuccinimide. The detection limit is 0.1 µg, the R_{st} is 0.3 with respect to bromocriptine Rf value.

A high performance tlc system has been developped for the in-process-control (28) using Merck silica gel HPTLC plates, no. 5628, as the stationary phase and tetrahydrofuran/chloroform/n-heptane/methanol/conc. ammonia 20:20:57:7:1 per volume as the mobile phase. The chromatography (6 cm ascending) is carried out without preceding chamber saturation. The compounds separated are visualized by uv at 254 and 366 nm, respectively, and by iodine vapour. Rf of bromocriptine is 0.27. The potential by-products yield spots at R_{st} 1.5 (2-bromo-α-ergocryptinine), and 0.55 (α-ergocryptine). 2-Bromo-lysergic acid remains at the starting point.

Beside the modified Dragendorff's reagent/hydrogen peroxide already mentioned, a variety of spraying reagents has been used for the visualization of bromocriptine. They are compiled in the following list.

spraying reagents for bromocriptine

reagent	colour	detection limit on silica gel Merck no. 5715
Dragendorff (Munier,Deboeuf) + 30 percent H_2O_2	brown	0.2 µg
0.05 percent aqueous permanganate	yellow	1 µg
van Urk's reagent	grey	10 µg
cinnamic aldehyde	yellow	1 - 2 µg
formaldehyde/hydrochloric acid	grey	2 µg
formaldehyde/sulphuric acid	violet	0.1 µg
chlorine/o-toluidine	violet	0.1 µg
folin/ammonia	grey	1 µg
ninhydrin/uv det. 360	red	0.1 µg
iodine/potassium iodide	brown	2 µg
Ehrlich's reagent	orange	1 µg

The identity of methanesulphonic acid may be determined by tlc on cellulose plates, Merck no. 5728, with ethanol/water/conc. ammonia 80:16:4 (v/v/v) as a mobile phase. Detection is achieved by spraying with acid-base indicators, e.g. bromocresol green or similar species. Rf of methane-sulphonic acid is 0.5 (that of bromocriptine base = 0.9).

7.43 Gas Chromatography

GC cannot be applied to the analysis of bromocriptine mesilate due to its low volatility and its thermal instabili-ty. A procedure according to 29 or 30, which claims excellent identification and quantitation on the basis of well-defined peptide section pyrolysis products, has not yet been attempted. However, GC is very useful determining the residual recrystallization solvent butanone-2.
The conditions are the following:

    Column: Porapak Q 80/100 mesh, in 1.8 m x 2mm glass
    Temperatures: injector 240 °C, column 130 °C
                  flame ionization detector, 240 °C.

7.44 High Performance Liquid

A series of HPLC systems have been utilized both for assay and purity of bromocriptine mesilate.
Satisfactory procedures have been accomplished both on straight phase (silica gel) and on reversed phase (octadecylsilanised silica gel) columns.

HPLC-conditions

straight phase:

stationary phase: silica gel, 5 µm, in stainless steel
                  25 cm x 3 mm i.d.
mobile phase: water-saturated dichloromethane/methanol
                  100:2 (v/v)
uv detection at 254 nm.

Fig. 11 shows a chromatogram of bromocriptine mesilate spiked
with dodecylbenzene, potential by-products and the precursor.
The flow was 1.7 ml/minute (31).

Key: 1 = dodecylbenzene, 2 = bromocriptinine, 3 = α-ergocryp-
tinine, 4 = bromocriptine, 5 = α-ergocryptine (precursor).

reversed phase, system I:

stationary phase: Merck RP-18, 10 µm in stainless steel
                  25 cm x 4.6 mm i.d.
mobile phase: gradient: 35 to 60 percent B within 60 min.
                  A = water, 0.2 percent triethylamine added
                  B = acetonitrile, 0.2 percent triethylamine
                     added.
uv detection at 280 nm.

This system was found optimal for the purity test.

Fig. 12 shows a chromatogram of the drug substance spiked
with potential by-products and the precursor. The flow was
set at 4.0 ml/minute.

Key: I = 2-bromo-α-ergocryptinine, II = α-ergocryptinine,
III = α-ergocryptine (precursor), IV = bromocriptine,
V = 2-bromo-lysergamide, VI = 2-bromo-lysergic acid,
VII = 2-bromo-isolysergamide.

reversed phase, system II (26):

stationary phase: Merck RP-18, 10 µm in stainless steel,
                  25 cm x 4.6 mm
mobile phase: 0.01 M ammonium carbonate or 0.05 M
                  ammonium hydrogen carbonate solution/
                  acetonitrile 35:65 isocratic,
                  flow 1.5 ml/min.

Figure 11.   High Performance Liquid Chromatogram
             of Bromocriptine Mesilate, spiked with
             Dodecylbenzene, Precursor and potential
             By-products.
             Adsorption Mode, isocratic,
             Uv-detection at 254 nm
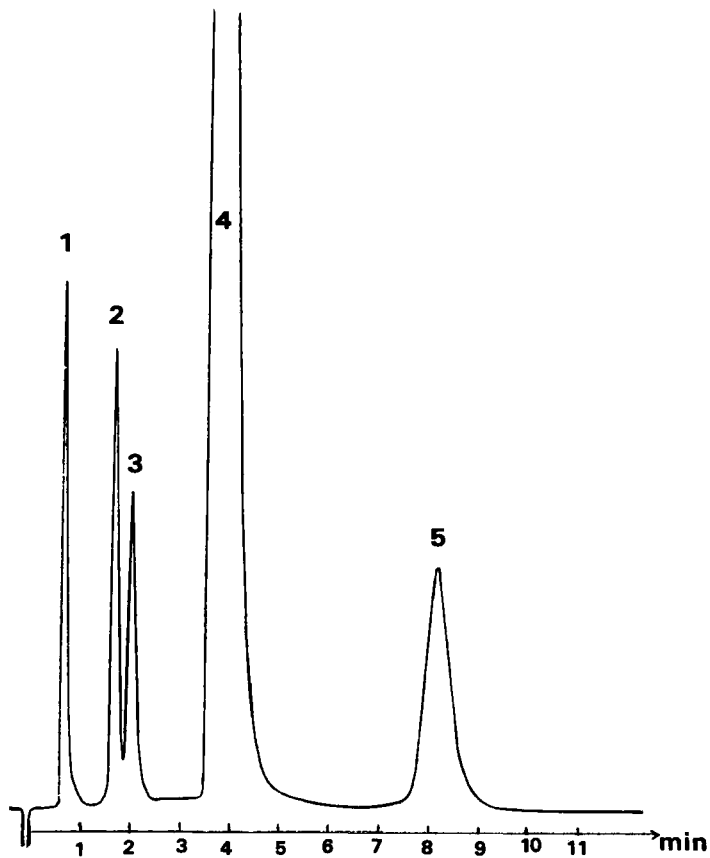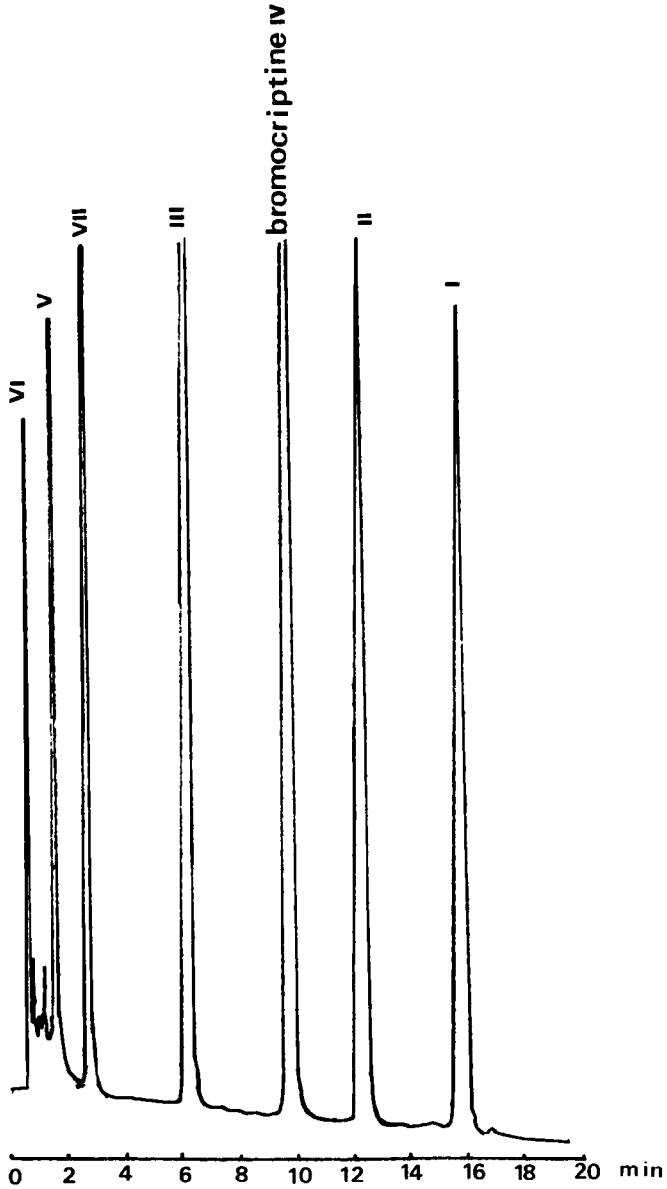
Figure 12.   High Performance Liquid Chromatogram
              of Bromocriptine Mesilate, spiked with
              Precursor and potential By-products.
              Reversed-phase Mode, Solvent Gradient,
              Uv-detection at 280 nm.

7.5 Differential Scanning Calorimetry

Differential scanning calorimetry cannot be applied to quantify the purity of the drug substance for the reasons mentioned in 2.31 and 2.34. However, it may be valuable in qualitative comparisons from sample to sample or batch to batch on the basis of their corresponding differential scanning calorimetry patterns.

7.6 Phase Solubility Analysis

For phase solubility analysis, acetonitrile appears to be the most suitable solvent. A typical plot is given in figure 13 along with the experimental conditions.

7.7 Analysis of the Dosage Form

The identification of bromocriptine mesilate in the dosage form can be carried out by thin layer chromatography using Merck plates with dichloromethane/methanol/formic acid 78:20:2 (v/v/v) and subsequent uv-visualization at 254 and 360 nm. Using this method, it is important to only air-dry the spot after application to the plate, since more vigorous evaporation of the solvent will give rise to artifacts (32).

Bromocriptine can also be identified as the base by ir spectroscopy after extraction from the dosage form with ethanol and removal of the solvent, both in solution and in a KBr pellet (33).

Bromocriptine mesilate in Parlodel® tablets may be assayed in a non-specific way by direct uv-spectrophotometry following extraction with methanol (32).

Fluorimetry in 0.1 N hydrochloric acid has been applied during the measurement of dissolution rate of the dosage forms with excitation at 335 and emission at 425 nm, respectively (26).

A specific assay of bromocriptine mesilate in the dosage form may be carried out by tlc followed by uv-spectrophotometry (26)(The system can also serve for identification purposes). The drug substance is extracted with methanol in the absence of light, the chromatographic conditions are: Merck plates F 254, mobile phase: dichloromethane/dioxane/ ethanol abs./conc. ammonia 180:15:5:0.1 per volume. The spot corresponding to bromocriptine is extracted with methanol, and the concentration is determined at about 300 nm by spectrophotometry.
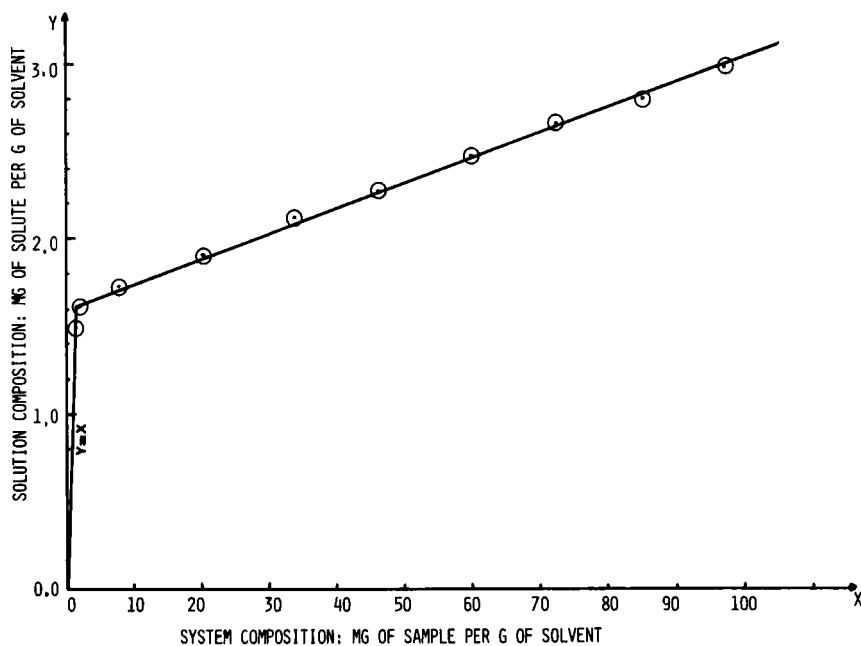
Figure 13.   Phase Solubility Analysis Plot of Bromocriptine
             Mesilate, dried in High Vacuum for 15 Hours.
             Solvent: dry Acetonitrile, Vibration for 24 hrs.
             in the Absence of Light. Slope 1.41 ± 0.05 %.

A further specific assay is the HPLC-determination of
bromocriptine mesilate following extraction with methanol
from the dosage form using RP-18 as the stationary and aceto-
nitrile/0.01 M ammonium carbonate solution 65:35 as the mobile
phase. Uv-detection wavelength is set at 300 nm (26).

For the detection and estimation of degradation products
in dosage forms, a solvent gradient, containing the components
mentioned above, is utilized with advantage.

7.8 Determination in Body Fluids and Tissues (23)

Due to its potent efficacy in the treatment of hyperpro-
lactinemia and acromegaly, bromocriptine is administered in
low doses leading to minute concentrations in body fluids and
tissues. Therefore, none but the most sensitive analytical
methods can be used to measure its concentration in biological
specimens. The only method so far applicable for pharmaco-
kinetic studies with bromocriptine is the use of the radio-
actively labelled drug, measurement of total radioactivity and
its fractionation by chromatographic separation techniques for
the assay of parent drug and major metabolites. Recently, a
radioimmunoassay kit for the analysis of picogram quantities
of unchanged bromocriptine in body fluids has become availa-
ble. Gas chromatography, mass fragmentography and liquid
chromatography also appear to be suitable for determining
bromocriptine in plasma from patients with Parkinson's disease
which are on treatment at high dose levels. These currently
developped procedures permit quantitative determinations down
to concentrations of 0.5 (GC), 1.0 (MF), and 10 ng/ml (LC),
respectively (34).

The pattern of metabolites in bile (animals only) and in
urine have been investigated using column chromatography
(Amberlite XAD 2 and Sephadex DEAE), tlc and reversed phase
HPLC in combination with radioactivity monitoring.
The principal metabolites have been isolated from the bile
of rats treated with high doses of bromocriptine. The
structure of the isolated metabolites was elucidated by means
of spectroscopic techniques.

8.   References

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Acknowledgments

CALCITROL

Eileen Debesis

1.   Description
     1.1 Name, Formula, Molecular Weight
          Calcitriol is 1 α, 25-dihydroxycholecalciferol.

MW = 416.65

$C_{27}H_{44}O_3$

     1.2 Appearance, Color, Odor
          Calcitriol is an odorless white crystalline
powder.

2.   Physical Properties
     2.1 Infrared Spectrum
          The infrared spectrum of calcitriol is shown in
Figure 1 (1). The spectrum was recorded on a Perkin-Elmer
Model 283 Grating Infrared Spectrophotometer and was measured
in a KBr pellet which contained 1 mg of calcitriol in 300 mg
of KBr.

          The following absorptions have been assigned for
Figure 1:
     a.   OH stretching (bonded): 3391 cm^{-1}
     b.   Aliphatic CH stretching: 2943, 2872 cm^{-1}
     c.   CH deformation: 1468, 1378 cm^{-1}
     d.   C-O stretching: 1056 cm^{-1}

     2.2 Nuclear Magnetic Resonance Spectrum (NMR)
          The NMR spectrum of calcitriol, recorded on a
Varian XL-100/Nicolet TT-100 pulsed Fourier Transform NMR
spectrometer, with internal deuterium lock, is shown in
Figure 2 (2). The spectrum was recorded using a solution of
0.84 mg of sample dissolved in 50 microliters of CD_3OD (100%D)
containing 1% v/v tetramethylsilane in a 1.7 mm capillary
tube. The spectral assignments are given in Table I.
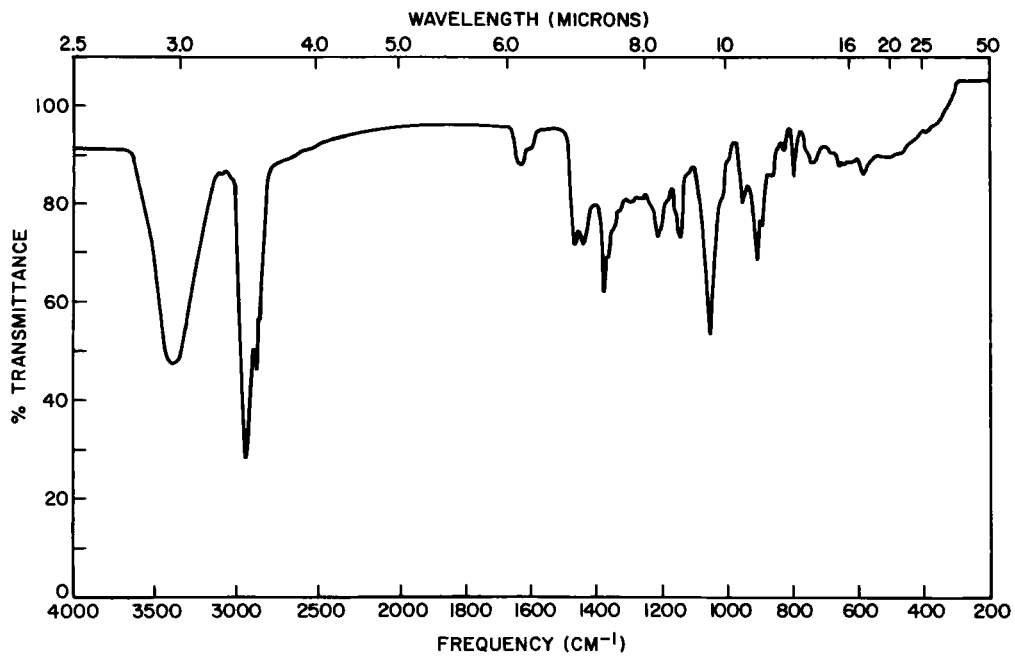
Figure 1
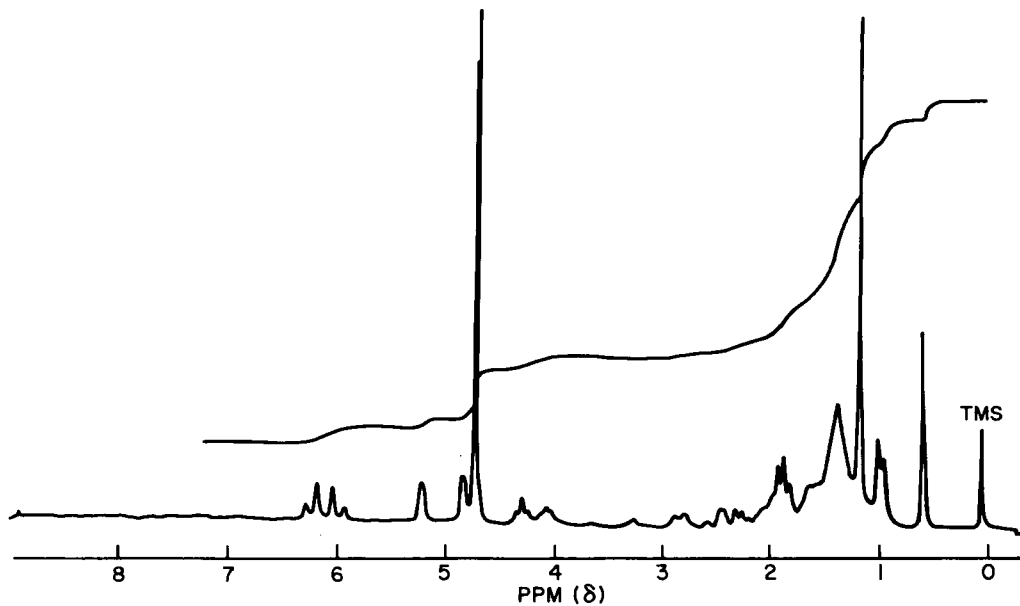
Infrared Spectrum of Calcitriol

Figure 2

NMR Spectrum of Calcitriol

Table I

NMR Spectral Assignments for Calcitriol

Proton	Chemical Shift (σ)	Multiplicity
$-CH_3(C_{18})$	0.57	Singlet
$-CH_3(C_{21})$	0.95	Doublet
$-CH_3(C_{26,27})$	~1.16	Singlet
$\searrow CH_2, \searrow CH$	~1.38-3	Complex
HO $\searrow CH$	4.08	Complex
HO $\searrow CH$	4.32	Triplet
$=CH_2$	4.90, 5.28	Complex
$\searrow C-C \searrow$ H H	6.07, 6.32	AB Quartet $J_{AB} = 11$ Hz
HDO + 3 x OH (exchangeable protons)	4.78	-

2.3 Ultraviolet Spectrum

The ultraviolet spectrum of calcitriol (1 mg of calcitriol/100 ml of absolute ethanol) in the region of 220 to 400 nm exhibits one maximum at 264 nm (ε = 1.9 x 10^4) and one minimum at 226 nm. The spectrum is shown in Figure 3 (3).

2.4 Mass Spectrum

The low resolution mass spectrum of calcitriol is shown in Figure 4 (4). The spectrum was obtained using a Varian MAT CH5 spectrometer, which was interfaced with a Varian data system 620 I. The data system accepts the output of the spectrometer, calculates the masses, compares the intensities to the base peak, and plots this information as a series of lines whose heights are proportional to the intensities.

The molecular ion was measured at m/e = 416. Other characteristic masses were observed at m/e = 398, 380 and 362, corresponding to the loss of one, two and three molecules of water, respectively, from the molecular ion; m/e = 383, corresponding to the loss of water and CH_3 from the parent peak; and m/e = 365, corresponding to the loss of two water molecules and one CH_3 from the molecular ion. The base peak is observed at m/e = 134, and corresponds to the

moiety
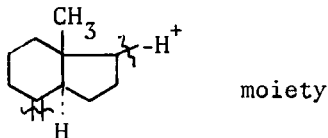
2.5 Melting Range

Calcitriol melts at 111-115°C (3).

2.6 Differential Scanning Calorimetry

Melting was accompanied by decomposition (5).

2.7 Thermogravimetric Analysis

Calcitriol was subjected to thermogravimetric analysis on a Perkin-Elmer Model TGS-1 Thermogravimetric Analyzer. The sample exhibited two overlapping weight losses. The initial weight loss, of 0.4%, began at 55°C and ended at 105°C, and was due to surface moisture or solvent. The second weight loss, due to decomposition, amounted to 2.5% at 205°C, 7.5% at 255°C, 33% at 305°C, and 93% at 355°C (5).

2.8 Hot Stage Microscopy

As observed on a Mettler Hot Stage FP 52 with FP5 controller, the sample appeared as birefringent needle-like crystals which melted from 115-117°C. The sample did not recrystallize from the melt (5).
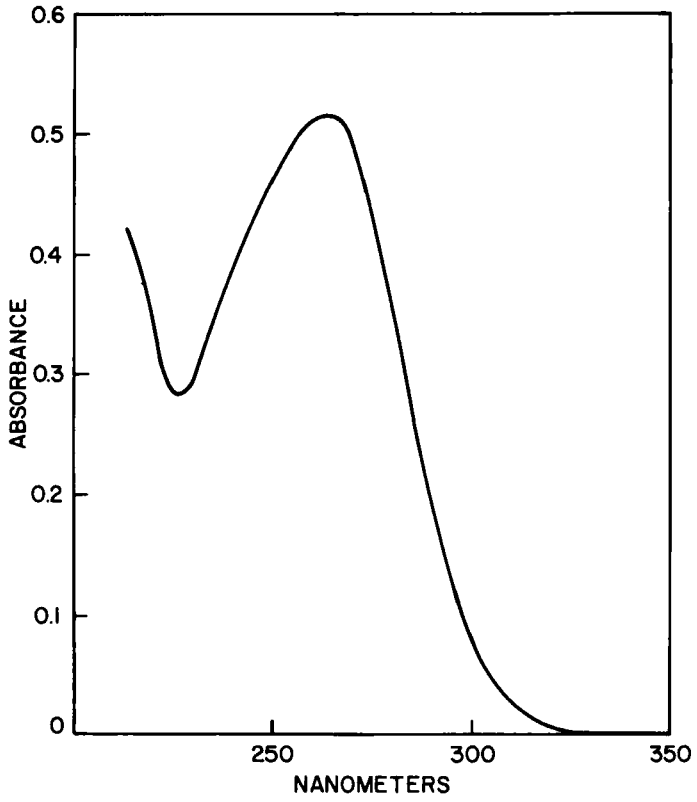
Figure 3

Ultraviolet Spectrum of Calcitriol
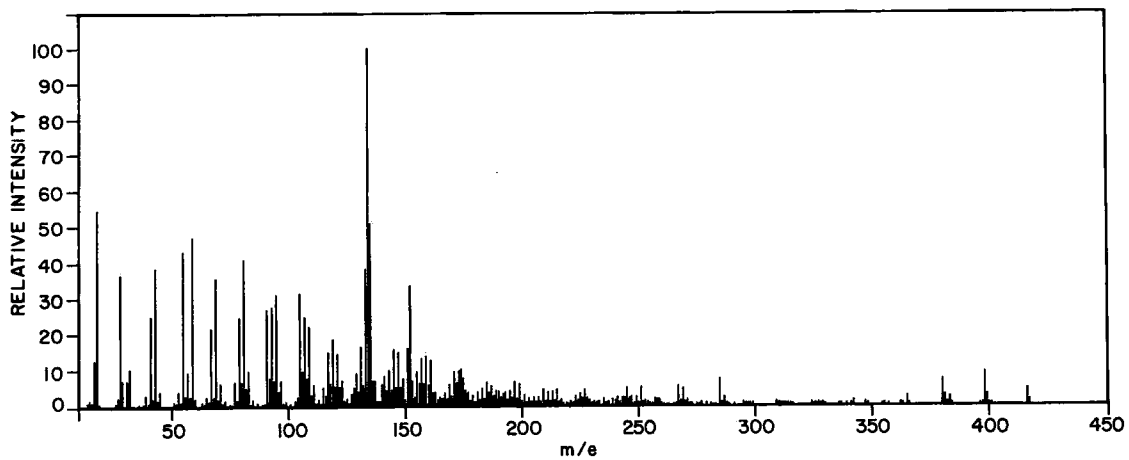
Figure 4

Mass Spectrum of Calcitriol

2.9 Solubility
       Extensive solubility data is not readily obtain-
able due to the scarcity of calcitriol. The material is
slightly soluble in methanol, ethanol, ethyl acetate and
tetrahydrofuran (3).

2.10 X-Ray Powder Diffraction
       The X-ray powder diffraction data for calcitriol
are presented in Table III (3); instrumental conditions are
given below. The diffraction pattern is shown in Figure 5.

Instrument and Operating Conditions

Instrument	Guinier-De Wolff Camera
Generator	GE XRD-6 50 KV, 12.5 mA
Detector	Film
Sample	As is
Densitometer*	Gelman DCD-16
Optics	Tungsten Lamp 575 nm Visible mode
Detector	Silicon phototransistor
Optical density	0.25
Beam exit slit	0.53 mm

*The diffraction pattern was recorded on film; the density
 of the individual lines in the pattern was determined by
 densitometry.
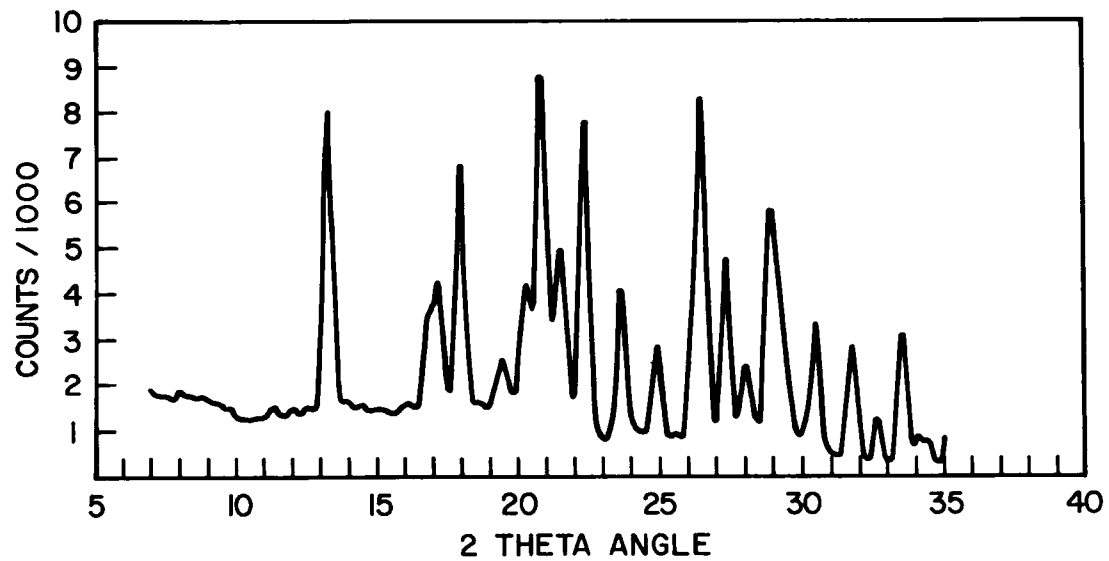
Figure 5

X-Ray Powder Diffraction Pattern of Calcitriol

TABLE III

Calcitriol Powder Diffraction Data

2θ	d (Å)*	I/Io**
26.52	4.993	100
13.33	9.863	90
22.41	5.892	88
20.89	6.316	82
18.01	7.314	70
28.97	4.578	66
27.33	4.847	49
23.70	5.577	44
33.55	3.968	36
30.54	4.348	34
31.77	4.183	33
17.19	7.662	33
24.95	5.302	26
21.54	6.129	26
28.04	4.727	15
32.66	4.072	12
19.46	6.776	12
20.35	6.480	12
16.07	8.192	2
11.37	11.559	2
8.03	16.343	2
34.18	3.896	2
12.07	10.889	1

*d $= \dfrac{n \lambda}{2 \sin \theta}$ (interplanar distance)

**I/Io = percent relative intensity (based on maximum intensity of 1.00)

2.11 Optical Rotation

The specific rotation of calcitriol in absolute methanol, measured at 589 nm and 25°C, was +48° (7).

3. Synthesis
    Several procedures for the synthesis of calcitriol have
been reported in the literature. Parren et al.(8) and Norman
et al. (9) described the preparation of calcitriol from
25-hydroxyvitamin D$_3$. Uskokovic and others (10-18) have
described lengthier syntheses utilizing more readily avail-
able starting materials. A typical reaction scheme, utiliz-
ing 1 α, 25-diacetoxy-7-dehydrocholesterol as the starting
material, is shown in Figure 6.

4. Stability and Degradation
    Calcitriol must be protected from air and light. The
drug substance exhibits good stability when stored at -15°C
to -25°C in an argon atmosphere. The material is stable
at room temperature when dissolved in a vegetable oil de-
rivative, containing antioxidants, such as is used in calci-
triol soft gelatin capsules (19).

5. Drug Metabolic Products
    Calcitriol may be absorbed directly into the intestine
or bone, or may be hydroxylated to form 1 α, 24, 25-trihydro-
xycholecalciferol prior to intestinal absorption (20-24).

6. Methods of Analysis
    6.1 Elemental Analysis
        A typical elemental analysis of a sample of calci-
triol is presented in Table IV (7).

TABLE IV

Elemental Analysis of Calcitriol

Element	% Theory	% Found
C	77.84	77.53
H	10.64	10.75
0	11.52	11.92
		(by difference)
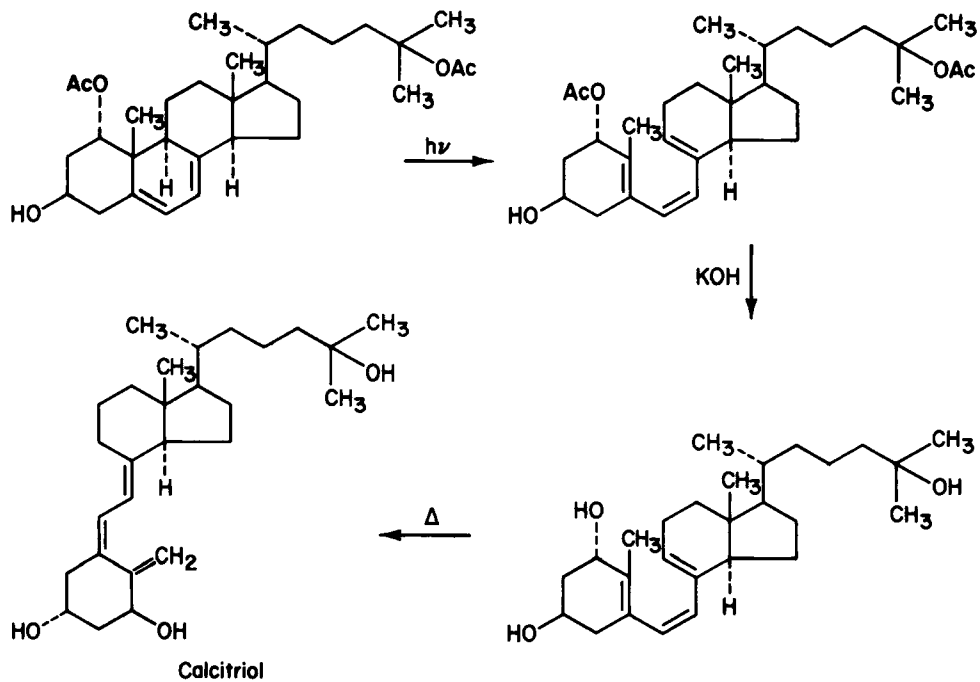
Figure 6
Synthesis of Calcitriol

6.2 Chromatographic Methods
6.21 Thin-Layer Chromatography (TLC)

The following TLC procedure is useful for determining the purity of calcitriol. It separates the previtamin, 1 α, 25-dihydroxyprecholecalciferol. A silica gel GF plate is activated by heating for one hour at 105°C and is then cooled in a desiccator. A low actinic all glass chromatographic chamber is equilibrated with the developing solvent, and 0.8 mg of calcitriol is applied to the plate from ethyl acetate. The plate is developed in an ascending mode in ethyl acetate:spectroquality heptane:methanol (100:10:2) for 15 cm. After air drying, the plate is viewed under shortwave ultraviolet radiation, then sprayed with a 15% w/v solution of phosphomolybdic acid in ethanol, followed by heating at 105°C for 10 minutes to develop the colors. The approximate R_f values are summarized in Table V (3).

TABLE V

Summary of TLC Data

Compound	Approximate R_f
1 α, 25-Dihydroxyprecholecalciferol	0.4
Calcitriol	0.5

6.22 High Performance Liquid Chromatography

High performance liquid chromatography is used to determine the purity of calcitriol, and to separate it from related compounds. Using a 10 micron silica column of 25 cm length, and a mobile phase of spectroquality heptane: ethyl acetate:methanol (50:50:1) at a flow rate of 1.7 ml/ minute, separation and quantitation are achieved. p-Dimethyl-aminobenzaldehyde may be used as an internal standard to compensate for variations in injection technique and instrumental conditions. With a 254 nm ultraviolet absorbance detector, 0.01 μg of calcitriol may be detected (3).

This procedure, with a mobile phase of spectroquality heptane:ethyl acetate:methanol (70:25:5) is also useful for analyzing calcitriol in soft gelatin capsules. The capsule fill solution may be injected directly. The amount of calcitriol in the capsule is determined by comparison to a calcitriol reference standard, prepared in a medium similar to the capsule fill (3).

6.23 Gas Chromatography - Mass Spectrometry

Halket and Lisboa (25) examined several Vitamin D derivatives by capillary gas chromatography coupled with mass spectrometry. This technique offered the advantages of great sensitivity and separating power. Retention times and fragmentation patterns for ergocalciferol, cholecalciferol and calcitriol were reported.

6.3 Biological Methods

6.31 Radioimmunoassay

Several workers (26-32) have reported on the use of radioimmunoassay for measuring calcitriol at very low (pg) levels in serum.

6.32 Protein Binding Assays

Various protein binding techniques are reported (32-42) for the determination of calcitriol in plasma. Generally, a preliminary purification step is required to avoid interference from other plasma components.

6.33 Bioassays

Stern, et al. (43,44) reported a bioassay technique based on fetal rat bone absorption of calcitriol. Parkes and Reynolds (45) developed an in-vitro bioassay using duodenal tissue from chicken embryos.

6.4 Polarography

Calcitriol drug substance may be analyzed polaro-graphically, using a glassy carbon working electrode. The limiting current of the observed oxidation wave ($E_{1/2}$ = 0.96 v) is linear with concentration in the 0.01 to 0.03 mM region (46).

7. Acknowledgments

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CHLORTETRACYCLINE HYDROCHLORIDE

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USASRG *

1. DESCRIPTION
    1.1 Drug Properties
         Chlortetracycline hydrochloride (CTC-HCL) is the
hydrochloride salt of an antibiotic substance produced by the
growth of **Streptomyces aureofaciens** (Fam. Streptomycetaceae).
It was discovered in 1948 by Duggar (1).

         CTC-HCl is a broad-spectrum antibiotic and anti-
protozoan active against many Gram-positive bacteria, some
Gram-negative bacteria, spirochetes, amebae, and certain
large viruses. Staphylococci have become generally resistant
to the drug, and drug-resistant strains may be found among
other bacterial genera and species generally sensitive to it.
Cross-resistance to other antibiotics of the tetracycline
family is automatic (2).
         Organisms may be considered susceptible if the
Minimum Inhibitory Concentration (MIC) is not more than 4.0
µg/ml and intermediate if the MIC is 4.0-12.5 µg/ml (see
Table 1). Tetracyclines are readily absorbed and are bound
to plasma proteins in varying degrees. They are concentrated
by the liver in the bile and excreted in the urine and feces
at high concentrations and in a biologically active form.
The mode of action against microorganisms involves the
inhibition of phosphorylation processes in bacterial cells (3).

TABLE 1 (5)
Antimicrobial Spectrum of Chlortetracycline

Microorganism	Minimum Inhibitory Concentration (µg/ml)
Micrococcus pyrogenes var. aureus 209P	0.292
Micrococcus pyrogenes var. aureus 1248A	0.39
Streptococcus pyrogenes	0.29
Streptococcus mitis	0.147
Streptococcus faecalis	0.39
Micrococcus flavus	0.292
Diplococcus pneumoniae	0.098
Sarcina lutea	0.147
Bacillus subtilis	0.195
Escherichia coli	1.45
Haemophilus influenzae	0.312
Klebsiella pneumoniae	0.29
Neisseria catarrhalis	0.098
Aerobacter aerogenes	1.17
Proteus vulgaris	4.6
Pseudomonas aeruginosa	25
Salmonella schottmuelleri	3.12
Salmonella typhi	1.17
Shigella dysenteriae	3.12
Brucella bronchiseptica	0.292
Mycobacterium tuberculosis	0.147
Mycobacterium friedmanii	0.122
Mycobacterium smegmatis	0.073
Mycobacterium sp.	0.58
Candida albicans	100
Clostridium butyricum	0.078
Pasteurella multocida	0.049
Vibrio percolans	0.098

1.2   Physical Description and Optical Crystallography
CTC-HCl is a yellow, odorless powder composed mainly of crystals in the shape of small hexagons, and has the following optical crystallographic characteristics (4):

α: 1.635; optic sign negative; $2\nu = 59°$ (calc.);

β: 1.706; orthorhombic; extinction parallel; and

γ: 1.730; symmetrical

It is stable in air, but is slowly affected by light (6).

1.3   Chemical Properties
CTC-HCl is the HCl salt of amphoteric CTC; it is multifunctional with two chromophores. It is a para-chlorophenol with an α,β-unsaturated ketone in conjugation. The second chromophore involves another α,β-unsaturated ketone that is in conjugation with an anomalously behaving amide (7). The tertiary amine is responsible for the basic character and the phenolic group is acidic. CTC is fluorescent and can be assayed polarographically (8).

A particularly intriguing aspect of the chemistry of the compounds of the tetracycline family is their ability to form metallic complexes. CTC shares in this intensively studied capability, which is very likely related to the therapeutic activity (9). This property is also used in the purification (10) and analysis (11) of CTC.

1.4   Structure
$C_{22}H_{23}ClN_2O_8 \cdot HCl$ Empirical Formula

515.35 Molecular Weight

7-Chloro-4-(dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-2-naphthacenecarboxamide monohydrochloride (CAS-64-72-2) (6,12-14) (Figure 1).

1.5   The FDA Chlortetracycline Standard
The current official FDA Chlortetracycline Working Standard is chlortetracycline hydrochloride, Lot #501-632B-95-1 (9/29/53), obtained from Lederle, which markets the antibiotic under the proprietary name Aureomycin. The current working standard has an assigned potency of 1000 µg/mg (the term µg applied to chlortetracycline means the chlortetracycline activity (potency) contained in 1 µg of the FDA Chlortetracycline Master Standard (Lot #990-107-141-1), which is also chlortetracycline hydrochloride).
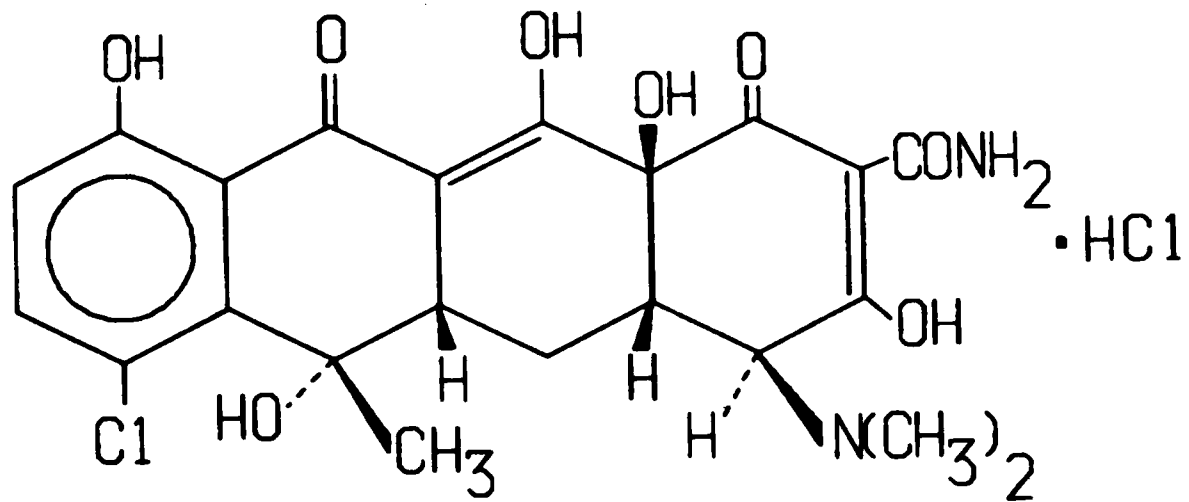
Figure 1. Structural formula of CTC-HCl.

The Working Standard is stored in lots of 250 mg at -20°C, protected from light and moisture, at the National Center for Antibiotics Analysis, Washington, DC.

2.   PHYSICAL PROPERTIES
   2.1   Thermal Properties
          Differential Thermal Analysis (DTA) and Thermal Gravimetric Analysis (TGA). CTC-HCl is stable until it begins to decompose exothermically at approximately 230°C (Figures 2 and 3) (15).   The compound does not lose any mass until the final decomposition takes place.   No polymorphs have been seen in the samples examined.

   2.2   X-Ray Powder Diffraction
          The X-ray diffraction pattern of the FDA Working Standard has been determined.   It demonstrates crystalline structure; the data are listed in Table 2 (16).

TABLE 2
X-Ray Diffraction Data

d(Å)	I/I_1	d(Å)	I/I_1	d(Å)	I/I_1
		3.34	17		
9.10	11	3.27	37	2.060B	13
8.48	12	3.23	16	D(1.985	11
D(7.75[a]	56	D(3.17	73	(1.962	13
(7.43	44	(3.12	74	1.897	12
6.65	13	3.095	38	1.880	10
6.38	43	2.982	8	1.868	13
D(5.72	81	2.948	9	1.856	13
(5.61	42	2.910	47	1.827	8
5.28	61	D(2.878	25	1.805	9
5.16	8	(2.854	31	1.784	6
D(4.96	8	2.788	35	D(1.748	8
(4.88	15	2.710	36	(1.737	11
4.68	3	D(2.653	14	1.705	9
4.54	40	(2.632	10	1.682	6
4.42	100	2.560	30	1.638	5
4.29	70	2.482	22	1.631	4
4.24	25	2.440	13	1.608	5
D(4.15	52	2.421	27	1.595	4
(4.09	78	2.390	12	1.583	4
3.88	37	2.343	24		
3.80	31	2.290	7		
3.71	59	2.235	14		
3.64	33	2.210	37		
3.57	16	2.170	13		
3.52	25	2.142	23		
3.37	23	2.093	21		

[a]D = Doublet.

120° 140° 160° 180° 200° 220° 240°
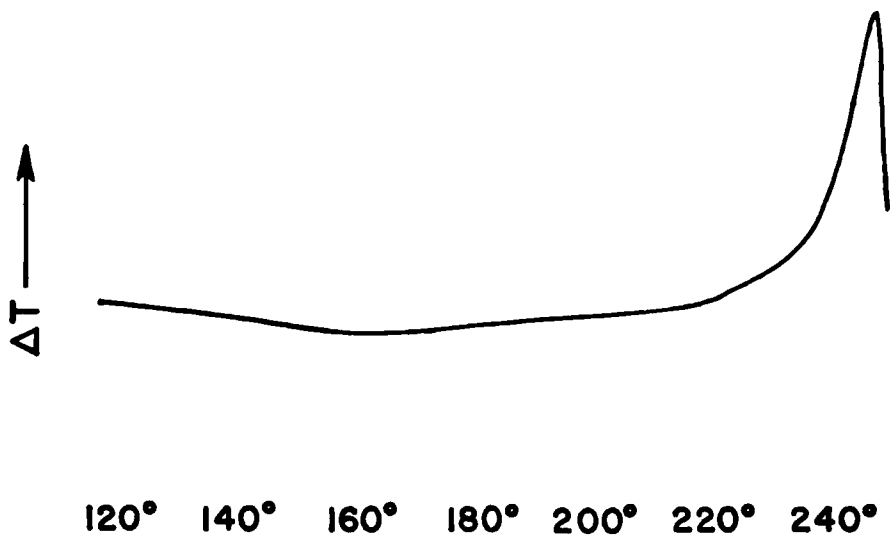
Figure 2.   Differential thermogram of CTC-HCl.

120°   160°   200°   240°
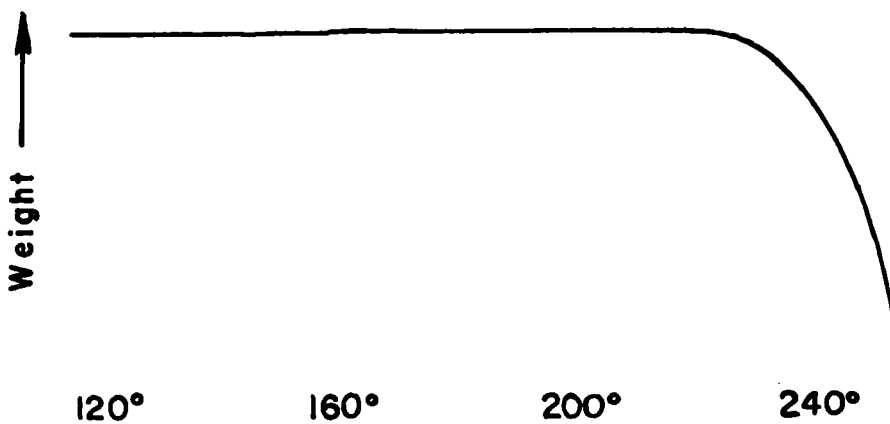
Figure 3.   Thermal gravimetric analysis curve of CTC-HCl.

2.3   Solubility
The solubility of antibiotics, including CTC-HCl, was reported by Andrew and Weiss (17). CTC-HCl is an amphoteric substance and consequently it is soluble in aqueous acid and base. However, it can rapidly degrade in these solvents. Its solubility in water is about 8 mg/ml and in methanol about 17 mg/ml. In higher molecular weight alcohols, the solubility of CTC-HCl is considerably less than in methanol. For practical purposes, it is insoluble in many common solvents such as the aliphatic hydrocarbons, benzene, ether, and chloroform. It is readily soluble in pyridine and to the extent of about 5 mg/ml in formamide. Pyridine is an undesirable solvent because of its basicity, and formamide is not desirable because of the difficulty in obtaining and maintaining it as a stable solvent.

2.4   Acid-Base Properties
CTC exhibits three acidic dissociation constants when titrated in aqueous solutions (18). Stephens et al. (19) identified the three acidic groups (Figure 4), and reported thermodynamic pKa values of 3.30, 7.44, and 9.27. Leeson et al. (20) assigned pKa values to the following acidic groups:

pKa	Assignment
3.30	Tricarbonylmethane System (A)
7.44	Phenolic Diketone System (B)
9.27	Dimethylamino System (C)

Kalnins and Belen'skii (21) verified the assignments by infrared (IR) spectroscopy.

The pH of a 10 mg/ml aqueous solution, as described in the Code of Federal Regulations monograph for CTC-HCl (22) should lie between 2.3 and 3.3.

2.5   Polymorphism
In recent years, with growing concern about the relative bioavailabilities of different samples of the same drug substance, polymorphism has become of prime interest. Miyazaki and co-workers (23) have reported the existence of two crystalline forms of CTC-HCl. The X-ray powder diffraction patterns, IR spectra, dissolution behaviors, and hygroscopicities that they reported were distinctly different and there were discrepancies in the bioavailabilities. In later work on two forms of CTC base prepared by crystallizing from water or methanol, the X-ray patterns and
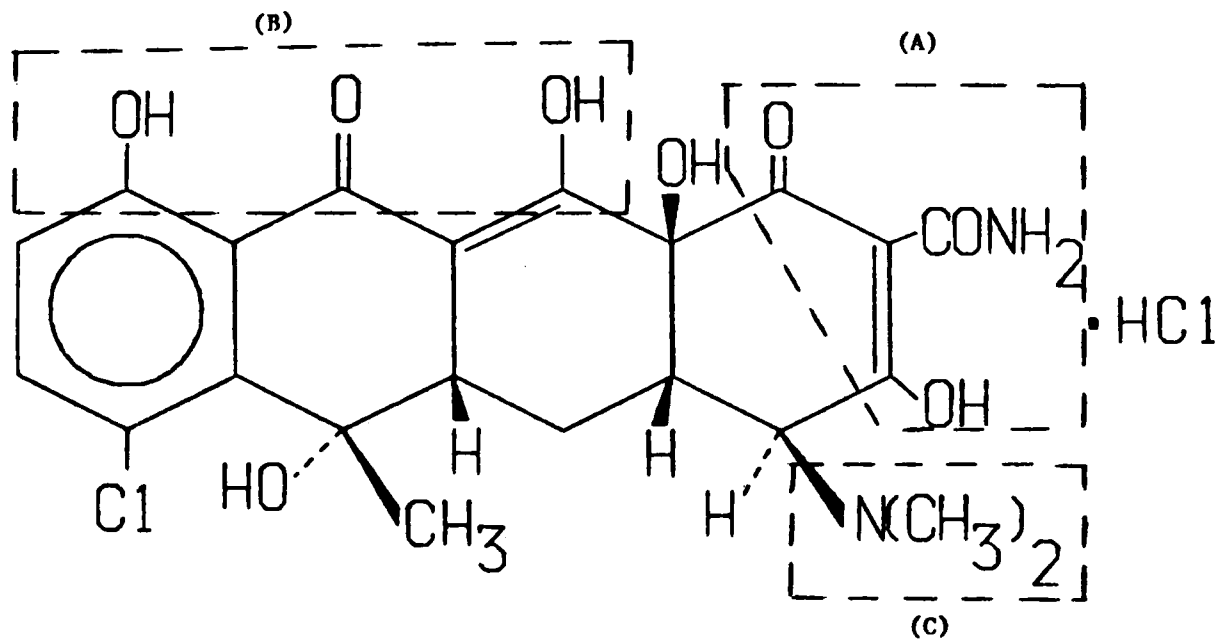
Figure 4. Acid-base properties of CTC.

water contents were different, but other physical properties
were indistinguishable. There appeared to be no difference
in bioavailability (24). No polymorphs were found in the FDA
CTC standard.

3.   SPECTRAL PROPERTIES (OPTICAL)
      3.1   Ultraviolet

Medium	Maxima (nm)
0.1N HCl	368, 340sh, 322sh, 265, 229
0.1N NaOH	345, 283, 253, 222
Methanol	372, 342sh, 322sh, 262sh, 253, 233

See Figure 5 (25).

      3.2   Infrared
            The IR absorption spectra of CTC-HCl are shown in
Figures 6 and 7. Spectra were run on a Perkin-Elmer Model 467
grating spectrophotometer as a KCl pellet (1 mg/200 mg KCl),
and as a Nujol mull (20 mg/3 drops). The spectra are essen-
tially identical. The CTC-HCl spectrum may also be found in a
compilation of IR spectra of Drug Reference Standards by
Hayden et al. (26). Major absorption frequencies have been
compiled (27) and assignments have been made on many of the
bands(28-31). Some of the major frequencies and band assign-
ments are:

cm^{-1}	λ	
3360	2.98	νNH of NH_2 (asym.)
3315	3.02	νNH of NH_2 (sym.)
3200-3450	2.90-3.12	νOH (alcoholic, masks NH)
2800-2400	3.57-4.17	tertiary amine halide salt, group of broad bands
1675	5.97	νC=O
1582	6.32	δNH_2
1450	6.90	δCH (bending of CH_3)
1368	7.31	νCN
1042	9.60	OH (deformation, alcohols)
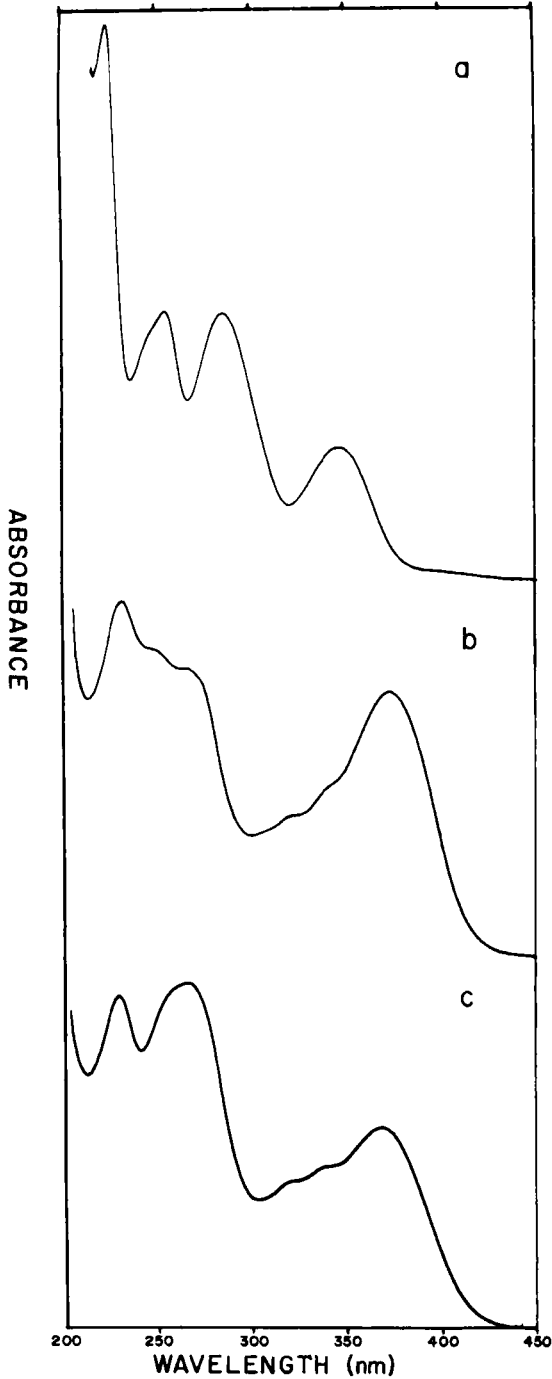
Figure 5.   Ultraviolet absorption spectra of CTC-HCl in
             a, 0.1N HCl; b, 0.1N NaOH; and c, methanol.
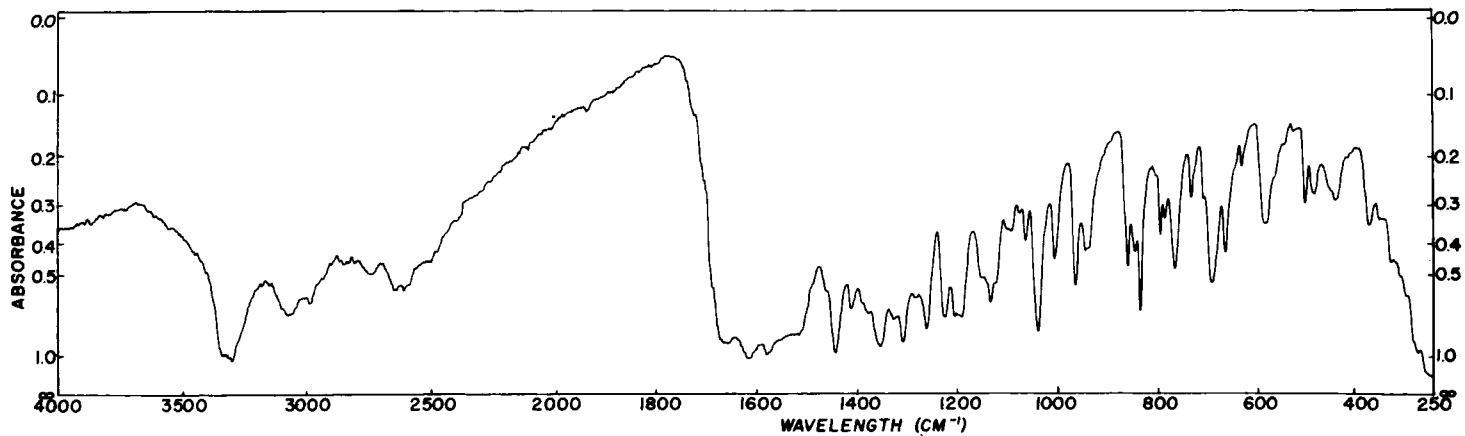
Figure 6. Infrared absorption spectrum of CTC-HCl as KCl pellet.
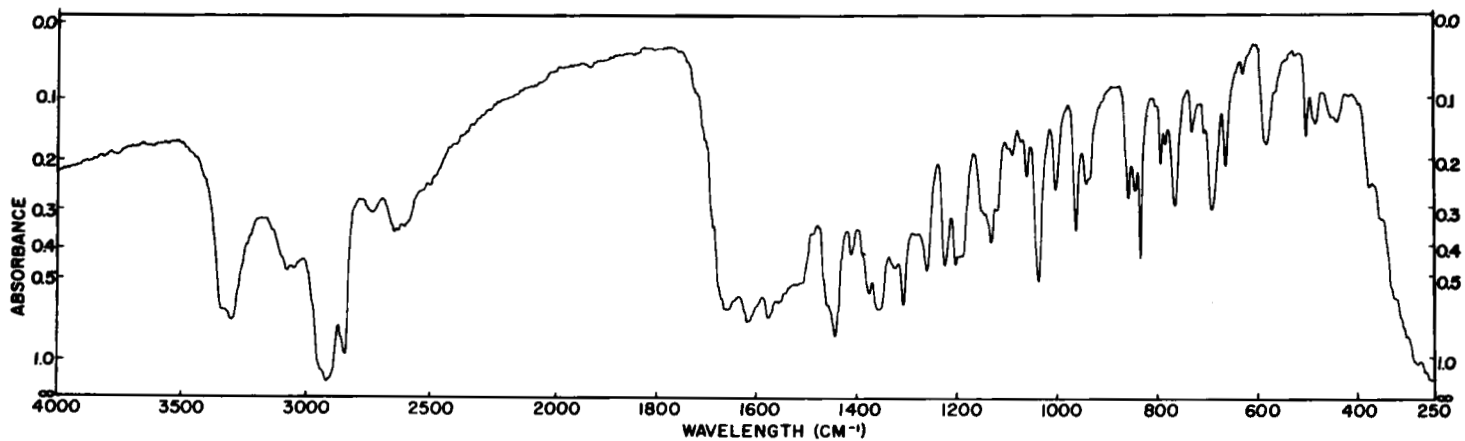
Figure 7. Infrared absorption spectrum of CTC-HCl as Nujol mull.

3.3   Spectropolarimetry

Because configurational information can be derived
from optical rotatory dispersion and circular dichroism scans,
considerable work has been conducted using these techniques to
study the tetracyclines (32). The absolute configuration of
CTC was determined using optical rotatory dispersion data (33).
Spectral curves are presented in Figures 8 and 9. The circu-
lar dichroism spectrum is similar to that presented by
Mitscher et al. (34), except that the values differ by a
factor of about 1.5. In Table 3, data obtained by Mitscher
and in FDA laboratories are compared.

3.4   Fluorescence

The CTC-HCl FDA Working Standard gives a yellow
fluorescence under longwave ultraviolet (UV) light (375 nm)
(35). The excitation (324 and 357 nm) and emission (443 nm)
spectra of this standard dissolved in 0.05N NaOH (11.4 mg/50
ml) are presented in Figure 10.

TABLE 3
Molecular Ellipticities of CTC

Wavelength (nm)	Mitscher's Value[a] $(\times 10^3)$	FDA Value $(\times 10^3)$	Ratio
236	+10	+23	0.43
254	−31	−48	0.64
288	+48	+88	0.54
318	−24	−32	0.73
355(sh)	−14	−12	1.09

[a]These values were obtained from the graph in Reference 34 .

4.   SPECTRAL PROPERTIES (OTHER)

4.1   Proton NMR

The 60 MHz NMR spectra of CTC-HCl in DMSO-d_6 and in
methanol-d_4 are shown in Figures 11 and 12, respectively. The
sharp signals due to the methyl groups are readily assignable;
their chemical shifts and intensities are consistent with the
structure. The 2 methylene protons at C-5 are not equivalent
and give two sets of multiplets. Only one multiplet is
assignable in the methanol spectrum: the signal centered at
2.20 ppm. In DMSO-d_6 these protons appear between 1.5 and 2.3
ppm. The only methine proton actually assigned is the one at
C-4. The close proximity to the positively charged N causes a
downfield shift away from the other signals. When D$_2$O is
added to the DMSO-d_6 solution, the H-4 signal remains constant.
However, the two amide protons, originally at 9.15 and 9.55
ppm, exchange with the deuterium and are no longer observed.
These signals were not observed in methanol-d_4, again, due to
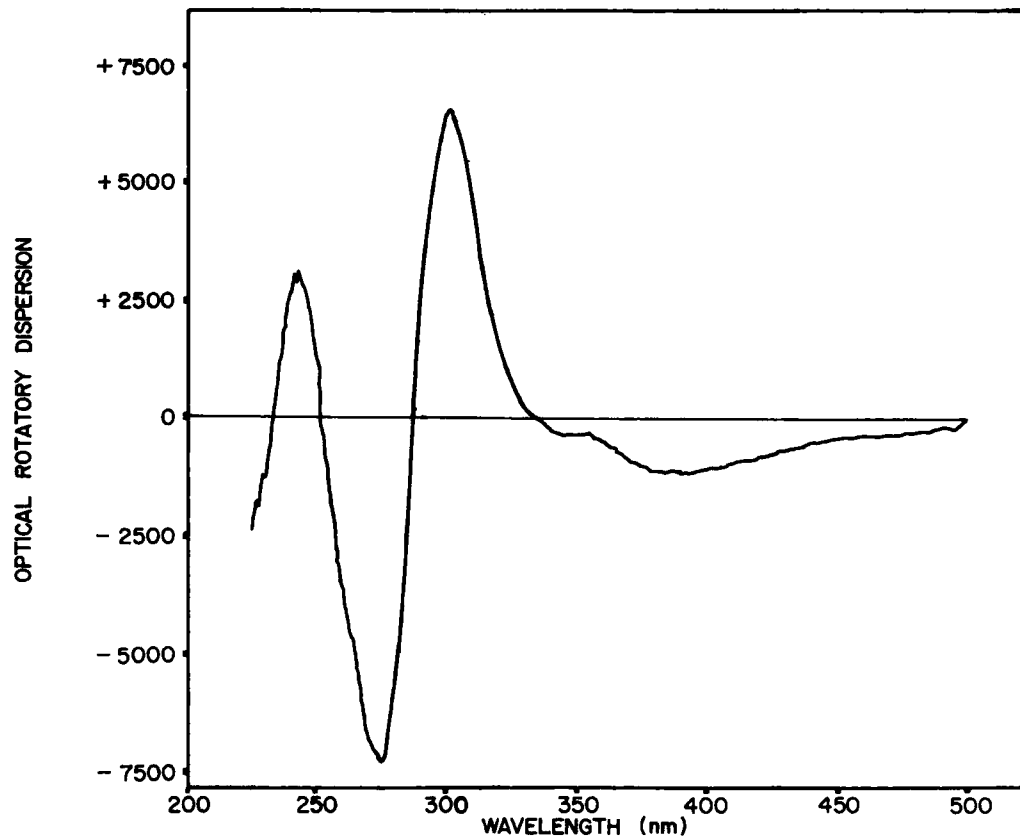
Figure 8. Optical rotatory dispersion spectrum of CTC-HCl.
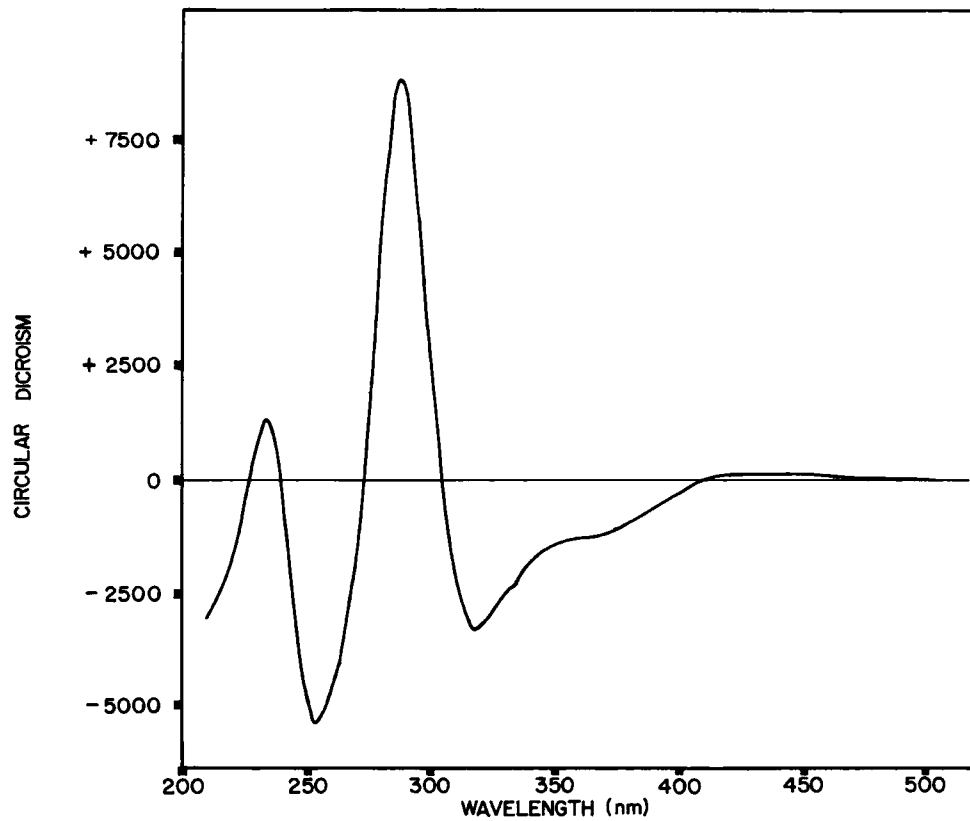
Figure 9. Circular dichroism spectrum of CTC-HCl.
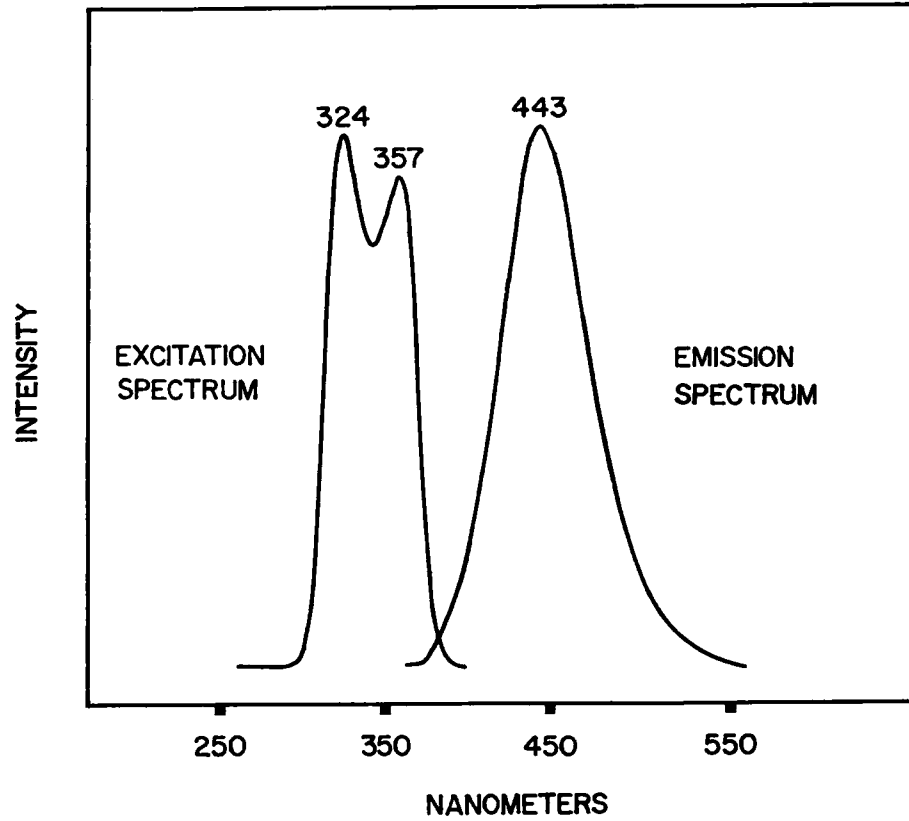
Figure 10.   Excitation and emission fluorescence spectra of CTC-HCl.
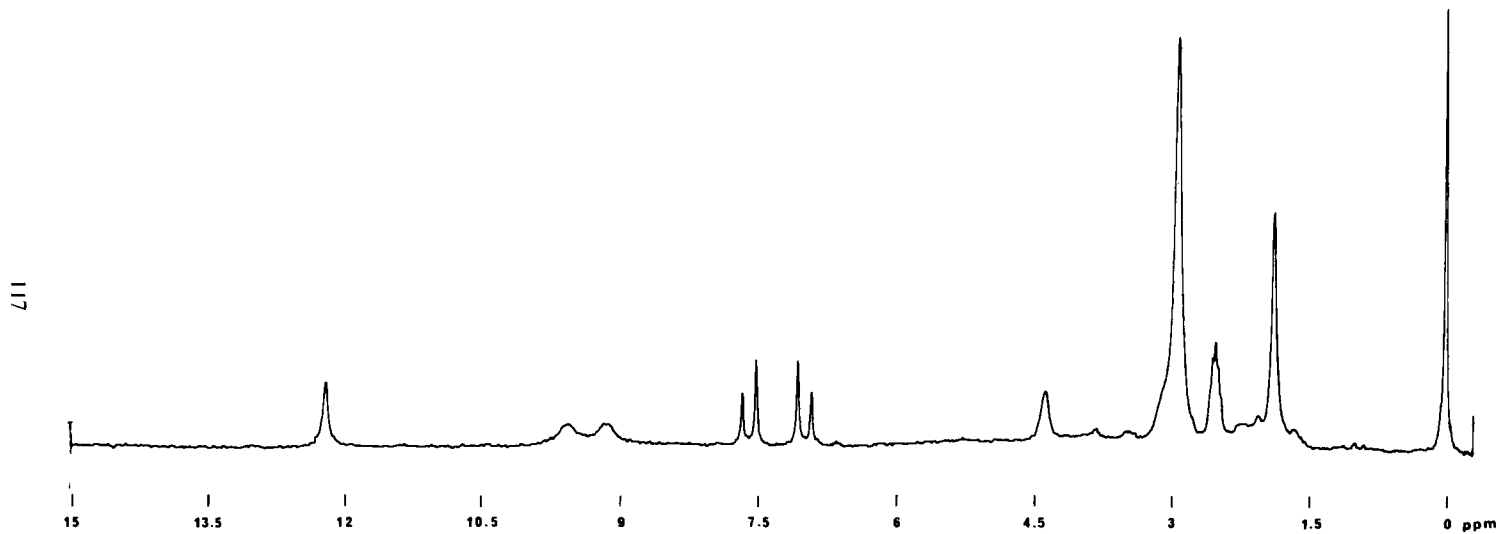
Figure 11. Proton magnetic resonance spectrum of CTC-HCl in DMSO-d$_6$.
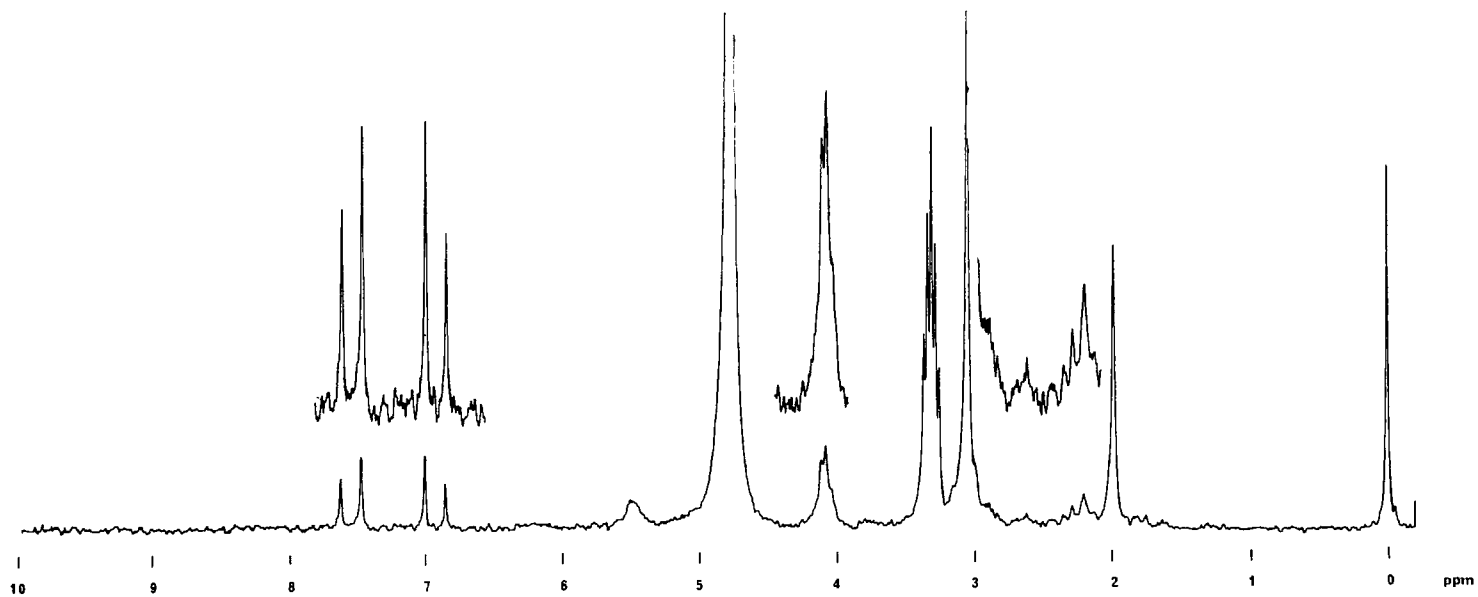
Figure 12.   Proton magnetic resonance spectrum of CTC-HCl in methanol-d_4.

deuterium exchange. Data from the literature (36), based on a
spectrum obtained in DMSO-d_6, are included in Table 4. As can
be seen from the table, these values are generally upfield
from our results. Although these differences were unexpected,
no explanation will be attempted here (37).

<div align="center">

TABLE 4

Chemical Shifts[a]

</div>

Proton	Literature (36)	Solvent	
		DMSO-d_6	Methanol-d_4
C_6-CH_3	1.7	1.88 (s)	1.98 (s)
C_4-$N(CH_3)_2$	2.7	2.93 (s)	3.05 (s)
H-8	6.4 - 7.0	7.58 (d)	7.55 (d)
H-9	6.4 - 7.0	6.98 (d)	6.93 (d)
H-4	4.0	4.36 (br. s)	4.10 (d)
H-5	1.7 - 2.1	1.5 - 2.3 (m,s)	2.20 (m)[b]
C_2-$CONH_2$	8.4, 8.9	9.15, 9.55	not observed[c]
H-4a	2.7	not assigned[d]	not assigned[d]
H-5a	2.7	not assigned[d]	not assigned[d]

[a]Spectra were obtained on a Perkin-Elmer R-12B equipped with a
 Nicolet TT-7 Fourier transform accessory. Chemical shifts are
 reported in ppm downfield from internal TMS. Multiplicities
 are indicated as s = singlet, d = doublet, and m = multiplet.

[b]This multiplet represents one of the H-5 protons. The
 chemical shift of the other one was not assigned.

[c]These protons exchange with the deuterium of the solvent and
 thus are not observed.

[d]The chemical shifts of these protons are not assigned. They
 are obscured by the -$N(CH_3)_2$ and/or the solvent.

4.2 ^{13}C NMR

Proton-noise decoupled and single-frequency off-
resonance decoupled carbon-13 NMR spectra were determined for
the CTC Working Standard (Figure 13).

The observed chemical shifts of the 22 carbon atoms
compare closely with those reported by Frank (38). The only
significant difference observed was that of the dimethylamino
carbons which were found at 48.5 ppm rather than at Frank's
reported value of 41 ppm. This difference in chemical shifts
can probably be ascribed to differing amounts of water present
in the DMSO-d_6 solvents and hence in the pH of these respec-
tive solutions.
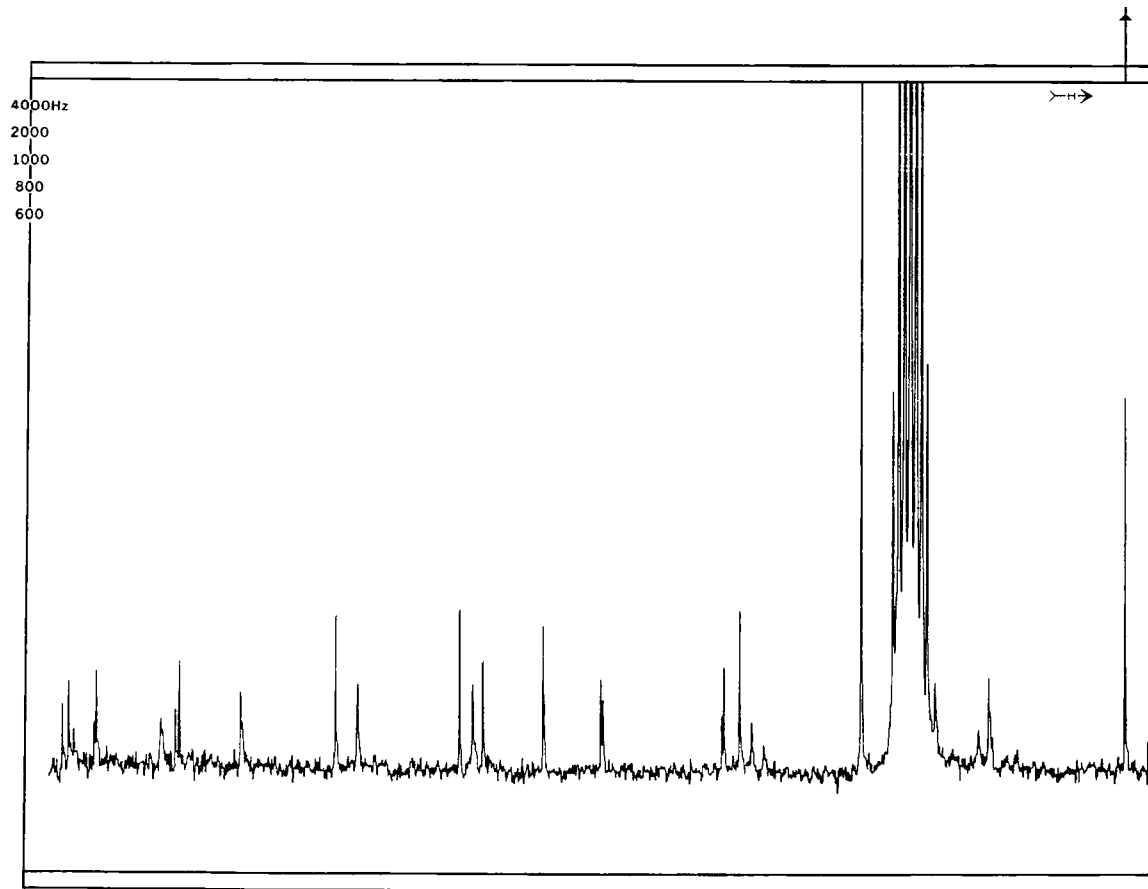
Figure 13. ^{13}C NMR spectrum of CTC-HCl.

Spectra were determined using a pulse width of 4 μseconds, which corresponds to a flip angle of 18° and a 1 second pulse delay time. The 4000 Hz spectrum was described using 8192 data points.

The observed resonance lines of CTC-HCl (39) and their assignments are shown in Table 5.

TABLE 5
Observed Resonance Lines of CTC-HCl and Their Assignments

Carbon Assignment	Chemical Shift[a]
CH_3	25.1 q
C-5	27.0 t
C-4a	34.9 d
C-5a	42.0 d
$N(CH_3)_2$	48.5 q
C-4	68.3 d
C-6	70.4
C-12a	73.3
C-2	95.5
C-11a	106.1
C-10a	117.0
C-9	118.9 d
C-7	121.3
C-8	139.7 d
C-6a	143.6
C-10	160.8
$CONH_2$	172.0
C-12	175.3
C-3	187.1
C-1	192.1
C-11	193.3

[a]In DMSO-d_6, parts per million from TMS (0.00 ppm). q = quadruplet, t = triplet, d = doublet.

4.3   Mass Spectrometry

Mass spectral studies, including low voltage techniques and accurate mass measurements, have been reported by Hoffman (40) on tetracycline and eight related compounds. As a result of good spectral correlations among these compounds, the major fragmentation processes are discussed in terms of two compounds in the series: 5α,6-anhydrotetracycline and dedimethylaminotetracycline, with only a selected number of ions reported for CTC. Further studies on these antibiotics, including CTC, were conducted by Morris and Cairns (41). However, little information on their results is included in the extended abstract of the meeting, and a full report has not

been published. Personal communications with the authors
indicate that the results of their work are in close agreement
with data reported here.

The fragmentation pattern of CTC can be discussed
in terms of three different mechanisms. These are:

a. Processes resulting from the capacity of the
dimethylamino group to direct losses of small fragments
together with its ability to retain and stabilize the positive
charge.

b. Fragmentation of the D ring system with charge
retention on the fused ring portion of the molecule.

c. Fragmentation of the D ring system with charge
retention elsewhere than on the fused ring moiety.

The mass spectrum of CTC, shown in Figure 14 (42),
is characterized by a reasonably intense molecular ion at m/e
478 with the concomitant isotope peak at P+2 representing
one chlorine atom in the ring system. Although it has been
suggested that this chlorine atom be employed as a tracer
via the ^{37}Cl isotope ratio for detection of species containing
the A ring and adjunct ring systems, many of these ions are of
such low relative intensity that this would have only limited
usefulness toward that end, except perhaps at higher mass
values.

The dominance of the fragmentation processes by the
presence of the 4-dimethylamino group gives rise to the
intense ions at m/e 44, m/e 58 $(CH_3)2-\overset{+}{N}=CH_2$, m/e 71 $(CH_3)_2\overset{+}{N}-$
$CH=CH_2$, and m/e 84 $(CH_3)_2-\overset{+}{N}=CH-CH=CH_2$. The ion at m/e 98
(C_5H_8NO+) appears to involve a cyclization of the dimethyl-
amino group with elements of the D ring. Of particular note
is the loss of 43 atomic mass units from the molecular ion to
give the intense ion at m/e 435 and from the ion at m/e 443
to yield m/e 400 (Figure 15).

The presence of the amide functional group is
indicated by observations of successive losses of NH_3 and OH
(as water) from the molecular ion to yield ultimately the ion
at m/e 443. The loss of water at m/e 460 appears to be
slightly favored over the competing loss of NH_3 at m/e 462
from the abundances of the respective ions.

Fragmentation of the D ring can provide the dual
purpose of determining the substituent at the 4 position
(dimethylamino in the case of CTC) and/or help to locate
functionalities elsewhere in the ring system. This, however,
requires a detailed examination of analogs such as in the work
of Hoffman (40).

The ion at m/e 365 will locate the substituents at
the 2 and 3 positions of the D ring by determination of the
mass of the lost substituent and also give evidence concerning
the extent of substitution on the A, B, and C rings. This

SPEC. NO. 20039 LS CHLORTETRACYCLINE HCL
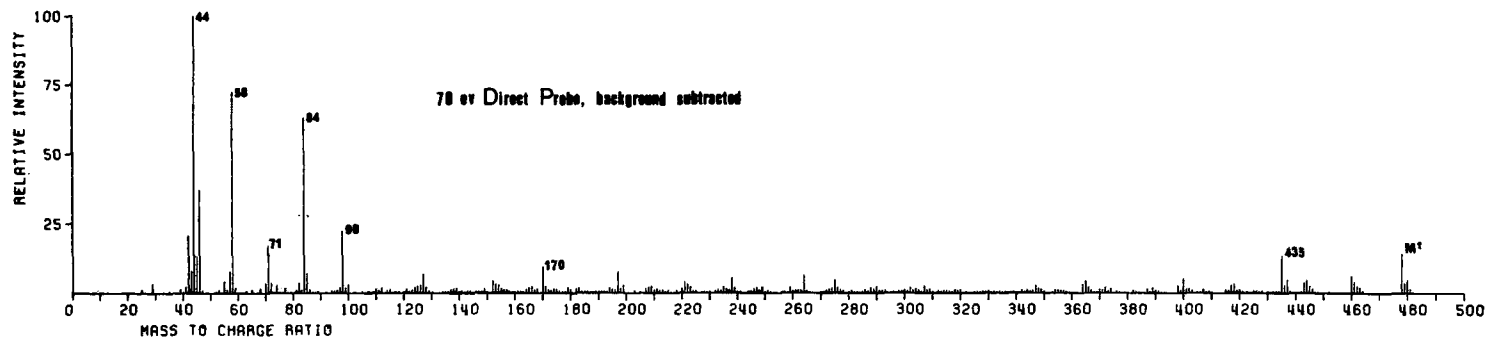
Figure 14. Electron impact mass spectrum of CTC-HCl. Instrument: Varian MAT 311; source temperature sufficient to produce vaporization. (Chemical ionization using ammonia as reagent gas gave MH^+ as base peak.)
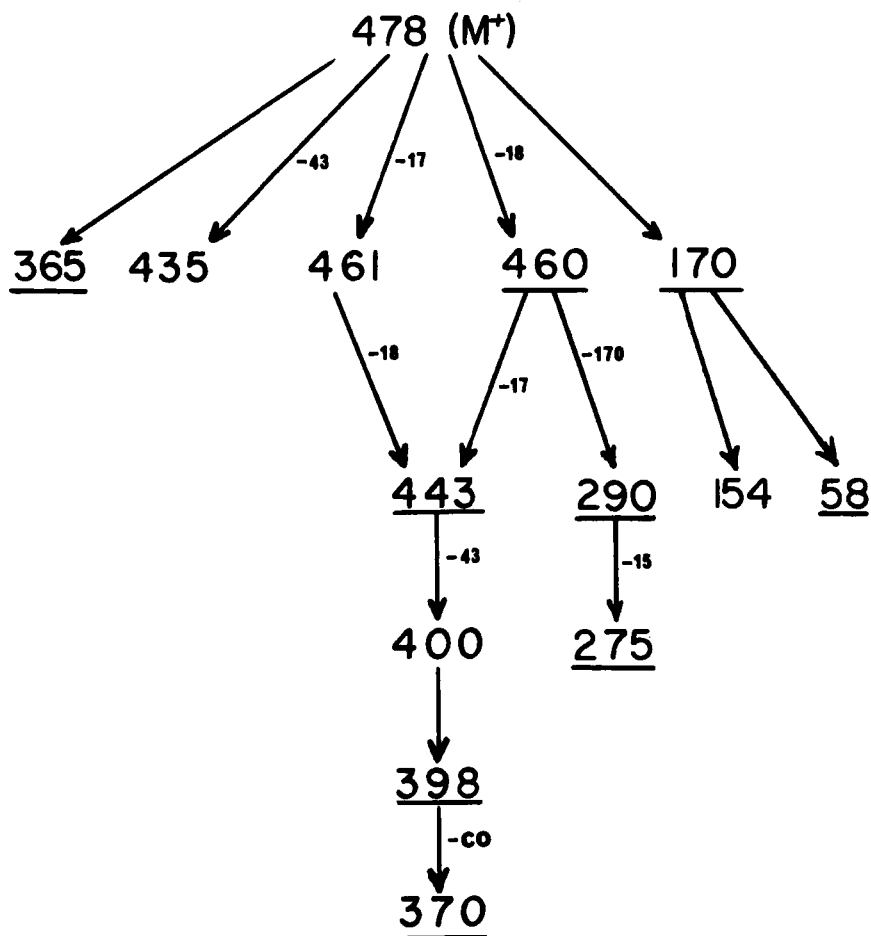
Figure 15.   Major fragmentation pathways of CTC involving
             molecular ion and other significant high mass
             ions.   Underlined m/e values indicate confirma-
             tion by accurate mass measurements.

knowledge plus the observed shift of the ion at m/e 170 should
suffice to allow postulation of the substitution (Figure 16).

5.   CHROMATOGRAPHY
     5.1   Paper and Thin Layer
           Some of the early reports on the chromatography of
the tetracycline antibiotics prior to 1957 are of limited
value. Fischbach and Levine (43) described a continuous
ascending technique and Berti and Cima (44) reported an
ascending method using aqueous sodium arsenite as the mobile
solvent. Other authors (45,46) reported descending techniques
and bioautographic means for locating the zones of activity.
           All of these methods fail to show the presence of
the epimeric form of the tetracyclines and in most instances
streaking of the spots is a problem. A basic improvement in
the paper chromatography of these antibiotics was achieved by
Selzer and Wright (47) and Kelly and Bryske (48) when they
reported methods for the pretreatment of the paper with com-
plexing agents to bind the metallic ions which may be present.
           The tetracyclines are well known for their abi-
lity to form complexes with polyvalent cations. This property
changes their solubility characteristics in the mobile sol-
vents and often results in troublesome streaking. To overcome
this difficulty, Selzer and Wright used paper dipped in
McIlvaine's buffer (pH 3.5) which contains citrate ions
capable of binding the metallic ions. The chromatograms were
developed with a mixture of nitromethane, chloroform, and
pyridine (20:10:3) on paper still damp from the treatment with
the buffer solution.
           For the same purpose, Kelly and Bryske used paper
impregnated with 0.1N disodium ethylenediaminetetraacetate
(EDTA) and two mobile solvents: the organic phase from a
mixture of n-butanol, ammonia, water (4:1:5) and the organic
phase from a mixture of n-butanol, acetic acid, water
(4:1:5). Disodium EDTA (0.1N) works as well as McIlvaine's
buffer when it is used to treat the paper in the method of
Walton et al. (49). A circular paper chromatographic method
also using paper dampened with McIlvaine's buffer (pH 4.5)
was reported by Urx et al. (50). They used a mixture of
chloroform and n-butanol (4:1) as the mobile solvent.
           Most of the methods in which the paper is treated
with a chelating agent are capable of showing a separation of
some of the tetracycline drugs from each other and from their
respective epimeric forms. They are also capable of revealing
the presence of common degradation compounds of these drugs.
           The usual method of detecting chromatographed
tetracycline antibiotics involves fuming the paper with
ammonia vapor and observing the yellow fluorescence under UV
light. As little as 0.2-0.5 µg can be visualized by this
technique.
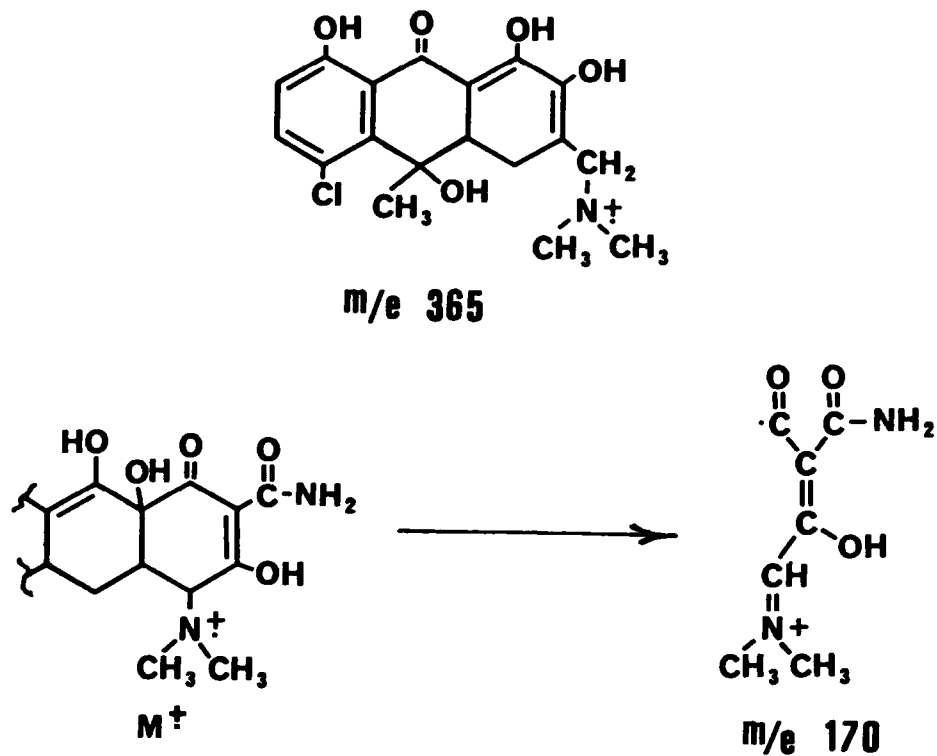
Figure 16. Proposed structures for ions at m/e 365 and m/e 170 for use in determination of ring substituents.

Some of the features that are successful for the
chromatography of the tetracyclines on paper have been
adapted to thin layer chromatography (TLC). Complexing agents
are almost always used in the preparation of the plates and, in
addition, several methods for hydrating the plates by the
addition of glycerin and/or polyethylene glycol have been
reported. Several investigators find it desirable to acid-
wash the inorganic support in order to remove metallic
cations. These TLC methods are much more time-consuming than
most of the paper chromatographic procedures because of the
special care often required to prepare and store the TLC
plates. For this reason, where applicable, the paper chroma-
tographic methods are preferable. However, for quantitative
analysis of the tetracycline epimers and degradation products,
TLC is usually considered to be better than paper
chromatography.

In 1964, Somanini and Anker (51) described a method
using Kieselguhr layers impregnated with a buffer solution
containing glycerin. Nishimoto et al. (52) reported the use
of silica gel layers treated with EDTA. In 1967, Ascione et
al. (53) published a method using layers made from acid-washed
diatomaceous earth. They prepared plates containing 0.1N
EDTA, glycerin, and polyethylene glycol 400. Other investi-
gators (54,55) have described methods which are modifications
of improvements of the methods previously published.

5.2   Gas and Liquid Chromatography
Investigators have found it quite difficult to
chromatograph the tetracyclines by gas-liquid techniques.
Often, only fragments of the original sample are obtained
(56). Tsuji and Robertson (57) did manage to chromatograph
silylated CTC using 3% methyl silicone on Gas-Chrom Q and
other stationary phases. However, with the advent of refined
high pressure liquid chromatographic (HPLC) techniques,
interest in gas chromatographic methods for the tetracyclines
has diminished.

A number of papers have appeared reporting the HPLC
separation of CTC from its isomers and/or other tetracyclines.
There is not a consensus of opinion as to the most satis-
factory approach; thus, it appears that at this time one must
still verify the optimal system for a particular instrument.
Methods found in the literature for CTC are described in
Table 6. EDTA is added to prevent the formation of complexes
of the tetracyclines with metallic surfaces.

Other chromatographic techniques that have been
applied to the tetracyclines, including CTC, involve low
pressure column chromatography. Ascione et al. (64) developed
a semiautomated system whereby sample solutions are automati-
cally injected onto a column of diatomaceous earth mixed with

TABLE 6
High Pressure Liquid Chromatography of CTC

Mobile Phase	Stationary Phase	Migration Time (min.)	Ref.
20% Methanol, 80% 0.05M ammonium carbonate, 0.02M EDTA	C8/Lichrosorb 10 μm, 25 cm x 3.2 mm	34.5	58
Phosphate buffer in 13% methanol, 0.85 ml/min., pH 2.5	Zipax-hydrocarbon polymer, 2.1 x 1000 mm	6	59
Aqueous perchlorate-citrate buffer mixed with CH_3CN	Sil-X, 13 μm, 5 x 125 mm	1.3	60
EDTA, NaCl in 30% methanol, NH_3, pH 9.9	Ion-X-SA, anion exchanger	3	61
EDTA, PO_4, isopropanol-water, pH 7.6, 2 ml/min.	μBondapak C_{18}, 300 x 4 mm	11.6	62
EDTA, NO_3, 9.6% ethanol, pH 9, 1 ml/min.	Zipak, 1.8 x 1000 mm	22	63

a solution of EDTA and polyethylene glycol. CTC is eluted
with the organic phase of a mixture of chloroform, benzene,
aqueous solution of EDTA, and polyethylene glycol. Ragazzi
and Veronese (65) separated CTC from other tetracyclines by
means of gel permeation chromatography.

6.   MANUFACTURE
      6.1   Fermentation
             A medium (containing corn steep liquor; calcium
carbonate; sucrose; ammonium, ferrous, manganese, and zinc
sulfates; and ammonium, cobalt, and magnesium chlorides) is
sterilized and diluted with water to the desired concentra-
tion. It is inoculated with Streptomyces aureofaciens, kept
at 27°C, and aerated and agitated for ∿60 hours, with lard
oil added to control foaming (66).

      6.2   Isolation
             The mash from the Streptomyces aureofaciens fermen-
tation broth is acidified and filtered. The filtrate is
adjusted to the desired pH, usually 7-8.5, and various floccu-
lating or chelating agents may be added (e.g., vinyl acetate-
maleic anhydride copolymer, sodium EDTA, ammonium oxalate,
Arquad). The precipitate is (1) stirred with filter aid,
filtered, stirred with HCl, refiltered, mixed with 2-
ethoxyethanol, filtered, washed, and the filtrates are
combined, acidified with HCl, NaCl is added, and the crystals
are collected, washed with 2-ethoxyethanol, water, and
ethanol, and dried (67), or (2) extracted into methyl
isobutyl ketone, the extracts are combined, filtered, and
acidified with HCl, and the crystals are collected and washed
with water, 2-ethoxyethanol, and isopropanol, and vacuum-
dried. If the crystals are greenish, they are treated with
sodium hydrosulfite at pH 1.8, filtered, washed, and dried as
in (1) above (68).

7.   STABILITY
      CTC-HCl, as a dry powder, is a stable yellow crystalline
material. The situation in aqueous solution, however, is
quite different. In sodium hydroxide solutions, CTC is
converted to iso-CTC on standing (69). The solution becomes
colorless and exhibits a strong blue fluorescence under UV
light. Dilute solutions of CTC, in pH 7.5 buffer, make the
same conversion at 100°C.
      In acid solutions, CTC is converted to anhydro-CTC (70).
This change is greatly accelerated by heating and results in
a yellow product that has a maximum absorbance at 445 nm.
      In addition, CTC undergoes reversible epimerization of
the 4-dimethylamino group (71). This occurs slowly in water
or methanol but is hastened in buffer solutions in the range

of pH 2-6. The rate of epimerization is undetectable in
solutions more acidic than pH 2 (72). The antimicrobial
activity of the epimer is probably zero. The slight activity
found for this material is probably due to the reemergence of
CTC under the test conditions.

In a fashion analogous to that of CTC, its epimer can be
converted to epianhydro-CTC in acid solution.

8.   ANALYTICAL METHODS
       8.1   Microbiological
              The microbiological methods used for the determina-
tion of CTC potency in body tissues and fluids, bulk products,
and pharmaceutical formulations can be separated into two
testing procedures: (1) agar diffusion plate method
(cylinder-plate) and (2) turbidimetric method.
              1.   Agar diffusion plate method (cylinder-plate):
              This method is employed for determining the
potency of CTC in human and animal pharmaceutical formula-
tions, bulk products, serum, tissues, urine, dairy products,
and animal feeds. The cylinder-plate procedure is described
by Grove and Randall (73) and the Code of Federal Regulations
(74). Additional methods using this assay are described by
Kramer et al. (75). The official final action method for CTC
assay in animal feeds is described in the Association of
Official Analytical Chemists' Official Methods of Analysis
(76).
              2.   Turbidimetric method:
              This method is used in lieu of the diffusion
plate method for human and animal pharmaceutical formulations
and bulk products. The turbidimetric method is described in
the Code of Federal Regulations (77).

       8.2   Chemical
              The physical structure of CTC has provided a good
source of characteristics useful for the analysis of this
antibiotic. Although titrimetric (78) and polarographic (79)
methods have been reported, the most useful procedures have
been based on the spectroscopic properties of CTC and its
derivatives (80).
              In 1949, Levine et al. (81) published two procedures
for the assay of CTC. One method was based on the conversion
of CTC by heating in acid to the more intensely yellow
anhydro-CTC derivative. The other method was based on measur-
ing the blue fluorescence of iso-CTC, which was prepared by
heating CTC in pH 7.5 phosphate buffer.
              Others have described modifications of these methods
for various purposes. Hiscox (82) suggested the direct spec-
trophotometric assay of CTC in either acid or alkaline solution
at various UV wavelengths. The possible contamination of CTC
with other tetracycline drugs was addressed by Chicearelli et

al. (83). They corrected for the possible presence of tetra-
cycline in CTC using the fact that the former is unchanged in
dilute alkali while CTC is converted to the colorless iso-
CTC.

        The tetracycline which may be present is then
converted to an anhydro derivative by heating in acid and is
measured spectrophotometrically. Feldman et al. (84)
developed the alkaline degradation method to measure CTC in
fermentation mash and Spock and Katz (85) used this method to
determine CTC in animal feed premixes.

        The natural fluorescence of CTC and its derivatives
has been used extensively to determine small amounts of CTC
in biological materials. Kohn (86) showed that the fluores-
cent complex formed by CTC with calcium ions and barbital
could be extracted from animal tissues into an organic solvent
and then measured spectrofluorometrically. The intense
fluorescence of anhydro-CTC was used by Hayes and DuBuy (87)
to determine CTC in animal tissues, tissue culture cells, and
bacteria. Poiger and Schlatter (88) extracted CTC from bio-
logical material into ethyl acetate as the CTC-calcium
trichloroacetate ion pair. The fluorescence of the antibiotic
was then enhanced by the addition of magnesium ions and a
base.

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DOBUTAMINE HYDROCHLORIDE

Rafik H. Bishara and Harlan B. Long

1. Description

1.1 Nomenclature

1.1.1 Chemical Name

(±)-4-[2-[[3-(4-Hydroxyphenyl)-1-methylpropyl]amino]ethyl]-1,2-benzenediol, hydrochloride

1.1.2 Nonproprietary Name

Dobutamine hydrochloride

1.1.3 Proprietary Name

DOBUTREX®

1.2 Formula

1.2.1 Empirical

$C_{18}H_{23}NO_3 \cdot HCl$

1.2.2 Structural
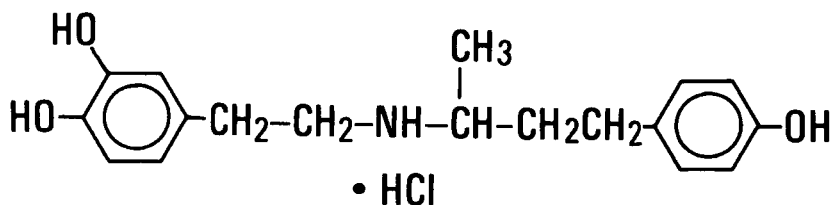
1.3 Molecular Weight

337.85

1.4 Appearance, Color, Odor, and Taste

White to off-white, odorless powder with a slightly bitter taste.

2. Physical Properties

2.1 Melting Range

189 - 191°C

2.2 Simple Solubility Profile

The sample is sonicated for one minute at ambient temperature.

Solvent	mg/ml
Water	>3.33
pH 1.2 (USP XIX)	>2.50
pH 4.5 (USP XIX)	>3.33
pH 7.0 (USP XIX)	>3.33
Ethanol	>5.00
Methanol	>10.00
Pyridine	>5.00
Octanol	<0.50
Diethyl ether	<0.50
Ethyl acetate	<0.50
Chloroform	<0.50
Benzene	<0.50
Cyclohexane	<0.50

2.3 pH Range

The pH of a 5% w/v solution in a water/ethanol (1:1) solution is about 4.9.

2.4 Dissociation Constant

The pKa in dimethylformamide/water (66:34) is 9.45.

2.5 Thermal Analyses

2.5.1 Differential Thermal Analysis

A DTA thermogram of dobutamine hydrochloride, at a heating rate of 5°C per min. in a nitrogen atmosphere of 40 cc per min., shows (figure 1) an endotherm at 196°C which appears to indicate a melt.

2.5.2. Thermogravimetric Analysis

A TGA thermogram of dobutamine hydrochloride, run simultaneously with the DTA, shows (figure 1) no weight loss until 233°C which results from decomposition.

2.6 Crystallinity

2.6.1 Crystalline Habit

Dobutamine hydrochloride generally crystallizes in a random manner usually from an oil (1). This results in a nondescript crystalline formation. In only few cases does the drug exhibit any crystalline habit of interest. Upon careful and patient crystallization small thin plates and/or small needles are formed.

2.6.2 X-Ray Powder Diffraction

The following data describe the pattern for dobutamine hydrochloride, where d is equal to the interplanar spacing measured in terms of Angstroms (Å). The ratio I/I_1 is the intensity of the X-ray maxima based upon a value of 100 for the strongest line. A b indicates a broad line resulting from failure to resolve two closely spaced diffraction maxima.
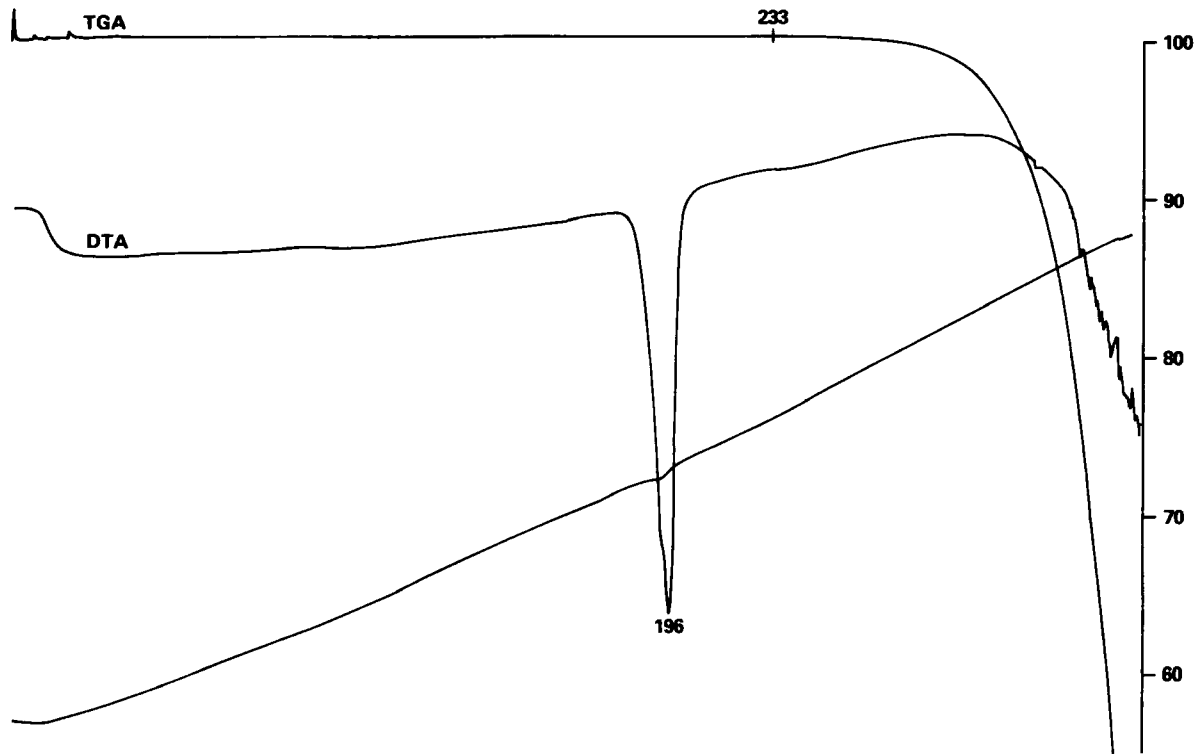
Figure 1.   Thermogravimetric Analysis and Differential Thermal Analysis
            Thermograms of Dobutamine Hydrochloride

Cu-Ni-λ1.5418 Å

d	I/I$_1$	d	I/I$_1$
9.07	7	2.74	14
6.87	5	2.63	9
6.37	7	2.51	16
5.27	18b	2.40	5
4.98	18	2.35	2
4.50	100	2.27	4
4.13	16	2.22	2
4.04	61	2.14	7
3.78	45	2.11	4
3.60	45	2.06	5
3.44	16b	2.01	7
3.18	18	1.99	7
3.11	7	1.95	7
3.01	14		
2.86	7		

2.7 Ultraviolet Spectrum

The ultraviolet spectrum of dobutamine
hydrochloride in methanol is given in figure 2.
The spectrum exhibits maxima at 281 and 223 nm with
molar absorptivities of 4,768 and 14,400, respec-
tively. When aqueous potassium hydroxide is added
to the methanolic solution of dobutamine hydro-
chloride the maxima at 281 and 223 shift to 293
(ϵ=6,100) and 240 nm, respectively. These shifts
are reversible by addition of hydrochloric acid.
When the absorption spectrum of the drug is
recorded in water rather than methanol, slight
shifts in peak positions and intensities are
observed:

λ max = 278 nm (ϵ = 4,112)

λ max = 220 nm (ϵ = 13,500).

2.8 Infrared Spectrum

The infrared spectrum of dobutamine hydro-
chloride in a potassium bromide disk is given in
figure 3. Major band assignments are as follows:
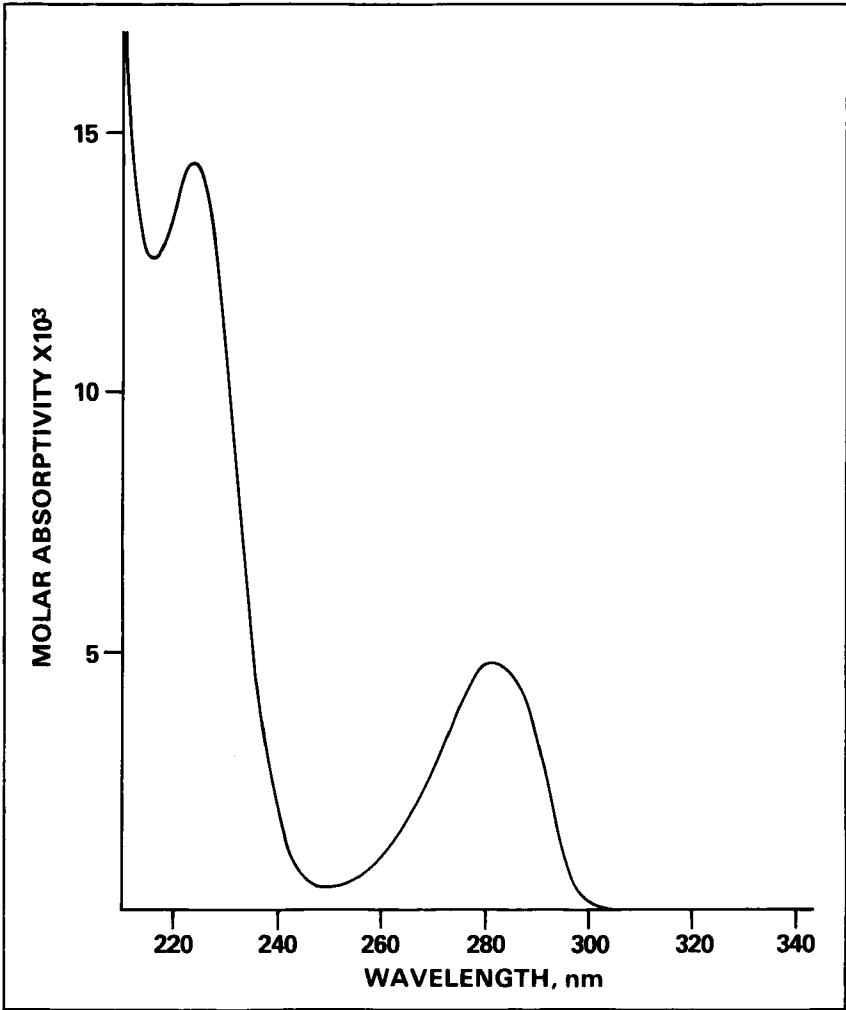
Figure 2. Ultraviolet Spectrum of Dobutamine
          Hydrochloride
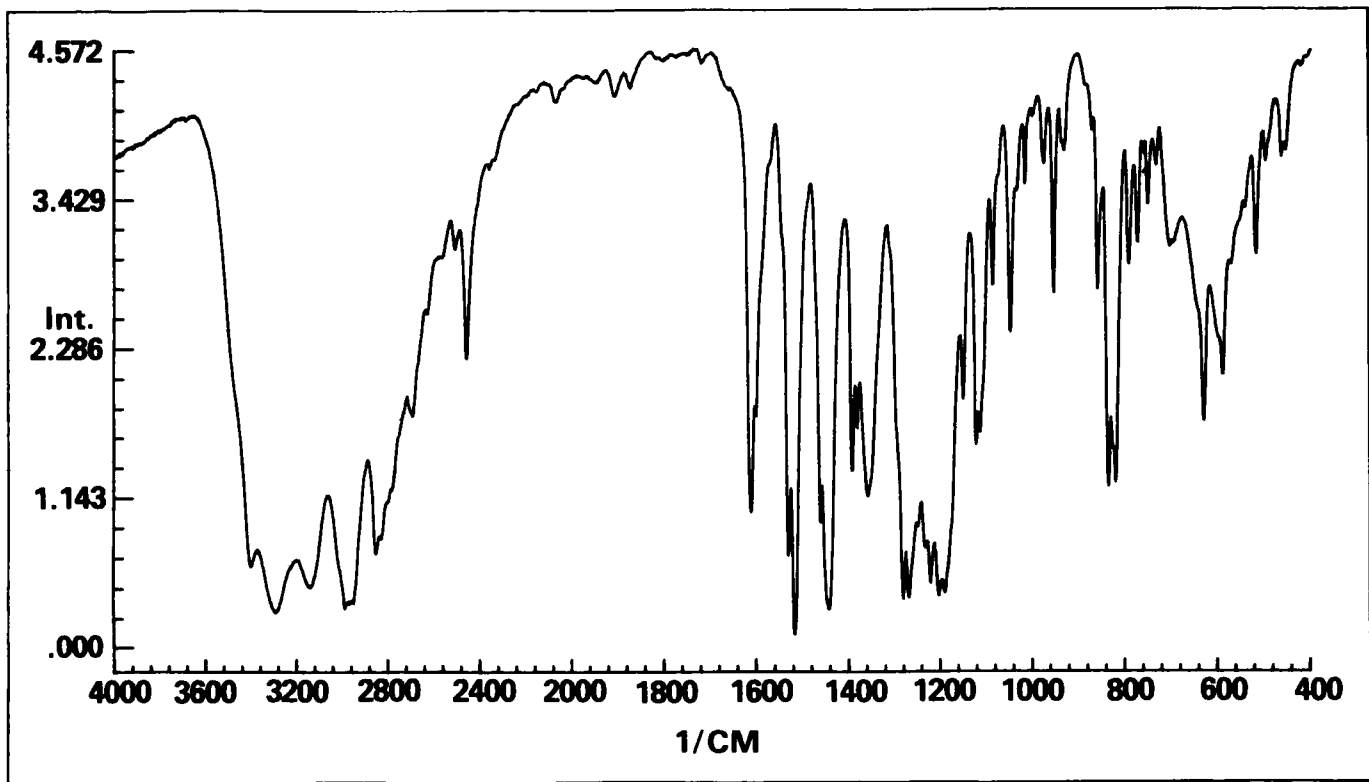
Figure 3. Infrared Spectrum of Dobutamine Hydrochloride

Band Position, cm^{-1}	Assignment
3400, 3300 and 3140	phenolic O-H stretching
2960 and 2840	C-H stretching (over-laping O-H bands)
2700 and 2450 (weak bands)	Mainly NH_2, NH stretching
1610, 1530, 1520 and 1450	Aromatic ring stretching
1440, 1390 and 1380	CH_2, CH_3, C-H bending
1360, 1280-1190 (several bands)	phenolic C-O stretching
1150 and lower	Mainly skeletal and aromatic C-H bending

2.9 Nuclear Magnetic Resonance Spectrum

The 60 MHz proton NMR Spectrum of dobutamine hydrochloride in deuterated dimethylsulfoxide is given in figure 4. Assignments of the bands are as follows:
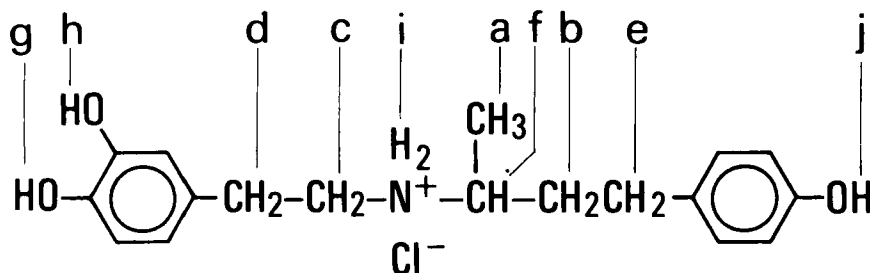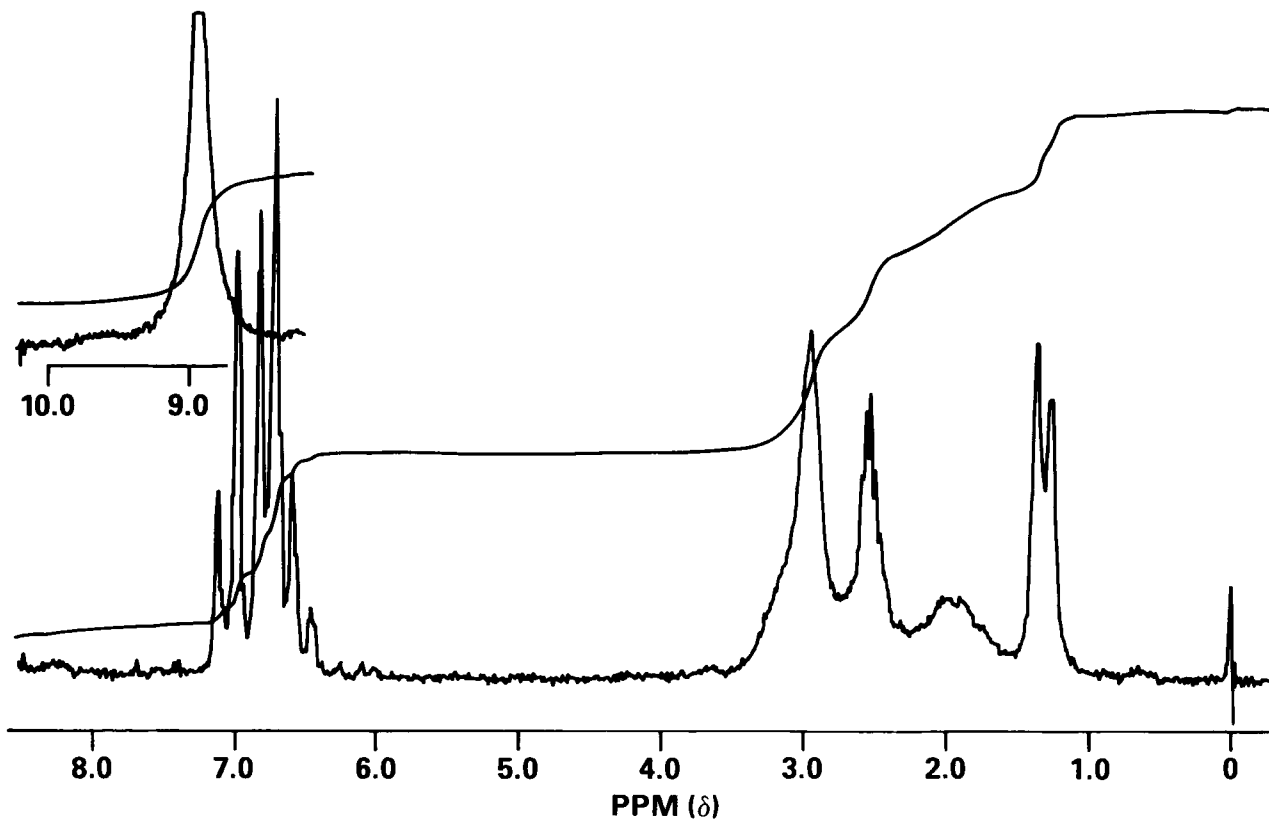
Figure 4. Nuclear Magnetic Resonance Spectrum of Dobutamine Hydrochloride

Chemical Shift (ppm)	Multiplicity	Area	Assignment
1.3	doublet	3	a
1.9	unresolved multiplet	2	b
2.5	unresolved (overlapping solvent)	2	c
3.0	unresolved triplets	5	d,e,f
6.8	overlapping multiplets	7	aromatic protons
8.9	very broad singlet	5	g,h,i,j

2.10 Mass Spectrum

The mass spectrum of dobutamine hydrochloride given in figure 5 shows the molecular ion of the free base at m/e 301. The major fragmentation consists of a cleavage beta to the nitrogen to yield peaks at m/e 123 and 178 with relative intensities, to the base peak, of 15.2 and 97.6%, respectively. The base peak is at m/e 107.

3. Synthesis

A mixture of crotonic acid, thionyl chloride, and a catalytic amount of dimethylformamide is stirred in a solvent such as benzene to give 2-butenoyl chloride (I). The Friedel-Crafts reaction of methoxybenzene (II) with (I) using $AlCl_3$ in carbon disulfide yields 1-(4-methoxyphenyl)-2-buten-1-one (III). 3,4-Dimethoxybenzeneethanamine (IV) is then condensed with (III) to give 3-[[2-(3,4-dimethoxyphenyl)ethyl]amino]-1-(4-methoxy-phenyl)-1-butanone (V). This ketone is reduced with hydrogen over Pd/C to give N-[2-(3,4-dimethoxyphenyl)ethyl]-4-methoxy-α-methylbenzene-propanamine (VI). An alternate synthesis for compound (VI) involves the reduction of 4-(4-methoxyphenyl)-3-buten-2-one (VII) with hydrogen over Raney nickel to yield the corresponding butanone (VIII), which is then condensed with (IV) to produce the imine (IX). Compound (IX) is then reduced again with hydrogen over Pd/C to give (VI).
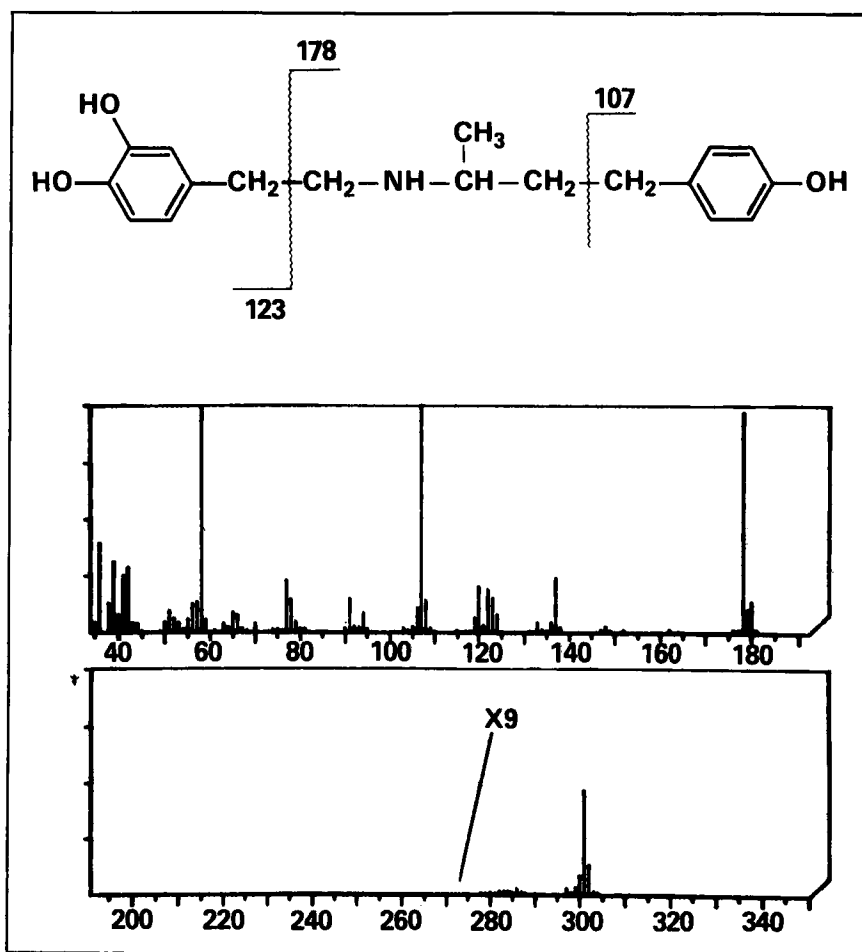
Figure 5.   Mass Spectrum of Dobutamine
            Hydrochloride

A second alternate synthesis of compound (VI) in-
volves the reaction of 4-methoxy-α-methylbenzene-
propanamine (X) with 3,4-dimethoxybenzeneacetic
acid (XI) at 200°C to yield 3,4-dimethoxy-N-[3-
(4-methoxyphenyl)-1-methylpropyl]benzeneacetamide
(XII), which is then reduced with borane in THF to
produce (VI). The trimethoxy secondary amine (VI)
is demethylated by refluxing its solution in glacial
acetic acid and HBr to yield dobutamine hydrobromide
(XIII). Compound (XIII) is added to aqueous meth-
anol then small amounts of hydrochloric acid are
added to produce dobutamine hydrochloride (XIV).
The flow diagram of the synthesis presented above
(2) is shown in figure 6.

4. Stability-Degradation

    Dobutamine hydrochloride is quite stable to re-
fluxing in acid and to heating in air for 20 hours
at 115°C (2). However, the drug is very rapidly
oxidized to the corresponding aminochrome at pH
11-13. Approximate kinetic measurements suggest a
half life of 30-45 minutes. This is similar to
catecholamines which produce aminochromes that
undergo further rapid and complex oxidations and/or
condensations. These reactions yield products of
unknown structure which finally are converted to
dark colored polymers related to the melanins.
Photolysis of an aqueous solution of the drug at
40-50°C for 5 days using a 325 w mercury lamp in
the presence of oxygen also produced the amino-
chrome as the photooxidation product.

5. Absorption, Metabolism, and Excretion

    5.1 In Dog

        The short plasma half-life of dobutamine
(1-2 minutes) was found by Murphy et al. (4) to be
due to the rapid redistribution of the drug from
the plasma to the tissue. However, plasma half-
life of radioactivity following the administration
of ^{14}C-dobutamine was 1.9 hours. The major circu-
lating metabolite is the glucuronide conjugate of
3-O-methyldobutamine. During a continuous intra-
venous infusion of dobutamine, the plasma level of
the parent drug reach a maximum within 8 to 10
minutes, while those of the metabolites peak be-

Figure 6.   Synthesis of Dobutamine Hydrochloride

tween 3 and 4 hours. Dobutamine and/or its meta-
bolites are eliminated via the urine and bile in
both the dog and rat (5). After 48 hours from
administering ^{14}C-dobutamine to dogs, 67% of the
radioactivity was excreted in urine and 20% in
feces. Dogs with canulated bile ducts excreted 30
to 35% of the administered drug in the bile. The
major urinary metabolites are the glucuronide con-
jugates of both dobutamine and 3-O-methyldobutamine.
At very high doses of the drug, small amounts of
hydroxylated dobutamine and hydroxylated 3-O-methyl-
dobutamine were observed in the urine. The exact
position of the extra hydroxyl group was not de-
termined. The metabolites were not observed at
therapeutic dosages.

5.2 In Man

Serum levels of dobutamine reached a max-
imum of 20 ng/ml during a 15-minute infusion of
dobutamine at a rate of 2 μg/Kg/min. and declined
to 3 ng/ml within 5 minutes after the infusion.
Rapid clearance from the plasma is indicated by its
short half-life of approximately 2 minutes. Dobu-
tamine is rapidly metabolized by methylation and
conjugation to 3-O-methyldobutamine and conjugates
of dobutamine. The major portion of the metabolites
are excreted in the urine within the first 2 hours
following infusion and the remainder within 6 hours.
Metabolism and excretion in man are similar to the
processes described above in the dog. For the
detailed pharmacological and biochemical properties
of dobutamine, the reader should consult the pro-
file by Weber and Tuttle (5).

6. Methods of Analysis

6.1 Elemental Analysis (As $C_{18}H_{23}NO_3 \cdot HCl$)

Element	Theory (%)
C	63.99
H	7.16
N	4.15
O	14.21
Cl	10.49

6.2 Chloride Identity

About 5 ml of dobutamine hydrochloride solution in water, 1 mg/ml, is acidified with two drops of concentrated nitric acid. When a drop of 0.1 N silver nitrate is added to this solution a white precipitate is formed which is readily solubilized by the addition of 3 drops of ammonium hydroxide. This indicates the presence of chloride ion.

6.3 Non-Aqueous Titration

The secondary amine function of dobutamine hydrochloride may be determined by potentiometric titration with perchloric acid using glacial acetic acid as a nonaqueous solvent. Mercuric acetate is used to tie up the chloride ion.

6.4 Chloride Determination

A sample of dobutamine hydrochloride containing at least 2 mg of chlorine is ignited in a Schöniger flask containing 20 ml of water. Three drops of diphenyl carbazone (5 mg/ml) in methanol are added to the solution of the completely burned sample. Mercuric nitrate, 0.5 N, is then used to titrate this solution to the first sign of rose color, using a 1 ml microburette:

$$\text{percent chlorine} = \frac{\text{ml mercuric nitrate X normality X 35.5 X 100}}{\text{mg sample}}$$

$$\text{percent purity of dobutamine hydrochloride} = \frac{\text{percent chlorine found X 100}}{\text{percent chlorine theory}}$$

6.5 Chromatography

6.5.1 Thin Layer Chromatography

The R_f value for dobutamine hydrochloride when chromatographed on a silica gel 60 F254 thin layer plate developed by ethyl acetate/n-propanol/water/acetic acid (100/40/15/5 v/v/v/v) in an unsaturated chamber is about 0.67. The spot of the drug may be visualized under short wavelength UV light (254 nm), or under white light after exposure to iodine vapors.

6.5.2 Gas Chromatography

Silylated dobutamine hydrochloride (by reaction with N-trimethylsilylimidazole) may be chromatographed on a 3 foot glass column packed with 3% OV-225 on Chromosorb G AW-DMCS (100/120 mesh). The column is operated at 230°C using helium as a carrier gas at the rate of 60 ml/min. The retention time of the drug is approximately 4 minutes. A flame ionization detector is used. n-Triacontane is used as an internal standard.

6.5.3 High Performance Liquid Chromatography

Dobutamine hydrochloride may be analyzed on a C_{18} reversed-phase column eluted with 75% 0.05M KH_2PO_4, pH 4.4, and 25% methanol at 2 ml/minute. The compound is detected at 280 nm. The retention time of the drug is approximately 6 minutes.

7. Analysis of Biological Samples

7.1 Enzymatic Assay

Plasma levels of dobutamine hydrochloride are determined by reaction of the drug with 3H-methyl-S-adenosylmethionine in the presence of catechol O-methyl-transferase. The radioactivity of the labeled methyl derivative is determined by a liquid scintillation counter using an external standard. The final recovery of added dobutamine as $3H-CH_3$-dobutamine is 24.9 ± 1.3% in the range of 2 to 170 ng/ml (4). When ^{14}C-dobutamine is administered the samples are counted by a double isotope method.

7.2 Chromatographic Assays

7.2.1 Thin Layer Chromatography

A Silica gel G plate is developed with a 15% aqueous solution of $NaHSO_3$. The R_f values in this system for dobutamine and 3-O-methyldobutamine are 0.50 and 0.35, respectively (4).

7.2.2 Gas Chromatography

Plasma and urine levels of the drug
are determined by chromatographing the trimethyl-
silyl derivative of dobutamine on a 6-foot column
packed with 3.0% UC-W98 silicon gum rubber (methyl-
vinyl) on Diatoport S operated at 260°C. The
hydrogen flame detector is maintained at 280°C.
Helium flow rate is 60 ml/min. The retention time
of dobutamine derivative (TMS) under these condi-
tions is 3.8 minutes. This method measures plasma
levels as low as 1 µg/ml (4).

The levels of free and conjugated
3-O-methyldobutamine in plasma and urine are de-
termined using electron capture detection of the
pentafluoropropionate derivative of the metabolite.
A 4-foot coiled column is packed with SP-2100 and
maintained at 240°C. The temperature of the [63]Ni
electron capture detector is 250°C. The retention
time of the pentafluoropropionate derivative is 1.6
minutes. Plasma levels as low as 50 ng/ml are
readily measured using this method. 3-Hydroxy-N-
3-(4-hydroxyphenyl)-1-methyl-N-propyl)phenethyl-
amine hydrobromide is used as an internal standard.

7.2.3. High Performance Liquid Chromatography

Dobutamine hydrochloride may be de-
termined in plasma levels, after extraction, on a
C_{18} reversed-phase column eluted with 22% aceto-
nitrile-78% 0.1 M phosphate buffer (pH 2.0) at
2 ml/minute. The drug and its metabolite are de-
tected by a fluorescent detector with an excitation
wavelength of 195 nm and a 330 nm emission cut off
filter. The retention times of dobutamine and the
3-methoxy metabolite are 5.2 and 7.9 min., res-
pectively. The lower limit of sensitivity is 10
ng/ml. Reproducibility is ± 5% over a 25-300 ng/ml
range. Nylidrin is used as an internal standard
(6).

7.3 Mass Spectrometry (GC/MS)

The biological samples are analyzed with an
LKB 900 GC mass spectrometer containing a 4-foot
coiled glass column packed with 1% UC-W98 on Gas
Chrom Q. The column is maintained at 240°C and the

flow rate of helium is 60 ml/min. The ion source
voltage is 70 eV. The retention times of the tri-
methylsilyl dobutamine and trimethylsilyl-3-O-
methyldobutamine are 3.8 and 3.6 min., respectively.
The mass spectrum of the dobutamine-TMS shows a
molecular ion at 517 and fragments at 250 and 267.
The spectrum of the derivatized metabolite has a
molecular ion at 459 with major fragments at 250
and 209. This fragmentation pattern confirms the
presence of the methyl group on the catechol
moiety.

8. Analysis of Pharmaceutical Formulations

8.1 Chromatographic Assays

8.1.1 Thin Layer Chromatography

        Samples are dissolved in methanol.
The insoluble excipients are removed by centrifug-
ation. The solution is applied on a silica gel
plate using the same conditions as listed previously
in section 6.5.1. Additional detection sensitivity
may be obtained by spraying with a 6% solution of
ferric chloride followed by a 2% solution of potas-
sium ferricyanide.

8.1.2 Gas Chromatography

        Samples are extracted into ethyl
acetate from pH 9.0 buffer.. After evaporation of
the solvent, n-triacontane, the internal standard,
in pyridine/chloroform is added to the residue.
The trimethylsilyl derivative is formed and chrom-
atographed according to the details mentioned in
section 6.5.2.

8.1.3 High Performance Liquid Chromatography

        Samples are dissolved in water to a
concentration of approximately 0.5 mg/ml and in-
jected directly into the liquid chromatograph with-
out additional preparation. The conditions of
section 6.5.3 apply also to the analysis of form-
ulations.

8.2 Spectrophotometric (UV)

Dobutamine hydrochloride may be determined spectrophotometrically in 0.5 M hydrochloric acid at the maximum of 278 nm. If excipients interfere, the drug may be extracted into ethyl acetate from pH 9 buffer followed by extraction into 0.5 M hydrochloric acid for the UV measurement.

9. Acknowledgments

The authors are thankful to Dr. A. Hunt, Mr. H. W. Smith and Dr. A. D. Kossoy for their help in generating and permission to use the UV, crystallinity and stability degradation data.

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The literature is surveyed through January, 1979.

ERYTHROMYCIN

William L. Koch

1. Description
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1.   Description

1.1   Name, Formula, Structure, and Molecular Weight

Erythromycin, erythromycin A
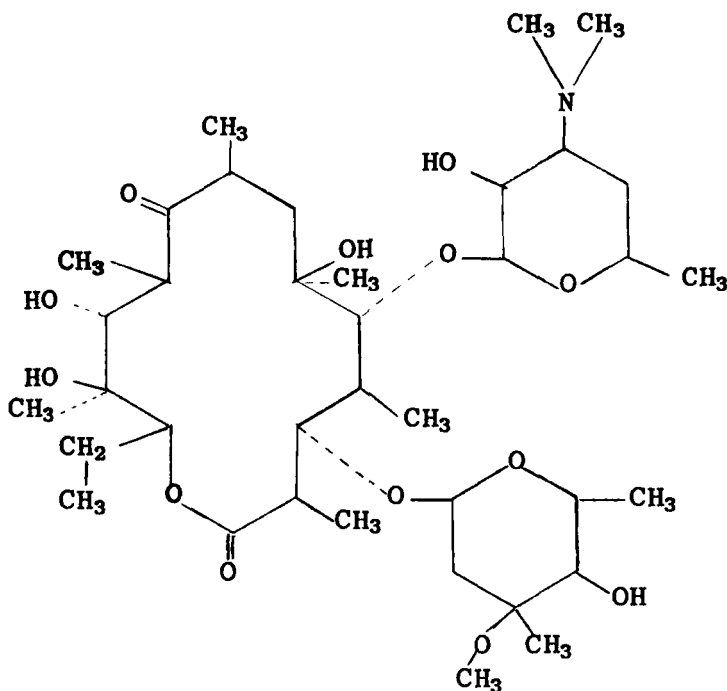
Erythromycin A

$C_{37}H_{67}NO_{13}$ Mol. wt.:   733.92

Erythromycin is a macrolide antibiotic consisting of the aglycone, erythronolide A; the aminosugar, desosamine; and the neutral sugar, cladinose.

Using NMR and CD studies,[1,2] the conformation features were deduced (Fig. 1).
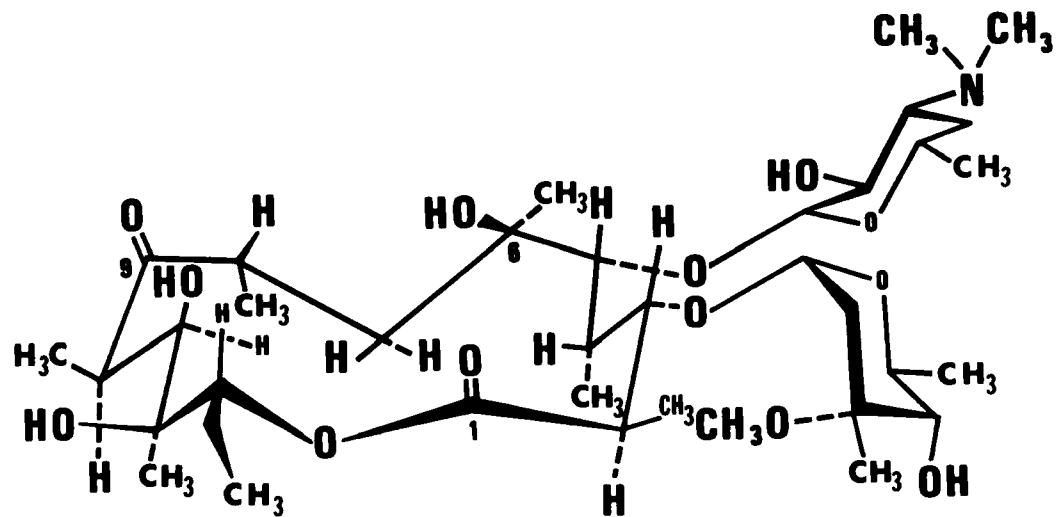
Fig. 1. Spatial arrangement of erythromycin

1.2   Appearance, Color, and Odor

The compound is a white crystalline powder, practically odorless, and has a bitter taste.

2.   Physical Properties

2.1   Solubilities

Weiss et al.[3] reported the solubility of erythromycin summarized in Table 1.

2.2   Infrared Spectrum

The infrared spectrum of erythromycin is commonly used for its identification. Figure 2 shows the spectrum of a 75 mg./ml. chloroform solution. The bands at 1685 and 1730 cm^{-1} are due to the ketone carbonyl and the lactone carbonyl, respectively. The absorption peaks between 1000 and 1200 cm^{-1} are due to the ethers and amine functions. The CH_2 bending is evidenced by peaks between 1340 and 1460 cm^{-1}, and alkane stretching peaks appear between 2780 and 3020 cm^{-1}. Hydrogen bonded OH and water appear as bands between 3400 and 3700 cm^{-1}.

2.3   Ultraviolet Spectrum

The ultraviolet spectrum of erythromycin in methanol exhibits λ_{max} at 288 nm. The ϵ value of 31.1 and the λ_{max} are consistent with the n $\longrightarrow \pi^*$ transition of C = 0.[4]

TABLE 1

Solubilities of Erythromycin

Solvent	mg./ml.
Isooctane	0.477
Petroleum ether	4.69
Cyclohexane	0.2*
Carbon disulfide	5.05
Carbon tetrachloride	> 20
Toluene	> 20
Benzene	> 20
Diethyl ether	> 20
Chloroform	> 20
Ethylene chloride	> 20
Methyl ethyl ketone	> 20
Acetone	> 20
1,4-Dioxane	> 20
Isoamyl acetate	> 20
Ethyl acetate	> 20
Isoamyl alcohol	9.65
Pyridine	> 20
Formamide	> 20
Benzyl alcohol	> 20
Isopropanol	> 20
Ethanol	> 20
Methanol	> 20
Ethylene glycol	> 20
Water	2.1

* Weiss reported this value as > 20. We
  found 0.2 in our laboratories.
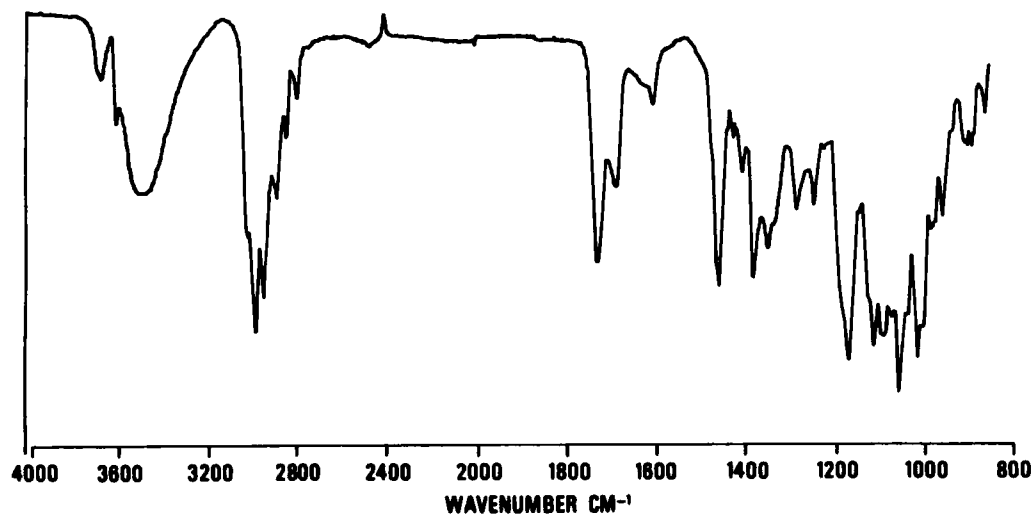
Fig. 2. Infrared absorption spectrum of erythromycin

2.4   Thermal Gavimetric Analysis

A TGA of erythromycin hydrate indicates a loss of volatiles of about 4%, then decomposition starting at 195°C.[5]

2.5   Differential Thermal Analysis

A DTA of erythromycin hydrate shows an endotherm at about 128°C., indicating simultaneous loss of volatiles and melting. A DTA of erythromycin anhydrate indicates melting starting at 193°C., then decomposition.[6]

2.6   X-Ray Diffraction

The x-ray powder diffraction patterns of erythromycin dihydrate and anhydrate are shown in Table II. Radiation: Cr/V, λ 2.2896.[7]

2.7   Nuclear Magnetic Resonance

The spectrum shown in Figure 3 was obtained as a CD_3OD solution using a Varian T-60A 60mHz instrument. General band assignments are listed in Table III according to Underbrink.[8]

2.8   pKa

The pKa for erythromycin in 66% DMF/34% water is 8.6.

TABLE II

X-Ray Powder Diffraction Data

Erythromycin

Anhydrate		Dihydrate	
d$\overset{\circ}{A}$	I/I$_1$	d$\overset{\circ}{A}$	I/I$_1$
19.02	50	11.84	10
15.63	10	9.01	50
13.40	70	8.57	100
11.14	60	7.26	10
10.11	60	6.73	100
9.74	80	6.40	20
8.43	100	6.12	40
7.79	60	5.43	30
7.24	70	5.05	40
6.79	15	4.99	30
6.43	60	4.60	30
6.31	60	4.42	20
6.02	15	4.25	20
5.71	70	4.12	20
5.46	20	4.00	10
5.20	70	3.90	10
5.01	10	3.78	10
4.85	60	3.39	10
4.78	50	3.30	10
4.57	20	3.16	10
4.51	30	2.99	10
4.43	40	2.90	10
3.91	20		
3.75	10		
3.58	05		
3.35	02		
2.98	02		
2.23	02		
2.06	02		
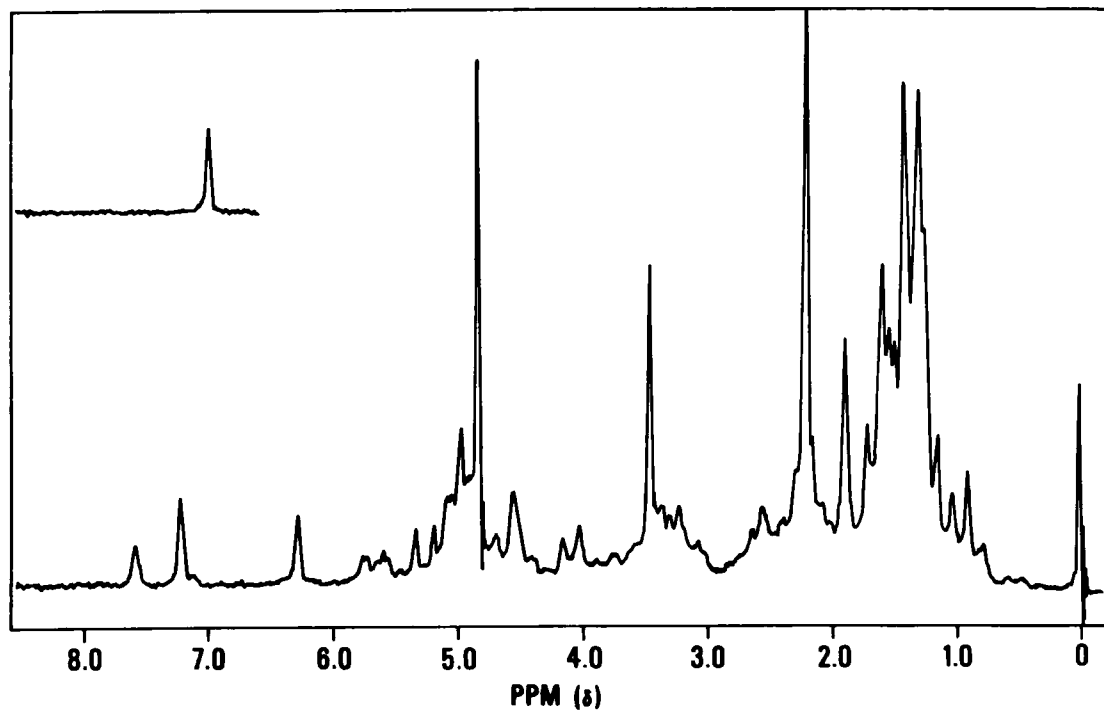
Fig. 3. NMR spectrum of erythromycin

TABLE III

NMR Spectral Assignments of Erythromycin

Solvent: CD_3OD

Instrument: Varian T-60A 60 mHz

Proton(s)	Resonance Position (PPM)	Peak Type
$C\underline{H}_3CH_2$ –	.9	Unresolved triplet
All methyls not listed above or below	1 –1.5	Overlapping singlets and doublets
$N(C\underline{H}_3)_2$	2.35	Singlet
– $OC\underline{H}_3$	3.35	Singlet (over-lapping solvent multiplet)

Other protons (methyne and methylene) give
unresolved and overlapping multiplets in
the 60 mHz spectrum of the CD_3OD solution.

3. Methods of Analysis

3.1 Ultraviolet Assay

The ultraviolet chemical assay for erythromycin remains largely unchanged from that described by Kuzel et al.[9] in 1954. This procedure is essentially as follows. The reference standard, alkali reagent, and buffer solutions are prepared prior to the assay.

Phosphate buffer pH 7.0 is prepared by dissolving 13.6 g. KH_2PO_4 (anhydrous) and 27.2 g. K_2HPO_4 (anhydrous) in sufficient purified water to make 5 liters.

The reference standard solution is prepared by dissolving about 35 mg. accurately weighed erythromycin standard in 100 ml. methanol in a 250 ml. volumetric flask. This is diluted with phosphate buffer pH 7.0 to 250 ml., mixed, and allowed to cool to room temperature, then again diluted to the mark and mixed well.

The alkali reagent is prepared by slurrying 42 g. $Na_3PO_4 \cdot 12H_2O$ in about 125 ml. 0.5N NaOH in a 250 ml. volumetric flask. An additional 100 ml. purified water is added and the slurry heated on the steam bath to aid in solution. The solution is cooled slowly to room temperature and diluted to 250 ml. with purified water, then filtered prior to use.

Four 10 ml. aliquots of the standard solution are pipetted into separate 25 ml. volumetric flasks, two are labelled standard and the others blank. One ml. of 0.5N H_2SO_4 is added to the blank flasks and they are allowed to stand after mixing at room temperature for 60 minutes ± 5 minutes. Two ml. of purified water are added to the standard flasks. At the end of this time, 1.0 ml. of 0.1N NaOH is added to the blank flasks and their contents swirled

to mix. Then, 2.0 ml. of alkali reagents are
added to all four flasks, they are swirled to
mix and placed in a 60°C. water bath for 15.0
minutes. The flasks are then cooled rapidly in
an ice bath, brought to room temperature, then
diluted to 25.0 ml. with purified water. The
UV absorbance is read at 236 nm. versus puri-
fied water in 1.0 cm. silica cells. The blank
values are subtracted from the standard values
and the average net absorbance used for calcula-
tion.

        Bulk erythromycin raw material is
treated the same as the standard. Formulations
are made up to the same concentration as the
standard in methanol and buffer and 10 ml.
aliquots used for chromophore development.

        The sulfuric acid treated aliquot
representing the blank forms a cyclic ether
anhydroerythromycin.[10] The alkaline treatment
causes the formation of an α,β unsaturated ketone
(9-keto-10-ene) having its absorbance maximum as
a shoulder at 236 nm. (ϵ 6000).[11,12] Thus, any
other UV absorbing species are measured with the
blank and subtracted from the absorbance before
calculation of the erythromycin concentration. A
typical spectrum is shown in Figure 4.

   3.2   Gas Chromatographic Assay

        Tsuji and Robertson[13] reported a gas
chromatographic procedure for erythromycin using
an OV-225 column or a PPE-20 column. The pro-
cedure involves silylating 10 mg. erythromycin
with a mixture of trimethylchlorosilane, N,O-bis-
trimethylsilylacetamide, and N-trimethylsilyl-
imidazole in pyridine for 24 hours at 75°C. Ten
micrograms are injected onto the column (3 mm. x
1850 mm., 3% OV-225 on GCQ100-120 mesh or 3 mm.
x 1850 mm. 3% PPE-20 on Supelcoport at 275°C.) of
an F and M model 400 gas chromatograph equipped
with a flame ionization detector. They report
being able to separate erythromycins A, B, C,
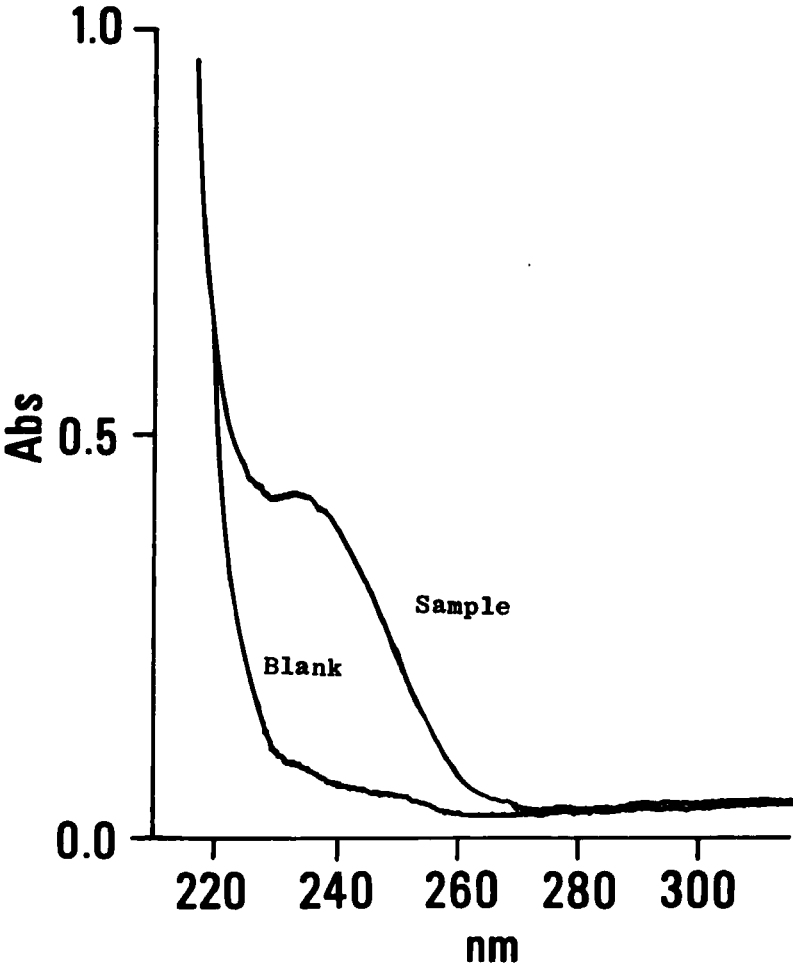
Fig. 4. UV spectra of sample and blank after chromophore development

anhydroerythromycin A, and erythralosamine.
Good agreement with the microbiological assay is
shown. However, the biggest drawbacks appear
to be in silylation time and the instability of
the GC column, 3 weeks at 275°C.

These authors[14] later report using the
GC method for enteric coated tablets of erythro-
mycin, giving a recovery of 99.8% and a coef-
ficient of variation of 2.3% based on placebo
tablets spiked with erythromycin.

3.3 Colorimetric Assays

Two procedures are worthy of note
here. The first, published in 1967 by Kuzel
and Coffey[15] is based on the ion pair dye com-
plex of bromcresol purple (5',5"-dibromo-o-
cresol-sulfonphthalein) and the desosamine moiety
of erythromycin in pH 1.2 buffer. The method
lacks specificity for erythromycin, measuring all
tertiary amines; however, it is quite sensitive
and precise, being routinely used for concentra-
tions of 250 mcg. erythromycin/ml. in tablets
and 20-100 mcg./ml. in fermentation broth. A
more recent method by Sanghavi and Chandramo-
han[16] is also based on a complex of the desosa-
mine moiety, but they use p-dimethyl amino
benzaldehyde as the coupling agent. The pro-
cedure is non-specific, but sensitive and linear
over a concentration range of 10-35 mcg./ml.

3.4 Thin Layer Chromatography

Egon Stahl[17] described four TLC systems.
Table IV summarizes the solvents and R_f's on
silica gel G.

TABLE IV

Solvent	R_f	Detection
Methanol	.16	Brownish-green color after
Chloroform-methanol 95 + 5	.03	spraying with 10% sulfuric acid and
Chloroform-methanol 50 + 50	.29	heating 5-10 minutes at 80°C.
Butanol-acetic acid-water 60 + 20 + 20	.39	

Spraying with 10% molybdophosphoric acid in alcohol, followed by heating produces a blue spot on a yellow background. The spot disappears in 2 hours.

Vilim et al.[18] devised a TLC identification system for erythromycin base, stearate, estolate and ethylsuccinate. This combination cannot differentiate between the estolate and ethylsuccinate.

Our laboratories[19] have developed a system separating erythromycin estolate, erythromycin base, and anhydroerythromycin on a silica gel 60 F-254 plate utilizing ethanol, methanol, triethylamine, 170:30:1. Visualization is made by spraying with 0.15% xanthydrol and 7.5% acetic acid in water. Table V summarizes the R_f's.

TABLE V

Component	R_f	Color
Erythromycin Base	0.30	Violet
Anhydroerythromycin	0.43	Violet
Erythromycin Estolate	0.60	Violet

3.5   Microbiological Analysis

Kavanagh and Dennen[20] report micro-
biological turbidimetric and plate assays for
erythromycin base in Analytical Microbiology,
Vol. 1. Staphylococcus aureus (ATCC 9144) is
used for the turbidimetric procedure. The bulk
raw material official assay is found in 21CFR –
452.10 and the official tablet assay is found in
21CFR452.110. The sample is diluted from 0.3 to
2.0 μg./ml. in pH 7.0 buffer and comparison is
made to a standard curve of 0, 0.3, 0.4, 0.6,
0.8, 1.2, 1.6, and 2.0 μg./ml. Sarcina lutea
(ATCC 9341) is used for the plate assay. A linear
response in the range of 0.5 – 2.0 μg./ml. is
obtained when pH 8.0 buffer is used for sample
and standard. In both methods, a small amount of
methanol is used to solubilize the erythromycin
prior to buffering at pH 7.0 or 8.0.

3.6   High Performance Liquid Chromatography

Omura et al.[21] used a reverse phase high
performance liquid chromatographic column, JASCO
PACK SV-02-500®, for macrolide antibiotics with
methanol, M/15 acetate buffer pH 4.9, and aceto-
nitrile (35:60:5) as solvent. A variable wave-
length UV detector using the absorption of the
individual compounds gave the required sensitivity.
Alterations of buffer pH and the composition ratio
of the mobile phase gave selectivity for separa-
tion of individual macrolide antibiotics.

Z. H. Hash[22] reported chromatographic
conditions for separating anhydroerythromycin from
erythromycin using a normal phase Corasil II®
silica gel column, with chloroform as mobile phase
and refractive index detection.

White et al.[23] devised a reverse phase
high performance liquid chromatographic procedure
for erythromycin. Refractive index detection was
used since the compound absorbs weakly in the UV.
A 10 μm C_{18}/Lichrosorb™ reverse phase column was
used with 80% methanol, 19.9% water, 0.1% ammonium
hydrochloride as the developing solvent.

Tsuji and Goetz[24] developed a quanti-
tative high performance liquid chromatographic
method for separating and measuring erythromycins
A, B, and C, their epimers and degradation prod-
ucts. This method uses a μBondapak® C_{18} reverse
column with acetonitrile-methanol-0.2M ammonium
acetate-water (45:10:10:25) as solvent. The pH
and composition of the mobile phase may be ad-
justed to optimize resolution and elution volume.
The authors utilized the procedure on USP reference
standard and report a relative standard deviation
of ± 0.64%.

4. Stability

Erythromycin is unstable in acidic or alka-
line solutions and shows its maximum stability
between pH 6.0 and 9.5[25]. Its aqueous, alcoholic
solution buffered at pH 7.0 - 8.0 is stable for
about one week under refrigeration.

5. Bioavailability

Maximum levels of erythromycin in serum are
obtained in 1 to 2 hours after a single dose.
The U. S. Dispensatory[26] reports maximum serum
levels of 0.2 ug./ml. 1 hour after administration
of a 250 mg. dose, 0.6 ug./ml. 2 hours after a
500 mg. dose, and 1.2 ug./ml. 2 hours after a
1 g. dose. Higher blood levels are achieved on
a multiple dosage schedule. Since it is acid
labile, a resistant coating is used in tablet
formulations to overcome the deleterious effect
of gastric fluid on erythromycin base; or the
stearate salt is prepared which does not dissolve
readily in the stomach.

The Bioavailability Monograph for Erythro-
mycin[27] provides data for comparison of several
manufacturers' tablets of erythromycin. The
criteria for bioavailability tests are discussed.

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GRAMICIDIN

Glenn A. Brewer

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1.   Introduction

Gramicidin is a peptide antibiotic first isolated by
Dubos in 1939[1] as a crude complex together with a second
peptide antibiotic known as tyrocidin. The mixture of the
two antibiotics was called tyrothricin. Although the mix-
ture was discovered about ten years after penicillin[2], tyro-
thricin was the first antibiotic utilized in clinical prac-
tice.

It was soon found that tyrothricin causes severe hemo-
lysis when administered parenterally and was destroyed when
given orally. The antibiotic complex or individual compo-
nents are effective topically and are used in various
cream, ointment, lotion and solution preparations alone
or in combination with other antibiotics or topical
steroids[3].

The aspect of gramicidin which is of most interest
to the analytical chemist is the continued study of the
structure of the antibiotic, from its discovery to the
present day. As more and more sophisticated separation
and structural elucidation techniques have been developed,
various scientists have applied these to the problem of
understanding the complete structure of the gramicidin
complex. Thus, we can trace the development of our under-
standing of the structure from the crude extract prepared
by Dubos[1] to our current knowledge of not only the struc-
ture of the various components of gramicidin, but the
conformation of the major fraction in solution.[4] It is
indeed interesting that so many scientists have applied
their knowledge and skill to solve this difficult struc-
tural problem, considering the relative minor role of this
material product in modern medicine.

2.   Chemistry

Tyrothricin was obtained by acidification of the
fermentation broth of Bacillus brevis to precipitate the
antibiotic activity along with various proteins and then
dissolving the antibiotic complex in alcohol. The alcohol
was removed under vacuum, the residue was washed with ether,
then redissolved in alcohol and finally reprecipitated with
1% sodium chloride[5,6].

It was soon recognized that tyrothricin was not a
pure compound but could be separated into a neutral frac-
tion called gramicidin and a basic fraction called tyroci-

dine by extraction with acetone and ether mixtures. The
individual fractions were thought to be homogeneous because
they were crystalline and had constant physical properties
on recrystallization[7,8,9,10,11,12]. At the time, these
constant properties were considered to be sufficient proof
to indicate chemical purity.

An empirical formula of $C_{74}H_{106}N_{14}O_{14}$ was proposed for
gramicidin based on elemental analysis and a Rast molecular
weight determination[8]. The application of various bio-
chemical methods led the investigators to the conclusion
that gramicidin was a peptide containing ten α-amino acid
residues and a saturated aliphatic fatty acid containing
14 to 16 cabon atoms. It was established that a hydroly-
sate of gramicidin contained tryptophane and that histidine,
arginine, tyrosine and ammonia were absent. The authors
further noted that about half of the amino acid residues
have the D-(unnatural)configuration. This was shown by
oxidation with d-amino acid oxidase[8,12].

Other workers began to study the structure of gramici-
din. Christensen and coworkers[12] isolated crystalline
tryptophane and leucine from a hydrolysate. They found no
evidence for a fatty acid component and established that
phenylalanine, proline and hydroxyproline were absent
from a hydrolysate. These workers isolated alanine diox-
pyridate from a hydrolysate and also established that grami-
cidin contained a compound with vicinal hydroxy and amino
groups. They speculated that this compound might be serine
or isoserine and proposed that gramicidin contains two
tryptophane, 2 leucine, 2 or 3 alanine and 1 hydroxyamino
residues or a multiple of this composition.

Hotchkiss[14] isolated optically and analytically pure
d-leucine from the hydrolysate. This was the first non-
enzymatic proof that d-amino acids actually occurred in
gramicidin. He also noted the presence of an amino-hydroxy
compound, but indicated that it was not isoserine.

Gordon, Martin and Synge[15] utilized their new and
elegant technique, chromatography, to establish the amino
acid composition of gramicidin. They proposed a 24 unit
cyclic peptide consisting of six moles each of leucine, and
tryptophane, 5 moles of valine, 3 moles of alanine and 2
moles each of glycine and unknown hydroxyamino compound.
They confirmed that the leucine was the d-form while the
tryptophane and alanine had the L-configuration.   The

valine appeared to be racemic.

Christensen[16] isolated the dipeptide valylvaline from completely hydrolyzed gramicidin. This worker later showed that he had isolated a racemic mixture of D(-)-valyl-D(-)-valine and L(+)-valyl-L(+)-valine rather than dipeptides containing one d and one L-residue.[17]

Synge[18], using starch columns, confirmed the presence of valylvaline and identified L-valylglycine in partial hydrolysates. This unexpected finding triggered a good deal of work on the kinetics of peptide hydrolysis in an attempt to develop a rational explanation.

Synge[21] isolated the elusive hydroxyamino compound by azeotropic distillation and identified it as 2-aminoethanol. He proposed a structure of 6 L-tryptophane, 6 D-leucine, 5 D and L-valine, 3 L-alanine, 2 glycine and 2 aminoethanol residues.

There was some evidence that the preparations of gramicidin used in this structural work were not completely homogeneous[15,22] but the evidence was not strong until Gregory and Craig[23] separated crystalline gramicidin into three major fractions by counter current distribution. Fraction B contained only 55% as much tryptophane as Fraction A based on an ultraviolet analysis. The third fraction was called gramicidin C.

Still using heterogeneous gramicidin, Synge[24] isolated the D-leucylglycine, L-alanyl-D-valine and L-alanyl-D-leucine from partial hydrolysates of gramicidin. He also had less conclusive evidence for the tripeptides alanyl-valylleucine or alanylleucylvaline.

In a review paper, Dr. Synge[25] recapitulated the early structural work on gramicidin and indicated that x-ray diffraction was incapable of distinguishing a gramicidin fraction purified by counter current analysis from the heterogeneous starting material.

Hinman, Caron and Christensen[26] corrected the earlier report by Christensen[16]. They reported that the dipeptides found on the hydrolysis of gramicidin were D-valyl-L-valine and L-valyl-D-valine.

James and Synge[27] speculated on the nature of the non-peptide bonds in gramicidin.

Hodgkin[28] examined crystals of gramicidins A and B by x-ray diffraction. She estimated that the molecular weight of gramicidin A was approximately 3800, Previous estimates by chemical methods were approximately 7000. In 1953, Cowan and Hodgkin[29] published a second report on gramicidin B.

A provisional structure for gramicidin was proposed by Gavrilov and Akimova[30].

Okuda and coworkers[31] determined the amino acid composition of gramicidins A, B and C. Ishii and Witkop[32] established the complete optical assignment of the amino acids in gramicidin A using enzymatic degradation and quantitative gas chromatography. The composition established was 4 moles of L-tryptophane, 4 moles of D-leucine, 2 moles of D-valine, 2 moles of L-valine*, 2 moles of L-alanine, 1 mole of glycine and 1 mole of aminoethanol. (*The authors actually found 1.6 moles of L-valine and 0.6 moles of L-isoleucine. This indicated the possibility of the non-homogeneity of gramicidin A.)

The evidence that gramicidin A was actually heterogeneous was obtained by Ramachandran[33] using counter current distribution with more than 1000 transfers. The new gramicidin which contained isoleucine was called gramicidin D. This was an unfortunate choice of terminology since the crude mixture of gramicidin fractions had been previously called gramicidin D (gramicidin Dubos) to distinguish it from other gramicidins (S and J).

Sarges and Witkop[34,35] established the amino acid sequence of gramicidins A and D. The same authors also established the amino acid sequences of gramicidins B[36] and C[37]. In addition, they synthesized gramicidins A and D[38].

Gross and Witkop[39] subjected commercial gramicidin to counter current distribution. They found that gramicidins A, B and C all consisted of a pair of congeners containing primarily valine-gramicidins (80-95%) with some isoleucine-gramicidins (5-20%) as minor components. In addition, they isolated a more hydrophilic, strongly antibiotic group of antibiotics which they called gramicidin D.

Urry and coworkers[40,41] proposed a left-handed helical structure for gramicidin A. This conformation can undergo ion induced relaxations which provides a mechanism for the movement of the ion along the channel. These workers confirmed this proposed structure by nuclear magnetic resonance spectrometry[42].

Using circular dichroism and nuclear magnetic resonance spectrometry, Veatch and coworkers[4] established that four conformational species of gramicidin A exist in solution. Two were postulated to be helixes of opposite handedness.

3.   Description

3.1   Composition, Formula, Molecular Weight

The gramicidin of commerce is a complex of at least four compounds. The identified fractions are called gramicidins A, B, C and D. The major component of the mixture is gramicidin A. (See Section 2.)

The mixture of gramicidins is called gramicidin [1405-97-6] or gramicidin D (Dubos) [1393-88-0]. The latter name is used to distinguish the gramicidin discovered by Dubos from gramicidins S and J.

As discussed in Section 2, the chemical structure of the various fractions has now been elucidated. The general formula for gramicidin is shown below, where R and R' are different amino acid residues depending on the particular type of gramicidin.

HCO-R-Glycine-L-Alanine-D-Leucine-L-Alanine-D-Valine-
L-Valine-D-Valine-L-Tryptophane-D-Leucine-R'-D-Leucine-
L-Tryptophane-D-Leucine-L-Tryptophane-NHCH$_2$CH$_2$OH

3.11   Gramicidin A [11029-61-1]

Gramicidin A consists of a pair of congeners containing primarily L-valine gramicidin A (80-95%) but also containing L-isoleucine gramicidin A (5-20%)[39, 34].

3.111 L-Valine Gramicidin A [4419-81-2]

R = L-valine
R'= L-tryptophane
$C_{99}H_{140}N_{20}O_{17}$
Molecular Weight 1882.349

3.112 L-Isoleucine Gramicidin A [5536-03-8]

R = L-isoleucine
R'= L-tryptophane
$C_{100}H_{142}N_{20}O_{17}$

Molecular Weight 1896.376

3.12   Gramicidin B [11041-38-6]

Gramicidin B consists of a pair of congeners containing primarily L-valine gramicidin B but also containing L-isoleucine gramicidin B[36].

3.121 L-Valine Gramicidin B [4422-52-0]

R = L-valine
R'= L-phenylalanine
$C_{97}H_{139}N_{19}O_{17}$
Molecular Weight 1843.312

3.122 L-Isoleucine Gramicidin B

R = L-isoleucine
R'= L-phenylalanine
$C_{98}H_{141}N_{19}O_{17}$
Molecular Weight 1857.339

3.13   Gramicidin C [9062-61-7]

Gramicidin C also consists of a pair of congeners containing primarily L-valine gramicidin C but also small amounts of L-isoleucine gramicidin C are present[37].

3.131 L-Valine Gramicidin C [58442-65-2]

R = L-valine
R'= L-tyrosine
$C_{97}H_{139}N_{19}O_{18}$
Molecular weight 1859.312

3.132   L-Isoleucine Gramicidin C

R = L-isoleucine
R'= L-tyrosine
$C_{98}H_{141}N_{19}O_{18}$
Molecular Weight 1873.339

3.14   Gramicidin D [1405-97-6]

As previously discussed, the term grami-
cidin D has been used to designate the entire gramicidin
complex of gramicidins A, B, C, D or the isoleucine com-
ponent of gramicidin A[33]. Gross and Witkop[39] have used it
to name a minor and still undefined, polar fraction of the
gramicidin complex.

In the rest of this Analytical Profile,
the physical and chemical properties which will be described
will be of the complex mixture of gramicidins A, B, C and D
unless otherwise noted.

3.2   Appearance, Color, Odor

Gramicidin is described as a crystalline powder,
which is white or nearly white and is odorless[43].

4.   Physical Properties

4.1   Spectra

4.11   Infrared Spectra

The infrared spectrum of gramicidin has
been reported by Hayden, et al.[44].

The infrared spectra of the U.S.P. grami-
cidin standard are presented in Figures 1 and 2[45].

4.12   Nuclear Magnetic Resonance Spectra

Nuclear Magnetic Resonance has been used
to establish the conformation of the pure fractions of the
gramicidin complex[42].

The proton resonance spectrum of the
U.S.P. gramicidin standard is shown in Figure 3[46].
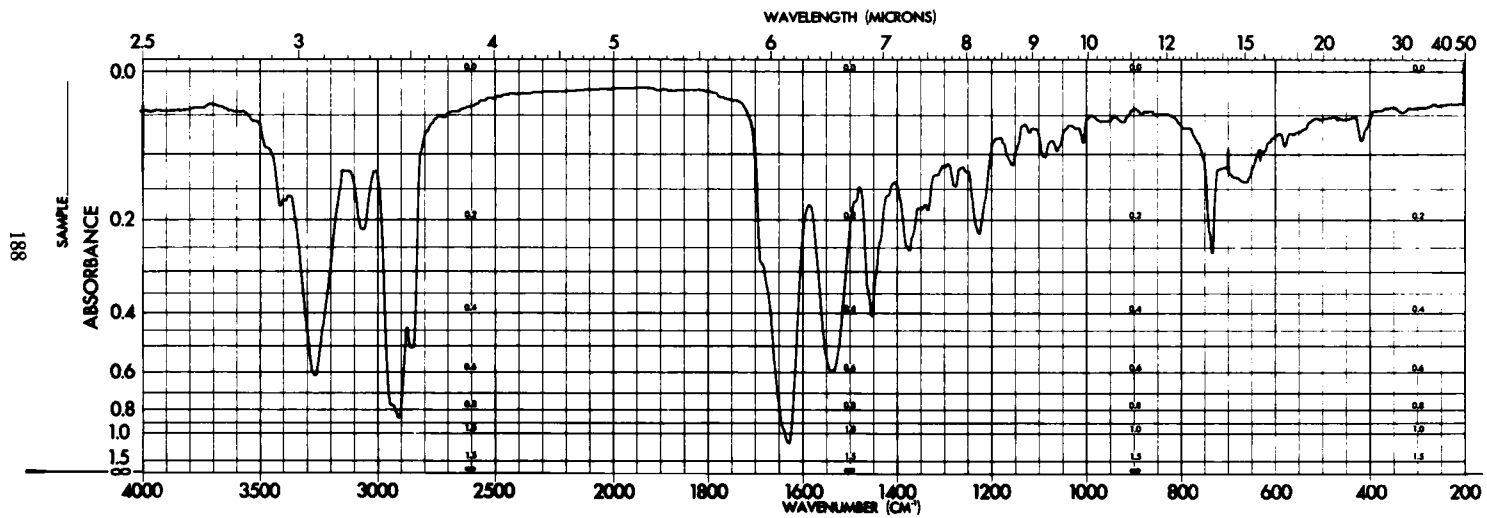
FIGURE 1
Gramicidin U.S.P. Standard
Mineral Oil Mull
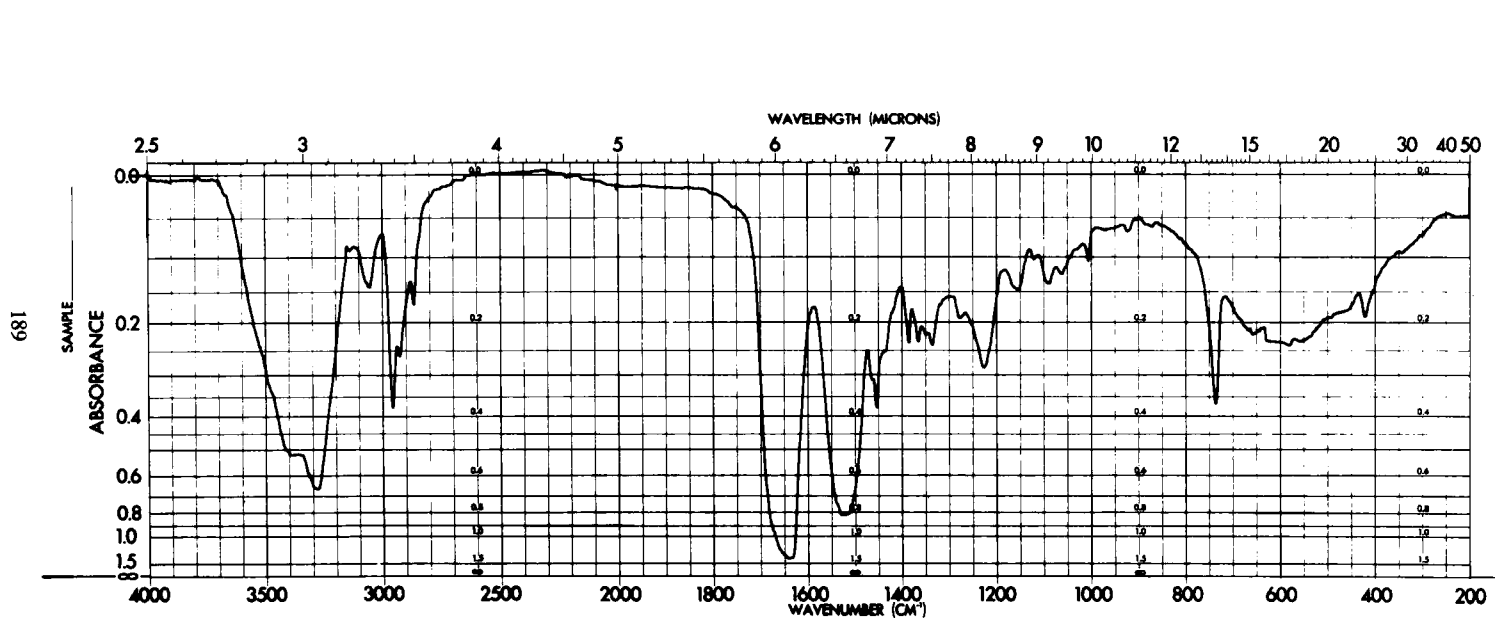
FIGURE 2
Gramicidin U.S.P. Standard
Potassium Bromide Pellet
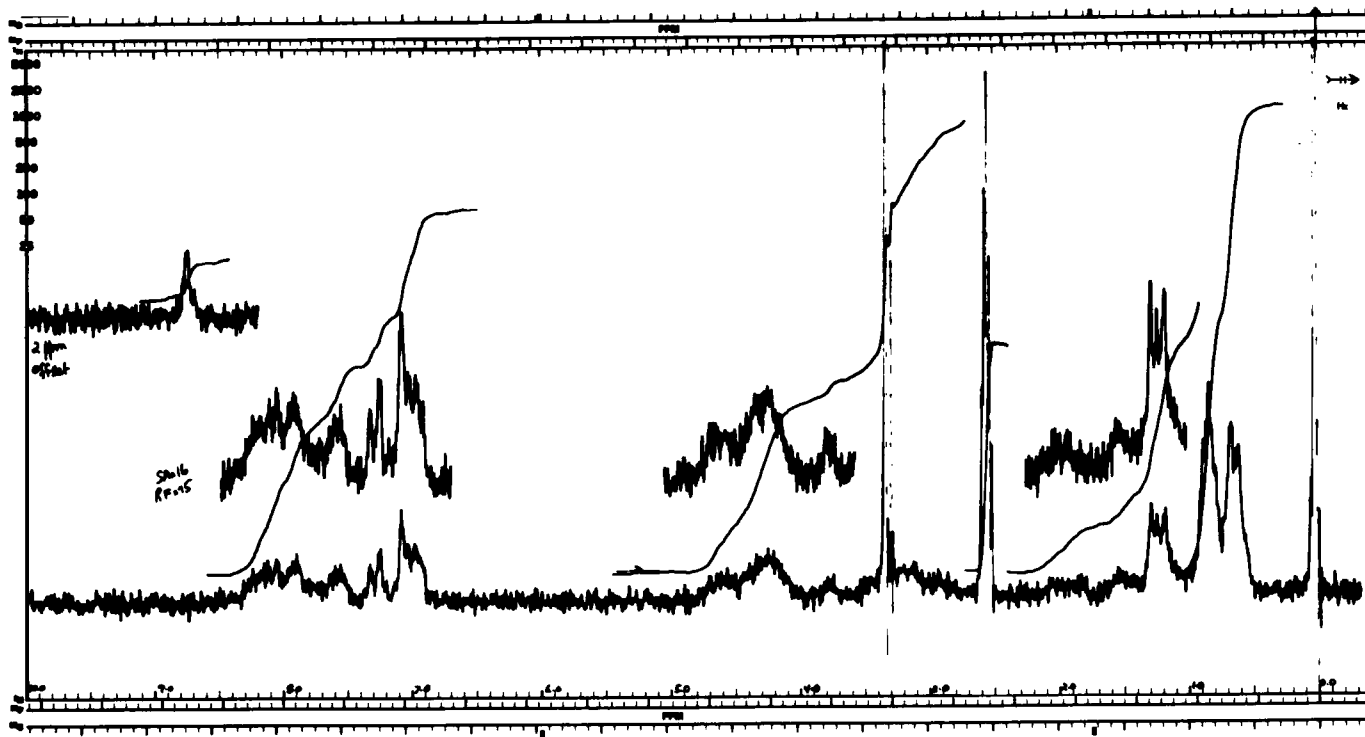
FIGURE 3
Gramicidin U.S.P. Standard
DMSO-d_6

4.13   Ultraviolet Absorption Spectra

The tryptophane content of the gramicidin complex was measured by the ultraviolet absorbance[47].

Cann has demonstrated the shift to longer wavelengths when acetic acid complexes with gramicidin[48].

The ultraviolet spectrum of the gramicidin complex has been reported by Hayden and coworkers[44].

Figure 4 presents the ultraviolet spectrum of the U.S.P. Reference standard taken in 95% ethanol[49].

4.14   Fluorescence Spectra

Sommermeyer and coworkers[50] reported that solutions of gramicidin exhibit fluorescence when irradiated with soft x-rays.

Gramicidin exhibits strong fluorescence in 95% ethanol. The excitation maximum is at 286 nm and the emission maximum is at 337 nm. The fluorescence intensity was linear with respect to concentration in solution[51].

4.15   Raman Spectra

Rothchild and Stanley studied the conformation of Gramicidin A using Raman spectroscopy[52]. Two types of conformation were found depending on the solvent used.

4.2   Crystal Properties

4.21   Crystalline Modifications

Dr. Synge noted that when gramicidin complex was crystallized from acetone, he obtained small crystals that were not suitable for x-ray diffraction[25]. When the sample was allowed to crystallize from alcoholic solution by slow evaporation, large chunky crystals were obtained. These gave very good x-ray diffraction patterns.

Olesen and Szabo obtained crystals from ethanol and acetone[53]. They found the crystals to have different solubility, melting point and x-ray diffraction patterns. Since acetone is retained in the crystalline lattice, it was indicated that the forms are pseudopolymorphs.
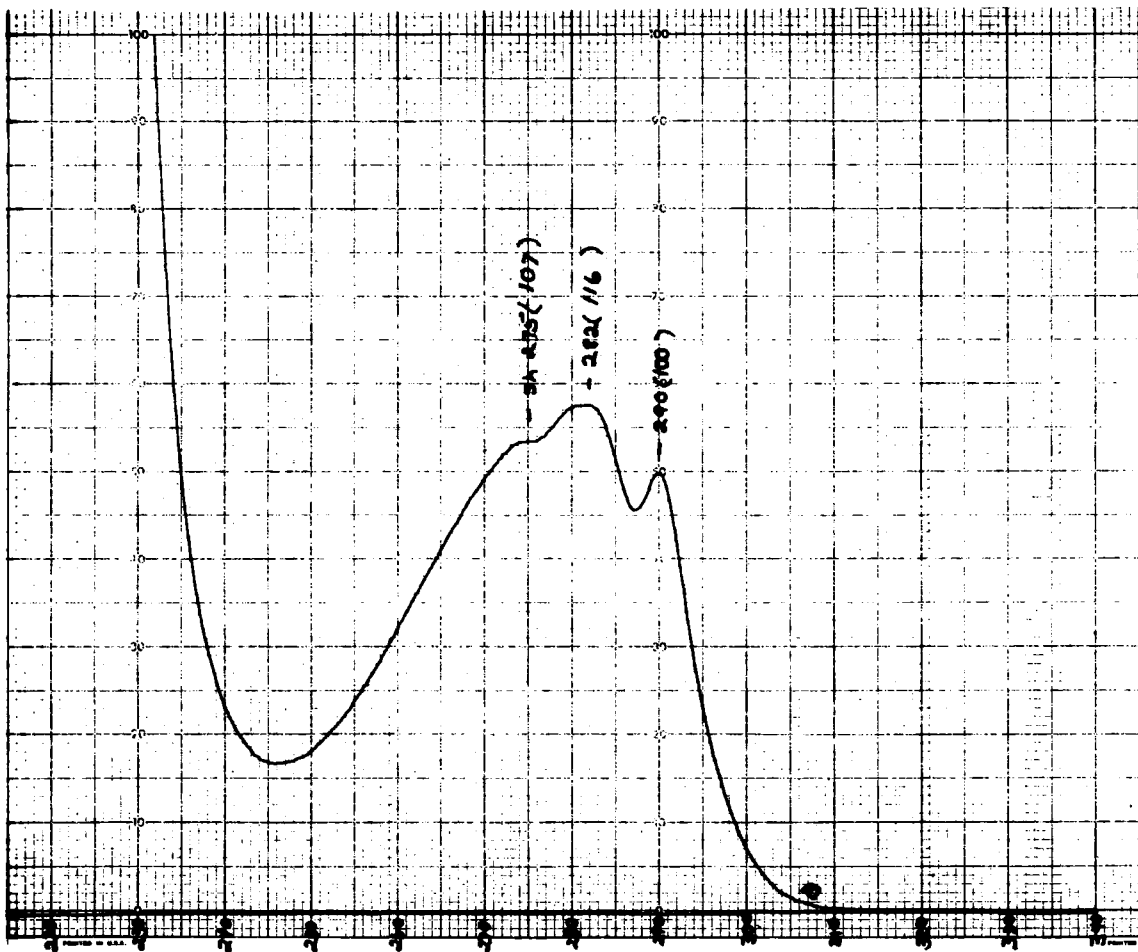
FIGURE 4
Gramicidin U.S.P. Standard
95% Ethanol

4.22   X-ray Powder Diffraction

As was mentioned in Section 2, x-ray diffraction was used in an effort to establish the structure of the gramicidin complex[25,28,29]. These studies were frustrated by the fact that the gramicidin was not chemically pure but was a mixture of components.

Belavtseva found that crystalline gramicidin was converted to amorphous gramicidin by the action of x-rays[54].

The powder x-ray diffraction pattern of the gramicidin U.S.P. reference standard is shown in Figure 5[55]. The relative peak intensities are presented in Table 1.

TABLE 1

Relative Peak Intensities of
U.S.P. Gramicidin Reference Standard as Measured
by Powder X-Ray Diffraction

2θ (DEG.)	D (ANGSTROMS)	PEAK	REL. PEAK	AREA	REL. AREA
6.64	13.31	143.5	1.000	850.0	1.000
7.40	11.95	104.1	0.725	791.4	0.931
9.44	9.37	28.4	0.198	301.2	0.354
13.69	6.47	31.1	0.217	327.6	0.385
14.46	6.13	28.6	0.199	246.0	0.289
16.75	5.29	46.5	0.324	349.9	0.412
16.92	5.24	45.3	0.316	274.6	0.323
17.86	4.97	48.7	0.339	473.6	0.557
19.39	4.58	63.8	0.445	716.2	0.843
20.07	4.42	50.7	0.353	414.9	0.488
21.26	4.18	38.8	0.270	288.3	0.339
21.68	4.10	39.5	0.275	250.9	0.295
22.28	3.99	37.2	0.259	275.6	0.324
23.55	3.78	36.2	0.252	312.1	0.367
23.89	3.72	34.6	0.241	306.0	0.360

4.23 Crystal Density

Low and Richards used a density gradient column to study the density of crystals of "mixed gramicidin fractions"[56].

4.24 Differential Thermal Analysis

The U.S.P. gramicidin standard shows a small endotherm at 161°C and a larger endotherm at 244°C. Both endotherms are broad[57].

4.3 Solubility

4.31 Solubility in Pure Solvents

The solubility of the gramicidin complex in a variety of solvents has been determined by Weiss and coworkers[58].

TABLE 2

Solubility of Gramicidin Complex
in Various Solvents

SOLVENT	SOLUBILITY mg/ml	SOLVENT	SOLUBILITY mg/ml
Water	0.140	Ethyl Acetate	11.90
Methanol	>20	Isoamyl Acetate	>20
Ethanol	>20	Methyl Ethyl Ketone	18.10
Isopropanol	>20	Acetone	18.80
Isoamyl Alcohol	14.10	Diethyl Ether	10.7
Cyclohexane	0.02	Ethylene Chloride	2.15
Benzene	0.19	1,4-Dioxane	>20
Toluene	0.04	Chloroform	>20
Petroleum Ether	0.007	Carbon Disulfide	0.100
Isooctane	0.005	Pyridine	>20
Carbon Tetrachloride	0.047	Formamide	>20
Ethylene Glycol	>20	Benzyl Alcohol	>20

4.32   Solubility in Solutions of Quaternary Ammonium Compounds

A number of reports have indicated that tyrothricin is more soluble in solutions of quaternary ammonium compounds[59,60].

When it was realized that tyrothricin was a mixture of antibiotics, the same principle was applied to gramicidin[61,62,63,64,65]. The addition of quaternary pounds to gramicidin formulations to increase the solubility of the antibiotic in aqueous solution has been utilized[66,67].

4.33   Solubility in Solutions of Other Surface Active Agents

A number of other materials have been shown to increase the solubility of gramicidin in aqueous solution. Alcohols[68], xanthocillin[69], fatty acid amides[70], fatty acid alcohols[71], aliphatic amines[72] and polyvinyl-pyrrolidone[73,74].

4.4   Physical Properties of Solutions

4.41   Diffusion

The diffusion constants for the gramicidin complex were determined in acetic acid and ethanol solu-tion[75]. The molecular weight range of gramicidin was calculated as 2800-5000.

Polson using a similar method obtained a molecular weight of 3000[76].

4.42   Surface Tension

Gramicidin decreases the surface tension of aqueous solution[77]. The bactericidal and hemolytic properties of gramicidin were destroyed by heat but the surface tension depression was not changed.

Kemp and coworkers found that when they partitioned gramicidin between water and heptane, it migrated to the walls of the vessel[78].

4.43 Specific Volume

        Derechin and coworkers noted the anomalous
behavior of gramicidin A in absolute ethanol[79]. The apparent
partial specific volume increased with decreasing concen-
tration of gramicidin. The same was not true for aqueous-
ethanol solutions.

4.44 Conformation

        The conformation of gramicidin in aqueous
solution has been extensively studied. A lipophilic left-
handed helical structure has been proposed for gramicidin
A[40,41]. It was proposed that the mode of action of grami-
cidin is due to the formation of ion transport channels
across biological membranes.

        Bamberg and coworkers have studied the
single channel conductance of gramicidins A, B and C[80,81].
Significant differences between gramicidin A and B were
found.

        Cabon 13 NMR has been used to confirm the
presence of a double helical dimer model[82].

        The conformation of gramicidin in various
organic solvents has also been established[4,83,84,85,86].

        Circular dichroism at high pressures has
been used to study the conformation of a derivative of
gramicidin A in trifluoroethanol solutions[87].

        Kyogoku and Kawano have prepared an exten-
sive review of the use of NMR techniques to study the con-
formation of gramicidin and other antibiotics in solution[88].

        Lotz and coworkers have used poly (γ-benzyl-
D-L-glutamate) as a stereochemical model to study the con-
formation of gramicidin A[89].

5.   Production

    5.1   Fermentation

        The gramicidin complex was originally isolated
by Dubos as a component of the antibiotic mixture called
tyrothricin formed by an aerobic sporulating bacillus[7].

Dubos and Hotchkiss found that a number of species of aerobic
sporulating bacilli produced gramicidin[10].

Stokes and Woodward established that Bacillus
brevis produces the antibiotic in stationary cultures of both
complex natural and synthetic media[90]. They noted that
production of the antibiotic occurred in an aerated syn-
thetic medium but not in an aerated complex nitrogenous
medium.

Konikova and coworkers compared the productivity
of two strains of bacilli in the production of gramicidin[91].
Lewis and coworkers studied the production of the antibiotic
complex by Bacillus brevis in both natural and complex
media[92]. They noted requirements for calcium, magnesium and
manganese ions. Stokes patented a submerged culture fermen-
tation production procedure utilizing a synthetic medium[6].
Appleby and coworkers studied the addition of vitamins to
synthetic medium[93]. Konikova and Dobbert studied the
effect of the addition of amino acids to a synthetic medium
on the production of tyrothricin[94]. They found that these
additions had no effect on antibiotic production although
growth was stimulated. Udalova and Fedorova have studied
the effect of different carbon sources on the yield of anti-
biotic[95].

A number of workers have described the production
of tyrothricin in a synthetic medium supplemented with
organic nitrogen compounds[96,97,98,99,100].

Several investigators have attempted to establish
the biosynthetic pathways for the production of tyrothri-
cin[101,102,103,104].

Akers, Lee and Lipmann have isolated two enzymes
from Bacillus brevis that are responsible for the synthesis
of the initial portion of the gramicidins[223].

5.2   Isolation

Hotchkiss and Dubos have utilized solvent extrac-
tion to separate gramicidin and tyrocidine[105]. Several
patents have been issued on procedures for isolating grami-
cidin from fermentation broth[106,107,108].

5.3   Derivatives

Shepel and coworkers have reported the properties of analogs of gramicidin A with shorter peptide chains[109].

It has been found that the treatment of gramicidin with formaldehyde results in the formation of a compound with the same antibiotic activity but with much less hemolytic activity[110,111,112,113,114].

Various esters of gramicidin have also been prepared[115,116,117]. Although these compounds have reduced hemolytic activity, they also possess less antibiotic activity.

6.   Stability

6.1   Stability in Solution

Nitti and Nislo have shown that gramicidin is stable to autoclaving in aqueous solution[118]. Certain aqueous solutions were patented because they produced stable gramicidin solutions[119].

Ishii and Witkop have found that treatment of gramicidin A with 1.5N hydrochloric acid in absolute methanol for one hour at room temperature selectively cleaved one peptide bond[120].

6.2   Effect of Light

The ultraviolet inactivation spectrum for gramicidin has been published by Setlow and Doyle[121]. Sugimoto and coworkers have presented infrared, ultraviolet, visible and ESR spectra for gramicidin solutions irradiated with various amounts of ultraviolet light[122].

6.3   Stability of Formulations

Buckwalter has indicated that gramicidin solutions in propylene glycol and carbowaxes are stable to autoclaving[123]. He also showed that the antibiotic can be solubilized in water with the aid of non-ionic wetting agents.

7.   Analytical Methods

      7.1   Identity Tests

            Brustier and coworkers utilized the Adamkiewicz-
Hopkins-Cole reaction as an identity test for gramicidin[124].
The test detects the indole ring structure of the trypto-
phane residue.

            Fischbach and Levine described the use of
Ehrlich's reagent as an identity test for gramicidin[125].
They also utilized a modified biuret reaction[125].

            Ramachandran reported on the reaction of hydra-
zine with gramicidin to yield formic hydrazide[126]. This
product was detected by a color reaction.

      7.2   Microbiological Assays

            Microbiological assays are the primary assay
method for antibiotics. They provide sensitive but non-
selective methods. A variety of microbiological methods
have been described for the assay of gramicidin. The offi-
cial method in the United States is the turbidimetric method
described in the Code of Federal Regulations[127,128].

            7.21   Dilution Methods

            Dilution assays are generally utilized as
early microbiological methods before well defined standards
are available. They have the advantage that one can com-
pare the activity of one preparation to another without
having a standard.

            Reedy and Wolfson have described a tube
dilution assay for gramicidin[129].

            An agar dilution assay using Micrococcus
lysodeikticus has been reported[130].

            7.22   Dye Reduction Methods

            The addition of a dye that is reduced by
the growing microorganisms gives a microbiological assay a
sharper end point.

Prévot reported on Janus green reduction methods with a number of different organisms[131,132].

DeFelip and coworkers use sodium resazurin as a dye to give a rapid assay in three to six hours[133].

7.23   Turbidimetric Assays

Ceriotti has reported a turbidimetric assay using Micrococcus lysodeikticus[134].

Berridge and Barrett have reported an assay with Streptococcus agalactiae which can be read after ½ hour incubation[135].

Kaiser and Camboni reported a turbidimetric assay utilizing Staphylococcus aureus[136].

Kramer and Kirschbaum have reported an assay with Streptococcus faecalis[137].

Leclercq has described a nephelometric assay for gramicidin[138,139].

Pain and coworkers have reported a turbidimetric assay utilizing lactic acid bacteria[140].

Kreuzig has described a turbidimetric assay utilizing Streptococcus faecalis[141].

7.24   Agar Diffusion Assays

Since gramicidin is not very soluble in aqueous solution, relatively few agar diffusion assays have been reported.

Ceriotti has reported an agar diffusion assay for gramicidin utilizing Micrococcus lysodeikticus[134].

Miller, Matt and Ciminera reported an agar diffusion assay for gramicidin[142]. Raitio and Bonn used this method to assay pharmaceutical preparations[143]. The Swiss Pharmacopeia utilizes an agar diffusion assay with Sarcina lutea[144].

Viola and Canestrini reported on an agar well technique with Sarcina lutea[145].

Kreuzig has studied the agar diffusion assay for tyrocidine in detail using gel chromatography as an analytical technique[146].

7.25   Potentiation of Microbiological Assays

Nisonger reported that the addition of hexadecylpyridinium chloride to gramicidin potentiates the activity found by microbiological assay[147].

Forni found that traces of cobalt chloride enhances the activity of gramicidin toward Escherichia coli and Staphlococcus aureus[148].

Gillissen indicated that while cationic surfactants like cetylpyridinium chloride have a synergistic effect, Tween 80 inhibits the activity of gramicidin[149]. This effect was confirmed by Barr and Tice[150].

Casilli and Ragni assayed gramicidin in the presence of cetrimide without interference using a cetrimide resistant strain of Staphlococcus aureus[151].

7.3   Chemical Assays

7.31   Colorimetric Assays

Rittenberg and coworkers used a colorimetric assay for tryptophane to determine tyrothricin in fermentation broth[152]. Kreuzig described a semi-automated colorimetric assay for gramicidin utilizing the reaction of tryptophane with perchloric acid-butanol and ferric chloride [141,153].

Patel and Naravane have published an assay utilizing p-dimethylaminobenzaldehyde and nitrite[154]. Ivashkiv has used this reaction to assay gramicidin and tyrocidine in fermentation broth[155]. The antibiotics are separated by a simple solvent partition.

7.32   Spectrophotometric Methods

Oberzill has described a spectrophotometric assay for gramicidin[156]. Thombs and coworkers have used the

absorbance of the peptide bond at 210 nm to measure gramici-
din[157].

7.33   Miscellaneous Methods

White and Secor have measured gramicidin
by measuring the Kjeldahl nitrogen[158].

7.4   Electrochemical Methods

Kramarczyk and Berg have described an indirect
polarographic assay for gramicidin[159].

7.5   Hemolytic Methods

As has been mentioned previously, gramicidin has
limited utility as an antibiotic because of its hemolytic
activity (Section 1.0). Various workers have utilized the
hemolytic property of the antibiotic as an assay tool. The
hemolytic activity of gramicidin is probably due to its
ability to form ion conducting channels in the membranes of
red blood cells. The loss of isotonicity causes the cells
to rupture.

Heilman and Herrell reported the first use of the
hemolytic assay to the assay of gramicidin[160]. Mann and co-
workers compared the hemolytic activity of gramicidin and
tyrocidine[161]. They reported that the addition of serum
inhibits the hemolytic activity of gramicidin.

Dimick reported that the hemolytic assay can be
used to measure the concentration of antibiotic in fermenta-
tion broth[162]. Other authors have reported on modified hemo-
lytic methods[163,164,165].

7.6   Enzymatic and Other Biochemical Methods

Many antibiotics have been found to have inhibi-
tory activity on enzyme systems. This inhibition can be
utilized as the basis of an assay system.

Creaser reported that Staphlococcus aureus forms
an inducible β-galactosidase[166]. The production of this
enzyme is inhibited by the addition of gramicidin. Strictly
speaking, gramicidin does not inhibit the enzyme directly but
this method could be used as the basis of a sensitive assay
method.

Gramicidin was found to uncouple the phosphorylation of ADP from the enzymatic reduction of ferricytochrome c[167,168,169,170,171,172,173,174,175].

Hinkson reported that gramicidin inhibited the photoreduction of NAD by photosynthetic bacteria[176].

7.7   Chromatographic Methods

7.71   Counter Current Distribution

Gramicidin was first shown to be heterogeneous by counter current distribution[23]. A water-methanol-chloroform-benzene (7:23:15:15) system was used to show the presence of two components. Craig later showed that gramicidin contained three crystalline components[177]. Stamm discussed the application of counter current distribution to the separation of gramicidins[178]. Ramachandran showed that there were at least four components in gramicidin and gave the fourth component the name gramicidin D[33].

Goss and Witkop separated each of gramicidins A,B and C into a pair of congeners and identified the major congener as valine-gramicidin and the minor component as isoleucine-gramicidin[39].

Okamoto and coworkers showed that gramicidin could be separated into three fractions (A, B and C) using droplet counter current distribution[179].

7.72   Paper Chromatography

Snell, Ijichi and Lewis published a series of paper chromatographic systems capable of separating various antibiotics including gramicidin[180]. Detection was by bioautography. Forni and Cavalli used the following systems on Whatman No. 1 paper to distinguish between bacterial peptide antibiotics[181].

t-Butanol-Acetic Acid-Water (74:3:25)

n-Butanol-Acetic Acid-Water (79:6:15)

Acetone-Water (70:30) + Ammonia

t-Butanol-Water (80:20) + Ammonia

Cunha and Baptista found that a butanol-acetic acid-water (50:25:25) system using Schleicher and Schull 2043a paper buffered to pH 3.0 gave the best separation of peptide antibiotics[182]. The same authors utilized salting out chromatography to separate gramicidin and tyrothricin[183].

Singh utilized anionic dyes to detect gramicidin on paper chromatograms[184]. Paris and Theallet described three paper chromatographic systems for gramicidin[185]. Ritschel and Lercher described two solvent systems for antibiotics[186]. The solvent systems were butanol-pyridine-acetic acid-water (15:10:3:12) and water saturated butanol-water saturated ethyl ether-acetic acid (5:1:1) on Schleicher and Schull 2043b paper. The antibiotics were visualized by ninhydrin.

DeFranca and coworkers described a system of 25 ml 9:1 acetone-water, 5 ml chloroform, 2 ml ethylene glycol and 1 ml of pyridine[187]. They claim the method is as accurate as the microbiological assay.

7.73   Thin Layer Chromatography

Nussbaumer utilized a solvent system of butanol-acetic acid-water (10:1:3) on acid Silica gel G[188]. Pitton described the following five thin layer systems for several antibiotics including gramicidin[189].

Water-Methanol-Butanol-Butyl Acetate-Acetic Acid
        (12:2.5:7.5:40:20)

Water-Butanol-Pyridine-Acetic Acid (14:30:20:6)

Water-Butanol-Pyridine-Acetic Acid (16:40:8:16)

Water-Butanol-Acetic Acid (39:55:6)

Aqueous phase of Methanol-Ammonia-Chloroform
        (10:10:20)

Guven and Ozsari reported some thin layer systems to use as identity tests for antibiotics including gramicidin[190]. McGilveray and Stickland described two thin layer chromatographic systems to use to identify several antibiotics including gramicidin[191].

Nekola published a thin layer chromatographic assay for gramicidin in fermentation broth[192]. The

broth was adjusted to pH 4.5 with HCl and the antibiotics
precipitated. The residue was dissolved in methanol and
purified on an alumina column. The eluate was chromato-
graphed on silica gel using water-methanol-butanol-butyl
acetate-acetic acid (24:5:15:80). The absorbance of the
gramicidin spot was then determined.

          Kreuzig reported a high performance TLC method
for gramicidin in fermentation broth[193,194]. A Kieselgel
60 plate is developed with acetic acid-butyl acetate-butanol-
methanol-water (40:80:15:5:12). The gramicidin spot was
visualized by spraying with ethanolic 4-dimethylaminobenzal-
dehyde-HCl and quantitated by scanning at 570 nm.

          7.74   Electrophoresis

          Cunha and Baptista reported an electro-
phoresis method to determine gramicidin in pharmaceutical
preparations[195]. Paris and Theallet have described two
paper electrophoresis systems for gramicidin[185]. Cunha and
Gomes have published paper electrophoresis methods for
various peptide antibiotics[196]. Lightbown and DeRossi
utilized agar electrophoresis to identify various antibiotics
including gramicidin[197].

          7.75   Column Chromatography

          Moses has patented a process for the separa-
tion of gramicidin from tyrothricin[198]. The 80% aqueous
methanol solution is passed through a cation ion exchange
resin in the hydrogen cycle followed by an anion resin in the
hydroxyl cycle.

          Bartley and coworkers separated gramicidin
from tyrocidine using Sephadex LH20[199].

          7.76   High Performance Liquid Chromatography

          Axelsen and Vogelsang have reported the
separation of gramicidins A, B and C by HPLC using Zorbax
ODS (5 μm) eluted at 60°C with methanol-5 mM ammonium sul-
fate (37:13)[200].

8.   Reviews

A large number of general reviews have been written on gramicidin[201,202,203,204,205,206,207,208,209,210,211,212,213 214,215,216,217,218,219,220,221,222].

These reviews cover the chemical properties, biological properties and medical use of gramicidin.

A review on the analytical methods for gramicidin and other antibiotics has been prepared by Brewer and Platt[224].

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GRISEOFULVIN

Edward R. Townley

1. Description
    1.1   Name, Formula, Molecular Weight
    1.2   Appearance, Color, Odor
    1.3   Compendial References, Other
2. Physical Properties
    2.01   Circular Dichroism
    2.02   Nuclear Magnetic Resonance
    2.03   Mass Spectrum
    2.04   Ultraviolet Spectrum
    2.05   Infrared SPectrum
    2.06   X-Ray Diffraction
    2.07   Fluorescence and Luminescence
    2.08   Photolysis
    2.09   Optical Rotation
    2.10   Melting Range
    2.11   Differential Scanning Calorimetry
    2.12   Thermogravimetry
    2.13   Electrophoretic Properties
    2.14   Solubility
3. Production and Synthesis
4. Impurities
5. Stability
6. Drug Metabolic Products
7. Methods of Analysis
    7.01   Identification
    7.02   Elemental Analysis
    7.03   Spectrophotometric Analysis
    7.04   Spectrofluorometric Analysis
    7.05   Colorimetric Analysis
    7.06   Iodometric Analysis
    7.07   Turbidimetric Analysis
    7.08   Polarographic Analysis.
    7.09   Chromatographic Analysis
        7.091   Partition Column Chromatography
        7.092   Paper Chromatography
        7.093   Thin-Layer Chromatography
        7.094   Gas Chromatography
        7.095   High Performance Liquid Chromatography
    7.10   Biological Methods of Analysis
8. Identification and Determination in Body Fluids
9. Analysis of Dosage Forms
10. Acknowledgments

1. Description

1.1 Name, Formula, Molecular Weight

Chemical Names

(2S-trans)-7-Chloro-2',4,6-trimethoxy-6'-methylspiro
[benxofuran-2(3H), 1'-(2) cyclohexene]3,4'-dione

7-chloro-4,6-dimethoxycoumaran-3-one-2-spiro-1'-(2'-
methoxy-6'-methylcyclohex-2'-en-4'-one)

Generic Names
Griseofulvin

Trade Names
Fulcin; Fulvicin U/F, Fulvicin P/G, Grifulvin; Gris-
actin; Grisovin, Gris-PEG, Grysio, Lamoryl, Likuden, Neo-
Fulcin, Poncyl; Spirofulvin; Sporostatin.

Formula and Molecular Weight
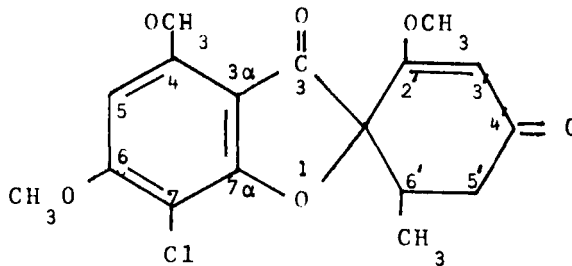
$C_{17}H_{17}ClO_6$ Molecular Weight 352.77

1.2 Appearance, Color, Odor
Griseofulvin is a white, odorless, crystalline pow-
der.

1.3 Compendial References, Other
Griseofulvin is listed in the following compendia:
The United States Pharmacopia (1) The British Pharmacoepia
(2) The Europeon Pharmacoepia (3) and the Merck Index (4). A
previously published review (5) is a good source for physical
and chemical data, production, use, occurrence and biological
information.

2. Physical Properties
     Physical measurements by Schering Corporation are provid-
ed for Batch Number UGFP-1961. This batch is chromatographi-
cally pure with the exception of 1.1% dechlorogriseofulvin.

   2.01 Circular Dichroism Spectra
         The circular dichroism spectra (Figure 1) was ob-
tained on a 0.0155 mg/ml solution in methanol with a Cary
Model 61 Circular Dichroism Spectrophotometer. The following
molar ellipticity values were obtained:

Table I

Wavelength nm	Molar Ellipticity $[\theta]$
345 shoulder	+ 7,520
326 shoulder	+ 19,100
314 shoulder	+ 23,200
294 peak	+ 43,700
236 peak	+110,000
218 peak	- 97,500

   2.02 Nuclear Magnetic Resonance Spectra
         The proton NMR spectrum of griseofulvin (Figure 2)
was obtained in DMSO-d_6 solution (conc. w/v = 10 mg/0.40
ml) containing TMS as internal reference utilizing the Varian
Associates CFT-20 Spectrometer operating at a frequency of
79.5 MHz. The chemical shifts (δ, ppm) are with reference to
TMS. The experimental conditions are:

    Sweep width   =   800 Hz
    Pulse width   =   6.0 μ sec (25°tip)
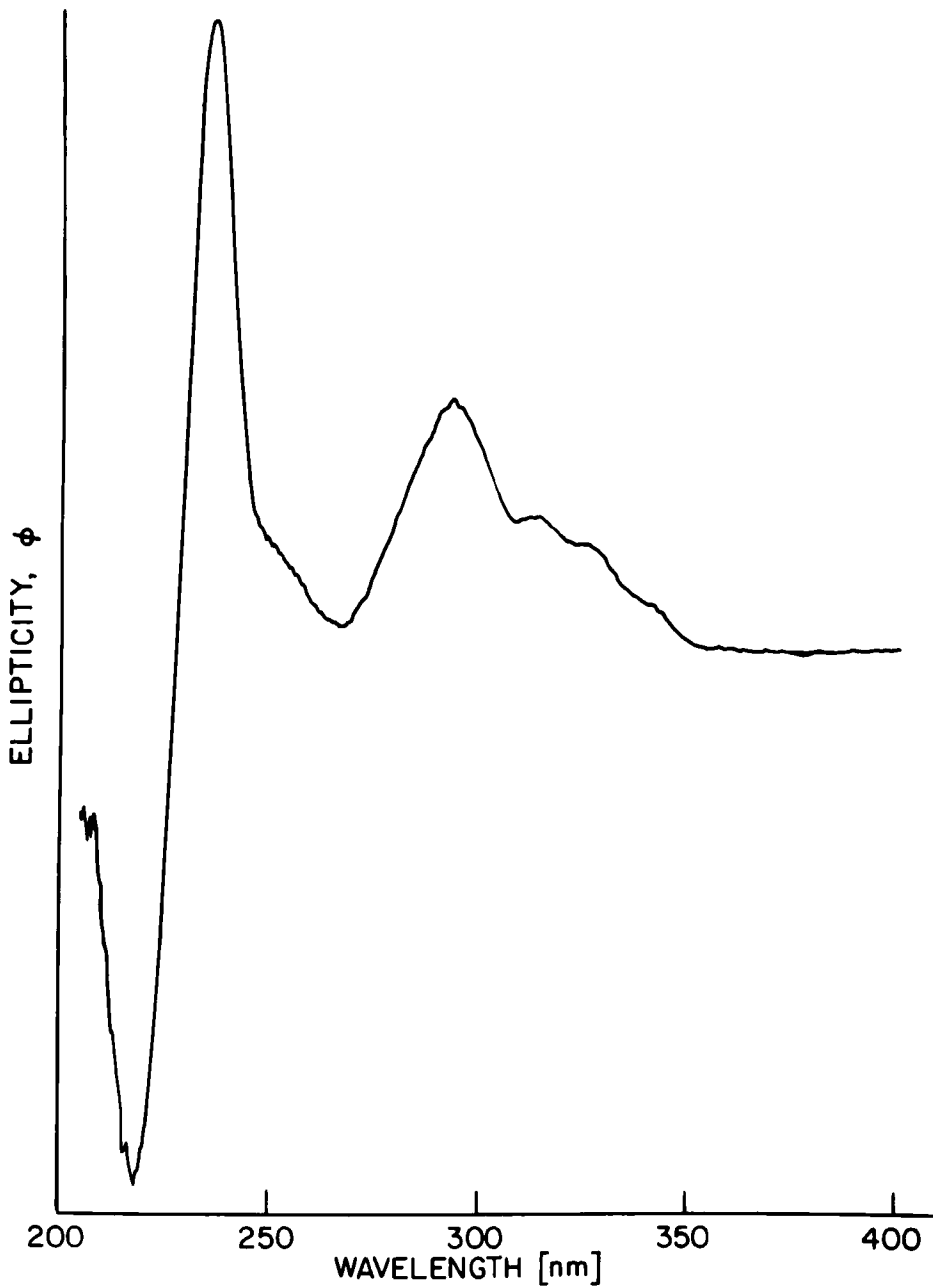   Acquisition time =   5.1 sec
    Data table   =   8 K

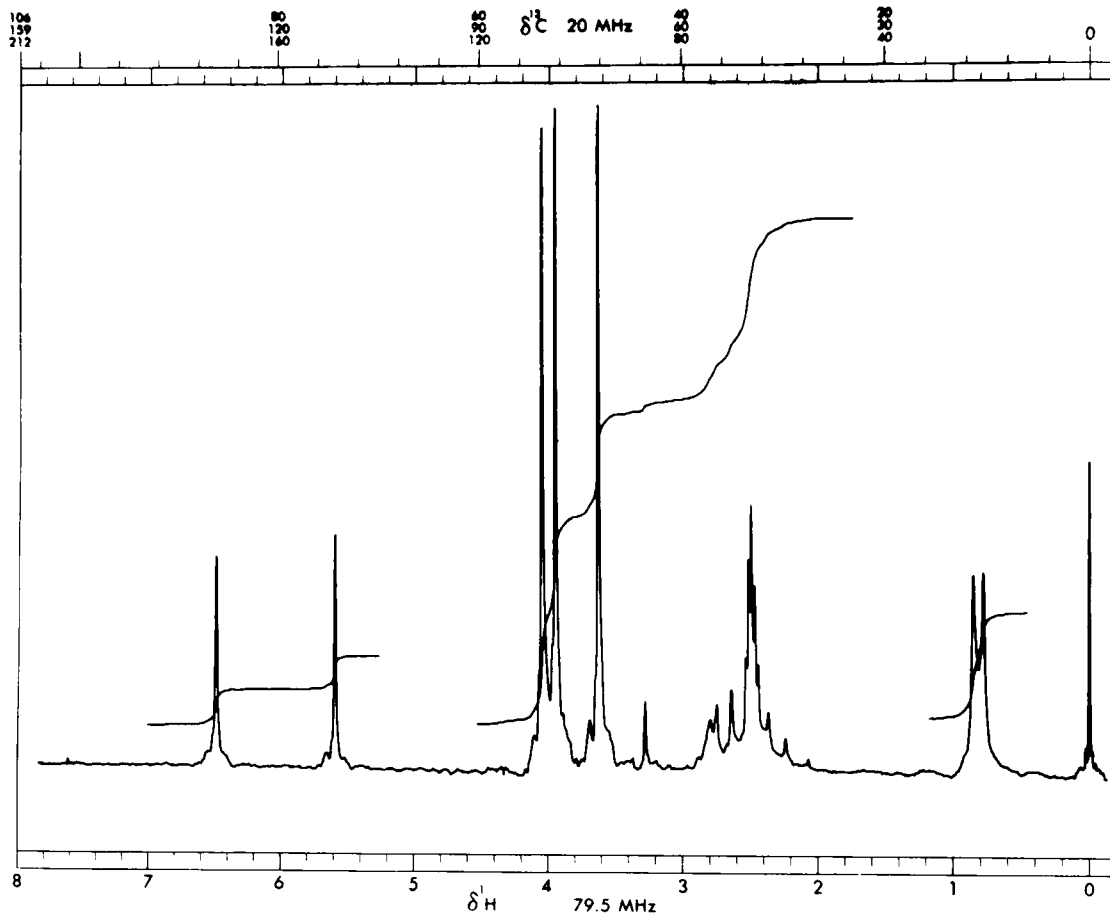FIGURE 1: Circular Dichroism Spectra of Griseofulvin

FIGURE 2:   Proton Nuclear Magnetic Resonance Spectra of Griseofulvin in DMSO d$_6$

Table II

Proton	Chemical Shifts (δ, ppm)		
6'-CH$_3$	0.82	d(J=6.5 Hz)	(3H)
5'-CH$_2$} 6'-CH	2.3-2.9	m	(3H)
2'-OCH$_3$	3.63	s	(3H)
4-OCH$_3$ } Aromatic 6-OCH$_3$	3.96 4.04	s s	(3H) (3H)
3'-H 5-H	5.59 6.50	s s	(1H) (1H)

d= doublet, s= singlet, m= multiplet.

The carbon-13 NMR spectrum of griseofulvin (Figure 3) was obtained at ambient temperature in DMSO-d$_6$ containing TMS as internal reference utilizing Varian Associates XL-100-15 spectrometer equipped with Fourier accessories. The system was locked to the deuterium resonance frequency of the solvent, and operated at a frequency of 25.2 MHz for carbon-13. The chemical shifts are reported (c, ppm.) from the internal standard TMS.

Sweep width	= 5500 Hz
Pulse width	= 15 μ sec (66° tip)
Acquisition time	= 1.6 sec
Acquisition delay	= 0.20 sec

Table III

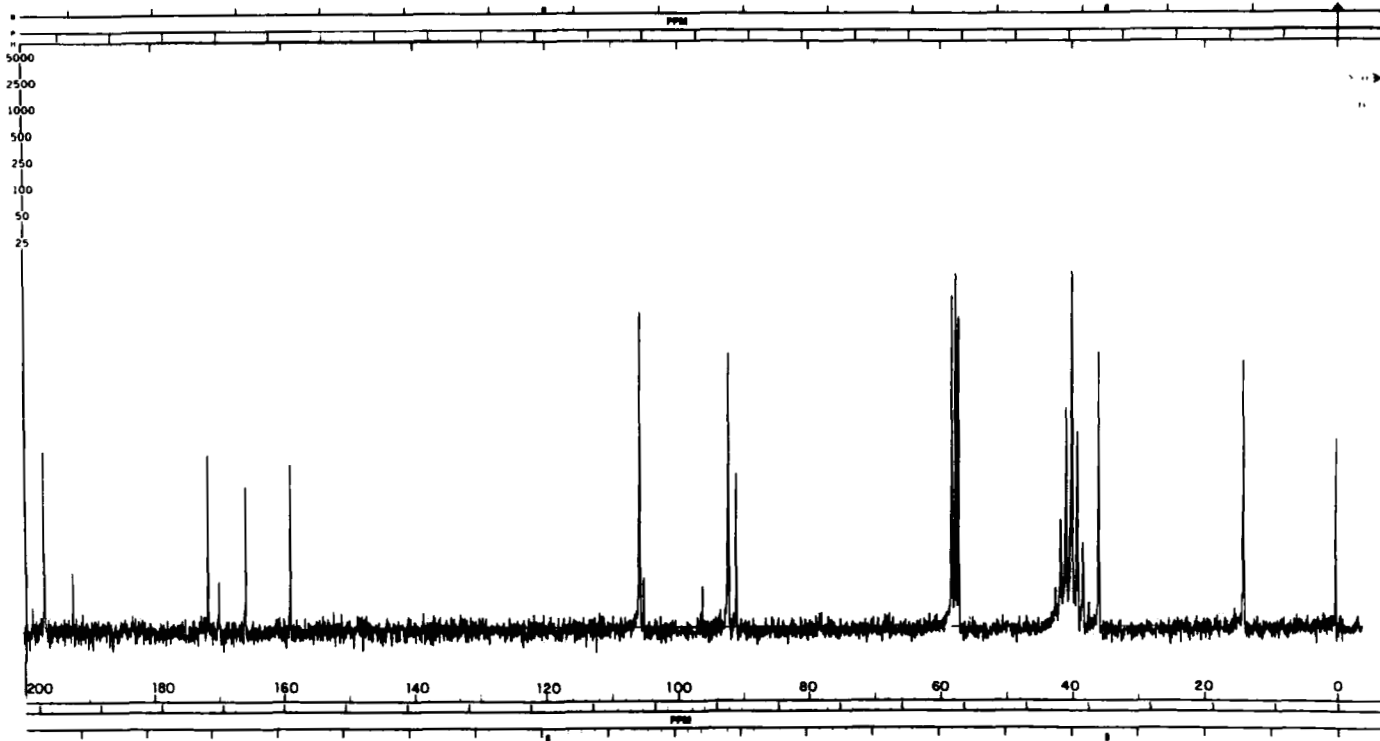| Carbon	Chemical Shift (δ_c)	Carbon	Chemical Shift (δ_c)
1'	90.11	6 OCH$_3$	56.52
2'	170.22	3	191.12
3'	104.60	3a	104.04
4'	195.45	4	157.59
5'	39.49	5	91.27
6'	35.52	6	164.44
6' CH$_3$	13.77	7	95.24
4' OCH$_3$	57.54	7a	168.57
2' OCH$_3$	56.97		

FIGURE 3: Carbon 13, Nuclear Magnetic Resonance Spectra of Griseofulvin in DMSO d_6

The spectrum is in substantial agreement with the data report-
ed by Wenkert et. al, (6). However, on the basis the proton
coupled carbon 13 NMR spectra, the assignments for 6 and 7α
are reversed [Brambilla (16)] from those previously reported.
The new assignments are based on long range H-C-O-C couplings.

   2.03 Mass Spectrum
          The medium resolution, electron-impact mass spectrum
of griseofulvin (Figure 4) was run on a Varian-Mat CH-5 Mass
Spectrometer. Instrumental conditions were; Electron Energy
70eV; Source Temperature 250°C; Sample Probe Temperature
140°C.

          The fragmentation ions, given below, are consistent
with the griseofulvin structure.

                            Table IV

Mass (amu)	Ions	Losses
352	M^+	
337	$(M-15)^+$	CH_3
321	$(M-31)^+$	CH_3O
310	$(M-42)^+$	CH_2CO
284	$(M-68)^+$	$MeC=CHCO$

215

138
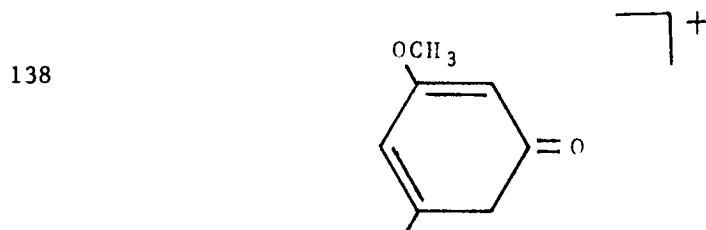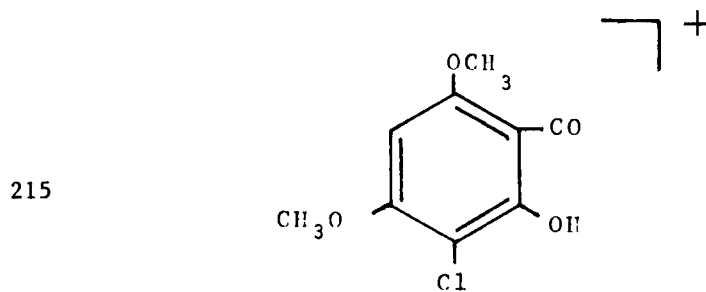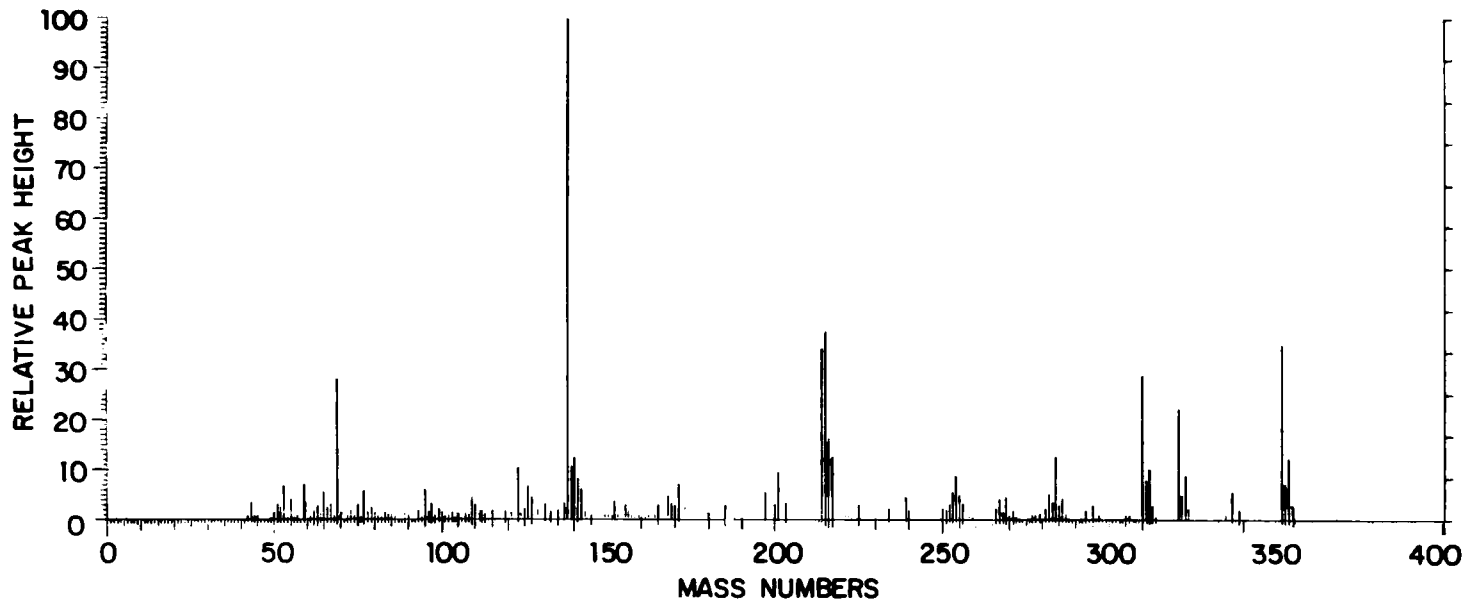
FIGURE 4: Mass Spectrum of Griseofulvin

123
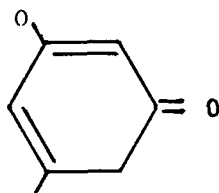
2.04 Ultraviolet Spectrum

The ultraviolet spectrum of griseofulvin in anhydrous methanol solution at 25°C gave the following absorptivity values

Wavelength maximum is at 324 nm; a = 15.5
Wavelength maximum is at 291 nm; a = 68.3
Wavelength maximum is at 235 nm; a = 64.0

The ultraviolet spectrum is shown in Figure 5.

2.05 Infrared Spectrum

The infrared spectrum of griseofulvin, obtained as a mineral oil mull, was run on a Perkin-Elmer Model 180 grating IR spectrophotometer. Important absorption assignments are given in Table V. The spectrum is given in Figure 6.

Table V

Wavenumber (cm^{-1})	Assignment
1703 (s)	C=O stretch; benzofuranone ring carbonyl
1658 (s)	C=O stretch; cyclohexenone carbonyl
1615, 1597, 1580 (s)	C=C stretch, aromatic and cyclic unsaturation
1501 (m)	C=C stretch, aromatic
1220, 1210 (s)	C-O stretch, aryl methoxyl

Intensity

s - strong
m - medium

2.06 X-ray Diffraction

The X-ray diffraction pattern of griseofulvin was obtained on a Phillips ADP-3500 X-ray Diffractometer using Cu Kα radiation (1.5405A°) and Ni filter. The data is given in Table VI.

2.06 X-Ray Powder Diffraction
                Pattern of Griseofulvin

Table VI

2θ	$d(\overset{o}{A})$ *	I/I' **
4.009	22.038	18
4.117	21.460	14
10.679	8.284	46
13.123	6.746	48
13.844	6.397	8
14.497	6.110	59
16.422	5.398	100
17.669	5.020	7
19.194	4.624	30
19.625	4.523	16
20.184	4.399	24
21.562	4.121	34
21.986	4.043	23
22.462	3.958	45
23.765	3.744	72
24.067	3.698	10
24.295	3.663	13
25.780	3.456	26
26.567	3.355	87
28.418	3.141	57
29.835	2.995	29
31.104	2.875	20
31.185	2.868	19
31.385	2.850	24
32.624	2.745	14
32.674	2.741	14
34.852	2.574	12
34.914	2.570	11
35.896	2.502	18
36.142	2.485	14
36.202	2.481	15
36.290	2.475	14
37.077	2.425	14
37.270	2.413	13
38.500	2.338	18
38.569	2.334	18
38.659	2.329	19

*d (interplanar distance) = n /2 sin θ
**I/I' = relative intensity (based on the highest
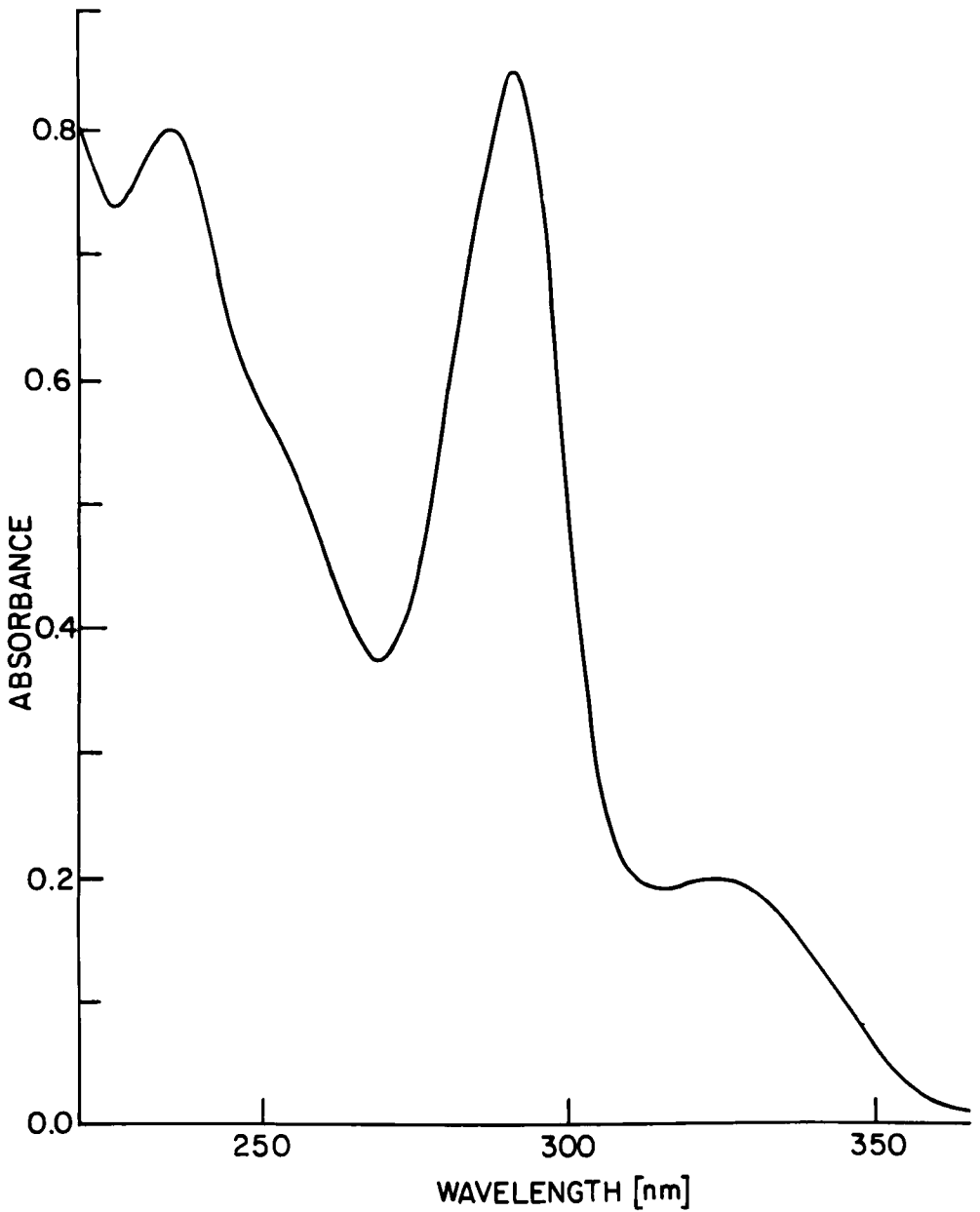 intensity of 100)

FIGURE 5: Ultraviolet Spectrum of Griseofulvin Obtained in
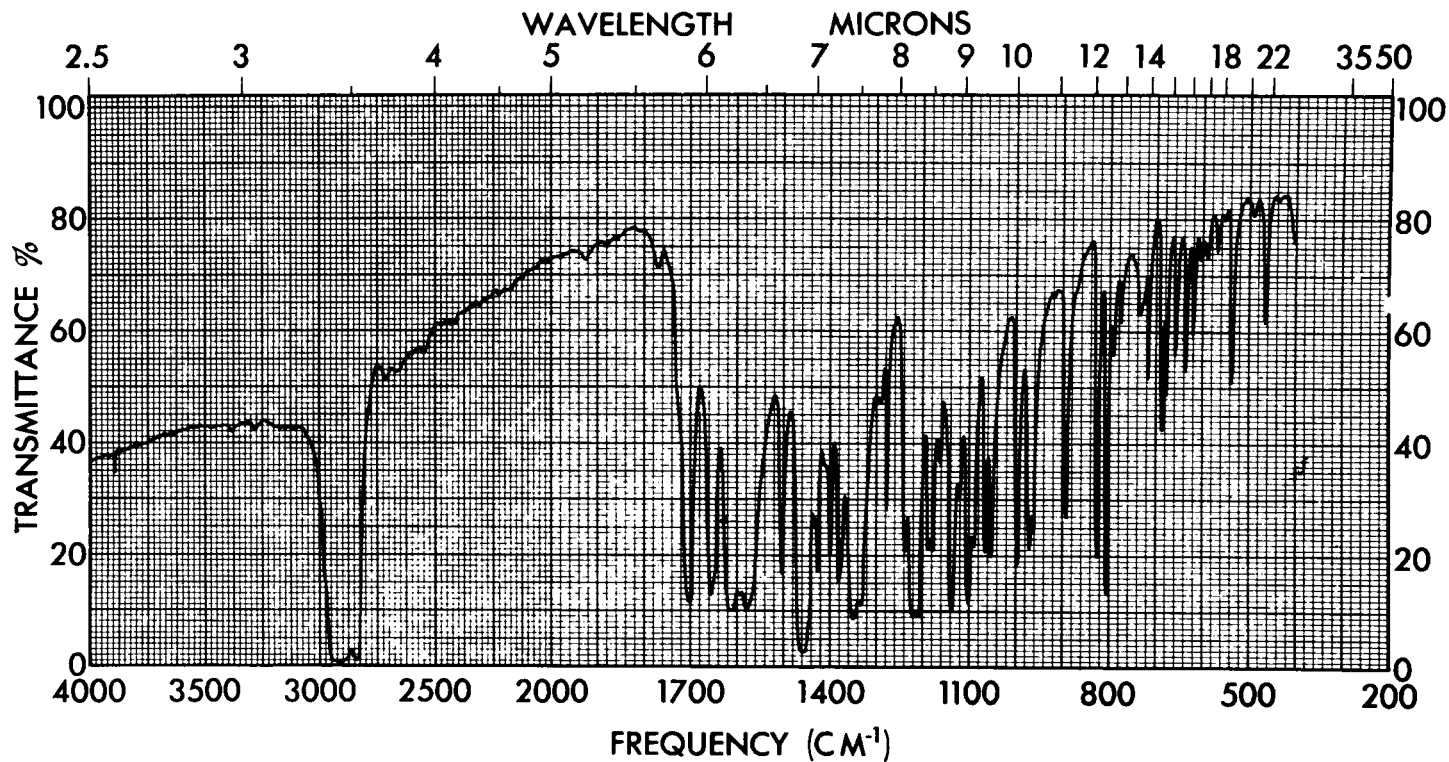   Anhydrous Methanol Solvent

FIGURE 6: Infrared Spectrum of Griseofulvin Obtained as a Mineral Oil Mull.

2.07 Fluorescence and Luminescence

Griseofulvin exhibits both fluorescence and luminescence. A report by Neely et al., (7) gives corrected fluorescence excitation (max. 295 nm) and emission (max. 420 nm) spectra, values for quantum efficiency of fluorescence (0.108) calculated fluorescence lifetime (0.663 nsec) and phosphorescence decay time (0.11 sec.). The fluorescence excitation and emission spectra are given in Figure 7.

2.08 Photolysis

There is no change in the thin layer chromatogram (single spot) or in the fluorescence or ultraviolet spectra after irradiation in methanol with a xenon lamp for 20 hours. It is therefore concluded that there is no significant photodegradation of griseofulvin under reasonable conditions of light exposure (7).

2.09 Optical Rotation

Griseofulvin exhibits the following optical rotation when dissolved in these solvents.

Table VII

Saturated chloroform	$[\alpha]_D^{17^O} = +370$	4
Acetone	$[\alpha]_D^{21^O} = +337$	5
Dimethylformamide	$[\alpha]_D^{26^O} = +358$	17
Dioxane	$[\alpha]_D^{26^O} = +302$	17

2.10 Melting Range

The Differential Thermal Gravimetry curve (Figure 9) demonstrates that the griseofulvin melting point takes place with decomposition. The melting range of griseofulvin from several sources is given in Table VIII.

Table VIII

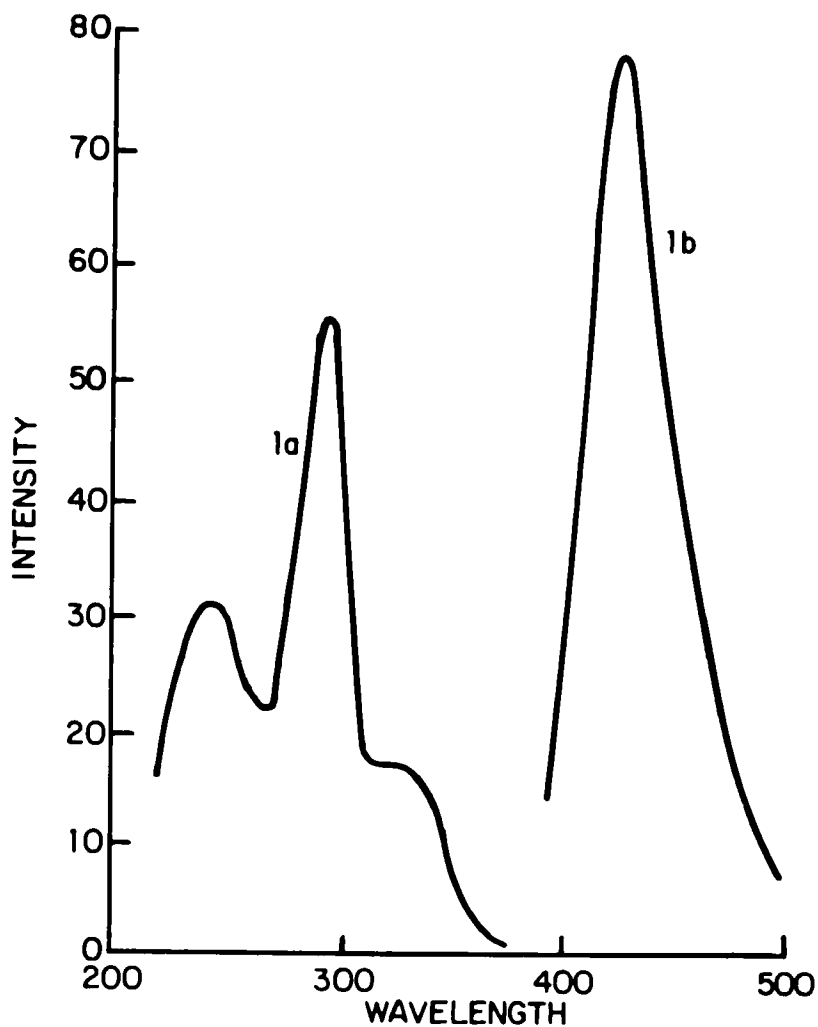| Melting Range ^{O}C	Reference
218 to 224	3
220	4
218	17
217-224	51

FIGURE 7:   Corrected Fluorescence and Emission Spectra of
            Griseofulvin: 1a, Excitation Spectrum with Emis-
            sion at 420 nm; 1b, Emission Spectrum with Excita-
            tion at 295 nm.

2.11 Differential Scanning Calorimetry
Figure 8 shows the DSC thermogram of griseofulvin obtained with a DuPont Model 900 Thermal Analyzer. A single sharp melting endotherm occurs for this substance with onset temperature at 216°C.

2.12 Thermogravimetry
Figure 9 shows the TG thermogram of griseofulvin obtained with a DuPont Model 950 Thermogravimetric Analyzer. The thermogram shows no weight loss from ambient to about 200°C followed by weight loss due to sublimation.

2.13 Electrophoretic Properties
Zeta potentials of dispersed griseofulvin have been studied both alone, and in the presence of surface-active agents, the latter at a controlled pH (8). A Mobility/Zeta Potential pH plot of = +25mV griseofulvin, shows a positive charge at pH 1.5 which rapidly decreases to zero at pH 2.4. There is then reversal of charge followed by an increase over the pH range 2.4 to 7.0. The zeta potential at the latter pH is -45 mV. The potential then stays constant over the pH range 7 to 10.

2.14 Solubility
The following data are given for the solubility of griseofulvin at 25°C; acetone 30 g/L, carbon tetrachloride 2 g/L, dichloroethane 80 g/L, dimethylacetamide, 40 g/L, dioxane 30 g/L, ethyl ether 0.7 g/L, heptane 0.3 g/L, methanol 0.4 g/L; mineral oil <0.1 g/L; propylene glycol 2 g/L; Span 80 0.2 g/L, Tween 80, 7 g/L water 0.2 g/L (17).

3. Production and Synthesis
Griseofulvin is biosynthetically manufactured by elaboration with Penicillium griseofulvum and related strains of Penicillia. The biosynthesis has been the subject of numerous chemical and biological studies, the latest of which is given by Harris, et. al. (9) Figure 10. Other proposed biosynthetic pathways are discussed.

Griseofulvin was first isolated in 1938 by Oxford (10) et. al., (1939); its total synthesis was accomplished in 1960 and following years in several laboratories (Brossi et al., 1960 (11) Grove, 1963; (12) Mutant strains of P. patulum are used for the commercial production of the antibiotic by fermentation (9).

4. Impurities
Some fermenter broth impurities have been listed by Holbrook, Bailey and Bailey (13) and repeated in a description

SAMPLE: UGFP-1961	SIZE 2.64 mg	ATM. N2 @ 100 ml/min	RUN NO. 78-DSC-062
	REF. Empty Pan	T | ΔT	DATE 3/16/78
	PROGRAM MODE Heat	SCALE 50 °C/m | 0.5 °C/m	OPERATOR CE
ORIGIN:	RATE 10 °C/m ,START RT °C	SETTING	ARN781535

EXO

ΔT

ENDO

0   50   100   150   200   250   300   350   400   450   500

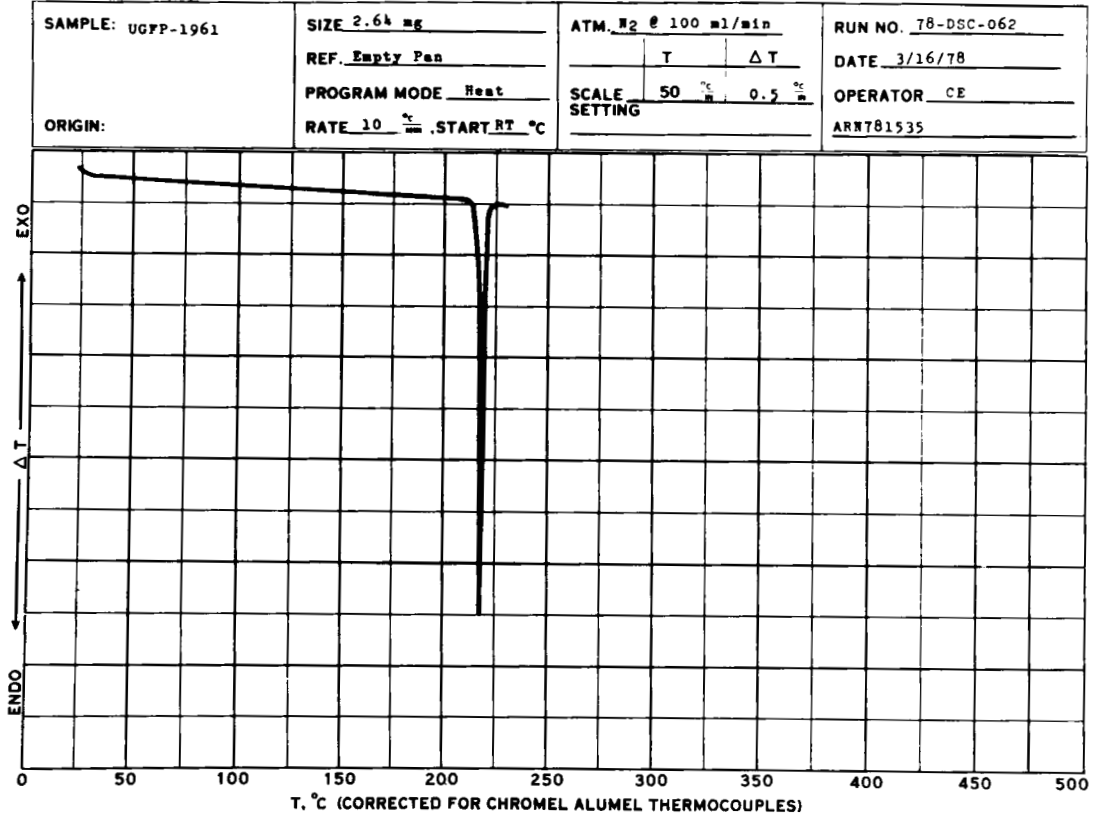T, °C (CORRECTED FOR CHROMEL ALUMEL THERMOCOUPLES)

FIGURE 8: Differential Scanning Calorimetry Curve of Griseofulvin
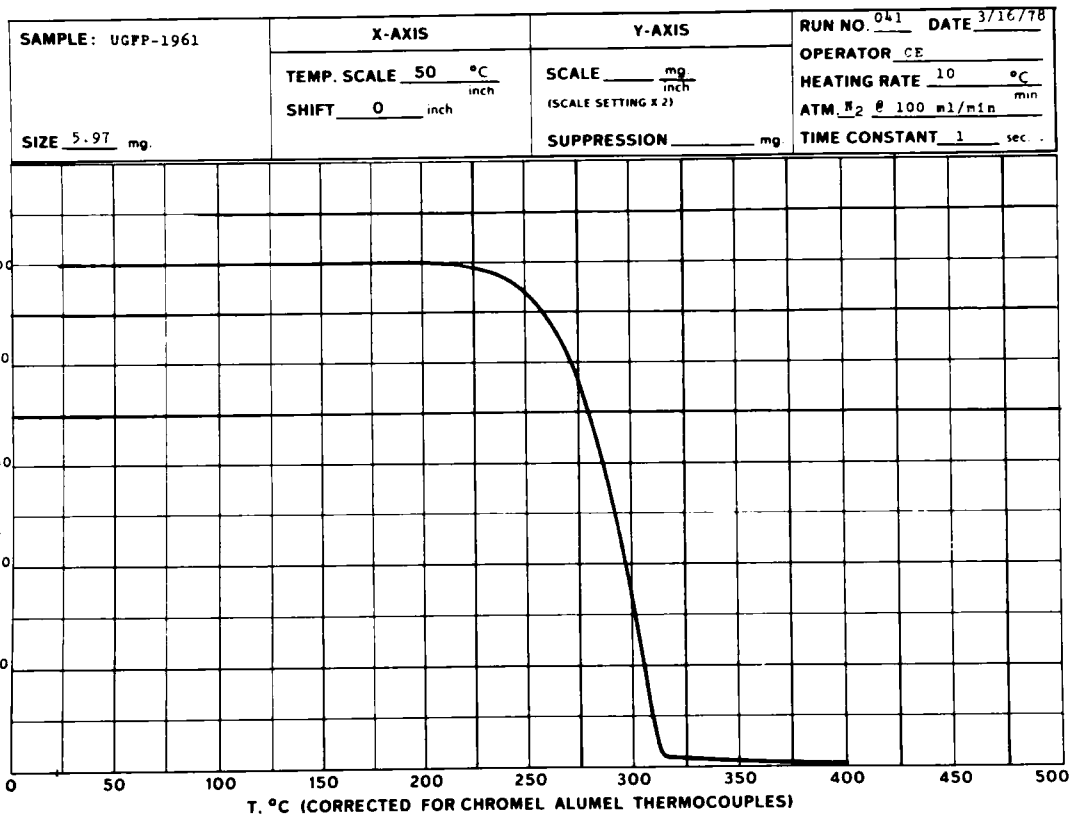
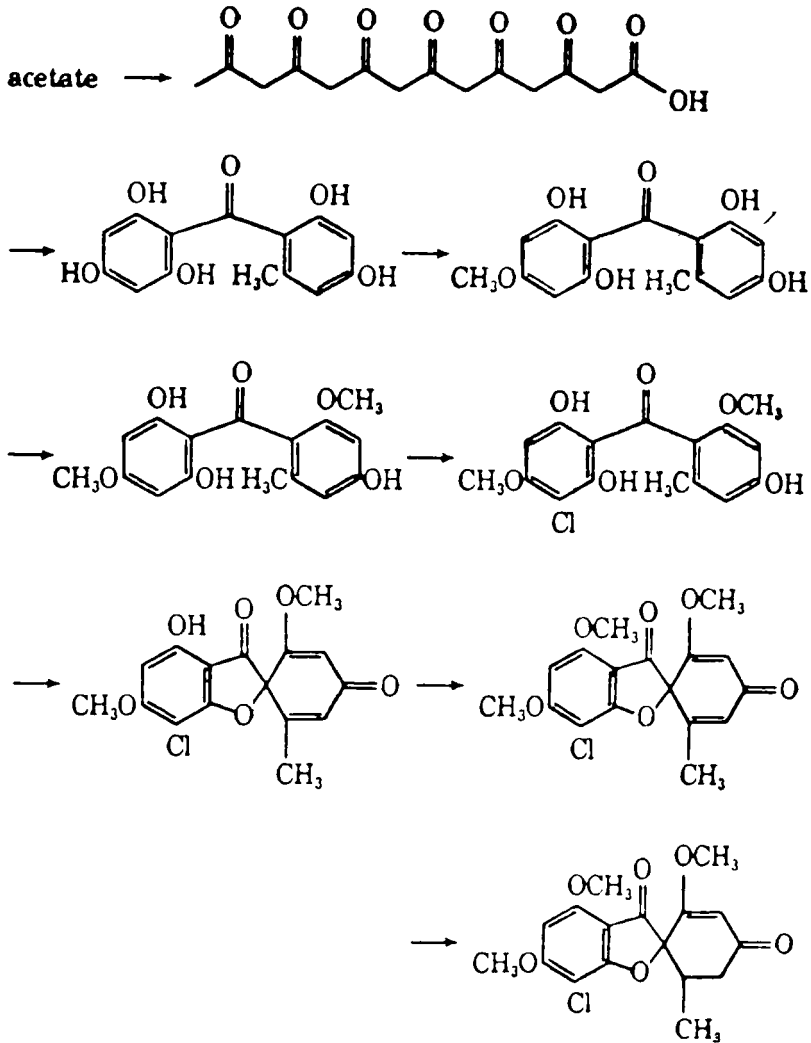FIGURE 9: Thermogravimetry Curve of Griseofulvin

FIGURE 10: A Biosynthetic Route for Griseofulvin.

of efficient liquid chromatographic separation systems by
Bailey and Brittain (14) and in a gas chromatographic separa-
tion system by Margosis (15). Structures are in Figure 11.

The common impurity found in commercial batches of griseoful-
vin is dechlorogriseofulvin (14,15) which appears to be in the
range of 0.5 to 3.5%.

5. Stability
    Griseofulvin is a stable drug substance. After 12 years
storage at room temperature no decomposition was detected by
differentiating LC methods (16). There is no photodegradation
under reasonable conditions of light exposure (7). Griseoful-
vin is converted to griseofulvic acid under acidic conditions.

6. Drug Metabolic Products
    The major human metabolite of griseofulvin is 6-demethyl-
griseofulvin and its glucuronide (17,18) which account for
about 65% of the intravenous dose (19) and 35 to 65% of the
oral dose (20,21). The 6-demethylgriseofulvin is also the
major metabolite in dogs (22) and rabbits (23) while both 4-
demethylgriseofulvin and 6-demethylgriseofulvin are major
metabolites in rats (24) and mice (25). These metabolites
can be determined by gas liquid chromatography via isopro-
poxyl derivatives (18) or trimethylsilyl ether derivatives
(26,27). The 6-demethylgriseofulvin has been measured in
urine by high performance liquid chromatography (28) and
ultraviolet spectrophotometry (19). Only trace amounts of
griseofulvin are found in the urine (28).

7. Methods of Analysis

    7.01   Identification
           A wine red color is produced when about 5 mg of
griseofulvin are dissolved in 1 ml of sulfuric acid with about
5 mg of powdered potassium dichromate (29).

    7.02   Elemental Analysis
           Analysis of griseofulvin, was determined for carbon,
hydrogen, and chlorine. The carbon, and hydrogen analysis was
performed on a Perkin Elmer Model 240 instrument. Analysis
for chlorine was performed by combustion of the sample and
coulometric titration using an American Instrument Co.
Chloride Titrator.

           The results from the elemental analysis are listed in
Table IX.
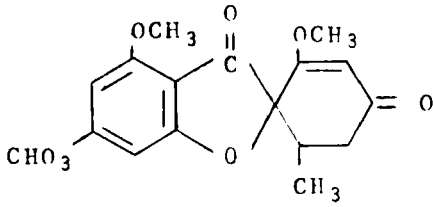
Dechlorgriseofulvin
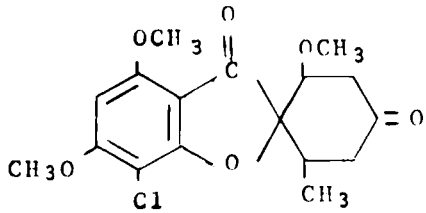
Dihydrogriseofulvin
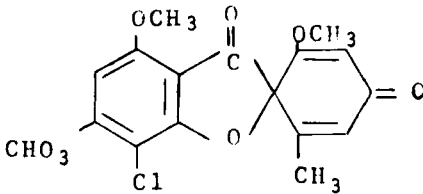
Dehydrogriseofulvin
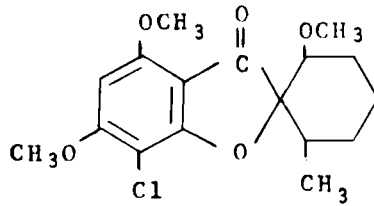
Tetrahydrogriseofulvin
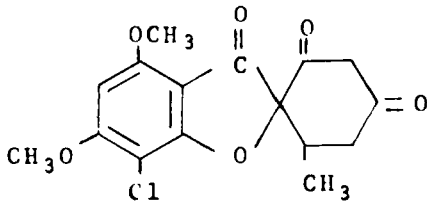
Griseofulvic Acid
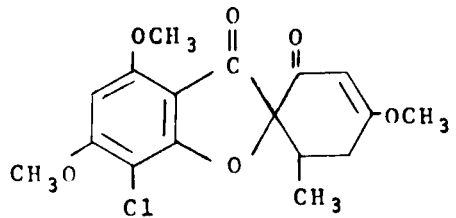
Isogriseofulvin

FIGURE 11: Impurities Found in the Fermenter Broth

Table IX

Elemental Analysis of Griseofulvin: Batch UGFP-1961

Element	% Theory	% Found
C	57.88	57.97
H	4.86	4.84
Cl	10.05	9.92

7.03 Spectrophotometric Analysis

Quantitative ultraviolet analysis of griseofulvin
may be performed by comparison to a Reference Standard. The
ultraviolet absorbance is described in Section 2.04 and
Figure 5.

7.04 Spectrofluorometric Analysis

Griseofulvin exhibits fluorescent properties which
have been utilized for highly sensitive analyses in blood and
serum (30-33) skin and sweat (34). Riegelman (32) has com-
bined TLC separation with a fluorimetric densitometer readout
to give a highly specific and sensitive griseofulvin deter-
mination in plasma. Other analyses are commonly performed in
either 1% aqueous ethanol (30), activation maxima 295 and 335
nm, fluorescence maxima at 450 nm or anhydrous methanol (31)
activation maxima unchanged at 295 and 335 nm, fluorescence
maxima 420 nm. Values are uncorrected. Other applications
to the analysis of bulk drugs, dosage forms or as a detection
method for high performance liquid chromatography are feasi-
ble.

7.05 Colorimetric Analysis

A colorimetric assay of griseofulvin, based on the
yellow-orange color (λmax=420 nm) which develops when griseo-
fulvin is heated with isonicotinic acid hydrazide in alkaline
medium has been described by Unterman (35) and the mechanism
investigated by Unterman and Duca (36).

7.06 Iodometric Analysis

Iodometric analysis has been applied to the deter-
mination of griseofulvin in stages of the manufacturing pro-
cess (37). The mycelium is extracted with chloroform and the
analysis carried out in alcoholic solution. The 0.01N iodine
solution is standardized with griseofulvic acid.

7.07 Turbidimetric Analysis

A turbidimetric assay for potency evaluation of
griseofulvin has been reported (38). The drug is dissolved in

ethylene glycol monomethyl ether (methyl cellosolve). Poly-
merization is induced with glycerol and guanosine-5'-triphos-
phate (GTP).

7.08   Polarographic Analysis

A study directed toward a comparison of the reduc-
tion potentials for griseofulvin homologs and analogs suggests
that polarography is a method of griseofulvin identification
(39). In ethanolic solution with 0.2 M KCl supporting elec-
trolyte, griseofulvin shows two polarographic waves with half-
wave potentials at about -1.58 V and -1.84 V. This system has
been applied with good results to the determination of finish-
ed products including tablets. The accuracy, precision and
selectivity of the method was compared with the iodometric
method (40). Isogriseofulvin and griseofulvic acid do not in-
terfere. (41,42)

7.09   Chromatographic Analyses

7.091   Partition Column Chromatography

Griseofulvin maybe separated from observed
structurally similar impurities in the fermenter broth by
means of partition column chromatography (13). A Celite col-
umn packing and solvent system consisting of methanol:water:
hexane:chloroform (8:2:9:1) was used. Tetrahydrogriseofulvin,
dihydrogriseofulvin, isogriseofulvin, dechlorogriseofulvin are
separated from griseofulvin. The structures for these com-
pounds have been given in Section 5.

7.092   Paper Chromatography

A paper chromatography system is given in
Table X. (43)

Table X

Solvent System	Paper	Detection	Reference
Benzene:Cyclohexane; Methanol:Water (5:5:6:4) Glacial acetic acid, 0.5%, was added to the organic phase of the solvent after equilibration.	Whatman No. 1	UV	43

7.093   Thin Layer Chromatography

Thin layer chromatographic systems are given
in Table XI. The detection method was U.V.

Table XI

Solvent System	Adsorbent	Tr	Reference
Methanol:n-butanol; 95% ethanol:conc. ammonium hydroxide (4:1:2:1, by vol.)	Silica Gel	0.64	44
Chloroform:isopropanol (3:1, by vol.)	Silica Gel	0.86	44
n-butanol:formic acid: water (77:10:13 by vol.)	Silica Gel	0.86	44
n-Butanol:95% ethanol conc. ammonium hydroxide:water (4:1:2:1 by vol.)	Silica Gel	0.64	44
Chloroform:acetone (93:7 by vol.)	Silica Gel	0.65	45
Chloroform:methanol (10:1 by vol.)	Silica Gel	0.82	46
Chloroform:acetic acid diethyl ether (17:1:3)	Silica Gel	-	47
Methanol:benzene (2:98 by vol.)	Silica Gel	0.50	48
Ethyl acetate	Silica Gel	0.50	17

7.094   Gas Chromatography

Successful griseofulvin analyses by gas chromatography are reported for simulated samples (49) fermentation extracts (45) and pharmaceutical bulk and dosage forms (50). Margosis (15) describes the application of gas chromatography to the purity determination of griseofulvin. Separation from the related compounds; dehydrogriseofulvin, isogriseofulvin and dechlorogriseofulvin was demonstrated. In a subsequent collaborative study (50) the accuracy and precision of the method was established. Although griseofulvin is thermally stable, the high GLC temperatures require precautions similar to those taken for steroids when preparing columns, column supports and associated equipment (45).

The specific and sensitive GLC determination
of griseofulvin in body fluids and tissues such as skin,
sweat, urine and plasma with electron capture detection has
been used by several investigators (34,51,52).

Gas chromatographic conditions are given in
Table XII.

Table XII

Column	Carrier Gas	Column Temp.	Internal Standard	Reference
150 cm x 4 mm I.D.; U-shaped stainless steel tubing, 1.5% QF-1 on Anakrom ABS	N_2	230°	diphenyl-phthalate	45,49
3 ft x 4 mm I.D.; coiled glass tubing, 3% OV-101 on Gas Chrom Q	He_2	245°	tetraphenyl-cyclopenta-dienone	15
5 ft x 4 mm I.D.; glass column, 3% OV-17 on Chromosorb W. or Gas Chrom Q.	10% Methane 90% Argon or N_2	270^oC	diazepam	34,50, 51,52
3 ft x 4 mm I.D.; glass coiled. % OV-17 on Gas Chrom. Q	He_2	225°	tetraphenyl-cyclopenta-dienone	15

7.095   High Performance Liquid Chromatography
Griseofulvin can be readily chromatographed on
either normal or reverse phase columns. Separation of de-
chlorogriseofulvin, the most common synthetic impurity is
accomplished on C/18, CN (17) or ETH columns. (15) Isogriseo-
fulvin, is separated on either CN (17) or ETH columns (15).

Liquid chromatography conditions are given in
Table XIII.

Table XIII

Column	Type	Mobile Phase	Internal Standard	Reference
μBondapak C/18 (octyl-decyl chemically bonded to silica)	Reverse phase	Methanol: water 3:2	n-butyl p-hydroxy benzoate	16
μBondapak C/18 (octyl-decyl chemically bonded to silica)	"	45% aceto-nitrile in 45mM KH_2PO_4 pH = 3.0	diazepan	53
Zorbax CN (cyano-propyl chemically bonded to silica)	"	Methanol: water 3:2	m-phenyl phenol	16
Permaphase ETH (C-7 ether chemically bonded to pellicular 30-50 mesh glass packing)	Normal phase	5% chloro-form in hexane		14

7.10 Biological Methods or Analysis

Microbiological procedures have been developed for assay of griseofulvin and applied to the analysis of bulk drugs and dosage forms (1). The cylinder plate agar diffusion method is the official microbiological method of determination (55). Microsporum gypseum (ATCC 14683) is the test organism.

8. Identification and Determination in Body Fluids and Tissue

Griseofulvin has usually been determined in body fluids and tissues by spectrofluorimetric (30-34) or gas chromatographic methods (45,46,48). More recently griseofulvin has been determined in plasma by high performance liquid chromatography (53,54).

9. Analysis of Dosage forms

Usual dosage forms of griseofulvin are capsules, tablets and boluses. These may be prepared for analysis by simple liquid solid extraction of drug substance. Margosis (15,50) used chloroform as an extracting solvent with gentle heat. A compendia procedure describes the extraction of griseofulvin from tablets with boiling alcohol. The analysis for extracted drug substance has been performed by several methods. Most common is a simple ultraviolet analysis (2,3,55). Polarography utilizing the system given in Section 7.08 has been used (39). Thin-layer, gas and liquid chromagraphy may also be

used utilizing systems described in Sections 7.093, 7.094, and
7.095 respectively. These latter methods are valuable because
of their specificity.

In the United States, griseofulvin drug substance and
dosage forms must conform to the regulations of the Federal
Food and Drug Administration concerning antibiotic drugs (55,
56). Microbiological assay results obtained by analytical
methods described in these compendia are conclusive.

10. Acknowledgements

References

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HALCINONIDE

Joel Kirschbaum

1 History, Description, Precautions and Synthesis

1.1 History

Halcinonide is a topical antiinflammatory agent.[1,2]
Introduction of a 9α-fluoro group[3] to the hydrocortisone mole-
cule resulted in an eight-fold increase in antiinflammatory
activity. Such other groups as 16α-hydroxy, 16α-methyl, 16β-
methyl, 16,17-acetonide and 6α-methyl moieties have been found
to diminish or eliminate mineralocorticoid activity resulting
from the 9α-fluoro substituent, while retaining the enhanced
antiinflammatory activity of the halo derivative[4]. Halcino-
nide lipophilicity is increased by masking the 16,17-hydroxyl
groups as the acetonide, which increases the availability of
the steroid at the site of action in the skin. The 21-chloro
group also increased antiinflammatory properties. Halcinonide
has systemic[3] as well as topical activity.

1.2 Names, Formula and Molecular Weight

Halcinonide is the United States adopted name[5] (USAN).
The preferred chemical names[6] are 21-chloro-9-fluoro-11β-
hydroxy-16α, 17-[(1-methylethylidene)bis(oxy)]pregn-4-ene-3,
20-dione, and 21-chloro-9α-fluoro-11β-16α,17-trihydroxypregn-4
-ene-3,20-dione cyclic 16,17-acetal with acetone. Other chemi-
cal names are 21-chloro-9-fluoro-11β-hydroxy-16α,17α-isopropyl-
idenedioxy-4-pregnene-3,20-dione, 9α-fluoro-21-chloro-11β, 16α,
17α-trihydroxypregn-4-ene-3, 20-dione 16,17 acetonide and 21-
chloro-9-fluoro-11β,16α,17-trihydroxypregn-4-ene-3,20-dione
cyclic 16,17-acetal with acetone.

$C_{24}H_{32}ClFO_5$
454.97 Daltons
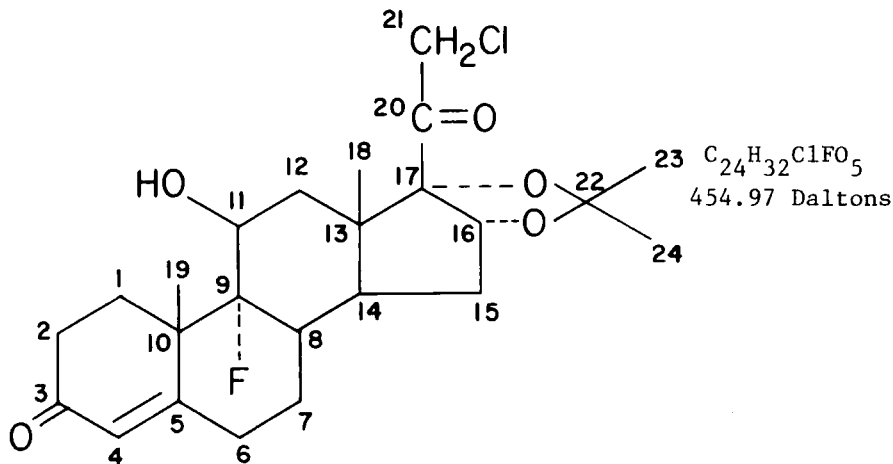
Halcinonide has also been called Halog and Squibb SQ 18,566.
The Chemical Abstracts systematic number is CAS-3093-35-4.

1.3 Appearance, Color and Odor

Halcinonide is a white or practically white, odor-less powder consisting of free flowing crystals.

1.4 Precautions

Since halcinonide is a corticosteroid, large doses of unformulated steroid are needed systemically before the un-wanted side effects appear.[7] Halcinonide is usually formula-ted at concentrations of 0.1%, or less. Normal handling pre-cautions are adequate.

1.5 Synthesis

The synthesis[8,9] of halcinonide is summarized in Figure 1, starting with 16α-hydroxy-9α-fluorohydrocortisone (Δ^4-pregnene-9α-fluoro-11β,16α,17α,21-tetrol-3,20-dione; dihy-drotriamcinolone, I), which is available commercially.[10-13] This tetrahydroxy steroid is slurried in acetone, and then 70% perchloric acid is added slowly. The acetonide, II (9α-fluoro-11β,16α,17,21-tetrahydroxypregn-4-ene-3,20-dione, cyclic 16,17-acetal with acetone; dihydrotriamcinolone-acetonide) precipi-tates spontaneously from solution. Mesyl chloride is added to the acetonide in pyridine to give the 21-mesylate derivative (dihydrotriamcinolone acetonide-21-mesylate, III). Compound III is dissolved in dimethylformamide, lithium chloride is ad-ded and the mixture is refluxed to produce halcinonide (IV), which is recrystallized from a solution of n-propanol in water.

2. Physical Properties

2.01 Single Crystal X-Ray Diffraction

The three dimensional structure was obtained[14] by means of single crystal X-ray diffraction. CuKα radiation, a graphite monochromator, and a photomultiplier tube were used to collect 1825 total reflections on an automated diffracto-meter. Of these, 1162 were used for the analysis. Figure 2 shows a computer generated drawing of halcinonide. The posi-tion of the chlorine atom was not clear from the Patterson map, but the direct method program "MULTAN" gave its position. Least squares refinement of coordinates together with aniso-tropic temperature factors, in the final stages, gave an R factor of 0.11.

Figure 1. Synthesis of Halcinonide
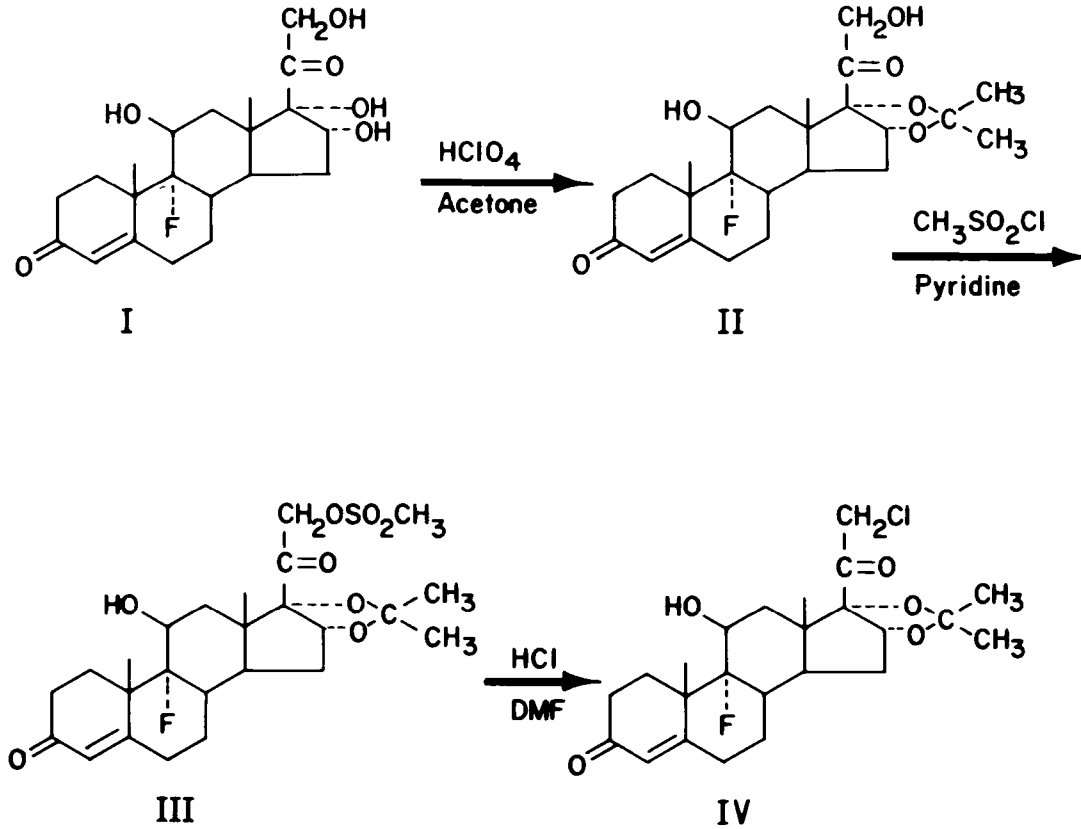
Figure 2.   Structure of Halcinonide, from X-ray Diffraction Data.
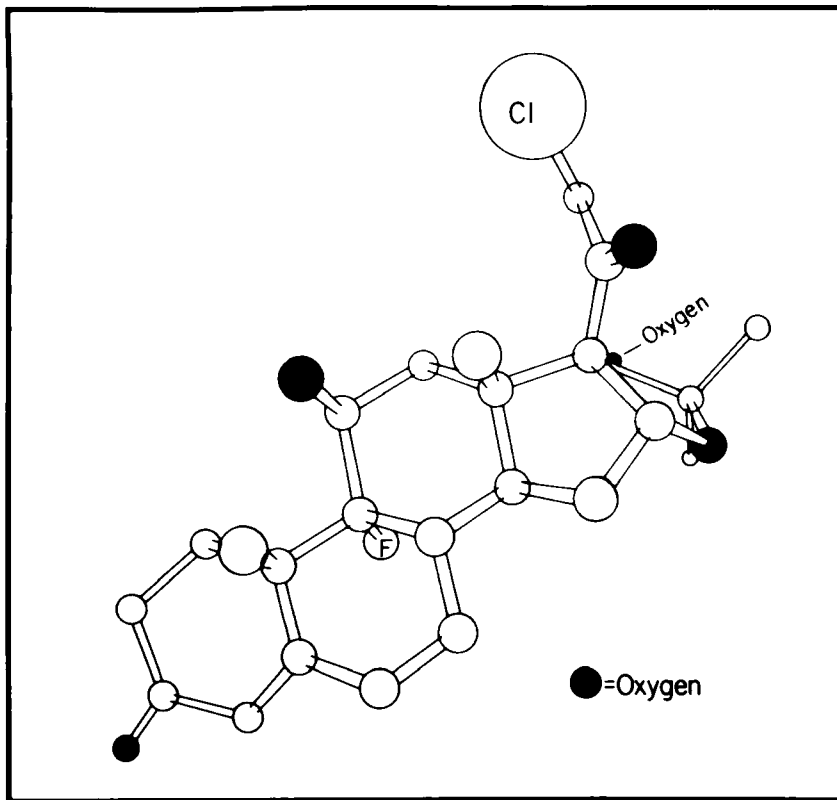
18,566 Computer Generated Drawing

2.02 Mass Spectrometry

Figure 3 is the plotted low resolution mass spectrum of halcinonide found using an AEI Scientific Apparatus Ltd, Model 902 mass spectrometer. Spectra were collected on frequency modulated analog tape and processed on a Digital Equipment Corp., PDP-11.

The table[15] below summarizes high resolution data from m/z 39 to the prominent molecular ion at 454.1922, which corresponds to $C_{24}H_{32}O_5FCl$. Each fragment ion containing chlorine appears as a doublet peak because chlorine is a mixture of two isotopes (^{35}Cl and ^{37}Cl). These peaks are two mass units apart with an intensity ratio of 3 to 1.

Fragmentation proceeds along several pathways, as shown below.
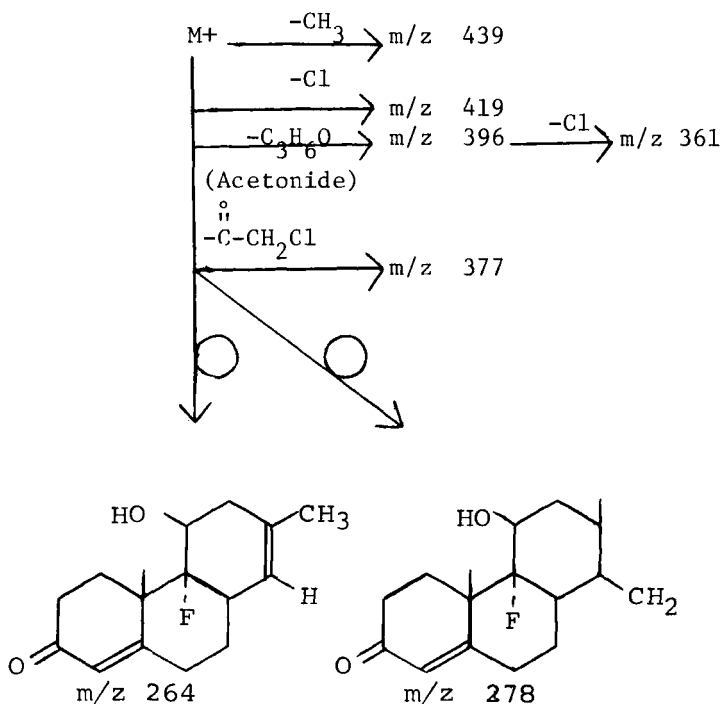
These structures do not necessarily represent the actual ionic structure but only the origin of the fragment.

The weak ions at m/z 121, 122 and 123 (C_8H_9O, $C_8H_{10}O$ and $C_8H_{11}O$) are characteristic of Δ^4-3-one steroids.

Figure 3

Low Resolution Mass Spectrum of Halcinonide. See text for details.
5433 SQ18,566 BA NN009NA 175 DEG
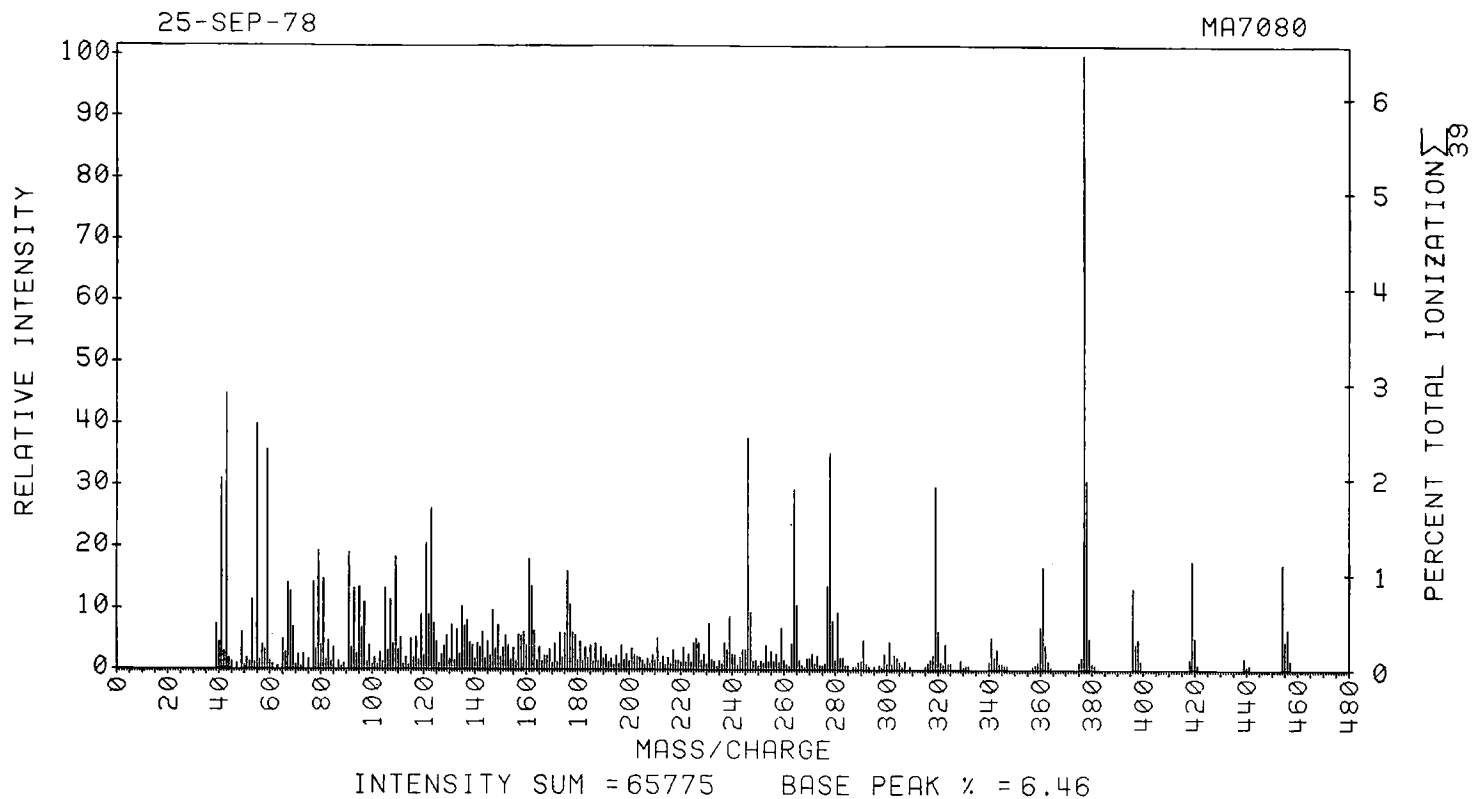
High-Resolution Mass[a] Spectrum of Halcinonide

Found Mass	Calc. Mass	Unsat.[b]	O/E[c]	C	H	O	F	Cl
454.1925	454.1922	8.0	O	24	32	5	1	1
439.1680	439.1687	8.5	E	23	29	5	1	1
419.2252	419.2233	8.5	E	24	32	5	1	0
396.1501	396.1503	8.0	O	21	26	4	1	1
377.2147	377.2128	7.5	E	22	30	4	1	0
361.1838	361.1815	8.5	E	21	26	4	1	0
319.1665	319.1709	7.5	E	19	24	3	1	0
278.1695	278.1682	6.0	O	17	23	2	1	0
264.1524	264.1525	6.0	O	16	21	2	1	0
246.1422	246.1420	7.0	O	16	19	1	1	0
135.0808	135.0810	4.5	E	9	11	1	0	0
123.0769	123.0810	3.5	E	8	11	1	0	0
122.0729	122.0732	4.0	O	8	10	1	0	0
121.0683	121.0653	4.5	E	8	9	1	0	0
76.9790	76.9794	1.5	E	2	2	1	0	1
43.0184	43.0184	1.5	E	2	3	1	0	0

[a] Only those peaks considered to be significant to the discussion are listed. A complete element map can be obtained on request.

[b] Number of double bonds and rings.

[c] O-odd electron ion; E-even electron ion.

2.03 <u>Nuclear Magnetic Resonance Spectrometry</u> (NMR)

2.031 ^{1}H-NMR

        Figure 4 is the 100 MHz NMR Spectrum of
halcinonide in deuterochloroform containing tetramethyl-
silane as internal reference at 0 Hz. The instrument used
is a Varian Associates, Inc., Model XL-100 NMR spectrometer.
Below is an interpretation of the various resonances.[16]

^{1}H-NMR Assignments

Proton at Carbon	Chemical Shift	Peak Appearance S = singlet D = doublet B = broad
C-4	5.78	S
C-11	4.58	B
C-16	5.05	D $J_{15,16}$= 4.0 Hz
C-18	0.87	S
C-19	1.51	S
C-21	4.19 4.57	AB quartet
β-Acetonide, methyl	1.15	S
α-Acetonide, methyl	1.44	S

    These data agree with the published results of the 21-
hydroxy-Δ^1 analog, triamcinolone acetonide.[17]

Figure 4

^{1}H-Nuclear Magnetic Resonance Spectrum of Halcinonide
See text for details.
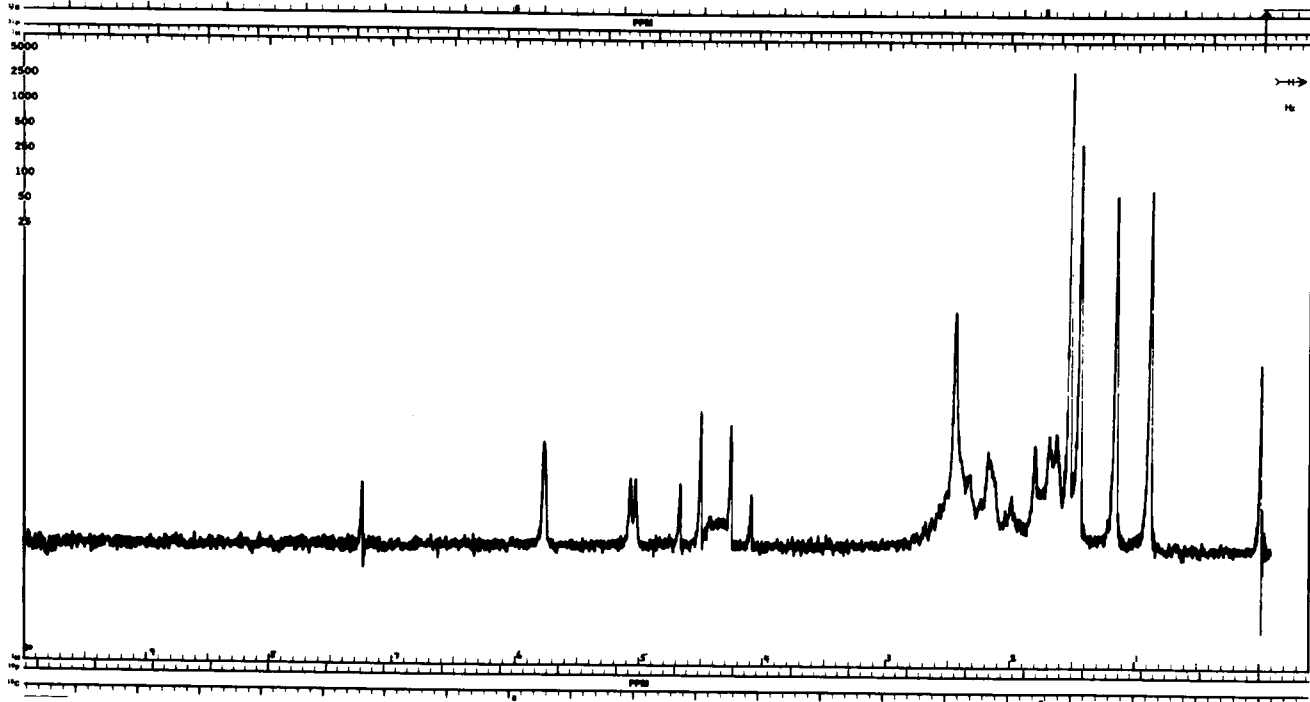
2.032 ^{13}C-NMR

The ^{13}C-NMR spectrum of halcinonide, Figure 5, was obtained[18] using a Jeol FX-60Q NMR spectrometer operating in the Fourier transform mode at 15 MHz.   Numbers on the various peaks refer to the assignments in the table below.   These assignments were made by comparison of chemical shifts and ^{13}C-^{19}F coupling constants to those assigned to such related steroids as 9α-fluorocortisol (9α-fluoro-11β,17,21-trihydroxy-pregn-4-ene-3,20-dione).

Four pairs of closely related peaks show chemical shift differences small enough to lead to possible reversals of their tentative assignments.   These pairs are the peaks assigned to the acetonide methyl groups (δ = 28.6 and δ = 25.1 ppm), the two carbonyl peaks (δ = 202.1 and 199.1 ppm), the methylene carbons assigned to C-1 and C-7 (δ = 26.2 and 28.5 ppm), and the methylene carbons assigned to C-2 and C-15 (δ = 33.2 and 33.7 ppm).   These assignments must be verified by additional experimentation.

^{13}C-NMR Assignments

Carbon	Chemical Shift δ (ppm)[a]	C-F Coupling Constant (Hz)
1	26.2 (28.5)	3
2	33.7 (33.2)	
3	199.1 (202.1)	
4	124.7	
5	169.0	
6	30.7	
7	28.5 (26.2)	3
8	33.1	19
9	99.0	175
10	43.7	21
11	70.2	
12	37.3	
13	44.8	
14	43.4	2
15	33.2 (33.7)	
16	81.9	
17	98.1	
18	17.0	
19	21.9	6
20	202.1 (199.1)	
21	47.1	
22	111.5	
23	28.6 (25.1)	
24	25.1 (28.6)	

[a]Referenced from center peak of deuterochloroform = 77.0 ppm from tetramethyl silane.   Numbers in parentheses refer to alternative assignments (see text for details).

Figure 5

[13]C-Nuclear Magnetic Resonance Spectrum of Halcinonide
See text for details.
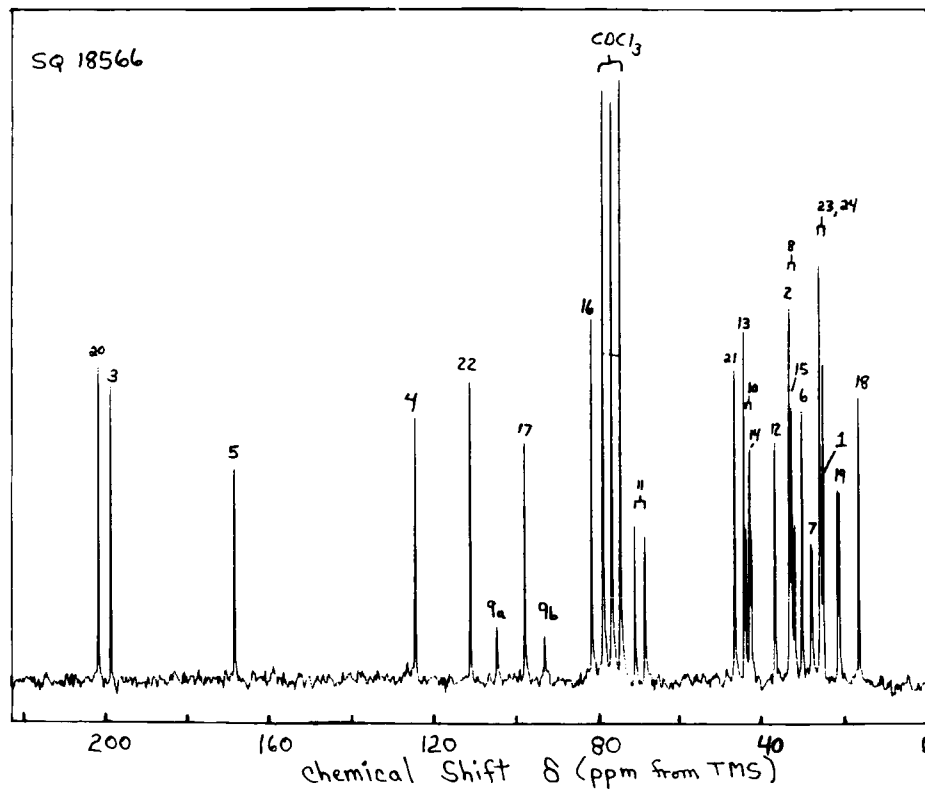
2.04 Infrared Spectrometry

Figure 6 shows the infrared spectrum of halcinonide run as a potassium bromide pellet, using a Perkin-Elmer Model 621 infrared spectrometer. Below are the interpretations of various absorbances.[19]

Frequency (cm^{-1})	Interpretation
3450	-OH Stretch
2950	-OH Stretch
1730	C_{20} Keto
1660	C_3 Keto
1620	Δ^4 C=C

2.05 X-ray Powder Diffraction

Figure 7 is the powder X-ray diffraction pattern of halcinonide as obtained on a Philips powder diffraction unit emitting Cu$K\alpha$ radiation at 1.54Å. Using a scintillation counter detector, the sample was scanned and recorded from approximately 2 to 40 degrees (2Θ). The table below is the sorted data.[20]

2Θ (Degrees)	'd'(Angstrom)[1]	Relative Area[2]
14.80	5.99	1.000
12.53	7.06	0.866
18.49	4.80	0.738
17.94	4.94	0.526
19.82	4.48	0.422
29.31	3.05	0.266
18.10	4.90	0.246
11.67	7.58	0.225
32.14	2.78	0.195
39.59	2.28	0.171
25.86	3.45	0.165
21.47	4.14	0.142
26.80	3.33	0.135
25.24	3.53	0.135
15.67	5.66	0.131
40.37	2.23	0.124
22.33	3.98	0.117
33.08	2.71	0.114
25.47	3.50	0.053
34.02	2.64	0.052

[1] Interplanar distance

[2] Relative area, or intensity, is based on highest intensity of 1.00 using Cu$K\alpha$ radiation.

Figure 6
Infrared Spectrum of Halcinonide, Potassium Bromide Pellet.
See text for details.
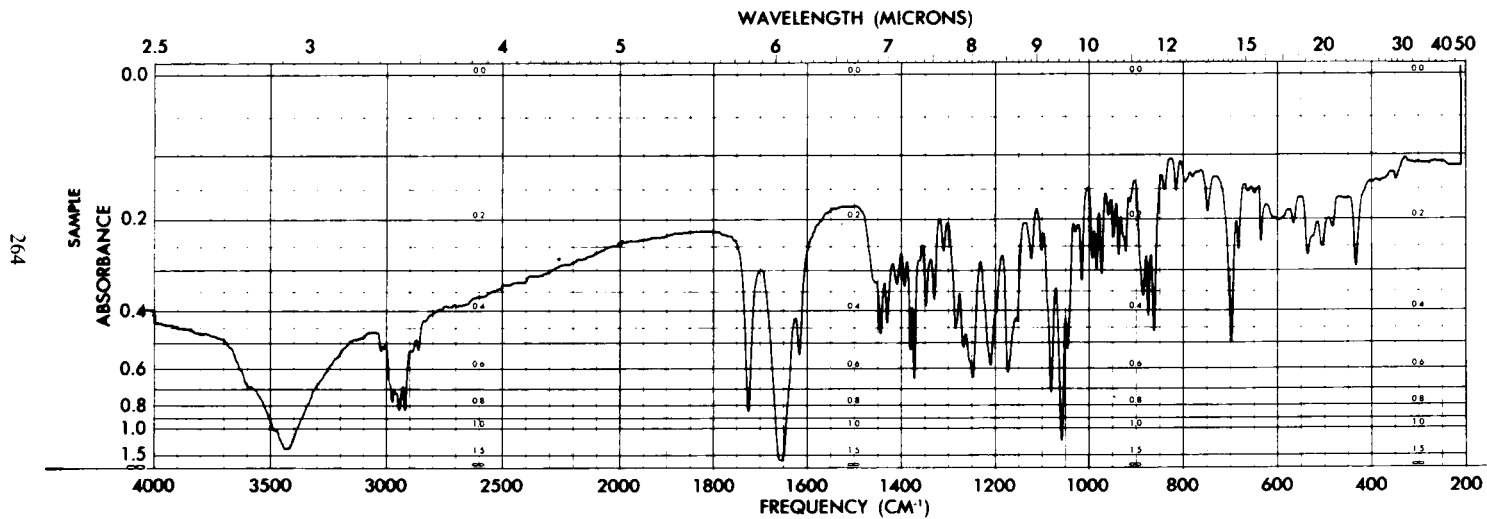
Figure 7

Powder X-ray Diffraction Pattern of Halcinonide. See text for details
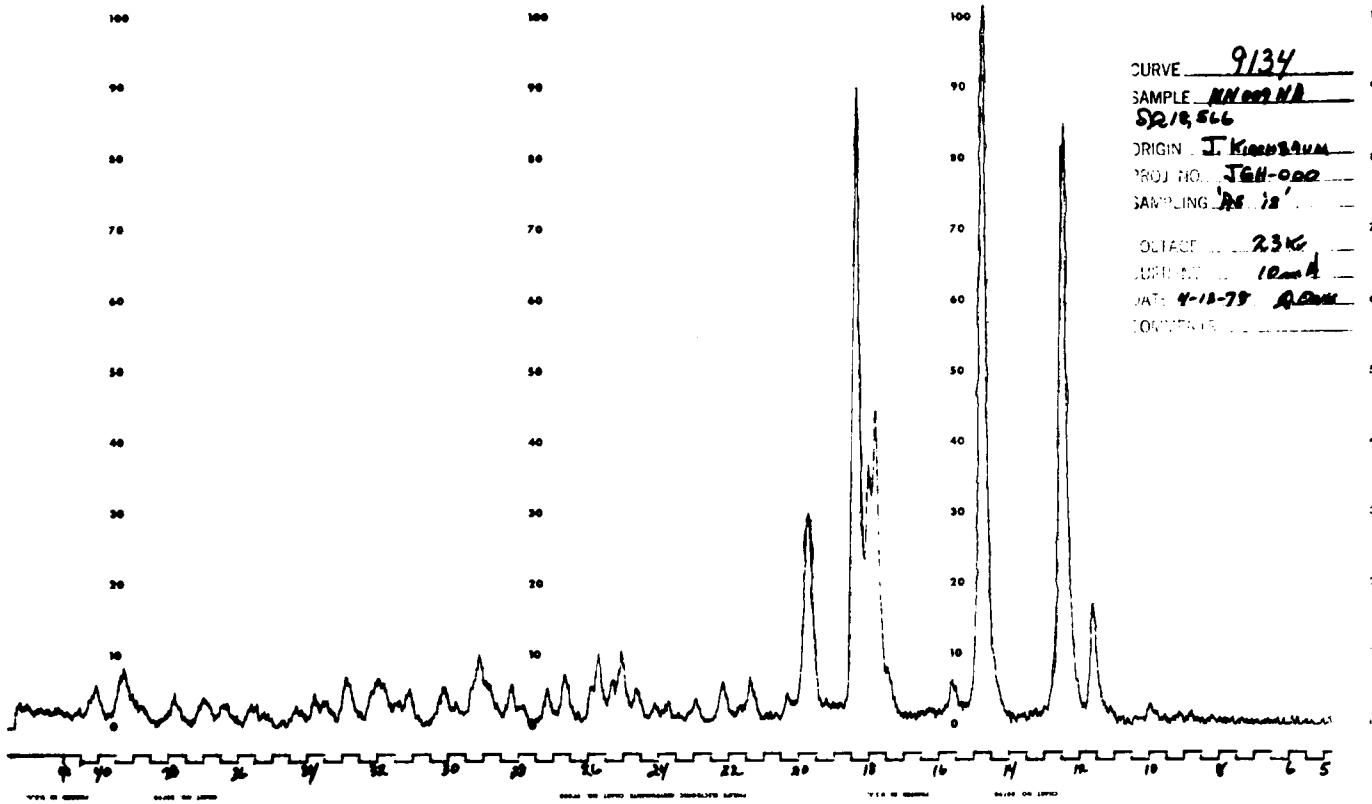
2.06   Ultraviolet Spectrometry

Ultraviolet spectra[21] of halcinonide were recorded on a Beckman Acta CIII spectrophotometer. The spectra in methanol, 0.1M methanolic hydrochloric acid and 0.1M methanolic sodium hydroxide show peak maxima at 238 nm, and remain unchanged after 24 hours. Absorbances are tabulated below using traditional $E_{1cm}^{1\%}$ notation.

Solvent	$E_{1cm}^{1\%}$		
	Initial	3 Hours	24 Hours
Methanol	379	374	373
0.1M Methanolic Hydrochloric Acid	370	372	370
0.1M Methanolic Sodium Hydroxide	380	382	380

The spectrum in 95% ethanol also has a peak maximum at 238 nm and appears stable for at least 3 hours. In acetonitrile and ethyl ether the maximum is shifted to 234 nm and 229 nm, respectively.

2.07   Optical Rotation

The specific rotations in alcohol[22] were determined[23] to be as follows,

Wavelength (nm)	Specific Rotation
589	+156
578	+164
546	+188
436	+348
305	+436

2.08   Fluorescence Spectrometry

Using a Perkin-Elmer Model 204 fluorescence spectrophotometer, no fluorescence[24] was found at either 1 or 1000 µg halcinonide per mL of methanol. No fluorescence was induced in 0.1M hydrochloric acid or 0.1M sodium hydroxide.

2.09 Melting Range

Following the USP procedure[25] for class 1A
compounds, the melting range·of halcinonide is 270–272°
(reference 21).   This is in excellent agreement with the re-
sults of differential thermal analysis, below, as well as
hot stage microscopy, section 2.12.

2.10 Differential Thermal Analysis

A duPont Model 900 Differential Thermal Ana-
lyzer shows halcinonide to have one endotherm[26] at 269°.
Decomposition on melting precludes differential scanning
colorimetry studies for purity.

2.11 Thermal Gravimetric Analysis

Using a Perkin-Elmer Model TGS-2 Thermogravi-
metric Analyzer, halcinonide was found[26] to lose 0.3% total
volatile material at 70°.   No further loss was found up to
150°.   The heating rate was 20°/minute under a nitrogen
atmosphere.

2.12 Microscopy and Crystal Type

Microscopically, the crystal type depends on
the method of preparation of halcinonide.   Slabs, either 2
to 4, 5 to 10, or 10 to 15 microns square, were found in
three lots of halcinonide, intermingled with needle-like
(acicular) crystals 5 to 10 microns long.[27]

Single crystal x-ray diffraction studies[14]
showed that the crystals of halcinonide recrystallized from
n-propyl alcohol-water azeotrope (79:22) are orthorhombic and
belong to the space group $P2_12_12_1$, with unit cell constants
of a = 10.007 Å, b = 11.875 Å and c = 19.460 Å.   Density is
1.330 gm/cm^3, as measured by flotation in a hexane-carbon
tetrachloride gradient.   The molecular weight calculated
from the unit cell volume and density is 461 daltons (theore-
tical is 455 daltons).

Hot stage microscopy[26] was performed using a Mettler
FP52 temperature controller at a rate of 3°/minute.   Melting
began at 268.2°, and by 271.4° all crystals were melted.   The
yellow melt slowly turned orange-brown.   Cooling showed no
recrystallization at ambient temperature.

2.13 Particle Size

By light scattering using a Royco instrument,
all particles are below 20 microns.[27] Coulter Counter an-
alysis of ground halcinonide suspended in a sodium chloride
solution shows 100% to be <10.2μ (μ = micron), 98.2% <6.4μ,
95.3% <5.1μ, 52.0% <2.6μ and 19.9% <1.6μ.

2.14 Surface Area

As measured by gas adsorption,[27] the surface
areas of three lots of ground halcinonide are 2.61, 3.85 and
4.08 m^2/g.

2.15 Polymorphism

There is no evidence for polymorphism from
infrared spectroscopy and differential thermal analysis, and
only inconclusive data from x-ray diffraction and microscopy
studies.

2.16 Hydration

The crystals of halcinonide are not solvated
with water, based on a total volatile content of 0.3% obtain-
ed by thermal gravimetric analysis, a correct elemental
analysis, and a loss-on-drying value of 0.6% (*cf.* section
4.1, Elemental and Inorganic Analyses).

3. Solution Properties

3.1 Intrinsic Dissolution Rate

The intrinsic dissolution rate was determined
after compressing powder under 2000 p.s.i.g. pressure using
3/8" diameter disc-shaped dies. In one liter of 0.1M hydro-
chloric acid at 37°, agitated at a rate of 50 r.p.m., the
intrinsic dissolution rate of halcinonide is 8.33 x 10^{-3} mg
min.$^{-1}$ cm^{-2} computed from data obtained by ultraviolet
spectrometry.[28]

3.2 Solubilities in Aqueous and Nonaqueous Solvents

Solubilities of halcinonide were determined
in various solvents.[29] Results are reported using the U.S.P.
definitions.[30]

Solvent	Solubility
Water	Insoluble
Hydrochloric acid, 0.1M	Insoluble
Sodium hydroxide, 0.1M	Insoluble
Acetone	Soluble
Acetonitrile	Sparingly soluble
Acetonitrile-water	Slightly soluble
Benzene	Slightly soluble
Chloroform	Freely soluble
Castor oil	Slightly soluble
Dimethylsulfoxide	Freely soluble
Ethanol	Slightly soluble
Ethyl ether	Slightly soluble
Glyceryl monooleate	Slightly soluble
Hexanes	Insoluble
Isopropyl myristate	Slightly soluble
Methanol	Slightly soluble
n-Octanol	Slightly soluble
Polyethylene glycol 200	Slightly soluble
Polyethylene glycol 400	Slightly soluble
Polypropylene glycol	Slightly soluble
Propylene glycol	Very slightly soluble

3.3 Partition Coefficients

Halcinonide was partitioned[31] between hexanes and methanol, and between hexanes and aqueous acetonitrile at apparent pH values of 2,4,6(unadjusted), and 10. After one hour of mixing, the steroid content was determined by ultra-violet spectrometry of both phases. In all cases, absorbance at the peak maximum of 239 nm was detected only in the acetonitrile-water or methanol layer. The aqueous acetoni-trile (pH 6) result was verified[32] using ^{14}C-halcinonide labeled at the 2- carbon of the acetonide moiety. Thus, the halcinonide is completely retained in either the acetonitrile-water or methanol layers, indicating the utility of these solvent systems for extracting the steroid from formulations.

Another study[33] involved isopropyl myristate and aqueous propylene glycol. After equilibration for two weeks at 37°, the layers were separated[34] and the aqueous glycol assayed for steroid using high-pressure liquid chromato-graphy (*cf.* section 4.31). The isopropyl myristate/aqueous propylene glycol partition coefficients are as follows: propylene glycol-water (4:1); 144; propylene glycol-water (2:3), 44.4; propylene glycol-water (3:2), 8.14, and propy-lene glycol-water (9:1), 0.82.

4. Methods of Analysis

4.1 Elemental and Inorganic Analyses

The elemental analysis[35] of halcinonide is carbon 63.38% (63.35%, theoretical); hydrogen 7.39% (7.10%, theore-tical); chlorine, 7.89 (7.79%, theoretical), and fluorine, 4.30% (4.17%, theoretical).

Emission spectrochemical analysis for metals was performed using a Spec Industries carbon arc A.C. unit with a Bausch and Lomb dual grating spectrograph. Data were recorded on glass plates and interpreted by means of a microphotometer. Halcinonide contained trace amounts ($\sim$ 60 μg/g total) of the following metallic impurities[36]; iron, manganese, copper, nickel, lead, zinc, aluminum, sodium, calcium, and magnesium. Residue-on-ignition[37] of the same lot[38] is less than 0.1%. Heavy metals content (reference 39, method II) is less than 0.003%.[38] After 3 hours at 100° in vacuum, the loss-on-drying[39] value[38] is 0.6%.

Four lots of halcinonide were analyzed for resi-dual solvents by dissolving portions in pyridine. After retention on a precolumn, water content was determined by

vapor phase (gas) chromatography using an authentic stan-
dard before and after each injection. Water contents of
0.6%, or less, were found by comparison with authentic
standards. n-Propanol has occasionally been found to be
present.

4.2   Identification, Ultraviolet and Colorimetric Analyses

Proposed compendial identification tests for the
United States Pharmacopeia involve comparing either the in-
frared or ultraviolet absorption spectrum of sample halcin-
nonide with that of an authentic sample[41]. To this author,
a chromatographic method is superior since elution time
usually depends on much of the molecule interacting with the
solid and mobile phases via weak bonding forces. Ultravio-
let absorption depends principally on the 3-one-4-ene, A and
B ring region being intact.

Halcinonide has been quantitated in various formu-
lations or as bulk powder by a differential ultraviolet,
borohydride reduction assay.[42] This differential assay in-
volves measuring the ultraviolet absorbance of an aliquot of
methanolic steroid solution containing sodium borohydride
decomposed prior to the addition of steroid. Its absorbance
is determined against a methanolic reference solution of
steroid reduced by sodium borohydride to destroy the 3-one-
4-ene chromophore. The utility of this procedure is that
many interferences from excipients and other, unconjugated,
steroids can be eliminated in the assay of a formulation.

The addition of halcinonide to various colorime-
tric reagents gives results typical of steroids with its
substituents.[43-45] As tested in our laboratory,[46] halcino-
nide reacts with acidic ethanolic 4-nitrophenylhydrazine[47],
after heating, cooling and the addition of sodium hydroxide,
to give a brilliant purple "plum" color. With methanolic
isoniazid[48], halcinonide gives a bright yellow color, with
no noticeable fading after two hours. Halcinonide added to
4-aminoantipyrine[49] in methanolic hydrochloric acid gives a
pale green color. Halcinonide added to ethanolic tetra-
methylammonium hydroxide[50], and heated, gives a cloudy amber
color. This can be the basis of a quantitative assay.[51] If
added to ethanolic tetramethylammonium hydroxide[52] and
picric acid, an orange-red (tea colored) solution results
with halcinonide. In concentrated sulfuric acid,[53] halcino-
nide gives a deep yellow color. Adding water slowly causes
a violet color to appear at the interface. Halcinonide
gives a royal purple color with blue tetrazolium[54] and a
yellow-brown color with aluminum chloride in nitromethane.

4.3   Chromatographic Analyses

4.31   High-Pressure Liquid Chromatography (HPLC)

Reverse phase high-pressure liquid chromato-
graphy is used to separate and quantitate bulk and formula-
ted halcinonide.[55]   Commercially available, prepacked octa-
decylsilane columns, 10 µm in particle size, *United States
Pharmacopeia* designation L-1 (such as Partisil, MicroPak or
µBondapak), are used. The mobile phase is aqueous aceto-
nitrile (1:1 to 1:3) with a flow rate of 0.3 to 1.0 mL/min.
Detection is at 254 nm. Figure 7 shows the elution of
halcinonide and progesterone internal standard. Using a
precision loop injector, repetitive injections gave a rela-
tive standard deviation of 0.6%. Without the progesterone
internal standard, the relative standard deviation is 0.8%.
Formulated halcinonide, in concentrations of 1 to 0.25 mg
steroid/g, is either diluted with mobile phase, extracted
into methanol, or partitioned into aqueous acetonitrile from
hexanes[55,56]. Halcinonide can be separated from kenalog[17]
(triamcinolone acetonide) by HPLC.[57]

Intermediates in the synthesis of halcinonide (*cf.*
section 1.5) have the following relative retention times;
Dihydrotriamcinolone, R_{RT} = 0.18; dihydrotriamcinolone ace-
tonide, R_{RT} = 0.32, and dihydrotriamcinolone acetonide-21-
mesylate, R_{RT} = 0.72 (Halcinonide, R_{RT} = 1.00)[46]. The purity
of dihydrotriamcinolone can be determined by HPLC using the
same reverse phase column and a mobile phase consisting of
0.8% ammonium nitrate, 0.23% monobasic ammonium phosphate,
32.5% methanol, 8% tetrahydrofuran and water.[58]   Cortisone
acetate is used as an internal standard.[59]   Impurities are
determined at a higher concentration.
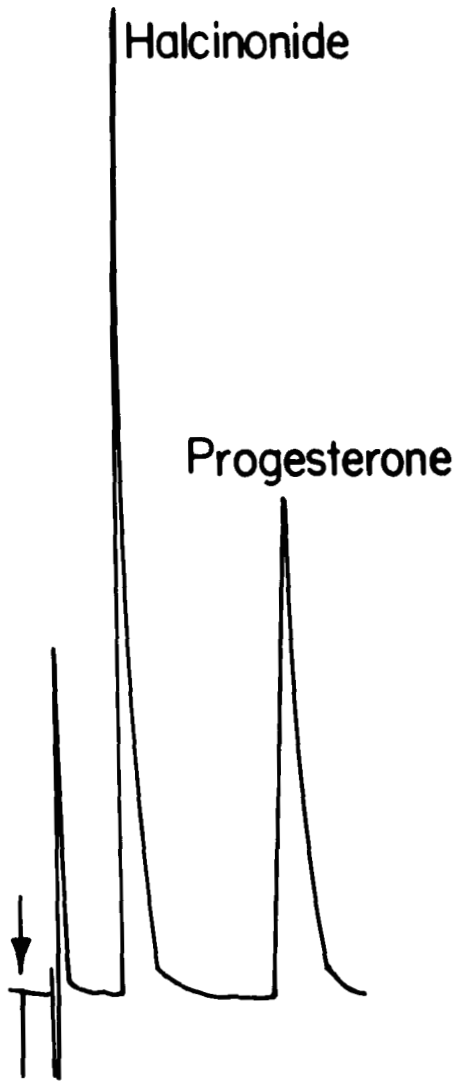
Figure 8

Reverse Phase High-Pressure Liquid Chromatography of Halcinonide. Progesterone is used as internal standard. See text for details.

4.32   Thin Layer Chromatography (TLC)

TLC, using silica gel GF 254 plates, can separate halcinonide from its synthetic precursors (*cf.* section 1.5). Using a developing solvent of chloroform-ethyl acetate (5:1), the following R_f values were found[60]: Dihydrotriamcinolone, R_f = 0.0; dihydrotriamcinolone acetonide, R_f = 0.08; dihydrotriamcinolone acetonide-21-mesylate, R_f = 0.18 and halcinonide, R_f = 0.36. Continuous TLC development gives respective relative R_f values of 0.02, 0.02, 0.48 and 1.0. With benzene (CAUTION)-acetone-water (70:30:07) the respective R_f values are 0.12, 0.42, 0.66 and 0.81. Traces of these intermediates have been found in some lots of halcinonide, as has dihydrotriamcinolone-21-acetate, an impurity occasionally found in some preparations of dihydrotriamcinolone. Elution from the TLC plate with ethanol permits quantitation of bulk halcinonide and halcinonide extracted from formulations. ^{14}C-Halcinonide (labelled at the acetonide carbon) was examined for impurities using silica gel TLC plates and chloroform-ethyl acetate (5:1)[61] mobile phase. The R_f value of halcinonide is 0.5.

4.33   Column Chromatography

A diatomaceous earth column was used to separate halcinonide from excipients[62]. The formulation is mixed with column packing material and then is transferred to the top of the column. Steroidal material is eluted with benzene (CAUTION) under a well-ventilated hood and then quantitated using thin layer chromatography[60] or tetramethylammonium hydroxide[51] colorimetry.

4.34   Paper Chromatography

Paper chromatography using Whatman No. 1 paper was once used to determine the homogeneity of halcinonide.[60] Twenty percent formamide in methanol comprises one stationary phase and methylisobutyl ketone-formamide (20:1) is the mobile phase. A second solvent system uses 25% propylene glycol-chloroform as the stationary phase and toluene saturated with propylene glycol as the mobile phase.

4.4   Polarographic Analysis

In dimethylformamide, halcinonide is reduced in two steps.[63] The 21α-chloroketo group exhibits a half-wave reduction potential of -1.17 volts *vs* Hg. This is easily distinguished from the half-wave potential of -1.62 volts *vs* Hg of the Δ^4-3-keto group. Thus, halcinonide can be

assayed in the presence of other 4-ene-3-keto steroids that
lack the 21-chloro group. This response requires a concentra-
tion between 0.5 and 2.0 mM of steroid per liter. The more
sensitive technique of differential pulse polarography[64]
should also be applicable to halcinonide.

4.5   Halcinonide in Tissues and Body Fluids

The antiinflammatory properties of such
topical agents as halcinonide are usually determined by a
vasoconstrictor assay. Topically applied corticosteroids
cause a blanching at the site of application, which can be
the forearm[65] or the upper back of healthy adults where
stratum corneum is removed with cellophane tape.[66] The test
areas, containing various concentrations of halcinonide, are
occluded with plastic wrap and are evaluated on an all-or-
none basis.[3] Percutaneous absorption studies[67] with 0.1%
[14]C-halcinonide cream, 1 g cream per dog or rabbit, showed
that the steroid is absorbed through intact or abraded skin.
In dogs, 0.4 to 0.5% is estimated to be absorbed through
intact and 4 to 10% through abraded skin. In rabbits, 6 to
16% of the ^{14}C-halcinonide was absorbed through intact and
14 to 23% through abraded skin.

Metabolism[68] was studied with halcinonide
labelled with carbon-14 in the 2-position of the acetonide
group. It was administered intravenously to dogs at a dose
of 5 mg/kg. The major portion of the radioactivity was
excreted in bile. Radio-autography of bile showed at least
10 distinct metabolites to be present. Four of the metabo-
lites were identified. The two most abundant metabolites,
that were identified, accounted for 43% (Figure 8, M1) and
30%(M2) of the radioactivity. The two minor metabolites (M3
and M4) accounted for 2% each. In dog urine, these four
metabolites (M1-4) accounted for 10, 15, 5 and 18% of the
radioactivity, respectively. In dog blood, unchanged hal-
cinonide and metabolites M3 and M4 each accounted for about
15% of the radioactivity. M1 and M2 were not detected.
These four metabolites, none of which contained chlorine,
were identified by comparing their ultraviolet, nuclear
magnetic resonance or mass spectra, and their thin-layer
chromatographic behavior (either underivatized, or as a
suitable derivative) with similar data obtained for authen-
tic reference samples. M1 was identified as the 21-carboxy
derivative of halcinonide ($-CH_2Cl \rightarrow -CO_2H$), M2 is the 6β-
hydroxy derivative, M3 is the 21-hydroxy derivative ($-CH_2Cl$
$\rightarrow -CH_2OH$) and M4 is the 6β-hydroxy derivative of M3. Figure
9 shows the proposed pathway. M3 is the acetonide deriva-

Figure 9
Proposed Metabolic Pathways for Halcinonide in the Dog.

tive in the synthesis of halcinonide (*cf*. **Fig. 2**) and it is also found as a degradation product in *in vitro* studies summarized below. This compound, dihydrotriamcinolone acetonide, possesses topical and systemic antiinflammatory activity.[69]

5. Stability:

Halcinonide dissolved in either methanol, deutero-methanol, aqueous ammonia-methanol, or deuterium oxide deuter-ated ammonia-methanol solvents appears stable after storage for six days at 50°, using nuclear magnetic resonance and mass spectrometry.[70]

Using thin layer chromatography (*cf*. section 4.32, first system)[60] no change was found in two lots of halcinonide after storage at 50° for six months in brown glass bottles. But the direct exposure to 900 foot-candles of light for one month caused an increase in impurities of two lots from 0.5% to 3.9% or 4.9%. Photolytic degradation of the A-ring is expected since hydrocortisone and prednisolone undergo rearrangements when solutions of these steroids in alcohol are exposed to ultraviolet radiation or ordinary fluorescent lighting.[71-73]

Formulated halcinonide, at a concentration of 0.1% in either a cream base or polyethylene glycol-water lotion, after storage at approximately 23° for 3 years, showed no loss on halcinonide content, using high pressure liquid chromatography.[46] The contents remained unchanged within 2% of labeled concentration.

One trial formulation, stored at 60° for 7 months showed degradation.[74] The one impurity that was isolated and identified (with the aid of mass spectrometry) is dihydrotriamcino-lone acetonide (Figure 1), an intermediate in the synthesis of halcinonide. This compound also possesses topical antiinflammatory activity.[69]

6. Acknowledgements

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HYDRALAZINE HYDROCHLORIDE

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1. DESCRIPTION

  1.1 Name, Formula, Molecular Weight
       The Chemical Abstracts name for hydralazine hydro-
chloride is 1(2H)-phthalazinone hydrazone monohydrochloride,
starting with volume 80; previously the name 1-hydrazino-
phthalazine monohydrochloride was used. The CAS Registry
No. is [304-20-1] for the hydrochloride salt, [86-54-4] for
the base.
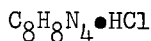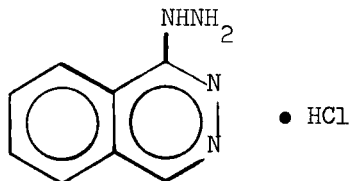
$C_8H_8N_4 \bullet HCl$ Molecular Weight: 196.64

  1.2 Appearance, Color, Odor
       Hydralazine hydrochloride is a white to off-white
odorless crystalline powder.

  1.3 History
       The ability of hydralazine and similar hydrazino
compounds to reduce blood pressure was reported by Gross
et al (1) in 1950. Methods of synthesis were published by
Druey and Ringier (2) in 1951, along with typical reactions
displayed by the compound, including several reactions
which later became the bases for analysis of hydralazine
and its metabolites. The drug has been widely used for
treatment of hypertension, and papers on its properties and
methods for its determination have been published in many
languages.
       Hydralazine is usually used in combination with
other drugs. In recent years, it has been found to be
especially useful when used with beta-adrenergic blocking
agents and diuretics (3,4).

2. PHYSICAL PROPERTIES

  2.1 Infrared Spectra

       The infrared spectrum of hydralazine hydrochloride
(Figure 1) was obtained with a Beckman IR-12 spectrophoto-
meter. A mineral oil dispersion between potassium bromide
plates was scanned from 420 to 4000 cm^{-1}, and a thicker
layer of the dispersion, supported on polyethylene film,

was scanned from 200 to 530 cm^{-1}. Some of the absorption
bands can be assigned as follows:

Frequency, cm^{-1}	Assignment (5)
3220	N-H stretch
3025	Aromatic C-H stretch
2800-3000	Mineral oil C-H stretch
1590-1600	C=C stretch
1460	Mineral oil C-H band
785	Out of plane bending, 4 adjacent H atoms on an aromatic ring

The mineral oil absorption at 2800 to 3000 cm^{-1} and at 1460
cm^{-1} obscures absorption bands of hydralazine hydrochloride
at 2810, 2920, and 2970 cm^{-1} (N-H$^+$ stretch) and a weak sharp
band at 1470 cm^{-1}; these bands can be observed in potassium
bromide dispersion spectra. The bands at 1070 and 1082 cm^{-1}
tend to merge into a single band in potassium bromide
dispersion spectra.
    The infrared spectrum of hydralazine hydrochloride
base in a potassium bromide dispersion (Figure 2) was
recorded from 400 to 4000 cm^{-1} , and the 200 to 550 cm^{-1}
region was obtained from a mineral oil dispersion supported
on polyethylene film. The spectra of potassium bromide
dispersions of the base are qualitatively identical to those
of mineral oil dispersions. The assignment of absorption
bands in the spectrum of the base is similar to that of the
hydrochloride except for the presence of N-H$^+$ stretch
absorption in the latter. A spectrum of the base has been
published (6).

    2.2 Ultraviolet Spectra
        Figure 3 is the ultraviolet absorption spectrum of
hydralazine hydrochloride in water solution, run on a Cary
Model 14 spectrophotometer. The solution contained 9.9 mg
of hydralazine hydrochloride per liter, and was run against
water (1 cm cells). The discontinuity in the spectrum at
219 nm is a change of absorption scale; the absorbance
scale range is 0.0 to 1.0 for the wavelength range 350 to
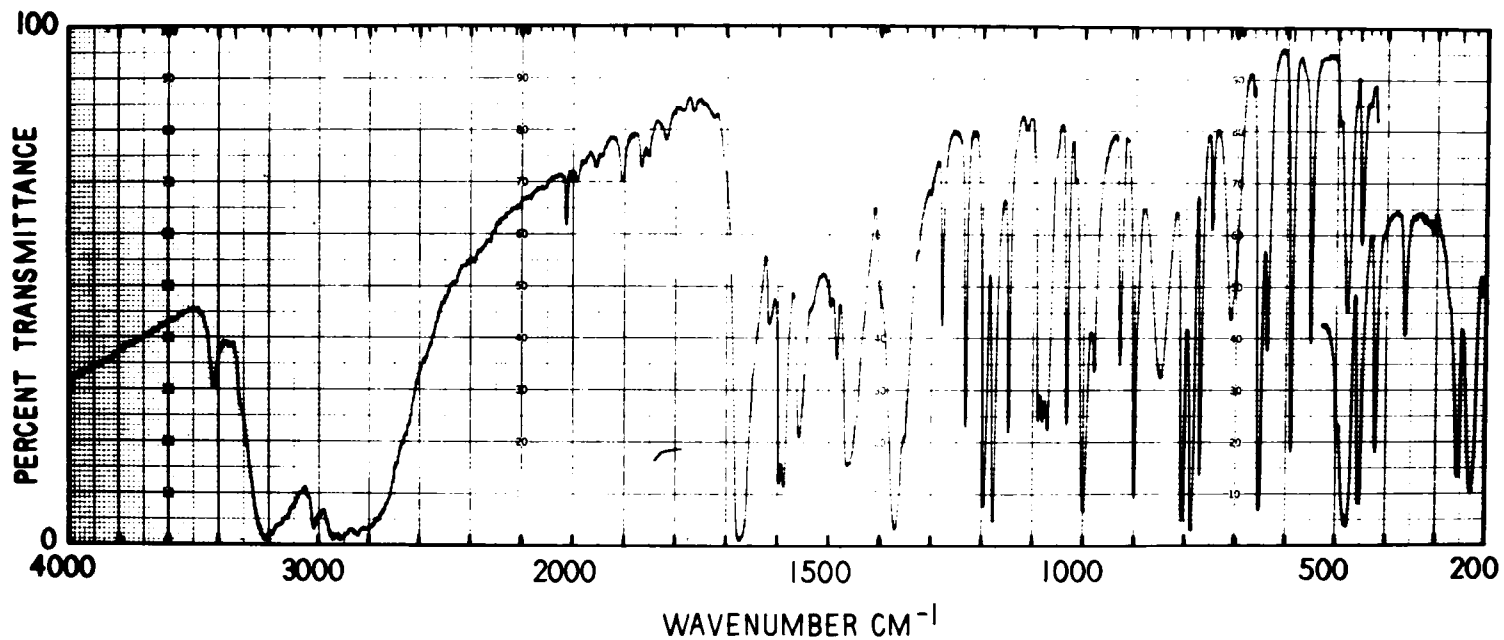
Figure 1. Infrared Absorption Spectrum of Hydralazine Hydrochloride, Mineral Oil Mull.
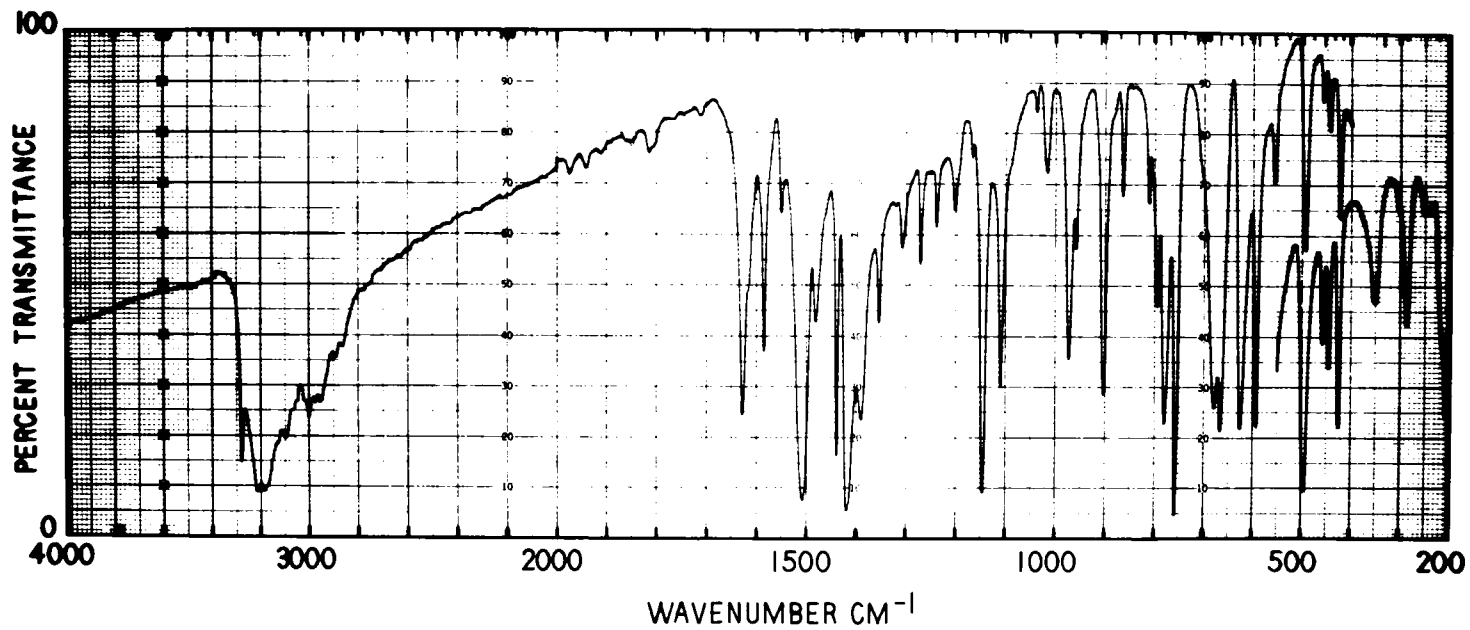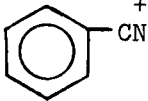
Figure 2. Infrared Absorption Spectrum of Hydralazine Base, Potassium Bromide Pellet and Mull.

219 nm, and 1.0 to 2.0 for the wavelength range 219 to 200
nm. A scan of water against water is also recorded on the
chart. The spectrum exhibits maxima at 315, 303, 260, 239,
and 211 nm, with respective absorptivities as follows:
4,200; 5,200; 10,600; 11,000; 33,800 l./mole cm.
         Druey and Tripod (3), Kuhnert-Brandstätter et al
(7), Sharkey et al (8), and Solomonova et al (9) reported
similar maxima and absorptivities, except that they did
not report the 211 nm maximum. Clarke (6) gives data
similar to that of the above investigators. Sunshine (10)
gives the spectrum in 0.1N hydrochloric acid, with maxima
at 312, 302, 259, and 233 nm, and the spectrum in 0.1N
sodium hydroxide, with maxima at 304, 271, and 262 nm.
Naik et al (11) gave partial ultraviolet spectra of the
base, the monohydrochloride, and the dihydrochloride:
(a) the base exhibited maxima at 305 and 273 nm, with a
third maximum near 262 nm; (b) the monohydrochloride
exhibited maxima at 315, 302, 290, and 260 nm; (c) the
dihydrochloride exhibited a maximum at 318 nm with a broad
shoulder at about 300 nm. The Merck Index (12) lists
maxima at 315, 304, 260, 240, and 211 nm for the aqueous
solution of the hydrochloride.

   2.3 Mass Spectrum
         Figure 4 shows the low resolution mass spectrum
of hydralazine hydrochloride. The data were obtained
with an LKB 9000S mass spectrometer, with an ionization
voltage of 70 eV, source temperature 250°C. Some of the
peaks may be assigned as follows (13):

Mass Charge Ratio	Assignment
160	M^+
131	$M^+ - \cdot N=NH$
129	$M^+ - \cdot NH-NH_2$
103	
76	$C_6H_4^+$
51	$C_4H_3^+$
36/38	HCl^+
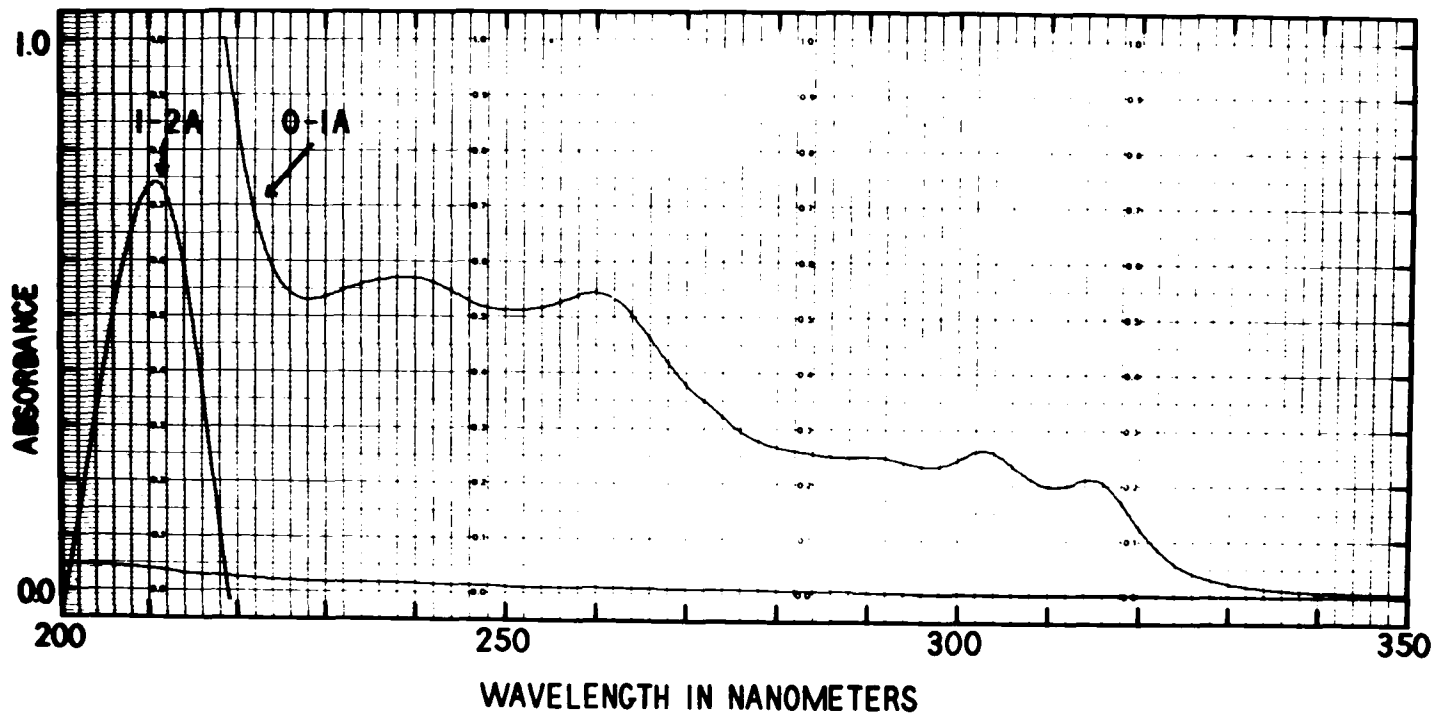
Figure 3.   Ultraviolet Absorption Spectrum of Hydralazine Hydrochloride, 9.9 mg per liter, Aqueous Solution.
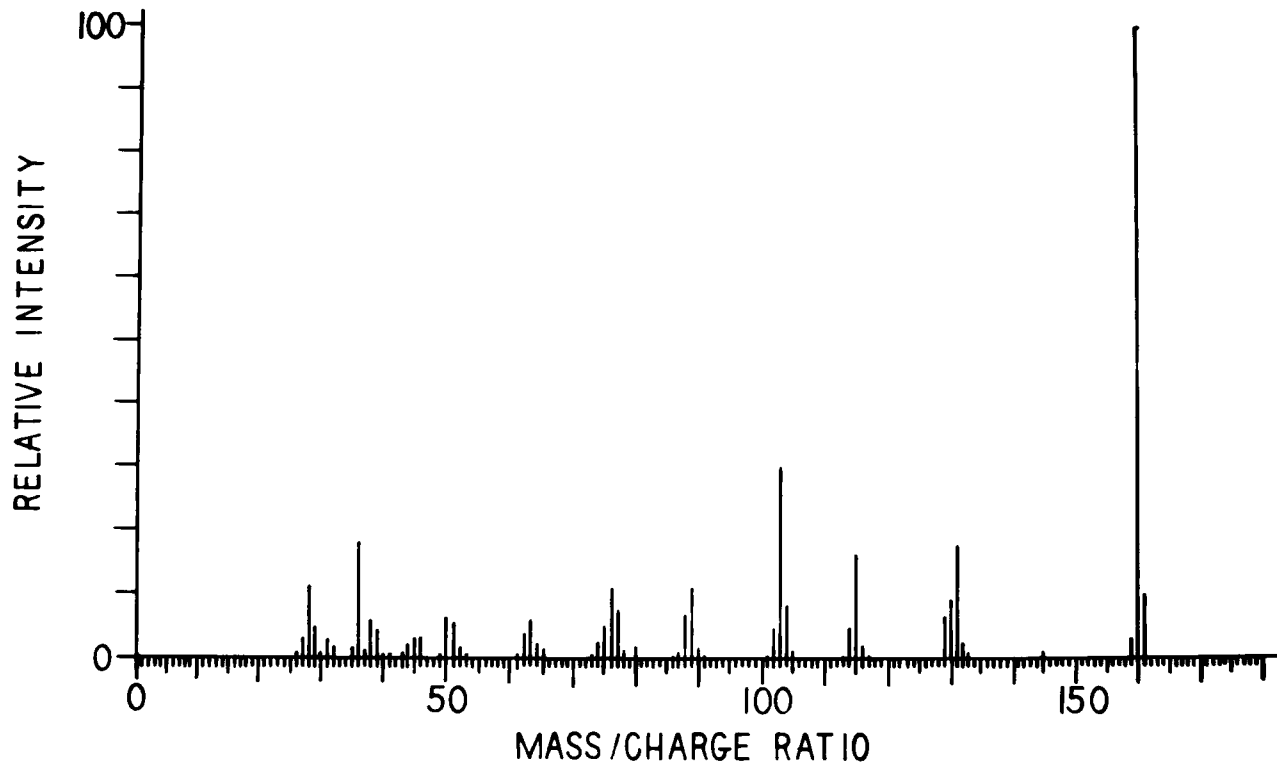
Figure 4.   Mass Spectrum of Hydralazine Hydrochloride.

2.4 Nuclear Magnetic Resonance Spectra

The NMR spectrum shown in Figure 5 was obtained by dissolving hydralazine hydrochloride in deuterium oxide containing 3-(trimethylsilyl)-1-propane-sulfonic acid sodium salt (DSS). The series of peaks at 0, 0.6, 1.8, and 3 ppm are all due to the DSS. The peak at 4.8 ppm is due to HDO which forms on exchange with the solvent and the peaks at 8.01 and 8.61 ppm are due to the aromatic protons. The NMR spectrum of the base (Figure 6) was obtained in a 1:1 mixture of dimethylsulfoxide-d$_6$:deuterochloroform. The peaks at 2.53 ppm are due to the solvent. The N-H protons are at 4.1 ppm and the aromatic protons are at 7.6 and 8 ppm. The spectra were produced using a Varian EM-360 NMR spectrometer.

2.5 Differential Thermal Analysis (DTA)

The DTA curves in Figures 7 and 8 were obtained with a DuPont Model 900 instrument. Figure 8 shows that the base melts sharply and Figure 7 shows that the hydrochloride melts with decomposition. Visually the hydrochloride melts at 274°C, but decomposition takes place over a large temperature range (Figure 7).

2.6 Crystal Properties

Chojnacki et al (14) investigated the crystal properties of hydralazine hydrochloride. Recrystallized from water, the crystals were found to be monoclinic, with the unit cell a = 9.408, b = 14.529, c = 6.643Å, β = 103.59°, Z = 4. The calculated density was 1.486, the measured density 1.479 g per cm^3. The space group was P2$_1$/c. Powder diffraction data, generally similar to that of Table 1, is also given.

X-ray powder diffraction data for hydralazine hydrochloride and hydralazine base are given in Table 1. These data were obtained with a Norelco diffractometer using nickel-filtered copper Kα radiation.

2.7 Microchemical Characterization

Sandri (15) reported the formation of characteristic crystals with cadmium bromide-hydrobromic acid, bismuth iodide-potassium iodide, and bismuth bromide-hydrobromic acid, with compositions $CdBr_2 \cdot HBr \cdot C_8H_8N_4 \cdot 4H_2O$, $HBiI_4 \cdot C_8H_8N_4$, and $HBiBr_4 \cdot C_8H_8N_4$, respectively.

Kuhnert-Brandstätter et al (7) reported the microscopic hot-stage characterization of hydralazine hydrochloride. Above 210°C, small rods, granules, and prisms sublime. The melting point is 268-275°C. Gas bubbles are evolved as melting begins, and melting proceeds with

TABLE 1

X-RAY POWDER DIFFRACTION PATTERNS OF
HYDRALAZINE HYDROCHLORIDE AND HYDRALAZINE BASE

Hydrochloride		Base	
$d(\overset{\circ}{A})$	I/I_o	$d(\overset{\circ}{A})$	I/I_o
7.74	100	7.37	100
7.25	93	5.87	11
5.92	9	5.62	49
5.54	2	5.04	11
4.82	2	4.86	17
4.62	18	4.48	18
4.37	47	3.98	2
3.99	15	3.74	10
3.87	12	3.66	2
3.63	54	3.47	15
3.39	9	3.28	100
3.38	11	3.24	17
3.32	31	3.12	11
3.29	65	2.96	2
3.22	92	2.80	7
3.15	7	2.71	1
3.05	9	2.67	2
2.99	4	2.60	2
2.95	2	2.49	2
2.93	2	2.44	2
2.85	6	2.38	1
2.77	22	2.30	2
2.72	2	2.28	1
2.64	2	2.25	2
2.58	4	2.18	1
2.44	7	2.12	3
2.41	2	2.07	2
2.39	1	2.04	3
2.34	16	1.99	1
2.24	2	1.95	2
2.22	2	1.87	2
2.18	2	1.85	1
2.16	2	1.83	1
2.10	3	1.79	1
2.06	2	1.78	1
2.02	1	1.76	1
1.99	2	1.72	3
1.97	4	1.67	1
1.94	4	1.62	1
1.89	2		
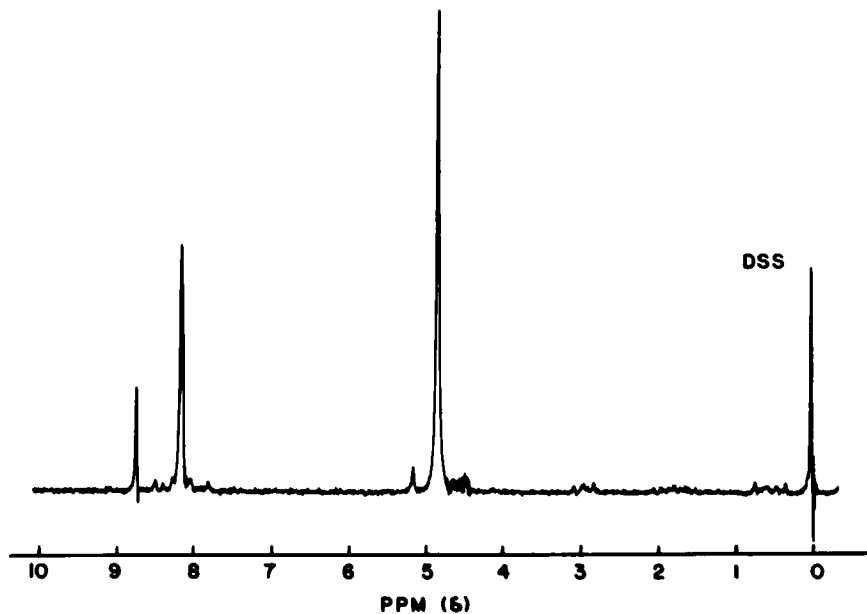
Figure 5. Nuclear Magnetic Resonance Spectrum of Hydralazine Hydrochloride.
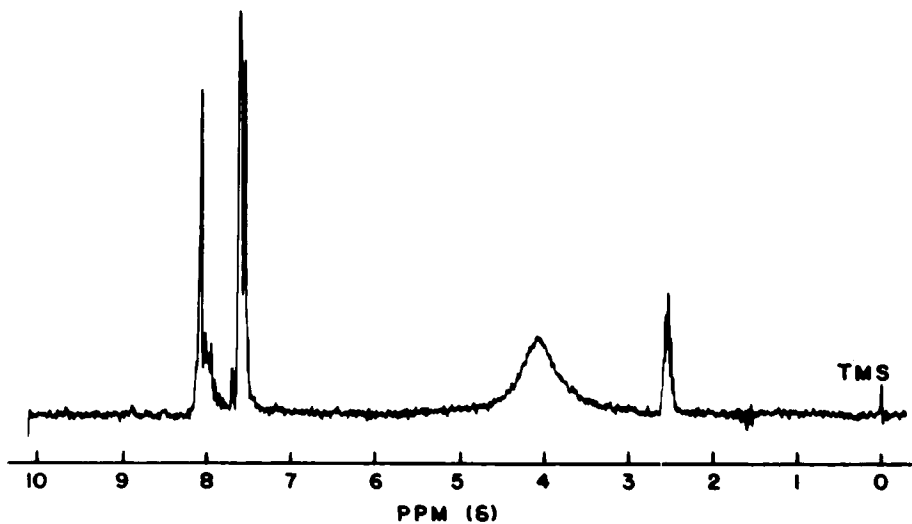
Figure 6. Nuclear Magnetic Resonance Spectrum of Hydralazine Base.
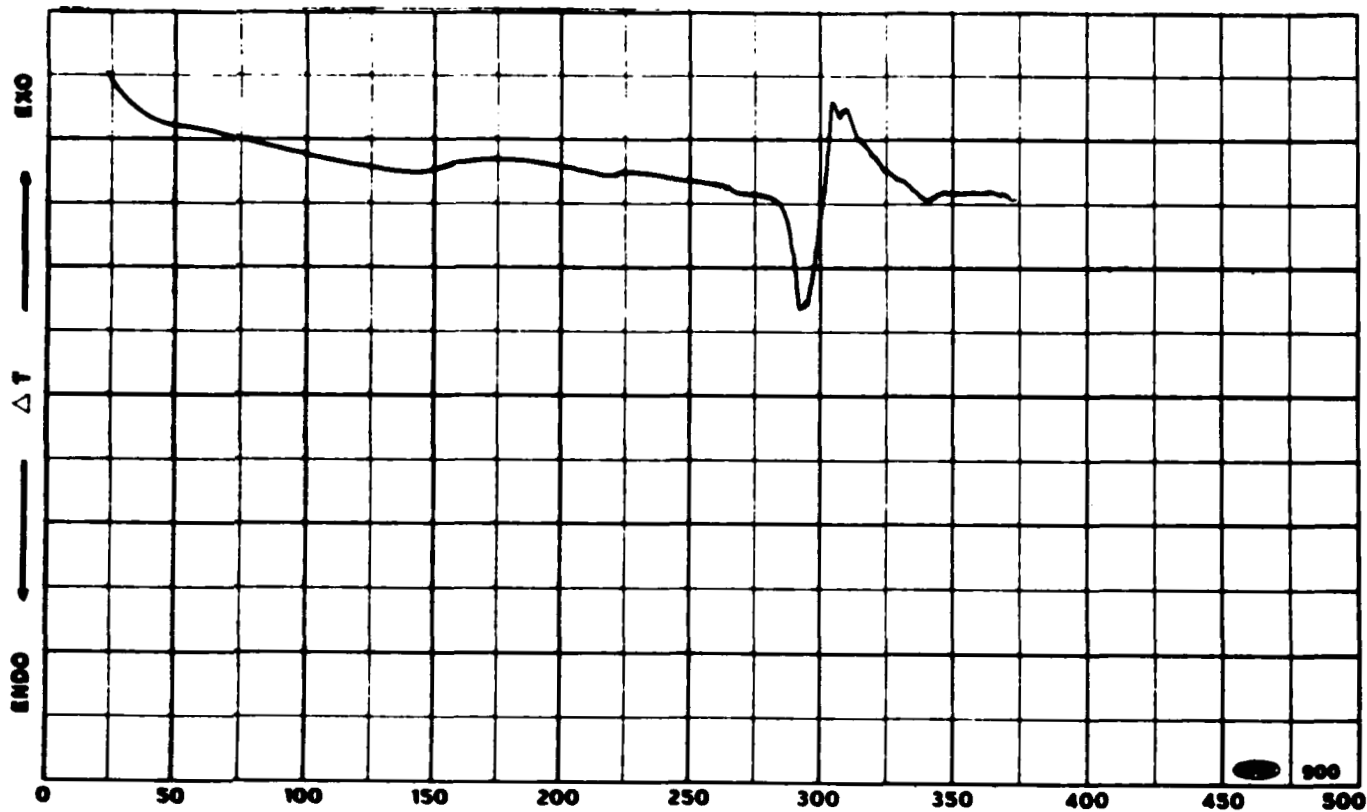
Figure 7. Differential Thermal Analysis Curve of Hydralazine Hydrochloride.
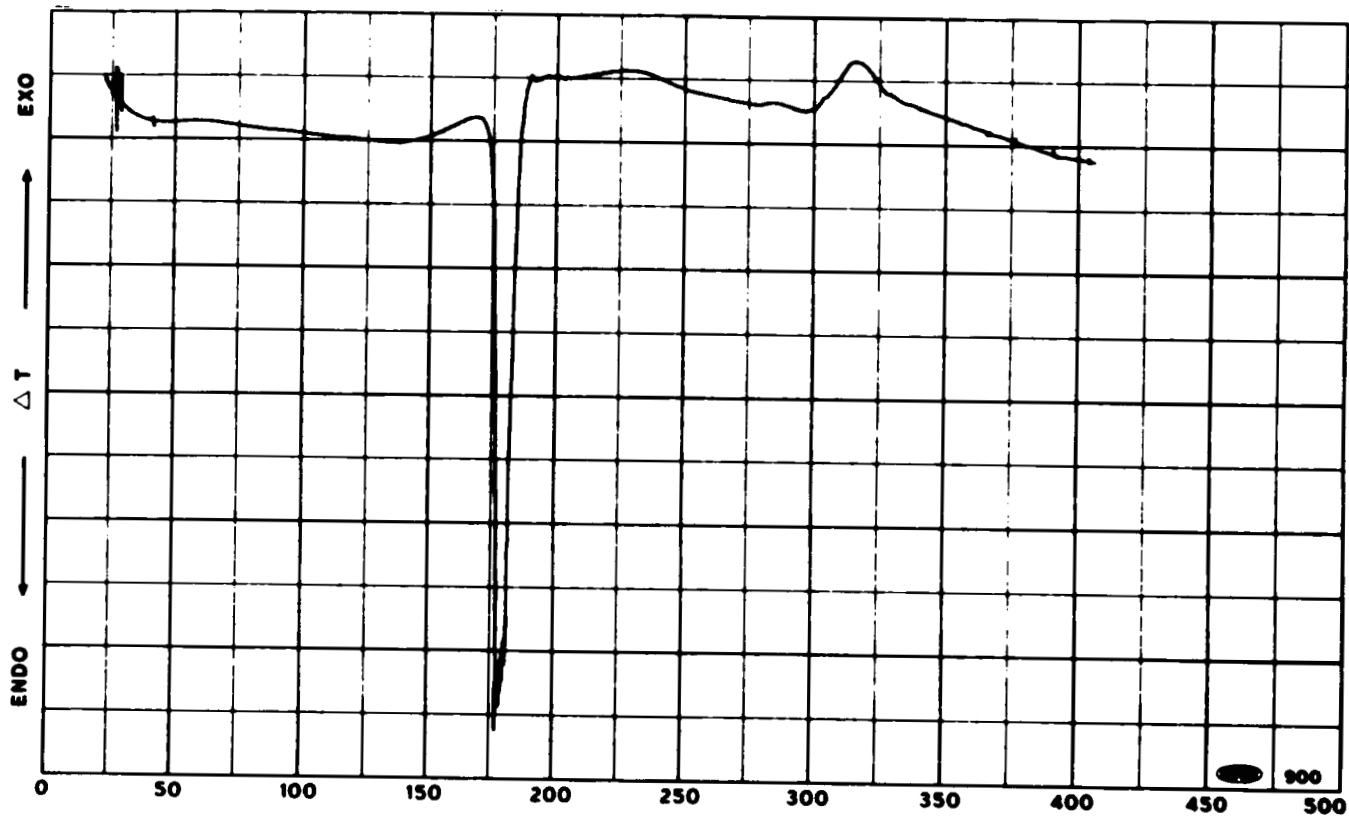
Figure 8.   Differential Thermal Analysis Curve of Hydralazine Base.

the separation of high melting (over 300°C) needles and
bushlike aggregates. The eutectic temperature with
p-acetamidophenyl salicylate was 181°C, with dicyandiamide
162°C. The picrate salt forms from aqueous solution as
long needles and columns that melt at 210 to 212°C with
decomposition. The styphnate forms needles and rosettes
with a melting point of 200 to 203°C.

      Sunshine (10) and Clarke (6) give two micro-
crystal tests, both from aqueous solution: (a) lead
iodide-potassium acetate reagent forms dense rosettes;
(b) iodine-potassium iodide (1:50) reagent forms clusters
or masses of needles.

   2.8 Solubility

      The Merck Index (12) gives the solubility in water
as 30.1 mg per ml at 15°C, and 44.2 mg per ml at 25°C. In
95% ethanol, the solubility is given as 2 mg per ml. These
are consistent with the following approximate solubility
data, determined at room temperature:

Solvent	Approximate Solubility, mg/ml
Water	39.
Methanol	6.7
Ethanol (95%)	1.9
2-Propanol	0.1
Chloroform	< 0.1
Ethyl ether (anhydrous)	< 0.1
Ethyl acetate	< 0.1
Acetonitrile	< 0.1

   2.9 Dissociation Constants

      Evstratova and Ivanova (16) reported a pK_a of 7.1.
Evstratova et al (17) reported a pK_a of 7.1 in water, a
pK_a of 4.7 in aqueous 90 percent acetone, and a pK_b of 15.6
in the latter solvent. Naik et al (11) reported a pK_a of
6.9 for the dissociation of the monohydrochloride and 0.5
for dissociation of the dihydrochloride, determined
absorptiometrically. Artamanov et al reported a pK_a of
7.2 (18).

2.10 <u>Melting Points</u>
    The melting point of hydralazine hydrochloride is
near 273°C, that of the base is near 173°C. Melting points
reported in the literature are as follows:

Melting Point of Hydrochloride (a), °C	Reference
270 - 280	(3,19)
273	(2,20,21,22,23)
271 - 272	(24)
265	(25)
273 - 274	(26)

(a)   The hydrochloride melts with decomposition.

Melting Point of Base, °C	Reference
172 - 173	(2,21,22)
165 - 172	(24)
172	(25)
173 - 175	(27)

3. SYNTHESIS

    Hydralazine has been prepared by various procedures
from 1-chlorophthalazine (2,21,22,24,28), phthalazine-1-
thione (20,23,25), 1,4-dichlorophthalazine (29,30,31),
1-(methylsulfonyl)-phthalazine (27), or 1-cyano-2-benzal
acetate (26). Phthalazone, used to prepare 1-chloro-
phthalazine, has been prepared from naphthalene (3,28) or
from phthalic anhydride (3). A comprehensive survey on
hydrazinophthalazines and related compounds has been
published by Druey and Tripod (3). Some of these procedures
are outlined in Figures 9 and 10.

4. STABILITY - DEGRADATION

    Hydralazine hydrochloride is quite stable as the
crystalline solid. At room temperature, it is stable in
distilled water solution for weeks (32). In aqueous
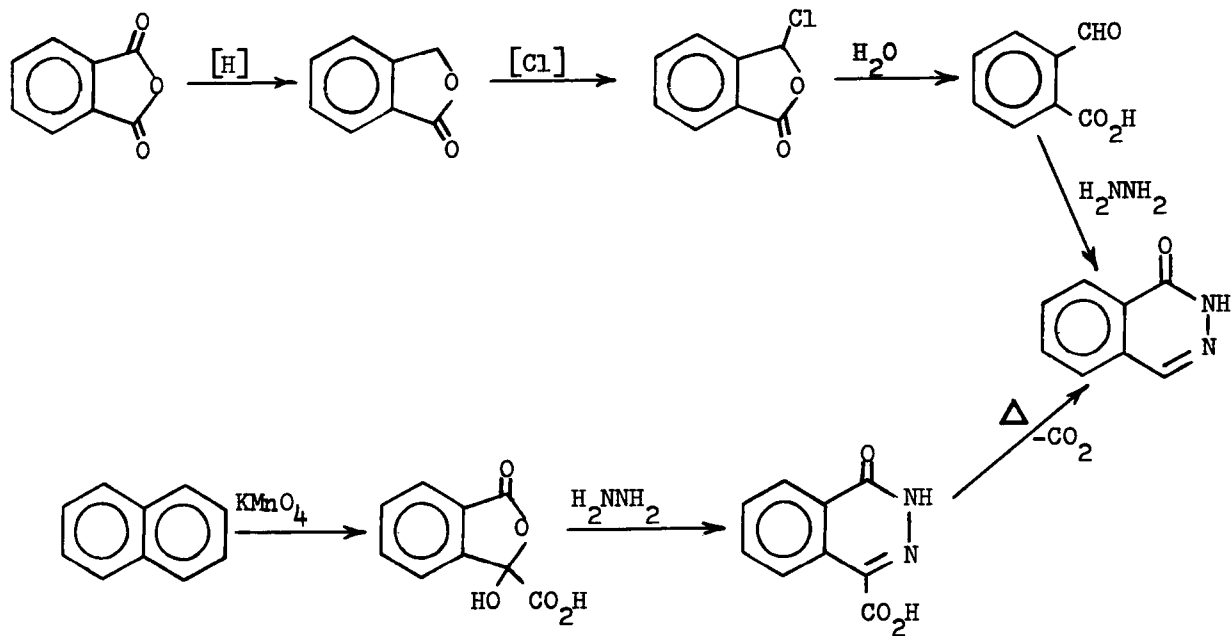
Figure 9. Syntheses of Phthalazone.
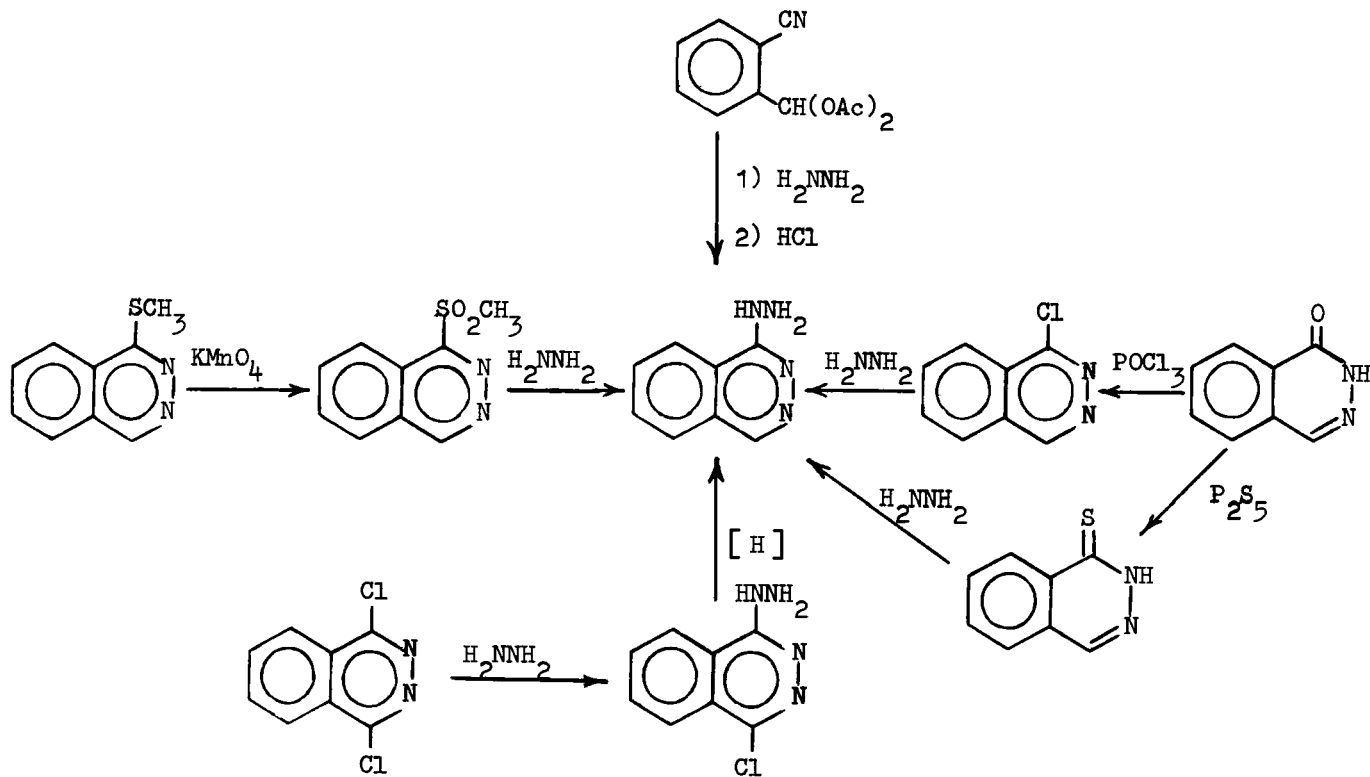
Figure 10. Several Syntheses for Hydralazine.

solution with a pH greater than 7, hydralazine decomposes, forming phthalazine; the rate of decomposition depends on pH, temperature, and the kind and concentration of anion present (33).

In biological samples the drug disappears rapidly at room temperature, apparently by enzymatic reactions.

The hydrazino group is highly reactive, forming hydrazones with aldehydes and ketones. It is also a reducing agent, and it forms complexes with many metal ions (34).

At 275 to 280°C it decomposes to hydrazine, ammonia, nitrogen, and 1,4-dihydro-1,1'-biphthalazine (35).

5. METABOLISM

Hydralazine hydrochloride is rapidly metabolized and excreted. Experiments with carbon-14 labeled drug in humans indicated that less than 10 percent of the intact drug was excreted (36). Within 5 days after a dose, 83 to 89 percent was excreted in the urine and 9 to 12 percent in the feces. Of the material excreted in the urine, 96 percent was recovered in the first 24 hours. Individuals who are slow acetylators exhibit higher hydralazine blood levels than fast acetylators, for the same dose (37).

Reported metabolic products of hydralazine in various species are as follows:

	Metabolite	Species	Reference
I.	3-Methyl-s-triazolo [3,4a]phthalazine	Man	(36,37,38,39, 40,41,42,43,44)
		Rat	(38,39,43,45, 46)
		Guinea pig	(45)
		Rabbit	(38)
		Pigeon	(45)
II.	N-(1-Phthalazinyl)- hydrazone of pyruvic acid	Man	(36,38,47,85)
		Rat	(38,46)
		Rabbit	(38)
III.	N-(1-Phthalazinyl)- hydrazone of acetone	Man	(47)
		Rat	(44,46)
IV.	N-(1-Phthalazinyl)- hydrazone of α- ketoglutaric acid	Man	(47)

V.	s-Triazolo[3,4a]	Man	(36,43,47,48)
	phthalazine	Rat	(46)
VI.	1(2H)-Phthalazinone	Man	(36,43,48)
		Rat	(46)
VII.	Phthalazine	Man	(37)
		Rat	(46)
VIII.	4-(2-Acetylhydrazino)-		
	phthalazinone	Man	(36)
IX.	9-Hydroxy-3-methyl-s-		
	triazolo[3,4a]		
	phthalazine	Man	(36)
X.	3-Hydroxymethyl-s-	Man	(36,43)
	triazolo[3,4a]	Rat	(46)
	phthalazine		

In cases where the metabolites above possess suitable functional groups, they are usually excreted as glucuronides or sulfates.

Figure 11 is a scheme illustrating the metabolism of hydralazine, based primarily on those proposed by Haegele et al (46) and Wagner et al (36).

6. METHODS OF ANALYSIS

6.1 Identification Tests

Hydralazine hydrochloride can be easily identified by the physical properties described in Section 2 above.

Where identification of hydralazine in formulations is necessary, the following tests may be useful.

Cooper (49) used spot tests on paper, reacting ferric ion or dimethylaminobenzaldehyde with the unknown. The iron reagent yields a blue color, and dimethylamino-benzaldehyde an orange color. The results obtained with 95 other drugs are given.

Belikov et al (50) described three reactions to identify hydralazine. Sodium nitroprusside reacts with an alkaline aqueous solution of hydralazine to yield a red color; when the mixture contains mineral acids, a green precipitate is formed, but with acetic acid, a red precipitate is formed. Cinnamaldehyde reacts with a hydrochloric acid solution of hydralazine to give a yellow precipitate, m.p. 197-200°C. p-Nitrobenzaldehyde reacts with an
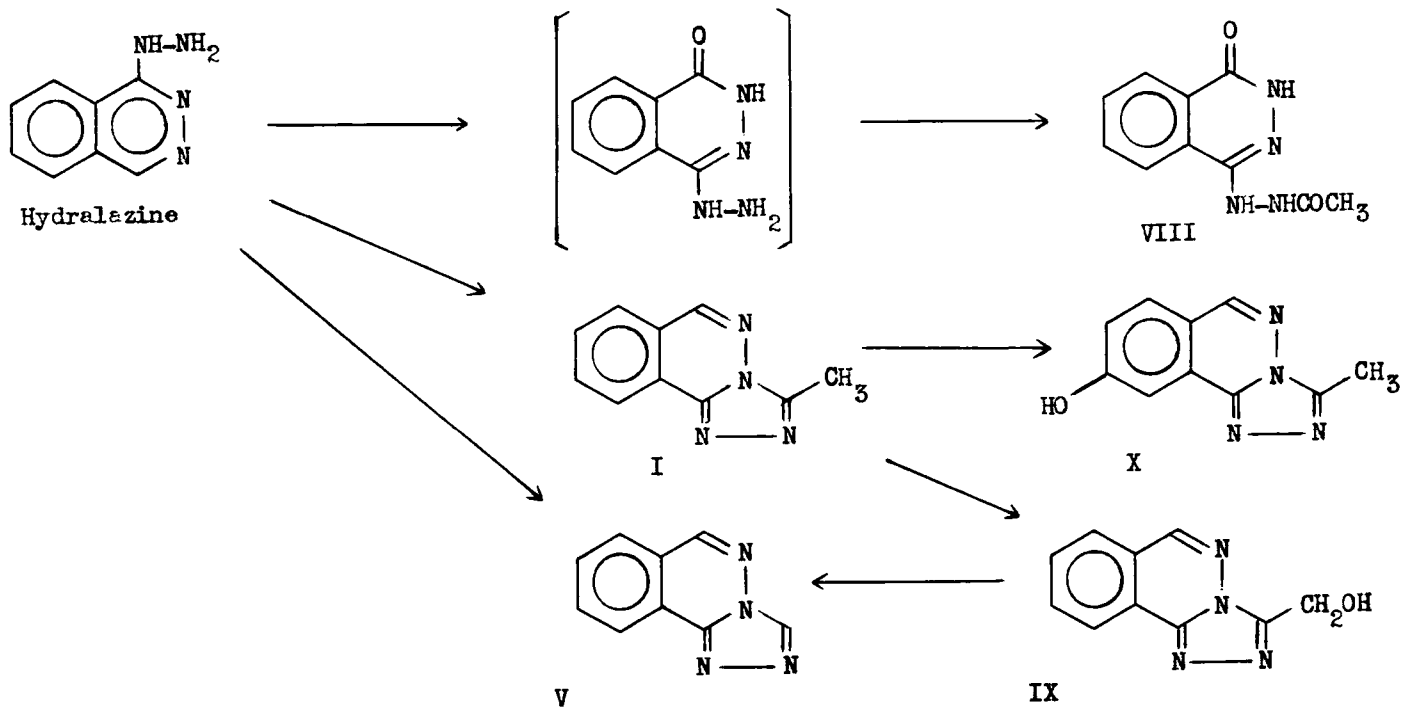
Figure 11a. Metabolic Products of Hydralazine.
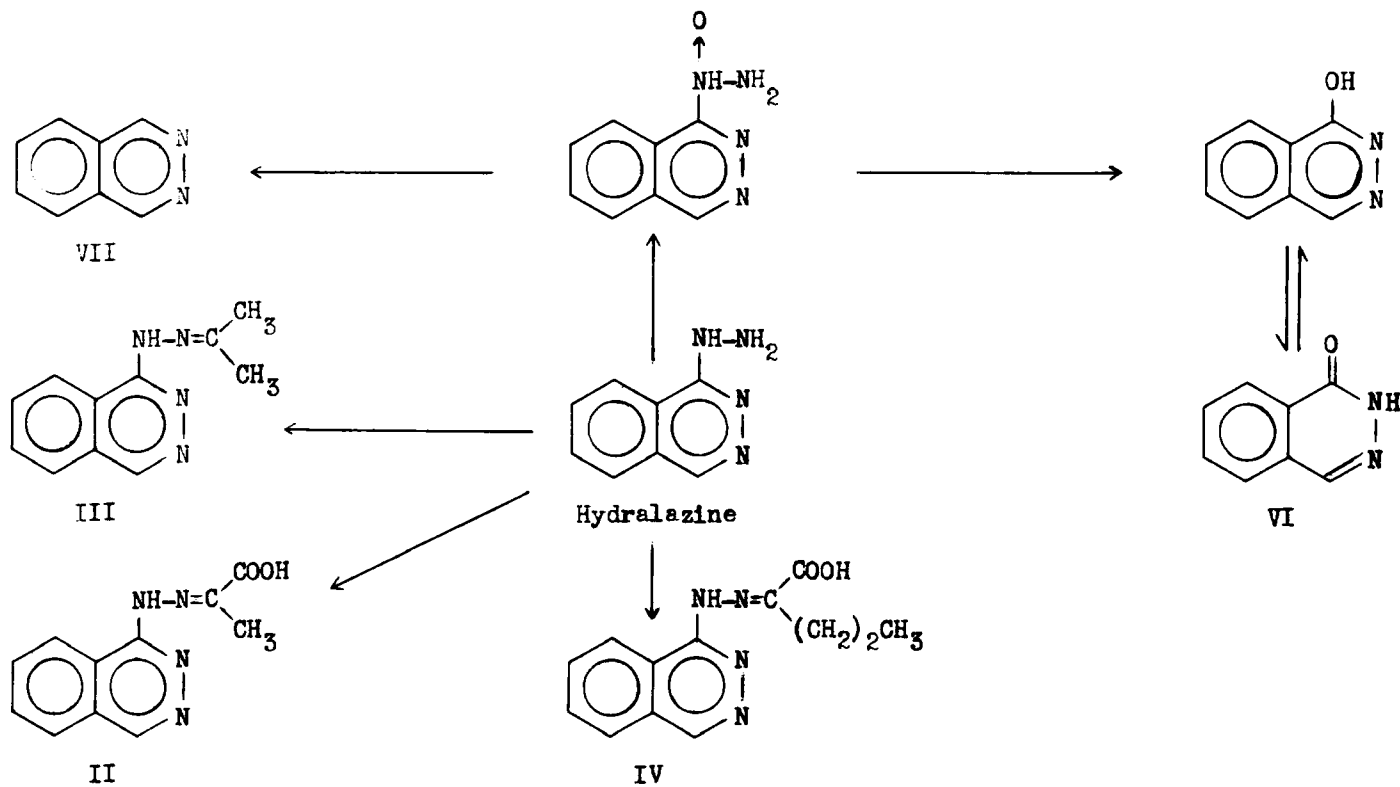
Figure 11b. Metabolic Products of Hydralazine.

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aqueous solution of hydralazine to give an orange precipitate, m.p. 260-261°C.

Modras (51) reported spot test reactions to differentiate hydralazine from closely related drugs. Reagents used were aqueous copper (I) chloride, aqueous ammonium molybdate, iodine in potassium iodide solution, aqueous cobalt (II) nitrate, alcoholic ninhydrin, and alcoholic bromophenol blue. The tests were performed on paper or on Silica Gel G.

Roshchenko et al (52) reacted hydralazine with p,p'-dichlorobenzene sulfonamidate sodium in water at 80 to 90°C to produce a crystalline precipitate $C_{20}H_{17}O_4N_5S_2Cl_2$ with a melting point of 196-197°C.

In the USP (19) method for identifying hydralazine in tablets and injections, o-nitrobenzaldehyde is added to form an orange precipitate.

Stohs and Scratchley (53) used thin layer chromatography to identify hydralazine in dosage forms and biological fluids (see Section 6.8).

6.2   Elemental Analyses

The elemental compositions of hydralazine hydrochloride and hydralazine base are as follows:

| Element | Hydrochloride | | Base | |
	Calculated	Found(2)	Calculated	Found(2)
Carbon	48.87	48.99	59.99	- - -
Hydrogen	4.61	4.83	5.03	- - -
Nitrogen	28.49	28.39	34.98	35.09
Chlorine	18.03	18.17	- - -	- - -

6.3   Spectrophotometric Methods

Solomonova et al (9) determined hydralazine in tablets by a direct ultraviolet absorption method.

Although the compound can be extracted from formulations with alcohol or water, other components of the mixture may interfere with direct ultraviolet absorption measurement by contributing to the observed absorption. The solutions usually cannot be cleaned up by extraction techniques because hydralazine decomposes in alkaline solution. However, there are many reactions that give rise to near ultraviolet or visible absorption bands suitable for quantitation.

A frequently reported spectrophotometric technique for the determination of hydralazine is based on reactions with aromatic aldehydes to form hydrazones with absorption in the visible region. Luk'yanchikova et al (54) used p-nitrobenzaldehyde; Wesley-Hadzija and Abaffy (55) and Ruggieri (56) used p-dimethylaminobenzaldehyde; Luk'yanchikova (57,58) used cinnamaldehyde; Schulert (33) used p-hydroxybenzaldehyde; and Zak et al (59) used p-methoxybenzaldehyde, after testing cinnamaldehyde, salicylaldehyde, 3,4,5-trimethoxybenzaldehyde, and 1-naphthaldehyde.

Perry (32), and Grabowicz and Brulinska (60) used ninhydrin to obtain a solution with an absorption maximum near 450 nm.

Ellert and Modras (61) treated hydralazine with ferrous ion in alkaline solution, and measured the color produced at 540 nm.

Ruggieri (56) reported a colorimetric test using 2-naphthoquinonesulfonate sodium to form a rose-violet color.

Urbanyi and O'Connell (62,63) developed an automated colorimetric method using blue tetrazolium for the analysis of hydralazine in the presence of reserpine and hydrochlorothiazide.

6.4 Fluorescence
Naik et al (11) extracted hydralazine hydrochloride from tablets with 50 percent aqueous methanol and mixed a portion of the extract with 99 volumes of concentrated sulfuric acid to obtain fluorescence at 353 nm with excitation at 320 nm. The fluorescence intensity varied linearly with concentration in the range 2 to 8 µg hydralazine hydrochloride per ml. Injections were analyzed similarly.

6.5 Titration Methods
Ruggieri (56) reported titrating hydralazine with perchloric acid.

Ruzhentseva et al (64) converted hydralazine to ammonia by heating with zinc in sulfuric acid, forming 3 moles of ammonia per mole of hydralazine. The mixture was made alkaline and the ammonia was distilled into boric acid solution, which was then titrated.

Sandri (15) tested three titration methods with good results. One was titration with potassium bromate in the presence of potassium bromide and hydrochloric acid, using a starch-iodine end-point. Another was addition of excess periodic acid-potassium iodide with sodium

thiosulfate back-titration. The third was addition of
sodium hydroxide and a concentrated solution of potassium
ferrocyanide, then acidifying and titrating with potassium
permanganate solution.

Perel'man and Evstratova (65) titrated hydralazine
hydrochloride in dimethylformamide solution to a potentio-
metric end-point with sodium methoxide solution.

Artamanov et al (18) determined hydralazine
hydrochloride by conductometric titration with sodium
hydroxide solution.

Goryacheva and Prikhodkina (66) titrated hydrala-
zine from tablets with N-bromosuccinimide solution. One
mole of hydralazine hydrochloride was equivalent to 2 moles
of N-bromosuccinimide. Methyl red was used as the
indicator.

Soliman and Belal investigated argentimetric
(67,68) and mercurimetric (69) methods. Hydralazine
precipitates silver from ammoniacal silver nitrate
solution. The silver is dissolved with hot nitric acid
and titrated with ammonium thiocyanate solution. Alter-
natively, mercury is precipitated from alkaline potassium
mercuric iodide solution. The precipitated mercury is
dissolved by adding excess standard iodine solution. The
excess iodine is back-titrated with sodium thiosulfate
solution after acidifying with acetic acid.

USP XIX directs that hydralazine be determined in
the raw material, tablets, and injections by potassium
iodate titration in strongly acid solution, using chloro-
form to detect the presence of iodine (19).

6.6 Gasometric Methods

McKennis and Yard (70) studied the nitrogen
evolution from a series of hydrazino compounds when treated
with $0.02\underline{M}$ KIO_3 in $0.2\underline{N}$ H_2SO_4 in a Warburg apparatus at
37°C. In an aqueous solution, hydralazine released 102
percent of the theoretical amount of nitrogen in 15 minutes.
Viala (71) determined hydralazine in solutions or tablets
by measuring the nitrogen freed from the hydrazine group,
using potassium permanganate in sulfuric acid, or iodine
in sodium bicarbonate solution.

6.7 Polarography

Polarographic studies of hydralazine and related
compounds were reported by Giovanoli-Jakubezak et al (72)
and by Modras (73). The reduction proceeds in two
2-electron stages with the formation of the tetrahydro
derivative. Hydralazine could be determined in the

presence of decomposition products, at a concentration of
about $0.001\underline{M}$ in $1\underline{N}$ hydrochloric acid containing a small
amount of gelatin. The half-wave potentials of the two
waves were -0.7 and -0.95 V against a saturated calomel
electrode.

6.8 Paper Chromatography

Ruggieri (56) and McIsaac and Kanda (38) describe
chromatography on Whatman No. 1 paper with butanol/acetic
acid/water (4:1:5) as the solvent. Detection was by
quenching of the background fluorescence of the paper under
ultraviolet light or by ammoniacal silver nitrate. The R_f
value for hydralazine was 0.90. Several investigators
(41,45,74) have used alkaline systems for paper chromatog-
raphy of hydralazine and its metabolites, but Lesser et al
(75) suggest that alkaline chromatography systems may not
be suitable for hydralazine itself.

6.9 Thin Layer Chromatography

Stohs and Scratchley (53) investigated several
thin layer chromatography systems for thiazide diuretics
and antihypertensive drugs, using silica gel G and a
variety of detection reagents.

Solvent System	Hydralazine R_f
Methyl ethyl ketone/n-hexane (1:1)	0.72
Methyl ethyl ketone/n-hexane (2:1)	0.62
Methyl ethyl ketone/n-hexane (3:1)	0.00
Chloroform/acetone/triethanolamine (50:50:1.5)	0.68

Reagents for detecting hydralazine were Dragendorff's
reagent, alkaline dimethylaminobenzaldehyde, anisaldehyde,
and Bratton-Marshall reagent.

Zak et al (59) used $3\underline{N}$ hydrochloric acid/methanol
ascorbic acid (44:6:1) on silica gel and found an R_f value
of 0.53 for hydralazine.

Lesser et al (75) used cellulose sheets with
fluorescent indicator, acetic acid/$0.01\underline{M}$ aqueous disodium
edetate (3:97), and observed an R_f value of 0.78 for
hydralazine. On silica gel with fluorescent indicator, the
R_f value was less than 0.05 with (a) chloroform/n-heptane/
acetic acid (6:4:1), and (b) cyclohexane/chloroform/
acetonitrile (1:2:1).

6.10 <u>High Pressure Liquid Chromatography</u>

Smith et al (76,77) analyzed hydralazine in a drug mixture containing hydralazine hydrochloride, hydrochloro-thiazide, and an impurity derived from the latter. The column was 1 m x 2.1 mm (ID) stainless steel, packed with a strong anion exchanger on 30 μm Zipax®. The mobile phase was pH 9.2 borate buffer containing 0.005$\underline{M}$ sodium sulfate (5% methanol), at 1.7 ml per minute. Detection was by ultraviolet absorption at 254 nm.

Honigberg et al (78) tested reversed-phase chromatography for separation of a number of drugs, including hydralazine. The columns contained either octadecyltrichlorosilane or diphenyldichlorosilane, bonded to 37 to 50 μm pellicular silica packing. Of the numerous mobile phases tested, the best for separating hydralazine, hydrochlorothiazide, and reserpine was acetonitrile/0.1% ammonium acetate (20:80), pH 7.35. The columns were 1.22 m x 2.3 mm (ID) and the flow rate was 1.4 ml per minute. Detection was by ultraviolet absorption at 254 nm.

6.11 <u>Gas Chromatography</u>

Jack et al (79) determined hydralazine in plasma. The sample was treated with nitrous acid, which reacts with hydralazine to form tetrazolo[1,5a]phthalazine (2). The derivative was extracted with benzene and determined by gas chromatography. 1-Hydrazino-4-methylphthalzine was used as an internal standard.

The same procedure, or modifications of it, was used by Zak et al (80), Talseth (42,82,82,83), and Haegele et al (46) for metabolic studies. Zak et al (80) point out that hydrolysis of conjugates of the drug may cause analytical results on biological samples to be variable, depending on the acid concentration during derivatization, and that selective analysis for unchanged hydralazine and acid-labile metabolites can be carried out by suitable adjustment of the acid concentration.

Smith et al (84) determined hydralazine in tablets. An aqueous extract of the tablets was treated with 2,4-pentanedione, forming 1-(3,5-dimethylpyrazole) phthalazine. The method was applied to stability studies of tablets subjected to elevated temperatures, where tablets could not be analyzed by the USP method.

7. ACKNOWLEDGMENTS

Table 2

GAS CHROMATOGRAPHY SYSTEMS USED FOR DERIVATIZED HYDRALAZINE DETERMINATIONS

Reference Number	Column	Carrier Gas	Column Temp., °C	Detector
(79)	1.5 m x 2 mm ID, glass, packed with 3% OV-225 on 80-100 mesh Chromosorb W-HP	N_2, 30 ml/min.	220°C	Pulsed electron capture (Ni-63, 10 mCi)
(80)	1.2 m x 2 mm ID, glass, packed with 3% OV-225 on Gas-Chrom Q	He, 35 ml/min.	220°C	Electron capture
(46)	1 m x 4 mm ID, glass, packed with 3% OV-17 on 80-100 mesh Chromosorb W	He, 40 ml/min.	Programmed, at 4°/min., 170-240°C	Mass spectrometer
(84)	1.8 m x 4 mm ID, glass, packed with 10% SE-30 on 80-100 mesh Gas-Chrom Q	N_2, 55 ml/min.	200°C	Flame ionization

interpretation, the library staff (Ms. J. McDonough and
Ms. G. Smith) for the literature search, and Mrs.
S. Willette for typing the profile.

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CALCIUM LEUCOVORIN

Leslie O. Pont, Andrew P. K. Cheung, and Peter Lim

1.   Description

1.1   Name, Structure, Empirical Formula, and Molecular Weight

Citrivorum factor (CF), folinic acid, and leuco-
vorin are all N-[4[[(2-amino-5-formyl-1,4,5,6,7,8-hexahydro-
4-oxo-6-pteridinyl)methyl]-amino]benzoyl]glutamic acid, I,
the Leuconostoc citrovorum 8081* growth factor first reported
by Sauberlich and Baumann.[1] Leucovorin refers to the chem-
ically synthesized material that contains both dL and lL
diastereomers; "citrovorum factor" and "folinic acid" gen-
erally apply to the biologically synthesized lL isomer.
Much of the data reported here was obtained on calcium leu-
covorin, the stable, isolatable, biologically compatible
salt.

Name	Empirical Formula	Molecular Weight
Leucovorin	$C_{20}H_{23}N_7O_7$	473.449
Calcium leucovorin	$C_{20}H_{21}N_7O_7Ca$	511.513

Calcium leucovorin is also identified by the National Can-
cer Institute number: NSC 3590.
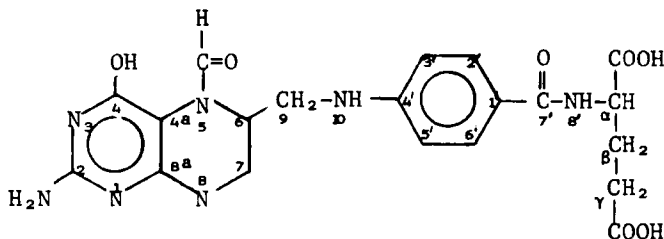
I

Citrovorum factor CF, folinic acid, leucovorin, $5fH_4F$
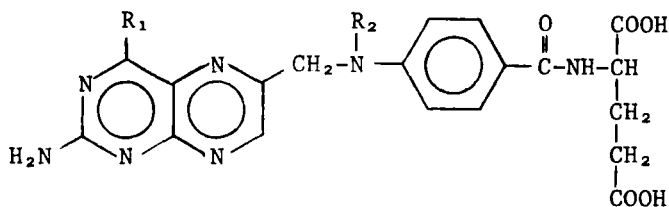
II

IIa	R_1 = OH	R_2 = H	Folic acid, FA
IIb	R_1 = NH$_2$	R_2 = CH$_3$	Methotrexate, amethopterin, MTX
IIc	R_1 = OH	R_2 = CHO	10 formylfolic acid, 10fFA
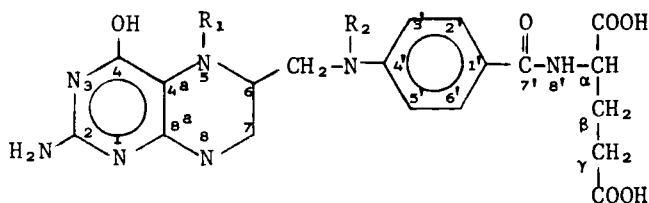
III

IIIa	R_1 = H	R_2 = H	Tetrahydrofolic acid, H$_4$F
IIIb	R_1 = CH$_3$	R_2 = H	5-methyltetrahydrofolic acid, 5mH$_4$F
IIIc	R_1 = H	R_2 = CHO	10-formyltetrahydrofolic acid, 10fH$_4$F

IV
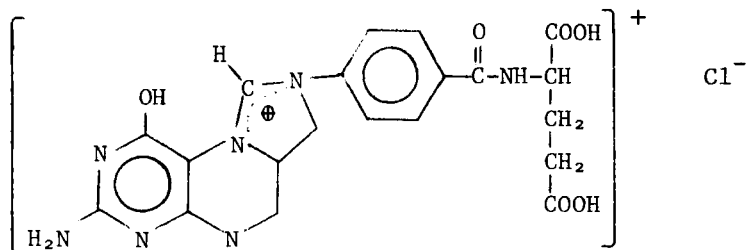
N^5, N^{10}-methenyltetrahydrofolic acid, anhydroleucovorin, 5,10-methenylH$_4$F

V
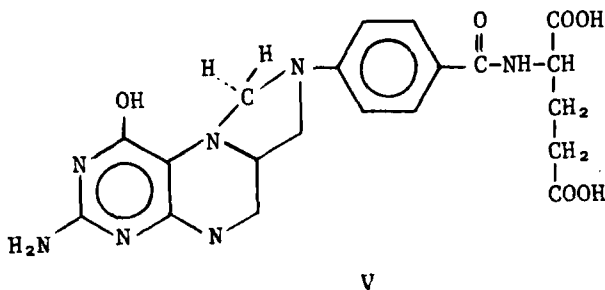
N^5,N^{10}-methylenetetrahydrofolic acid, 5,10-methylene H_4F

1.2 Appearance, Odor, Color

Calcium leucovorin occurs as an amorphous, odor-less powder that is off-white to light beige in color. The salt is not isolated in an anhydrous form, but generally contains 10-15% water (3-5 molecules of hydration).

1.3 Isomeric Forms

Two asymmetric carbon atoms, the α carbon in the glutamic acid portion of the molecule and the C_6 carbon in the tetrahydropteridine ring, allow four possible isomers. Since synthetic procedures would undoubtedly start with L-glutamic acid, the isomeric possibilities are reduced to the dL and lL diastereomers. Of these, the biologically more active form is the lL; separation of the diastereomers is effected by solubility differences of the calcium salts.[2]

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* Reclassified as Pediococcus cerevisiae.

Although a pure dL sample had not been obtained, a sample
possessing a high positive rotation and lower biological
activity had been isolated.[2]

2.   Physical Properties

   2.1   Infrared Spectrum

        Figure 1 is the infrared spectrum taken from a
mineral oil suspension of a representative sample of cal-
cium leucovorin.   A common feature of folate derivative ir
spectra obtained in this laboratory is the absence of sharp
absorption bands, which is usually attributed to lack of
crystallinity.   Below are listed the major absorption
assignments:

Ir absorption (μ)	Assignment
$\sim$ 3.0	H_2O, N-H(2)
6.0-6.7	H_2O, amide I, aryl systems, CO_2^-, amide II
$\sim$ 7.1	CO_2^-
11.9	(tentative)
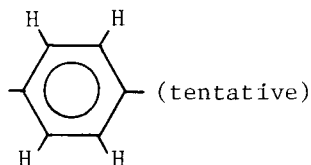
   2.2   Proton Magnetic Resonance Spectrum

        Due to the ionic nature of calcium leucovorin, an
[1]H nmr spectrum could not be obtained in an aprotic solvent;
therefore, the spectrum was obtained from a deuterated
aqueous solution.   The following assignments have been made
relative to TMS = 0.00 ppm from a 100-MHz Varian XL100 spec-
trum (Figure 2):

Assignment	Chemical Shift (ppm)	Multiplicity	J (Hz)
H β, γ	1.70-2.60	m	
H 7, 9	2.95-3.80	m	
H α	4.00-4.60 (obscured by HOD)	q	
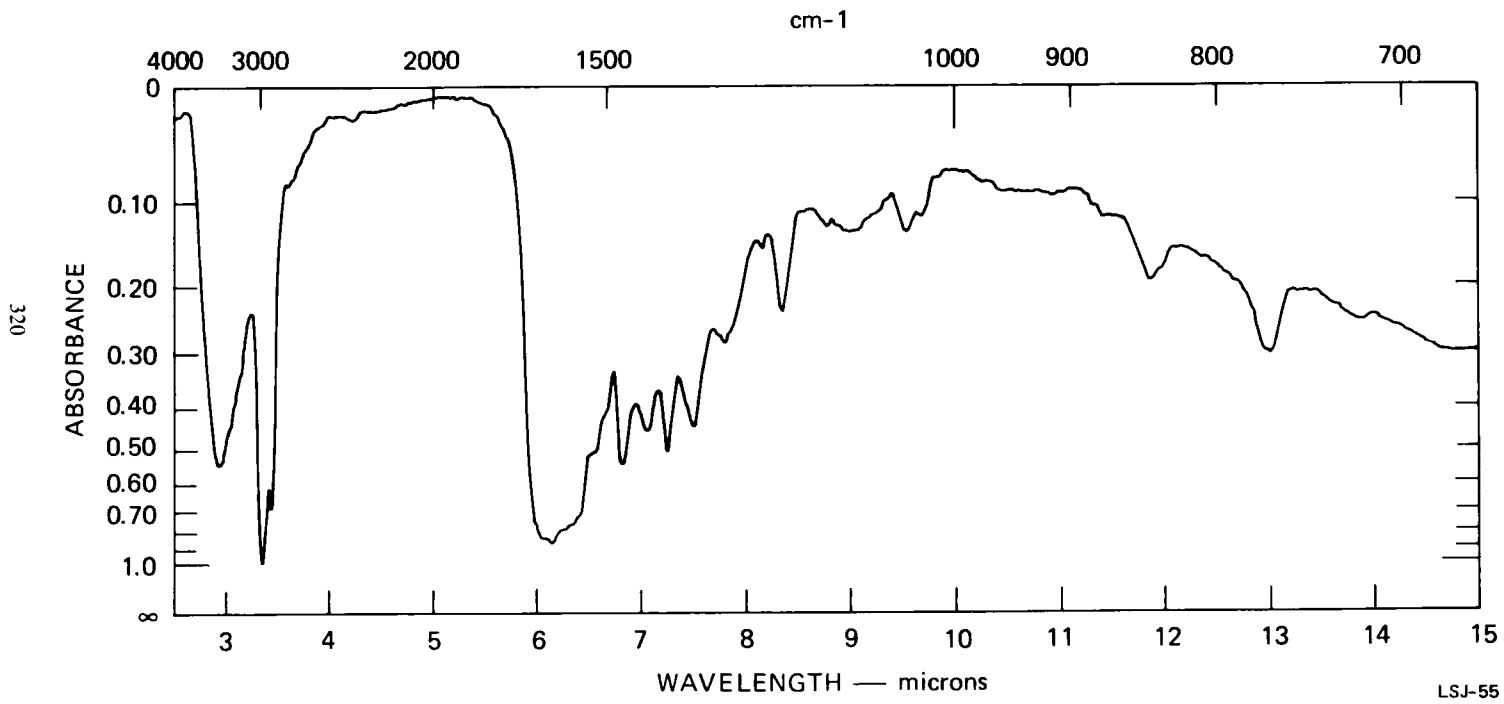
FIGURE 1   INFRARED SPECTRUM OF CALCIUM LEUCOVORIN

LSJ-55
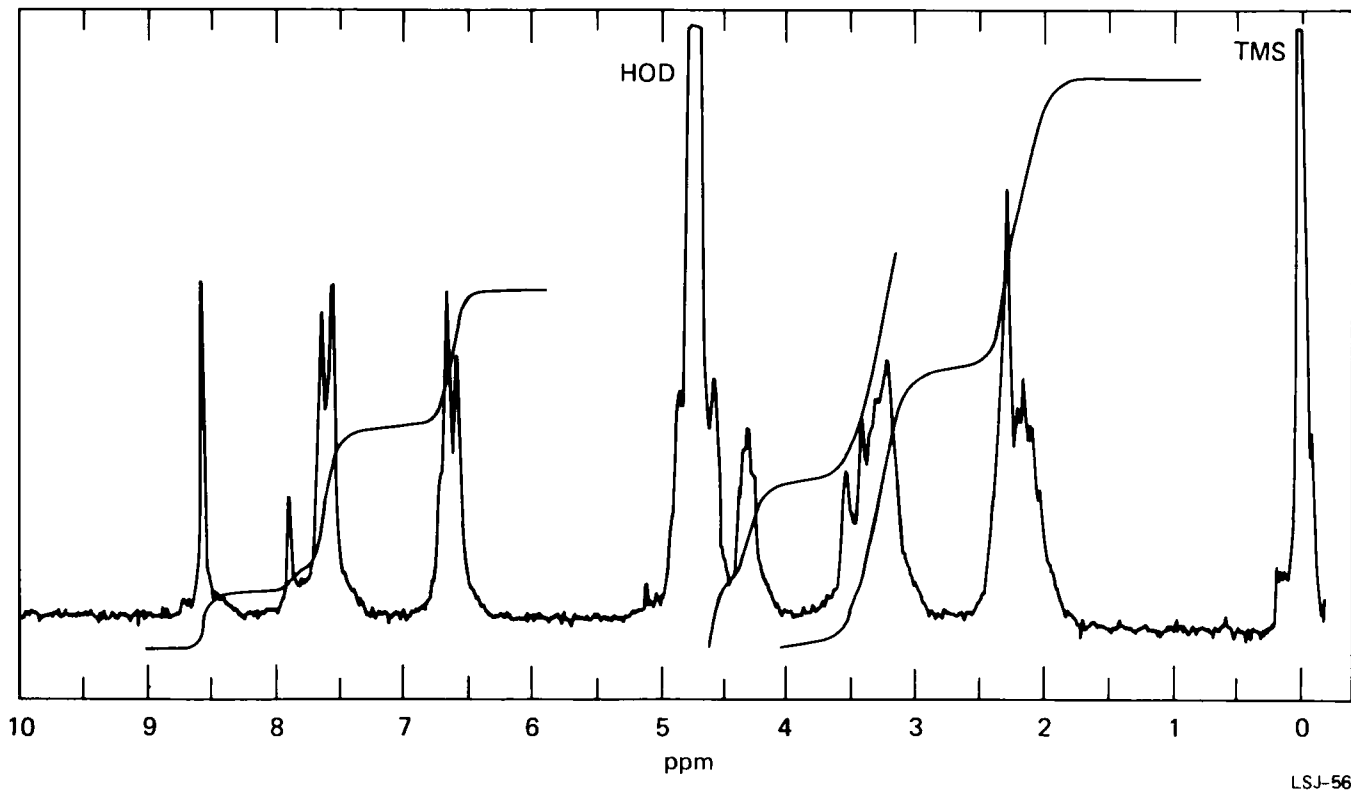
FIGURE 2 ^{1}H NMR OF CALCIUM LEUCOVORIN

LSJ-56

Assignment	Chemical Shift (ppm)	Multiplicity	J (Hz)
H 3', 5'	6.64	d	8
H 2'c 6'	7.60	d	8
$\overset{\overset{O}{\|\|}}{H-C-},5$	8.59	s	
unknown	7.89	s	
H, 6	∿ 4.8	m (broad)	

The above assignments are in general agreement with those of Pastore[3] for folates. The C_6 proton is obscured by the large HOD peak, and its location was confirmed by shifting the HOD absorbance at 80° C. The peak at ∿ 7.9 ppm due to an unknown species has been present in all calcium leuco-vorin samples. The identity of the species responsible for this singlet remains unknown, but at the present time these authors hypothesize that the dL diastereomer's formyl group contributes this peak. Since chemically prepared calcium leucovorin is assayed at this laboratory, an ^1H nmr spec-trum has not been obtained on a biologically or enzymati-cally prepared material. Either should be folinic acid and therefore have the 1L configuration. Investigations to resolve this problem are continuing.

2.3   Carbon-13 Magnetic Resonance Spectrum

The ^{13}C nmr spectrum reproduced in Figure 3 was obtained from a $D_2O/NaOD$ solution of calcium leucovorin on a Varian XL100 Spectrometer. The assignments on the TMS scale and referenced to dioxane are listed below:

Assignment	Chemical Shift (ppm)
C-2	93.7
C-4	102.0
C-6	0.0
C-7	- 23.3
$\overset{\overset{O}{\|\|}}{-C-H}$	98.2
C-9	- 25.0

DIOXANE

+120 +100 +80 +60 +40 +20 0 −20 −40 −60
ppm

LSJ-57

FIGURE 3 ^{13}C NMR OF CALCIUM LEUCOVORIN

Assignment	Chemical Shift (ppm)
C-4a or C-8a	25.2
C-8a or C-4a	86.5
C-1'	54.7
C-2', C-6'	62.4
C-3', C-5'	45.7
C-4'	84.8
C-7'	102.8
C-α	- 10.8
C-β	- 37.9
C-γ	- 32.3
α-COO$^-$	112.6
γ-COO$^-$	115.6

The above assignments are in reasonable agreement with those previously reported for pteridines.[4]

2.4 Ultraviolet Spectrum

Figure 4 represents the uv spectrum of calcium leucovorin in pH 7.0 phosphate buffer (0.10 M), λ_{max} = 286 nm, λ_{min} = 243 nm, and in 0.10N NaOH (pH 13), λ_{max} = 282 nm, λ_{min} = 242 nm. These spectral features are similar to those reported in the literature.[5-12] The most commonly reported absorptivities for leucovorin or folinic acid were obtained from solutions prepared in 0.1N NaOH; these values vary from $\sim$ 2.7 x 10^4 M^{-1} cm^{-1}[7] to 3.26 x 10^4 M^{-1} cm^{-1}[11]. Robinson[12] reports ε_{282} approximately 5% lower (at pH 8.4) than those in basic solution. The single absorption is due to contributions from p-amino-benzoylglutamate and the unsaturated portion of the pteridine ring. Loss of absorbance at shorter and longer wavelengths in basic solution (256 nm and 368 nm) is characteristic of reduced folates. Spectral data obtained from solutions prepared in acids do not reflect leucovorin absorbances because the compound is not stable. Leucovorin dehydrates under acidic conditions to produce anhydroleucovorin, N^5,N^{10}-methenyltetrahydrofolic acid, IV. This compound has been isolated in more than one form, depending on pH.[13]
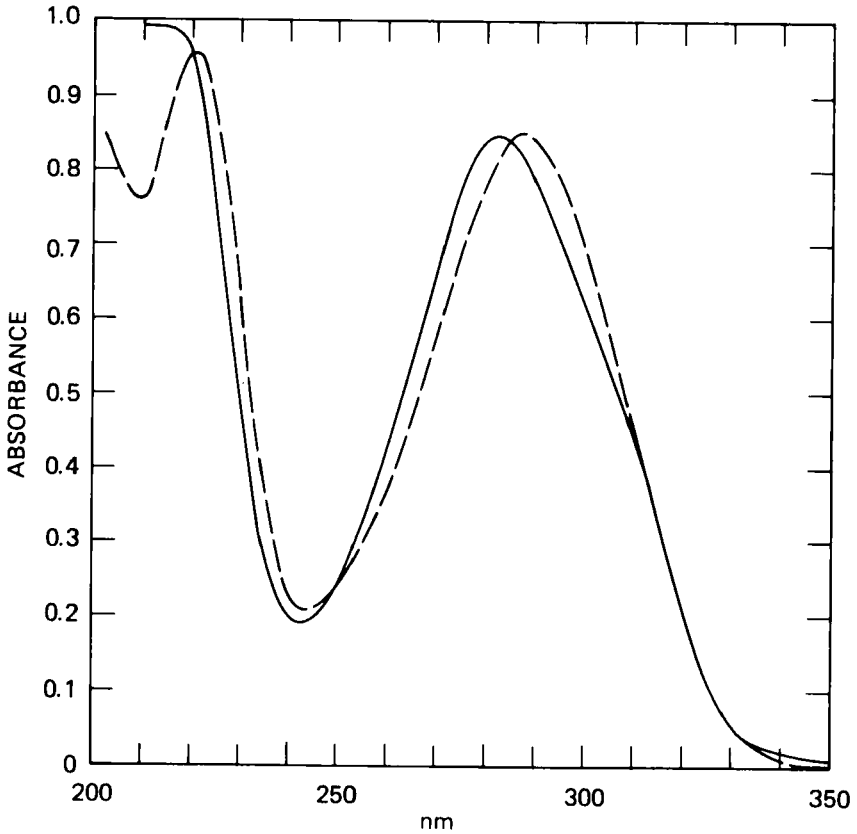
LSJ-58

FIGURE 4   ULTRAVIOLET SPECTRUM OF CALCIUM LEUCOVORIN
0.1N NaOH ————
pH 7 Phosphate buffer —— ——

2.5 Mass Spectrum

An electron impact mass spectrum of calcium leuco-
vorin has not been obtained because the compound is not suf-
ficiently volatile. It would be difficult to isolate the
free acid without first dehydrating the compound. Due to
its ionic nature, calcium leucovorin will not dissolve in
common silylating reagents. Field desorption, another mass
spectral technique, generally lends itself more to compounds
like leucovorin. Indeed, this technique has been applied
successfully to methotrexate and other folic acid analogs.[14]

2.6 X-ray Crystallographic Data

Crystallographic data have not been published for
calcium leucovorin, but literature values do exist for the
barium salt.[7] Diffraction patterns could not be obtained
on all samples although they had been recrystallized sev-
eral times. Interplanar spacings are listed, but these num-
bers appear to change with the amount of moisture present
in the sample.

2.7 Optical Rotation

$$\alpha]_{589}^{21°} = + 14.3 \pm 0.4° \text{ (}\underline{c}\ 1,\ N/10\ NaOH)$$

$$\alpha]_{546}^{21°} = + 17.9 \pm 0.4° \text{ (}\underline{c}\ 1,\ N/10\ NaOH)$$

$$\alpha]_{589}^{21°} = + 14.9 \pm 0.4° \text{ (}\underline{c}\ 1,\ H_2O)$$

$$\alpha]_{546}^{21°} = + 18.8 \pm 0.4° \text{ (}\underline{c}\ 1,\ H_2O)$$

The above values were obtained on an average lot of calcium
leucovorin examined in this laboratory. Corrections were
made for water content. Differences in rotation values may
partly reflect solubility in the two solvents; the compound
was more difficult to dissolve in N/10 NaOH. The H_2O rota-
tion is close to the literature value: $\alpha]_D = + 14.26°$ ($\underline{c}$,
3.42 H_2O).[2]

2.8 Circular Dichroism

The following data were obtained on an average sample of calcium leucovorin:

Solvent	λ_{max} (nm)	$[\theta]$(deg $M^{-1}cm^{-1}$)
0.1N NaOH	282	3.89×10^3
H_2O	287	3.47×10^3

The molar ellipticities are calculated for leucovorin free acid and have been corrected for water content. These values may be quite different from those of folinic acid. Although a single value is reported for each solvent, both spectra contain more than one absorption band. Whether the extraneous absorptions are due to impurities or are actually due to leucovorin's absorption behavior is not known at this time.

2.9 Dissociation Constants

Three pKa values have been reported for leucovorin (free acid); they are 3.1, 4.8, and 10.4, as determined by electrometric titration.[6] The first two values are attributed to the glutamyl carboxyls, and 10.4 is assigned to the hydroxyl group at the 4 position,[8] by comparison to model compounds.

2.10 Solubility

The ionic nature of calcium leucovorin severely restricts its solubility in common organic solvents (solubility in DMSO << 1 mg/ml). In water, the solubility is large ($\sim$ 100 mg/ml), but in 0.1N NaOH it drops significantly (< 20 mg/ml). Acid solubility is difficult to interpret since the solute may no longer be leucovorin.

3. Synthesis

With slight modifications in procedure, one basic synthesis of leucovorin and the subsequent isolation of its salts have remained unchanged for nearly thirty years. The method involves hydrogenation of folic acid (pteroylglutamic acid) in the presence of a platinum or palladium catalyst, as first described by O'Dell et al.[15] This

reduction step is carried on concurrently or after[16-19]
initial formylation of folic acid in formic acid. The pH
of the reaction mixture is adjusted with base in the pres-
ence of an antioxidant and heated (incubated 50-150° C
with or without reduced pressure). Purification by column
chromatography is followed by precipitation of the desired
salt by addition of the appropriate cation and organic sol-
vent. The calcium salt is precipitated from an aqueous
solution by addition of $CaCl_2$ followed by ethanol.[20]

IIIc

incubation
50-150° C

I

CaCl₂ or Ca(OH)₂
Ethanol

Calcium leucovorin
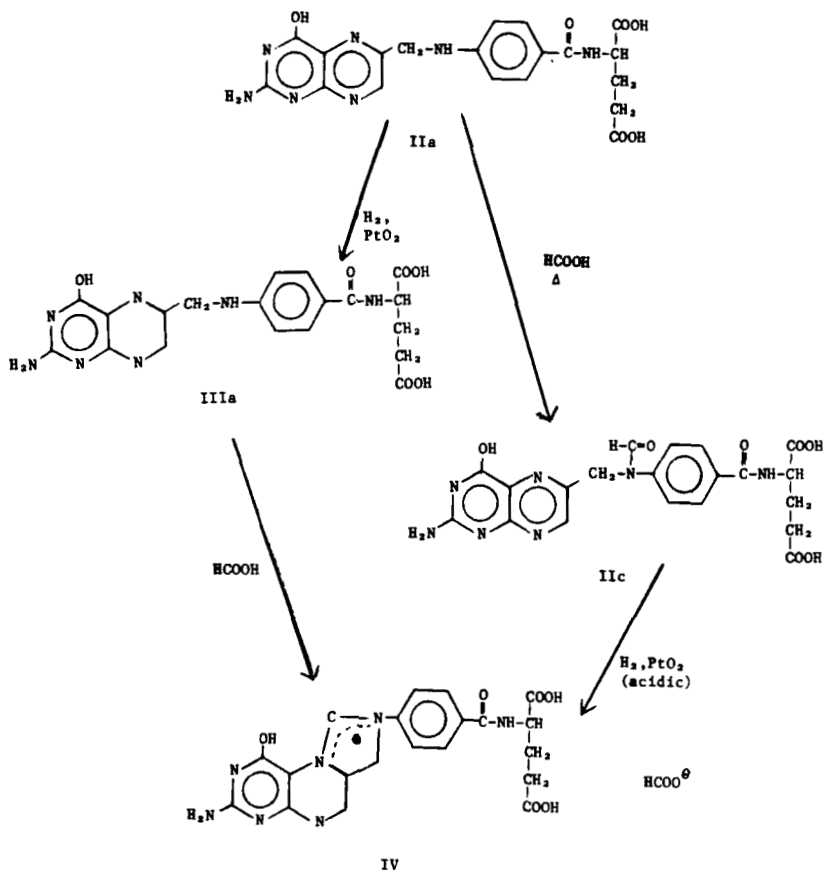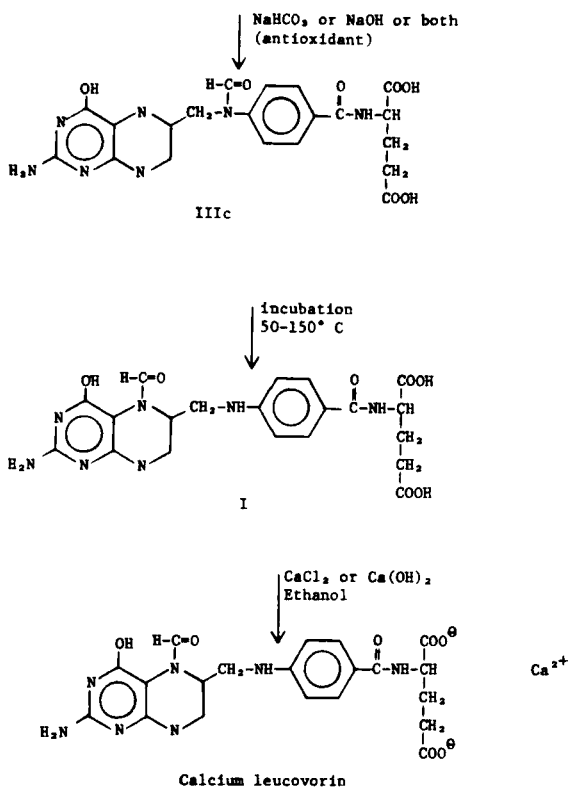
Early syntheses[7,16] employed ascorbic acid as the anti-
oxidant to protect the newly formed tetrahydro ring. But
Roth et al.[9] found that ascorbic acid had no effect on
leucovorin yield (40-50%) as long as incubation was per-
formed under anaerobic conditions. More recent syntheses
have used 2-mercaptoethanol[21] or a stream of nitrogen[22] to
to protect the tetrahydro system.

    Isolation of the active component is carried out chro-
matographically. Roth et al.[9] used a magnesol (magnesium
silicate) column to absorb colored impurities, followed by
a Darco G-60 activated carbon column to eliminate sodium
formate and inorganics. Elution with an alcohol/ammonia
solvent was followed by rechromatographing in magnesol.

Flynn et al.[6] used a potato starch column, elution with
n-butanol/water/ethanol/glacial acetic acid, 100/50/40/
0.4, followed by a Florisil column, treated with pH 5.5
acetate buffer, $CaCl_2$ and ascorbic acid and elution with
distilled water. Subsequent recrystallizations were made
from water. Other authors prefer ion-exchange cellulose
chromatography. Zakrzewski and Sanson[21] use DEAE cellu-
lose in the OH⁻ form, eluting with a 2-mercaptoethanol/
ammonia gradient. Beavon and Blair[22] recommend 1-mm
cellulose plates developed in n-propanol/NH_4OH/H_2O,
200/1/99.

The chemical synthetic methods of obtaining leuco-
vorin or its salts are discussed above, but of course the
originally identified material came from biological syn-
thesis of citrovorum factor in animal liver.[1] Early iso-
lation techniques included electrolysis of liver concen-
trates in the presence of acetic acid.[23] Folinic acid
synthesis has been reported in a variety of biological
systems, including rat liver,[24] avian liver homogenates,[25,26]
microorganisms, (i.e., bacteria[27,28,29] and virus[30]) and
plants.[31] Factors affecting synthesis (e.g., temperature,
pH, antioxidants) are discussed in several of these papers.
In living systems, folinic acid can be synthesized ulti-
mately from folic acid by reduction to tetrahydrofolic
acid followed by addition of a 1-carbon fragment to the
molecule (N^5,N^{10}-methylenetetrahydrofolate, V). After a
2-step oxidation, the formyl group resides either at the
N^5 or N^{10} position or as an equilibrium mixture. The
essential reactions are summarized below:[32]

$$\text{Folic acid} \xrightarrow{\text{H}} \text{dihydrofolate} \xrightarrow[\substack{\text{dihydrofolate} \\ \text{reductase}}]{\text{H}} \text{tetrahydrofolate}$$

+ serine

N^5,N^{10}-methenyltetrahydrofolate $\xrightleftharpoons[\text{NADPH}]{NADP^+}$ N^5,N^{10}-methylenetetra-
hydrofolate + glycine
"active formaldehyde"[33]

N^5-formyltetra- ↔ N^{10}-formyl-
hydrofolate   tetrahydrofolate

NADH NAD⁺

N^5-methyltetrahydrofolate
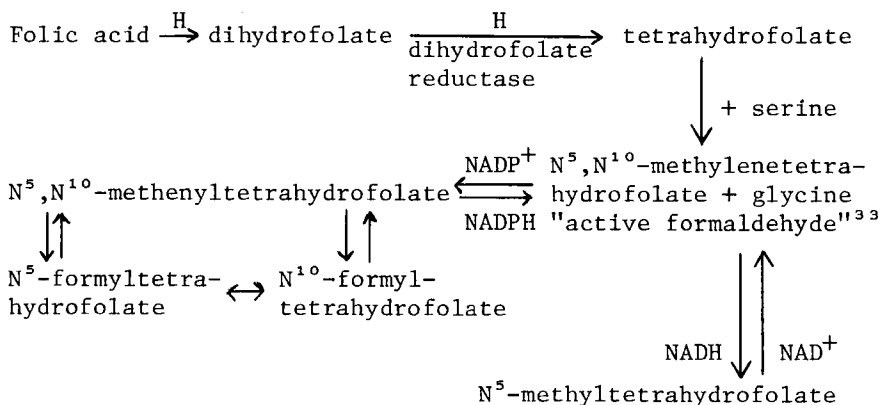
Recently, the enzymatic formation of folinic acid has been utilized to synthesize radioactively labeled products.[34]   The preparation of 5-formyl tetrahydrofolate, 9,3',5'-^{3}H and 5-formyl-^{14}C-tetrahydrofolate starts with tritiated folic acid, which is reduced to dihydrofolate, incubated in the presence of formaldehyde, dihydrofolate reductase, and NADPH, and finally incubated with 5,10-methylenetetrahydrofolate dihydrogenase. The product, N^5,N^{10}-methenyltetrahydrofolate (with ascorbic acid) was adjusted to neutral pH, autoclaved, and stored at $-20°$ C prior to column purification on DEAE and G-15 Sephadex. These labeled products are the biologically active diastereomers, and they are used to study the metabolism of folinic acid in cells, tissues, and animals.

4.   Stability

    4.1 Bulk

        Calcium leucovorin is stable in bulk form after four weeks' storage at $60°$ C. The stability was monitored by HPLC in this laboratory, using the conditions discussed in Section 6.6.4.

    4.2 Solution

        Citrovorum factor has been reported to be stable under severe alkaline conditions by Broquist et al.[35] and earlier by Lyman and Prescott.[36]   Steam-heating for 30 minutes in 0.2N NaOH did not decompose the compound. Alkaline stability is not surprising when one considers that many of the synthetic procedures require autoclaving at high pH as a final step in converting anhydroleucovorin. Investigations conducted in this laboratory in 0.1N NaOH (pH $\sim$ 13) and in deionized water (pH $\sim$ 6 show leucovorin to be stable at room temperature and under laboratory illumination for at least 24 hours (concentration 1 mg/ml).   Stability was monitored by high-pressure liquid chromatography using conditions found in Section 6.6.4. As stated previously, leucovorin is not stable under acidic conditions.[13]   Depending on the pH, different forms of anhydroleucovorin have been isolated: pH $\leq$ 1.3 isoleucovorin chloride; pH = 2 anhydroleucovorin A; pH = 4 (hot) anhydroleucovorin B. All three forms convert back to leucovorin when dissolved in NaOH under anaerobic conditions.

5.   Metabolism

Citrovorum factor functions (primarily) as a 1-carbon donor in the synthesis of serine from glycine.[37-40] The N^5 formyl group is incorporated from the 5,10 methylene compound.[33,39] Silverman et al.[41] have shown that the citrovorum factor, when in the presence of a large excess of glutamic acid and a hog liver enzyme, loses its formyl group to form the N-formylglutamic acid and tetrahydrofolic acid. These authors dismiss the possible formation of 10fH₄F or 5,10-methenyl H₄F in their reactions. However, Peters and Greenberg[42] reported separating an enzyme from sheep liver, citrovorum factor cyclohydrase, which converted 5fH₄F to a compound much like, but not identical to, 5,10-methenyl H₄F. Anhydroleucovorin, the 5,10-methenyl H₄F compound, exists in equilibrium with the 5 and 10 formyl compounds (see biosynthetic scheme). The latter, 10fH₄F, has been reported to donate its formyl group to methionine, which is associated with an esterified species A transfer RNA, which in turn continues in the synthesis of proteins.[43] In the presence of appropriate enzymes, formyltetrahydrofolates donate formyl groups for purine synthesis[44]:
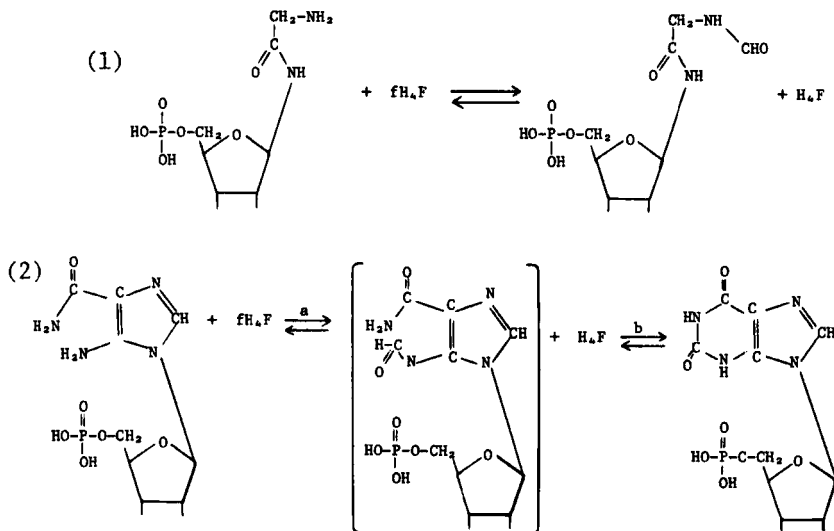
Equation 1 is catalyzed by glycinamide ribotide (GAR) trans-
formylase and Equation 2 is catalyzed by aminoimidazole-
carboxamide ribotide (AICAR) transformylase.

     In humans and rats, early investigations showed that
large doses of folic acid resulted in increased excretion
of a substance that stimulated the growth of P. cerevisiae
and that was presumed to be citrovorum factor.[45] This
response is now also associated with other reduced folates.

     Recently a great deal of effort has been spent in study-
ing the metabolism of leucovorin in vivo. This emphasis
was prompted by the chemical stability of this folate and
by the observation of a reduction in toxicity of methotrex-
ate when it was given in conjunction with leucovorin. Fol-
inic acid is found in human liver, but it is not the major
circulating folate, which is 5-methyltetrahydrofolate.
Most of the folates that occur naturally in man are poly-
glutamates,[46] although they are transported as monogluta-
mates.[47]

     Several groups investigating human metabolism of
folinic acid have used a differential microbiological
method for determining folates in bile, serum, and
urine.[47,49] Using three microorganisms that respond to
different folates, they deduced the identity of some meta-
bolic products. Generally, L. casei responds to all mono-
glutamates, S. faecalis also measures monoglutamates
except $5mH_4F$, and P. cerevisiae growth quantitates tetra-
hydrofolates, again excluding $5mH_4F$. Pratt and Cooper[48]
showed a large increase in L. casei response and a less
pronounced increase in S. faecalis growth in bile and
plasma after oral administration of leucovorin to patients.
Little, if any, increase was noticed in P. cerevisiae.
From these observations they concluded that $5fH_4F$ is
rapidly metabolized to $5mH_4F$, although a very small amount
may be absorbed as the intact 5-formyl compound. Perry
and Chanarin[49] found similar results in urine samples.
With the synthesis of the doubly labeled radioactive com-
pound,[34] 5-formyl-^{14}C-tetrahydrofolate-^{3}H, investigators
have been able to trace the metabolic products when the
drug is administered orally or intravenously.[50]

    Nixon and Bertino have done an extensive study of
folinic acid metabolism in human subjects given the drug
both ways. When given orally, after 75 minutes the major-
ity (90%) of ^{3}H in serum was found in 5mH$_4$F, with 20% of
^{14}C associated with the same compound; 8 to 9% of each
label was found as 10fH$_4$F or 5,10-methenyl H$_4$F. Due to
the labile nature of the formyl group, 70% of the ^{14}C was
not absorbed on Sephadex as folate under the chromato-
graphic conditions used, and the authors presumed that
radioactivity had been incorporated into amino acids.
Virtually no radioactive 5fH$_4$F was found. However, the
radioactivity associated with urine (0-1 hour after admin-
istration) was ∿ 40% ^{3}H-labeled 5fH$_4$F. Less than 20% of
the remaining ^{3}H was identified as 5mH$_4$F or p-aminoben-
zoylglutamate, less than 40% as 10fH$_4$F or 5,10-methenyl H$_4$F.
The ^{14}C was excreted as a mixture of the three folates. At
longer time intervals, these proportions varied. Ninety
minutes after injection, 60% ^{3}H was found as 5mH$_4$F, 40% of
radioactivity as 5fH$_4$F, and 40% ^{14}C was no longer assoc-
iated with serum folate. Urine showed most radioactivity
as 5fH$_4$F initially, and several hours later a rise was seen
in labeled 10fH$_4$F and/or 5,10-methenyl H$_4$F. From this
series of studies, the authors concluded that most ^{14}C was
removed from circulating folates, most of the ^{3}H was assoc-
iated with 5mH$_4$F, most of the drug was absorbed by tissues,
and the majority of 5fH$_4$F was converted to 5mH$_4$F. Equally
important was the site of metabolism. Since the 5f → 5m
conversion was more rapid in patients receiving oral admin-
istration, the authors felt that the reaction took place
in the intestine, a view supported by others.[51] The
voided folates seemed to be 10fH$_4$F or 5,10-methenyl H$_4$F.
Another interesting suggestion was that 5mH$_4$F was con-
served over 5fH$_4$F by the kidneys.

    Although leucovorin is rapidly absorbed and metabo-
lized in the intestine, its metabolic product, 5mH$_4$F, is
concentrated in cerebrospinal fluid (CSF). In a study
with dogs, Levitt et al.[52] found that injected labeled
folates disappeared from serum and appeared as 5mH$_4$F in
CSF following first-order kinetics. With doubly labeled
5fH$_4$F, the authors were able to trace the ^{14}C formyl
moiety to serine or methionine and the ^{3}H portion to
5mH$_4$F. The uptake of drug occurred in two steps:
a) equilibration in extracellular fluid with nonlabeled

compound,[53] followed by b) cellular uptake and metabolism.
Early papers reported a natural concentration of folate in
CSF that was three times the equilibrium concentration in
serum. This could indicate the importance of folates in
neural metabolism, and there have been some reports regard-
ing the therapeutic treatment of neuropsychiatric disorders
with folates.

The recent interest in leucovorin is due to its ability
to reduce methotrexate (MTX) toxicity when both are admin-
istered to cancer patients. Early studies[54] in mice showed
that most benefit occurred when leucovorin was given 12-24
hours after MTX infusion. The delay in administering leu-
covorin reduced the toxic effects of MTX without reducing
the latter's antitumor activity. Therefore, an enhance-
ment of MTX effect can be produced by larger dosages with-
out the toxicity associated with these amounts. When given
concurrently, leucovorin reduced MTX toxicity at the ex-
pense of tumor activity. However, recent work[55] has shown
some advantage in concurrent administration. In the case
of MTX-sensitive cells, "folinic acid protection" increases
the cell survival percentage. Rescue procedure, or leuco-
vorin given after a delay period, was most effective (mea-
sured in percentage of cell survival) in treating MTX-
resistant cells. The therapeutic effect of leucovorin
lies in an earlier resumption of DNA synthesis than if MTX
were administered alone.[56] Nahas et al.[57] attribute this
resumption to several possible actions of leucovorin (as
studied in L1210 leukemia cells): a) efflux of MTX is
increased in the presence of $5fH_4F$; b) $5fH_4F$ could deblock
dihydrofolate reductase bound by MTX by being a potential
supply of dihydrofolate; c) the intact drug could possibly
displace some of the MTX; and d) $5fH_4F$ competes with MTX
for cellular uptake. Cellular uptake seems to be enhanced
by methyl or formyl groups at N^5 on reduced folates, by
amino substitutions on C_4 of oxidized folates, and by ter-
minal glutamates.[47] Thus $5fH_4F$, $5mH_4F$, and MTX appear to
use the same carrier-mediated transport into cells, a sys-
tem used by folinic acid to a lesser extent. Another
author[58] describes the transport of folates across mem-
branes as an exchange phenomenon because although leuco-
vorin competes with MTX for cell uptake, it can also stim-
ulate influx of the latter if the cells are already

preloaded with folinic acid. The influx is noted even in
cases where dihydrofolate reductase has been inactivated
by previous MTX dosage.

6.   Methods of Analysis

     6.1 Elemental Analysis

          Below are listed elemental analysis results
obtained from a typical sample of calcium leucovorin:

Element	% Theoretical	% Found[*]
C	46.96	46.87
H	4.14	4.11
N	19.17	19.07
Ca	7.84	6.94[†], 7.71[‡]

[*] Corrected for 10.4% H_2O.
[†] Found by atomic absorption.
[‡] Found by sulfate ash.

The higher calcium value obtained by the ash method is no
doubt due to the presence of sodium (the counter ion for
acetate commonly found in some lots).

     6.2 Equivalent Weight Determination

          Because synthetic products are isolated as the
barium or, more frequently, the calcium salt of leucovorin,
common acid-base titrations are not reported. If this type
of titration or one in which the cation is exchanged were
feasible, the results would require careful interpretation
because impurities containing the glutamic acid moiety
would respond similarly to leucovorin when the carboxyl
groups are being analyzed.

     6.3 Biological Assay

          6.3.1 Microbiological Assay

               Several microbiological procedures for
assaying CF have been described in the literature. In
general, these methods are designed to measure CF content
in a biological fluid or specimen. They all make use of
the growth promotion of L. citrovorum 8081, or P. cere-
visiae, as it has been renamed. Before CF was isolated

or leucovorin was synthesized, Sauberlich and Baumann[1] measured enhanced growth of the microorganism in the presence of various biological extracts, especially liver concentrates. Quantitation came from turbidometric readings and/or NaOH titration of acid produced. Their results are reported in terms of citrovorum units that corresponded to the half-optimal growth in a standard reticulogen sample. The authors realized the limits of their assay method since folic acid additions caused enhanced growth after an initial lag. Winsten and Eigen[59] tried to improve the method by chromatographing the samples on paper before testing for P. cerevisiae growth, but they, too, found more than a single active component. The basis of this particular technique is still being used. Cooperman[60] refined the procedure somewhat, but continued to measure turbidity. Folic acid interference is minimized by incubation for short periods. Quantitation is in terms of mg/ml, using commercial samples of leucovorin as standards. In one recently published method,[61] paper discs are impregnated with the test solutions and CF content is found by manually measuring L. citrovorum ATCC 8081 growth on agar plates. The advantage of this method is that CF can be quantitated in solution with a large excess of amethopterin because the microorganisms are resistant to the latter. Statistical variation on this method is $\sim 10\%$.

6.3.2 Enzyme Assay

Silverman et al.[41] purified a hog liver enzyme that catalyzed the transfer of the formyl group of CF to glutamic acid. If not protected from oxidation, the tetrahydrofolic acid formed would spontaneously decompose to p-aminobenzoylglutamate and pteridine. Arylamine formation could be monitored by the Bratton-Marshall method. This method gives a better indication of enzyme activity than CF purity. The technique developed by Peters and Greenberg,[42] later refined by Greenberg,[62] is perhaps more straightforward. The test solutions are mixed with ATP, $MgSO_4$, and enzyme isolated from sheep liver in a buffered aqueous solvent. Absorbance at 343 nm is read in a spectrophotometer at 30° C. The 5,10-methenyltetrahydrofolate being formed should give a direct measure of the CF or leucovorin present.

The above biological assays are intended to measure
CF in biological sources rather than to directly measure
leucovorin or CF purity. In fact, commercial samples of
leucovorin are usually used to determine standard curves.

6.4   Polarographic Assay

Leucovorin, since it is totally reduced, is
polarographically inert in a pH 9 buffered solution.[63]
After acid treatment, three polarographic waves are
generated, corresponding to an anodic oxidation of a tet-
rahydro compound and two cathodic reductions of unreduced
pteridines; presumably at least one of these three is a
dihydro species. Polarography is useful as a technique in
structural elucidation, but analytical data would be dif-
ficult to obtain from an acid-treated solution containing
several species, each with its own polarographic behavior.

6.5   Spectrophotometric Analysis

6.5.1   Fluorometric Analysis

In 1957, Duggan et al.[64] reported that
maximum natural fluorescence of folinic acid occurred at
pH 7, with excitation at 370 nm and emission at 460 nm.
A concentration of 0.15 µg/ml gave a fluorescence of 10%
full-scale deflection at maximum instrumental sensitivity.
These authors explored analyzing folinic acid in the pres-
ence of folic acid and found that excitation at 290 nm
effectively shifted the emission band of the compound of
interest to 370 nm, thus enabling analysis of a mixture.
A later paper[65] reported a fluorescence maximum for leuco-
vorin at 365 nm when excited at 314 nm in a pH 7 solution;
the concentration was 5×10^{-5} M. Variation between these
data and other values was attributed to sample impurity,
pH of solution, and quenching. The authors made an attempt
to correlate structure and fluorescence of reduced folates.
Similarity between tested compounds and p-aminobenzoyl-
glutamate lead them to conclude that this portion of the
molecule is responsible for maxima at 360–425 nm when
excited at 300–320 nm. They suggested that intensity dif-
ferences may arise from various substitutions on the tetra-
hydropteridine moiety.

In 1964, Netrawali et al.[66] described fluorescence
measurements of calcium leucovorin in $NaHCO_3$ solution after
paper chromatography and reaction with acidic orcinol. The
gray-blue fluorescent species formed on the developed paper-
gram was very sensitive for leucovorin, showing a detection
limit of 0.1 µg. In the eluting solution, however, the
required concentration increased to 0.5 µg. Recovery by
this method was 100 ± 15%, and results obtained from fluor-
escence were lower by 10 to 20% than those obtained by
microbiological methods.

6.5.2   Ultraviolet/Visible Analysis

Ultraviolet spectroscopy does not lend
itself to leucovorin analysis for two reasons. First,
because commercial samples are frequently contaminated with
uv-absorbing impurities, a reliable molar absorptivity has
not been determined for leucovorin. Recently, in this
laboratory a value of 3.09×10^4 M^{-1} cm^{-1} was derived from
thorough analysis of a relatively pure sample. This value
is in reasonable agreement with that of Zakrzewski and
Sanson.[21]

Second, leucovorin is known to dehydrate under acidic
conditions to form anhydroleucovorin, 5,10-methenyl H_4F,
which absorbs at 352-353 nm. In the absence of inter-
fering species,* leucovorin may be analyzed by acidifica-
tion with 0.1N HCl followed by uv measurement after 1.5-
2.0 hours. Purity may be determined relative to a sample
of known purity or relative to literature values:
$\varepsilon_{352} = 2.39\text{-}2.41 \times 10^4$ M^{-1} cm^{-1}.[21]

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*
Absorption interferences would arise from compounds other
than leucovorin that convert to anhydroleucovorin at
acidic pH. An example of such a compound is $10fH_4F$,
although it is not expected to contribute to absorption
due to its instability. However, 10-formyl-7,8-
dihydrofolate is a possible contaminant. Additional
absorption could also result from unsaturated pteridines.

6.6   Chromatography

6.6.1   Paper Chromatography

Both paper and thin-layer chromatography
serve as separation techniques before quantitation of
active components.   Paper chromatography has been used by
many early investigators to separate biological samples
before detecting by microbiological assay, the bioauto-
graphic method.   Winsten and Eigen[59] used 2,4,6-
trimethylpyridine to develop papergrams on Whatman No. 1
or No. 4.   Colón[29] developed his chromatograms in isoamyl
alcohol:5% dibasic sodium phosphate, 1:2.   Netrawali et
al.[66] used ethanol:n-butanol:water:25% NH_3, 50:15:35:5,
on Whatman No. 1 and detected leucovorin by forming a
fluorescent orcinol derivative.   The mobile phases in
these systems are neutral to basic due to the acid
instability of leucovorin.

6.6.2   Thin-layer Chromatography

Thin-layer chromatography on cellulose
has been used as an intermediate clean-up procedure and
final isolation technique in the synthesis of leucovorin.[22]
A cation exchange/cellulose support was prepared by Copen-
haver and O'Brien,[67] and the mobile phase was 15% $Na_2HPO_4 \cdot$
$12H_2O$ (pH 8.5) containing 0.1 M mercaptoethanol.   Unfor-
tunately, this system did not separate 5f and 10fH_4F from
each other or from 5,10-methenyl H_4F.   Separation of the
former two from the latter was achieved with 1% $Na_2HPO_4 \cdot$
$12H_2O$.   Leucovorin was detected with a 6N $HCl/ZnCl_2$/sodium
citrate spray.

Brown et al.[68] have developed a cellulose plate with
a fluorescent indicator.   Compounds are developed in 3.0%
(w/v) NH_4Cl and detected by fluorescence quenching.   These
authors also use 0.5% mercaptoethanol in their mobile phase,
but this is only to prevent oxidation of the labile reduced
pteridines, which are not adequately protected by substi-
tution at the N^5 position.   Since neutral or alkaline solu-
tions of leucovorin are relatively stable in air, this
precaution may not be required for routine assay.

6.6.3 Column Chromatography

Leucovorin has been chromatographed on columns of different packing materials since it was first synthesized. Early investigators used magnesium silicate and activated charcoal,[9] starch,[6] and--more recently-- DEAE cellulose[21] in their purification and isolation of leucovorin. Column techniques have been coupled with various detection methods to identify and quantitate CF and other folates in naturally occurring products. Noronha and Silverman[69] used L. casei, P. cerevisiae, and S. faecalis to detect folates in chicken liver extracts chromatographed on DEAE cellulose. A similar method was used by Rohringer et al.[70] to monitor folates in healthy and infected wheat leaves. An earlier publication[71] reported anion-exchange chromatography on Dowex-1-chloride using uv absorption of collected fractions for detection. Due to its instability in acid, leucovorin was eluted with NaCl rather than HCl, which is commonly used for other folates. This method was limited to synthetic products rather than biological mixtures. The authors felt that sensitivity could be gained by microbiological detection.

More recently, attention has been placed on gel permeation techniques. Sephadex G-15 and G-25 columns coupled with uv detectors were used to separate folates found in rat kidney extracts.[72] Kás and Cerná[73] used Sephadex G-10 to separate folates in cow's milk, utilizing spectroscopic and microbiological detection. Nixon and Bertino[74] used fluorescence ratios and markers on DEAE Sephadex A-25 to separate folate coenzymes. In separating folates from biological sources, several authors treated their samples with the appropriate conjugase to hydrolyze polyglutamates. Radioactivity measurement has been used to detect doubly labeled $5fH_4F$ and its metabolic products.[50]

6.6.4 High-Pressure Liquid Chromatography

A natural extension of column chromatography is high-pressure liquid chromatography, which combines separation, detection, and quantitation (and isolation in the case of preparatory work). Perhaps the single
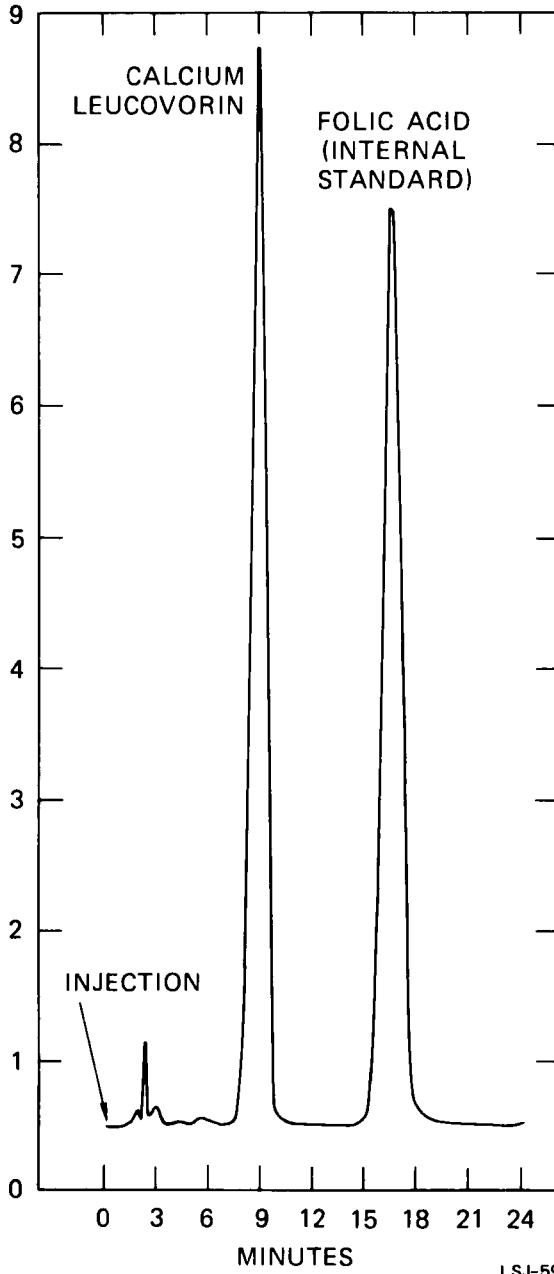
FIGURE 5   HIGH PRESSURE LIQUID CHROMATOGRAM OF
          CALCIUM LEUCOVORIN

most attractive feature of this technique is speed, once
the chromatographic conditions have been determined.
Various modes are available, but folates have been
restricted to ion exchange or partitioning due to limited
solubility in organic solvents. Detection is usually by
uv absorption, although in the case of leucovorin, sensi-
tivity is increased several orders of magnitude by electro-
chemical detection. HPLC has been used routinely in this
laboratory since 1973, and among the compounds analyzed
are folates.[75] The most versatile system that these
authors have found is a chemically bonded C_{18} column with
0.1 M KH_2PO_4 (pH adjusted to 4.0) containing varying
amounts of methanol (see Figure 5). An earlier system
using the same reverse phase C_{18} column with tris buffer,
2-amino-2-(hydroxymethyl)-1,3-propanediol at pH 6.7, was
abandoned in favor of the above phosphate eluent which
better resolves contaminants from the major component.
A similar set of conditions was utilized by Reif et al.[76]
to analyze folic acid, using leucovorin as an internal
standard. Other authors[77] have used similar conditions
to study extensively the identity of possible leucovorin
contaminants. In addition to these partition/ionization
surpression methods, anion exchange hplc has been used to
separate CF from other naturally occurring folates,[78] and
some correlation has been made between the number of
glutamyl residues and retention time.

6.6.5   Affinity Chromatography

Although affinity chromatography has not
been used directly as an analytical method, it may be
modified in the future to produce a viable technique.
Leucovorin has been used as an effective spacer in obtain-
ing active samples of dihydrofolate reductase.[79] If the
enzyme could be immobilized without losing its activity,
perhaps it could be used to separate folates.

6.7   Radioassay

Recently, radioassay methods have been refined
to measure folates in biological samples. These tech-
niques use radioactively labeled folates and competitive
protein binding.[80] Johnson et al.[81] compare this method
with traditional microbiological assay with L. casei.

Although this method was designed for folates in general, differential microbiological testing could give an indication of reduced folate concentration.

7. Acknowledgments

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METHIMAZOLE

Hassan Y. Aboul-Enein and A. A. Al-Badr

METHIMAZOLE

1. Description

    1.1   Nomenclature

          1.11   Chemical Names
                  1,3-dihydro-1-methyl, 2-H-Imidazole-2-thione.
                  1-methylimidazole-2-thiol
                  1-methyl-2-thioimidazole
                  1-methyl-2-mercaptoimidazole

          1.12   Generic Names   Methimazole, thiamazole

          1.13   Trade Names   Thiamethazole, Bazolan, Dananti-
                  zol, Faristan, Frentorox, Mercazole, Metazole,
                  Tapazole, Thacapazol, Thycapazol, Strumazol,
                  Metothyrine.

    1.2   Formulae
          1.21   Empirical $C_4H_6N_2S$

          1.22   Structural
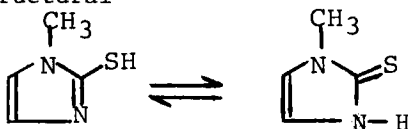
    1.3   Molecular weight   114.17

    1.4   Elemental composition
          C 42.08%,   H 5.30%,   N 24.54%,   S 28.09%

    1.5   Appearance, color, odor
          White to pale buff, crystalline powder, having a
          faint characteristic odor. Its solution is practi-
          cally neutral to litmus.

2. Physical   properties

    2.1   Crystal properties

          2.11   Crystallinity
                  No detailed studies on the crystal structure
                  of methimazole is reported in the literature.
                  Methimazole can be microscopically identified
                  using Kofler's method, the occurrence of poly-
                  morphous modification is indicated in methi-
                  mazole.

2.12 Melting point
USP XIX (1) specifies a melting range for
methimazole between 144 and 147°.

Leaflets from alcohol m.p. 146-148°(2), b.p.
280° (some decomposition).

2.2 Dipole Moments
The dipole moment of methimazole was determined in
benzene and 1,4 dioxane solution at 25° (3) and
reported to be 4.74 D and 5.53 respectively. The
results were discussed in terms of tautomerism and
molecular conformations.

2.3 Solubility
Freely soluble in water, in alcohol and in chloro-
form, slightly soluble in ether, petroleum ether
and benzene.

2.4 Identification
The following tests are cited from USP XIX (1) :-

a) The infrared absorption spectrum of a potassium
bromide dispersion of it exhibits maxima only at
the same wavelength as that of a similar pre-
paration of USP methimazole Reference Standard.

b) Mercuric chloride TS produces in a solution (1 in
200) a white pricipitate, but no precipitation is
produced by trinitrophenol TS. The solution is
coloured intensely blue by molybdo-phosphotungs-
tate TS.

c) Methimazole can be identified by forming crystals
of with gold bromide/HCl solution. Plates, often
in crosses (sensitivity : 1 in 1000); potassium
tri-iodide solution - bunches of rods or needles
(sensitivity 1 in 1000) Fig. 1.

2.5 Spectral Properties

2.51 Ultraviolet spectrum
Methimazole in 0.1N sulfuric acid shows
maxima at 211 nm (E 1%, 1 cm 593) and 251.5 nm
(E 1%, 1 cm 1528). In neutral aqueous solution,
methimazole absorbs ultraviolet radiation at
251.5 nm (Fig. 2). Hayden _et al_ (5) published
a report on the relation between the spectra
and structure of methimazole and some related
compounds. The replacement of an oxygen
atom at C_2 by sulfur causes a big shift to
longer wavelength with increased absorption(6).
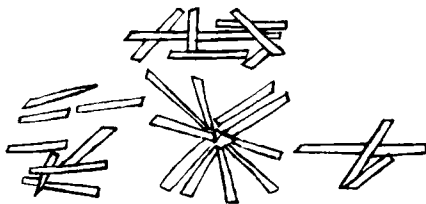
Fig. 1 - Methimazole - KI/I$_2$ crystals.
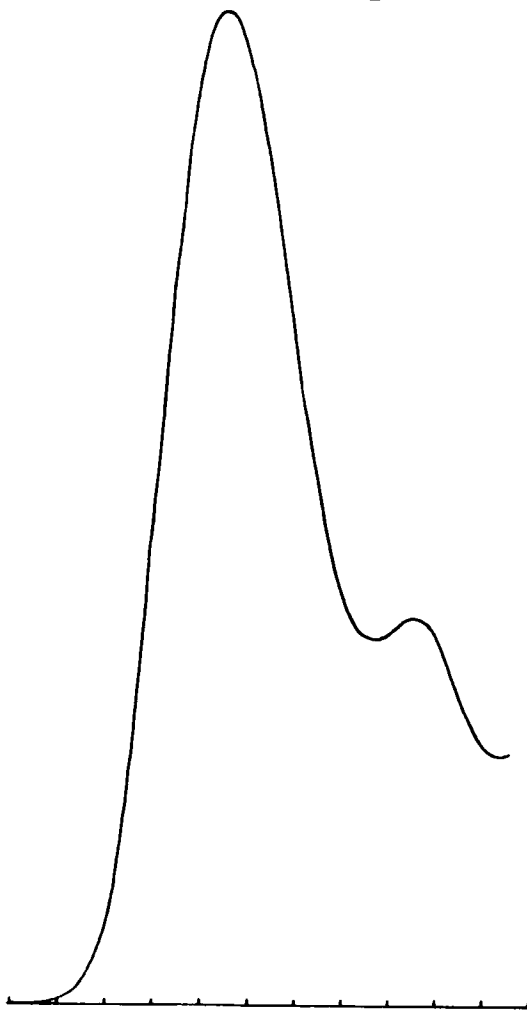
Fig. 2 - Ultraviolet spectrum of Methimazole in methanol.

2.52   Infrared spectrum
       The infrared spectrum of methimazole is shown
       in Fig. 3. The spectrum was obtained from
       Nujol mull.

       The structural assignments have been correlat-
       ed with the following band frequencies:

           Frequency (Cm^{-1})   Assignment

           2500 - 2450 broad weak   - SH

           1580   C = N aromatic

       Other fingerprint bands characteristic to
       methimazole are at 1466, 1570 and 1271 Cm^{-1}
       as shown in Figure 4.

       Further information with regards to the in-
       frared spectra of methimazole is given in
       several references (4,5,7).

2.53   Nuclear Magnetic Resonance Spectrum
       A typical NMR spectrum of methimazole is shown
       in Fig. 5. The sample was dissolved in $CDCl_3$.
       The spectrum was determined on a Varian T-60
       A NMR spectrometer with TMS as the internal
       standard. The following structural assign-
       ments have been made for Fig. 5.

           Chemical Shift (δ)   Assignment

           Singlet at 3.63   N-CH_3

           Singlet at 6.70   aromatic H_4 and
                                     H_5 of the imida-
                                     zole ring system.

       Further information   concerning the NMR
       spectrum of methimazole can be obtained from
       Sadtler NMR catalog (8) and also from CRC
       Atlas of spectral data (7) and Aldrich NMR
       catalog (9).

2.54   Mass Spectrum and Fragmentometry
       The mass spectrum of methimazole obtained by
       electron impact ionization shows a pronounced
       molecular ion M^+ at m/e 114 (Fig. 6).

       Bowie et al (10) studied the mass spectra of
       several imidazoles and   methimazole was
       included. They discussed the fragmentation
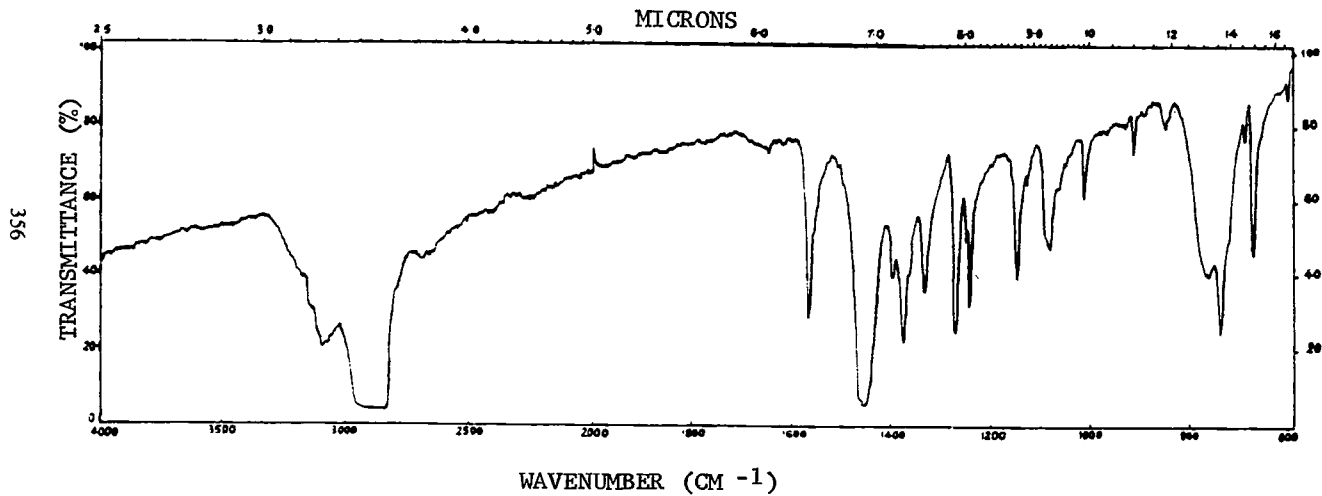
Fig. 3 – Infrared spectrum of methimazole in Nujol mull.
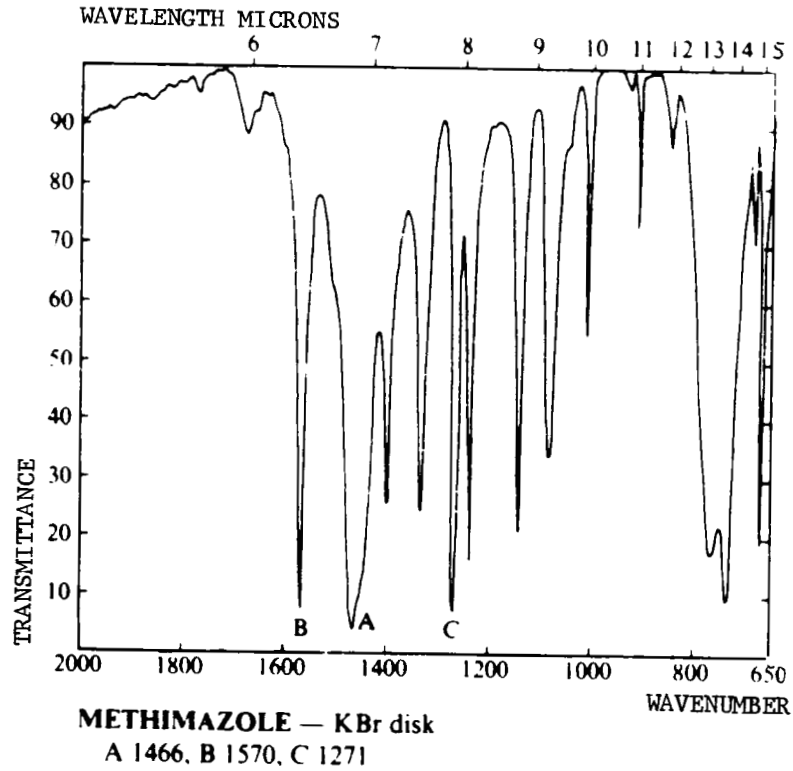
Fig. 4 – Fingerprints bands of methimazole, in KBr.
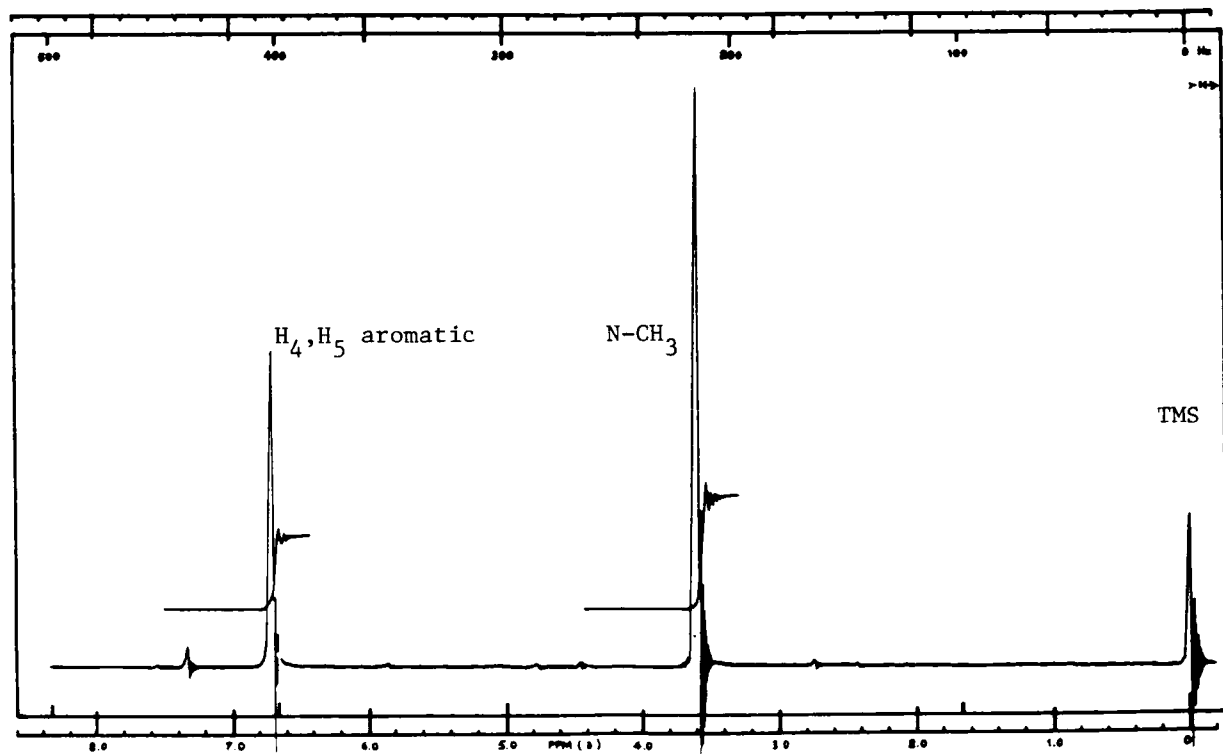
Fig. 5 — NMR spectrum of methimazole in CDCl$_3$ containing TMS as internal standard.

METHIMAZOLE
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SCAN 33 SIGMA=35 RT=0.7 BACK=185 X 100% = 1924000
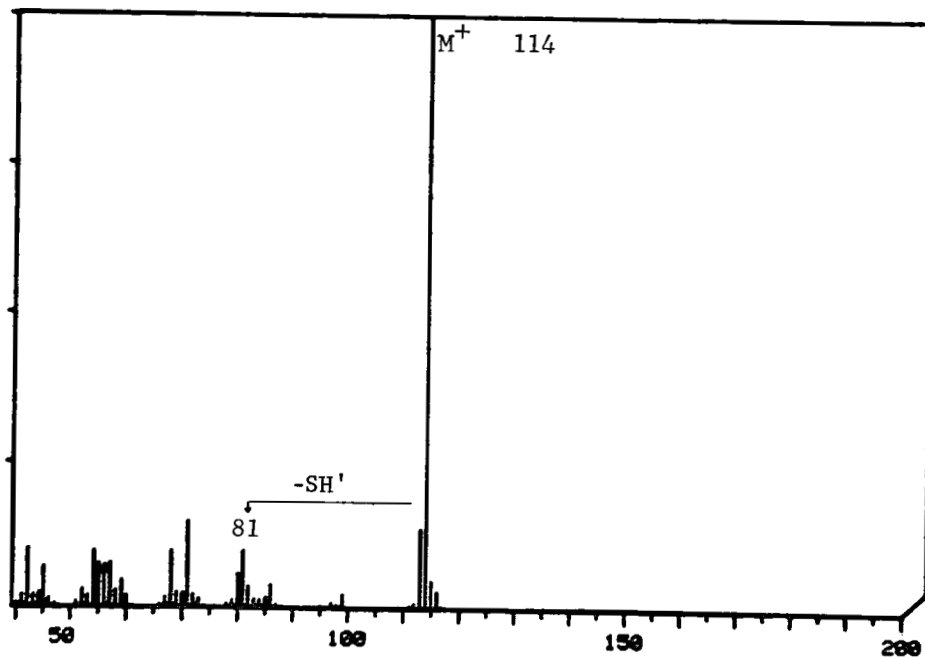
Fig. 6 — Mass Spectrum of Methimazole (EI).

patterns and modes which is substantiated by
deuterium labelling, exact mass measurements
and appropriate metastable ions. Skeletal
rearrangements are rare in the imidazole ring
system.

3. Synthesis
The method of preparation of methimazole illustrates the
general synthesis of 2-mercaptoimidazoles by ring closure
from α -amino-aldehydes or ketones and alkylisothiocyan-
ates. This reaction gives 1-alkyl 2-imidazolethiols, and
the simpler compounds unsubstituted in the 1-position can
be made by the use of simple metallic thiocyanate in
place of the alkyl compound. In the original synthesis
of Wohl & Marckwald (Ber., 22, 1354, 1889) of which a
number of variations have since been introduced. Methi-
mazole is made by condensing methyl isothiocyanate with
amino-acetal, $NH_2-CH_2CH(O\ Et)_2$ (a convenient substitute
for aminoacetaldehyade) and cyclizing the product by
heating with acid. The 2-mercaptoimidazols can be alkyl-

$$S = C = N + H_2N-CH_2CH(OC_2H_5)_2 \longrightarrow S = C \underset{NH-CH_3}{\overset{NH-CH_2-CH(OC_2H_5)_2}{\Big<}} \longrightarrow$$

$$\longrightarrow \quad \underset{N}{\overset{CH_3}{\underset{|}{N}}} SH$$
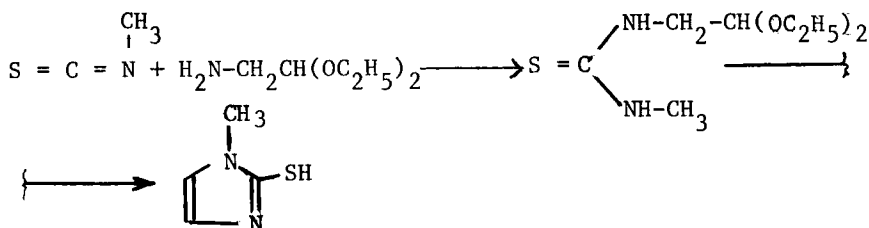
ated or acylated on the sulphur or the second nitrogen
atom or the whole SH group can be removed by oxidation
with HNO_3 to give a simple imidazole.

Methimazole is also prepared (12,13) by the reaction of
$CH_3-NHCH_2-CH(O-Et)_2$ with KSCN in the presence of dil. HCl.
Hydrochloric acid (300 ml., 2N) was added gradually to a
mixture of 72.5 gm of $CH_3-NHCH_2-CH(O\ Et)_2$ and 56.4 gm of
KSCN, the mixture kept 12 hours, evaporated to dryness,
the residue refluxed 1 hour with 200 ml. of anhydrous
acetone. The mixture filtered, the precipitate washed
with 50 ml acetone and the solution evaporated to give
63-8% of methimazole m.p. 147-8° Yield (75-80%).

$$CH_3-NH-CH_2-CH(OCH_2CH_3)_2 \quad + KSCN \xrightarrow{\text{dil HCl}} \quad \underset{N}{\overset{CH_3}{\underset{|}{N}}} SH$$
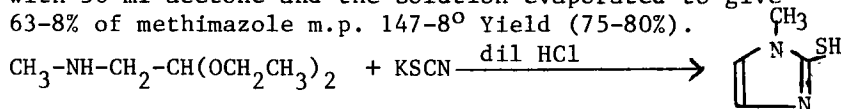
4. Stability, Decomposition product and metal complexes
Methimazole is a relatively stable compound at room
temperature. However, it is recommended that it should

be kept in a well-closed container protected from light
and in a dry place.

Methimazole forms metal complexes with heavy metals e.g.
Cu^{+2}, Al^{+3}, Fe^{+3} ions. Foye and Lo (14) studied the
chelating properties of some imidazoles and other related
heterocyclic thiones, and reported a relation between the
metal binding strengths and their antimicrobial activity.

5. Metabolism
When 20 mg/kg of methimazole was administered i.p. or
orally to rats, urinary methimazole glucuronides accounted
for 36-48% of the dose in 24 hours. The only other uri-
nary metabolite accounted for 10-20% and was not character-
ized. An additional 14-20% of methimazole was excreted
unchanged in 24 hour urine. The bile contained methimazole
glucuronide and two unidentified metabolites. One of
which was the same as the unidentified urinary metabolites.
Plasma proteins bound 5% of methimazole which had no
affinity for any specific tissue. Methimazole had a much
greater $CHCl_3/H_2O$ partition coefficient and H_2O solubility
than did propylthiouracil. Between 77 and 95% of the methi-
mazole was excreted in the urine and approximately 10% in
the bile. Since fecal excretion was neglegible; an enter-
ohepatic circulation was present. The half life of uri-
nary excretion was 5-7 hours regardless of the route of
administration (15).

^{35}S - labelled methimazole given i.p. to rats accumulated
in the thyroid gland where it was mainly oxidized to
methimazole sulphate (16). Most of the label was excreted
in the urine. The same results were found in man too (17).
Pittman et al (18) showed that both the thyroid and adrenal
glands had the highest organ to plasma ratios of the drug
after four days of i.v. administration in rats. It was
reported that methimazole in man, is more slowly absorbed
and excreted than propylthiouracil. The plasma half-life
in hours of methimazole ^{35}S was 2 or 5 times that of label-
led propylthiouracil. The blood radio activity curve
after the oral administration of carbimazole – ^{35}S was
very similar to that of methimazole. It was suggested by
Alexander et al that the renal function may have a more
important influence on the biological half-life of the
drug than the thyroid status (19). However, the identi-
fication of methimazole metabolites still need more inves-
tigation.

6. Methods of analysis

   6.1. Titrimetric methods

      6.11 Aqueous
            Several titremetric methods were developed
            for the analysis of methimazole

         i) Silver nitrate method (1)

         a) for the standard methimazole
             Dissolve about 250 mg of methimazole, accu-
             rately weighed, in 75 ml of water. Add from
             a buret 15 ml of 0.1 N NaOH, mix and add with
             agitation, about 30 ml of 0.1 N $AgNO_3$. Add
             1 ml of bromothymol blue TS, and continue the
             titration with the 0.1 N NaOH until a perma-
             nent, blue green color is produced. Each ml.
             of 0.1N NaOH is equivalent to 11.42 mg of
             $C_4H_6N_2S$.

         b) For methimazole tablets
             Weigh and finely powder not less than 20 methi-
             mazole tablets. Weigh accurately a portion
             of the powder equivalent to about 120 mg of
             methimazole and place in 100 ml volumetric
             flask. Add about 80 ml of water, insert the
             stopper and shake by mechanical means or occ-
             asionally by hand during 30 minutes, dilute
             with water to volume and mix. Filter and
             transfer 50.0 ml of the filtrate to a 125
             ml conical flask. Add from a burett 3.5 ml of
             0.1N NaOH, mix, and add with agitation about
             7 ml of 0.1N $AgNO_3$. Add 1 ml of bromothymol
             blue T.S. and continue the titration with
             0.1N NaOH until a permanent, blue green color
             is produced. Each ml of 0.1N NaOH is equiva-
             lent to 11.42 mg of $C_4H_6N_2S$.

         ii) Iodometric methods

         a) Kossakowski et al (20) developed the follow-
             ing method for determination of methimazole
             (thiamazole) in substances and tablets.

             A solution containing 10-60 ug thiamazole was
             buffered at pH 5.6, treated with NaN_3 and then
             with 0.02 M iodine solution, left 10 minutes
             and titrated for unchanged iodine with 0.02 M
             Na_3AsO_3. The method was useful in determining
             methimazole in compound drugs provided they

contain no S^{-2} ions.

b) By the method Blazek et al (21)
   The determination is done by oxidation of the
   -SH group with 0.1N iodine solution in $NaHCO_3$
   solution visually with respect to strength or
   potentiometrically (the equivalence point is
   difficult to recognize) or in NaOH solution
   potentiometrically, which is more favorable.
   The error of the method for a weight of 10mg
   amounts is approximately 0.5%.

iii) Iodine complex method (22).
   The potential colorimetric use of the iodine
   complexes of methimazole in $CHCl_3$ and CCl_4 is
   studied. The absorption maximum of methima-
   zole-iodine complex at 269 nm could be used
   analytically.

iv) Bromometry method
   The method was described by Varga et al (23).
   To a 2-5 mg sample in aqueous solution, 10 ml
   of water, 10 ml of 0.1N $KBrO_3$, and exactly
   0.5 gm. KBr are acidified with 1.0 ml 50%
   H_2SO_4. After 15 min. 5 ml 20% KI is added,
   with starch indicator and the iodine titrated
   with 0.1N $Na_2S_2O_3$. 1 ml 0.1 $KBrO_3$ = 0.0009517
   methimazole. The bromometric determination of
   methimazole and other similar compounds is
   markedly influenced by the presence of excess
   bromide. In general, the greater the amount
   of bromide present the lower the percentage
   of the compound found. The deviation appro-
   aches a limit which is different for each
   compound (24).

v) Cerimetry method
   The method was developed by Varga et al (23)
   0.03 - 0.10 gm sample in 5 ml water is cooled
   to 0° and titrated with 0.1N Ce $(SO_4)_2$,using
   p-ethoxychrysoiodine or (not reversible)
   methyl orange, methyl red, or thymol blue as
   indicator. 1 ml 0.1N $Ce(SO_4)_2$ = 0.011417 gm
   methimazole. The error is less than $\pm$ 1%
   Vehicles of tablets do not interfere.

6.12. Non-aqueous titration

A non-aqueous titration method was developed
(25,26) for the determination of basic com-
pounds with thio(-S-) and mercapto (-SH-)
groups. The reaction of the S group with
Hg (OAc)$_2$ in acetic acid makes this possible.
Methimazole and other compounds were all tit-
rated with HClO$_4$ in acetic acid using gentian
violet as indicator.

6.2   Spectrophotometric methods

6.21   Infrared spectrophotometric method

A method recommended for adoption as official,
first action (27). The method in which methi-
mazole is separated from tablet excipients by
column chromatography on Celite 545 with
chloroform as the eluent and then quantitativ-
ely measured and identified by IR spectropho-
metry. This method was studied collaborative-
ly by 10 analysts; average recoveries from two
simulated and two tablets mixtures ranged from
96.6% ± 1.0 to 101.1% ± 0.9 (28).

6.22   Nuclear magnetic resonance spectrometric

An NMR procedure is described by which methi-
mazole is determined in pure and tablet for-
mulation. The method is rapid, accurate,
precise (S.d. = 0.95%) and also provides a
specific identification of methimazole.

The spectrum was run in 10% methylene chloride
in carbon tetrachloride with use of benzoic
acid as an internal standard, using the N-CH$_3$
protons of methimazole at 3.56δ and the aro-
matic protons of benzoic acid at 7.5 and 8.13δ
as criteria for analysis (29).

6.23   Ultraviolet Spectrophotometric method

In this method, absorption spectra have been
determined for methimazole (mercazolyl) in
aqeuous acid and alkaline solutions. Aqeuous
solutions of methimazole have an absorption
band in the medium wave region with a max at
250 nm. In strong acid and alkaline media
hypsochromic and hypochromic effects are obser-
ved (30).

6.3   Chromatographic Analysis

6.31   Thin Layer Chromatography
Methimazole was determined in chloroform
extracts of rat urine by thin layer chromato-
graphy on silica gel G. (containing 1% Zn
Silicate phosphor) plates with $CHCl_3/CH_3OH/H_2O$
(32:8:5) as developing solvent and 2,6-dichlo-
roquinone chlorimide as detection reagent by
densitometric scanning (31). This method is
suitable for routine analysis (better than
GLC because of the instability of the S. meth-
ylmethimazole derivative).

6.32   Gas Liquid Chromatography
This method is used for S-methyl methimazole
on a 10% Apiezon L + 5% KOH/Chromosob W column
at 100° with N carrier gas. The method is
more sensitive and more precise than TLC
method, but duplicate measurements must be
made on the same day due to instability of S-
methylmethimazole (32). Clarke (4) reported
retention time of 0.43 relative to diphenhydra-
mine under condition of methimazole described
in the monograph.

6.33   Paper Chromatography
Clarke (4) described several solvents systems
used for paper chromatography of methimazole
as shown in Table 1.

Table 1.

Solvent System	Visualising Agent	R_f	Ref.
Acetate Buffer (pH 4.58)	ultraviolet iodoplatinate spray (white)	0.27	33
Phosphate Buffer (pH 7.4)	ultraviolet iodoplatinate spray (white)	0.00	34,35
Citric acid: H_2O: n-butanol (4.8g: 130 ml: 870 ml).	ultraviolet iodoplatinate	0.72	36,37

6.4   Colorimetric   Analysis
       In this method the reaction of diphenylpicrylhydra-
       zine with methimazole (thiamazole) was sufficiently
       rapid for its use in a spectrophotometric assay.
       When the concentration of the reagent was 4×10^{-5}
       M and the molar ratio of methimazole to the reagent
       was 2 : 10, decoloration stopped after 90 minutes
       at 26°. The reaction mixture then contained bis
       (1-methylimidazol-2-yl) disulphide, the reagent,
       diphenylpicrylhydrazine. After several hours the
       decoloration started again giving rise to a multi-
       plicity of colored compounds some of which were
       also formed in a methanolic solution of the reagent.
       Reaction mechanisms were proposed for equimolar
       amounts of the reactants as well as for the reaction
       of methimazole with excess of the reagent and for
       the reaction of the reagent with the disulphide of
       methimazole (38).

       Szabo et al (39) described method for identificat-
       ion and determination of methimazole by conversion
       into its coloured 1:1 Cu^{+2} complex which has an
       absorption max. at 614 nm. The complex is not
       stable, but on treatment with HCl, a 2:1 methimazole
       CuCl complex is precipitated as   white crystals.

6.5   Potentiometric Analysis
       Methimazole was analysed in pharmaceutical prepara-
       tions potentiometrically using 0.1N chloramine.
       The method can detect amounts of up to 5 mg of the
       drug with an accuracy of 98-99.5% (39).

       An alternative method was described by Pinzauti
       et al (40) for determination of several antithyroid
       drugs potentimetrically with 0.01 M mercuric ace-
       tate with use of a mercuric sulfate reference
       electrode and an amalgamated gold or a silver indi-
       cator-electrode. The method is rapid and the re-
       sults are reproducible; the errors are all within
       $\pm$ 0.38%.

6.6   Coulometric determination
       Certain thiols, including methimazole, were deter-
       mined coulometrically by direct titration with
       electro-generated Hg^{+2} (42). The coulometric assay
       is carried out in a cell having three compartments
       separated from each other by sintered-glass discs.
       One compartment contains the mercury for the electro-

generation of Hg^{+2}, the mercury indicator electrode
and the S.C.E. (enclosed in a Perley tube contain-
ing 4 M-sodium nitrate to avoid interference with
Cl^-); the second is filled with supporting electro-
lyte (an aqueous soln. 0.05 M in $Na_2B_4O_7$ and 0.5M
KNO_3 and of pH 9.3); and the third acts as an auxi-
lliary compartment. The counter-electrode is of
polished platinium.

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ACKNOWLEDGEMENT

NALIDIXIC ACID

Patricia E. Grubb

1.   Description

     1.1   Name, Formula, Molecular Weight

          Nalidixic acid is 1-ethyl-1,4-dihydro-7-methyl-4-
     oxo-1,8-naphthyridine-3-carboxylic acid.
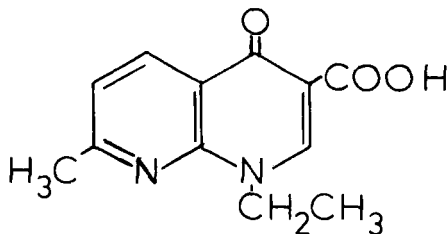
          Molecular Formula: $C_{12}H_{12}N_2O_3$
          Molecular Weight: 232.24[1]

     1.2   Appearance, Color, Odor

          Nalidixic acid is a white to slightly yellow, odor-
     less crystalline powder.[1][2]

2.   Physical Properties

     2.1. Spectral Properties

          2.11 Mass Spectrum

               The low resolution mass spectrum of nalidixic
          is presented in Figure 1. It was obtained on a
          Joel JMS 01SC Mass Spectrometer at an ionization
          potential of 75 eV. The fragmentation pattern
          is presented in Figure 2. The molecular ion
          (m/e=232) is present at an intensity of 18%; the
          loss of CO_2 gives a fragment of mass 188 which is
          the most abundant ion.

          2.12 Infrared

               The infrared spectrum of nalidixic acid in a
          KBr pellet is presented in Figure 3. The spectrum
          was obtained on a Perkin-Elmer Infrared Spectro-
          photometer Model 21. It agrees with the spectrum
          presented by Salim and Shupe.[2]

MASS SPECTRUM
of Nalidixic Acid

m/e	%
232	18
215	1.6
188	100
173	7.6
160	18
145	7
132	12

m/e
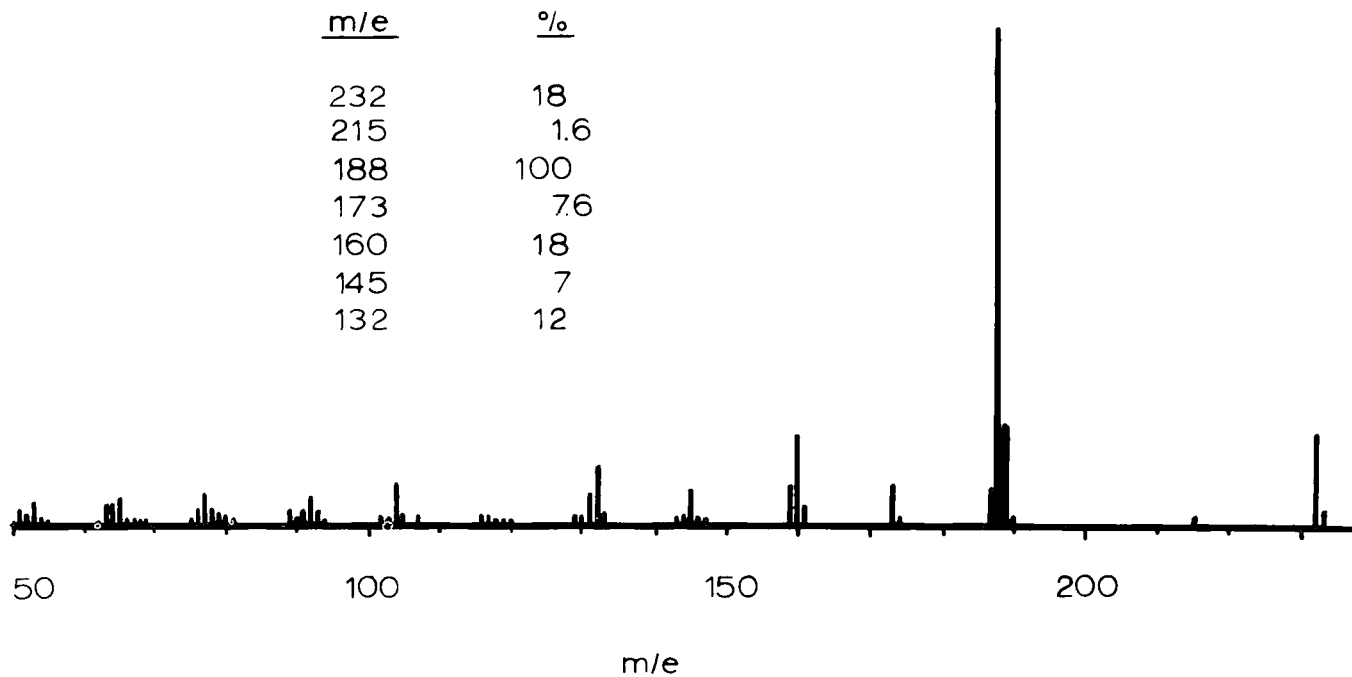
Figure 1

Figure 2

Fragmentation Pattern
of Nalidixic Acid
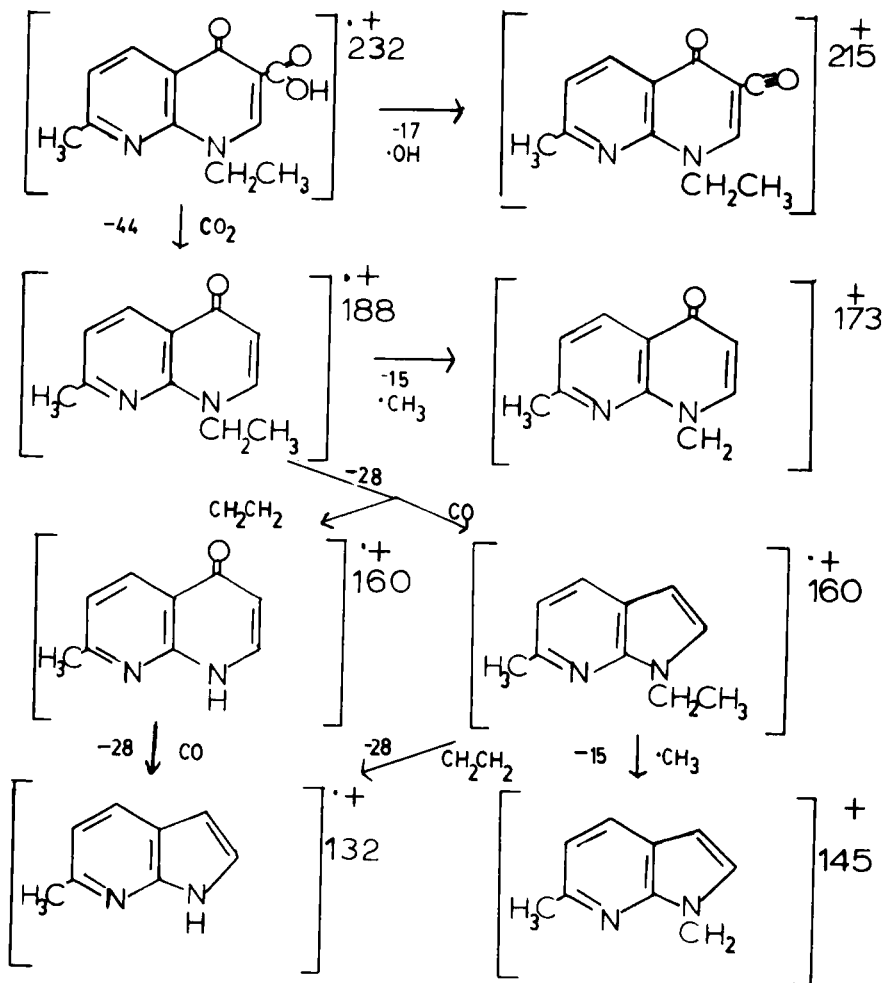
Figure 3

INFRARED SPECTRUM
of Nalidixic Acid
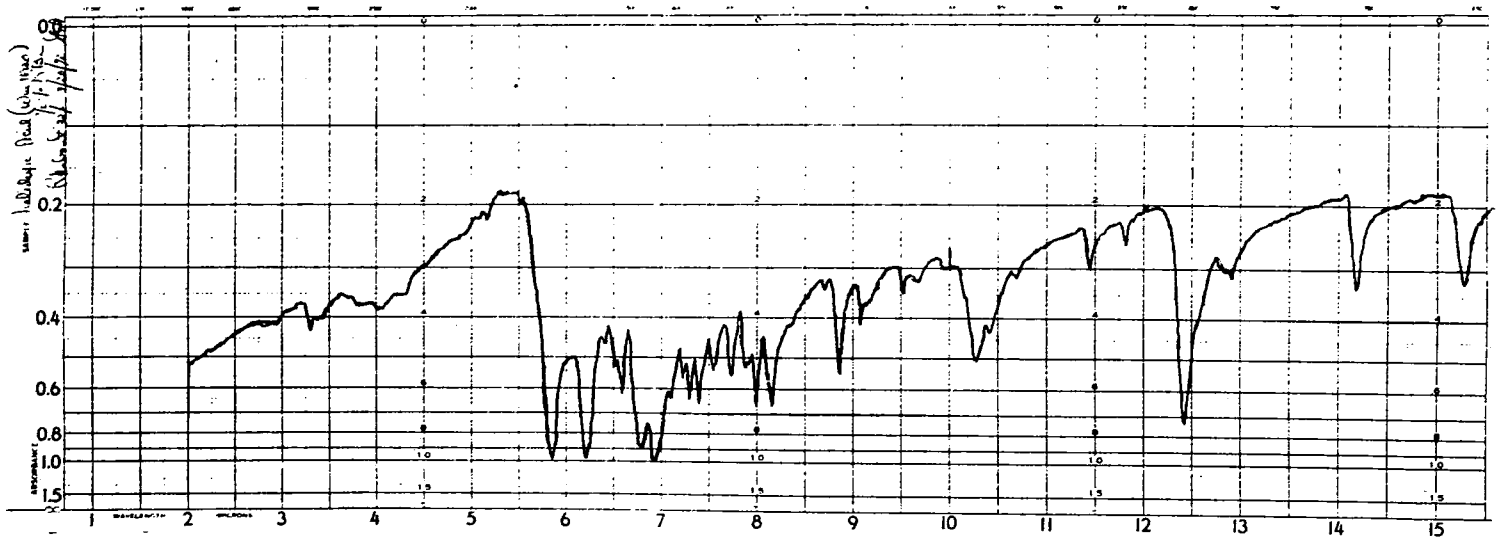
The carboxylic acid OH bands in the region of 3300-2500 cm^{-1} are weak and broad, indicating that hydrogen bonding with the carbonyl may be present.[3] The intense peak at about 1715 cm^{-1} is probably due to the C=O stretching of the carboxylic acid. The peak at 1620 cm^{-1} may be due to the C=O stretch of the carbonyl at position 4 or the C=C stretch of C-2 and C-3, conjugated with the carbonyl, or a combination of these two vibrations.

2.13 NMR

The NMR spectrum of nalidixic acid is presented in Figure 4. It was obtained on a Varian A60 spectrometer. This spectrum is in agreement with the spectrum presented by Hamilton and co-workers.[14] The following assignments have been made.

ppm	# protons	description
1.75	3(triplet)	$-CH_2CH_3$
2.98	3(singlet)	$-CH_3$(aromatic)
5.12	2(quartet)	$N-CH_2-CH_3$
7.85	1(doublet)	C(6)H
8.85	1(doublet)	C(5)H
9.47	1(singlet)	C(2)H

2.14 Ultraviolet Spectrum

Figure 5 shows the ultraviolet spectra of nalidixic acid at about 7.5 mcg/ml in 0.1 N NaOH, methanol, and chloroform, obtained on a Perkin-Elmer 323 recording spectrophotometer. The intensity, position, and fine structure present in each spectrum is related to the solvent polarity. These spectra are in agreement with the spectra published by Salim and Shupe.[2] Zubenko and Shcherba also report that there are two bands in methanol and 0.1 N NaOH, at 258 nm and 324 or 332 nm, respectively.[5] Gafari[6] has reported three bands in methanol: 213-216 nm (^{1}La), 255 nm (^{1}Lb) and 320-322 nm ($\propto$). Gafari also reports an absorbance in 0.1 N NaOH at 279-281 nm and 325 nm. These wavelengths are about 3-10 nm lower than those reported by other sources.[2][5][7]

NMR SPECTRUM
of Nalidixic Acid

offset
100 Hz
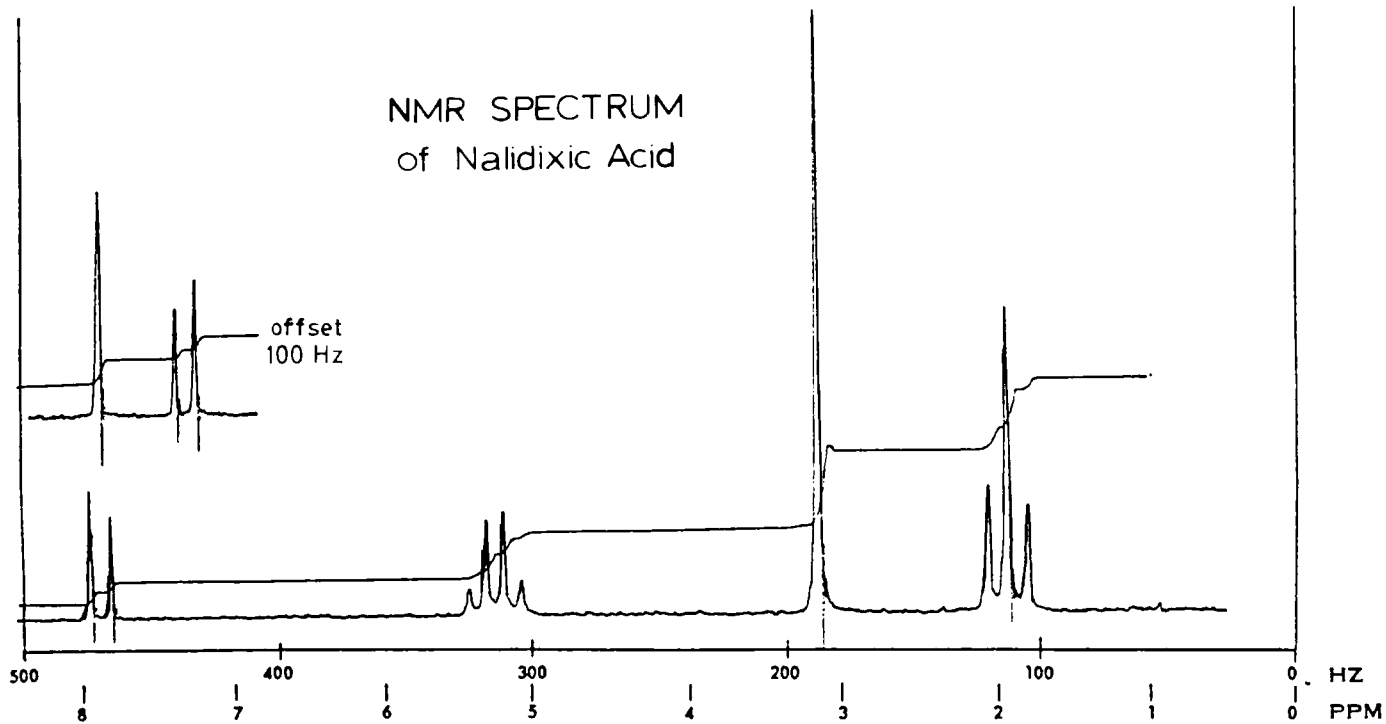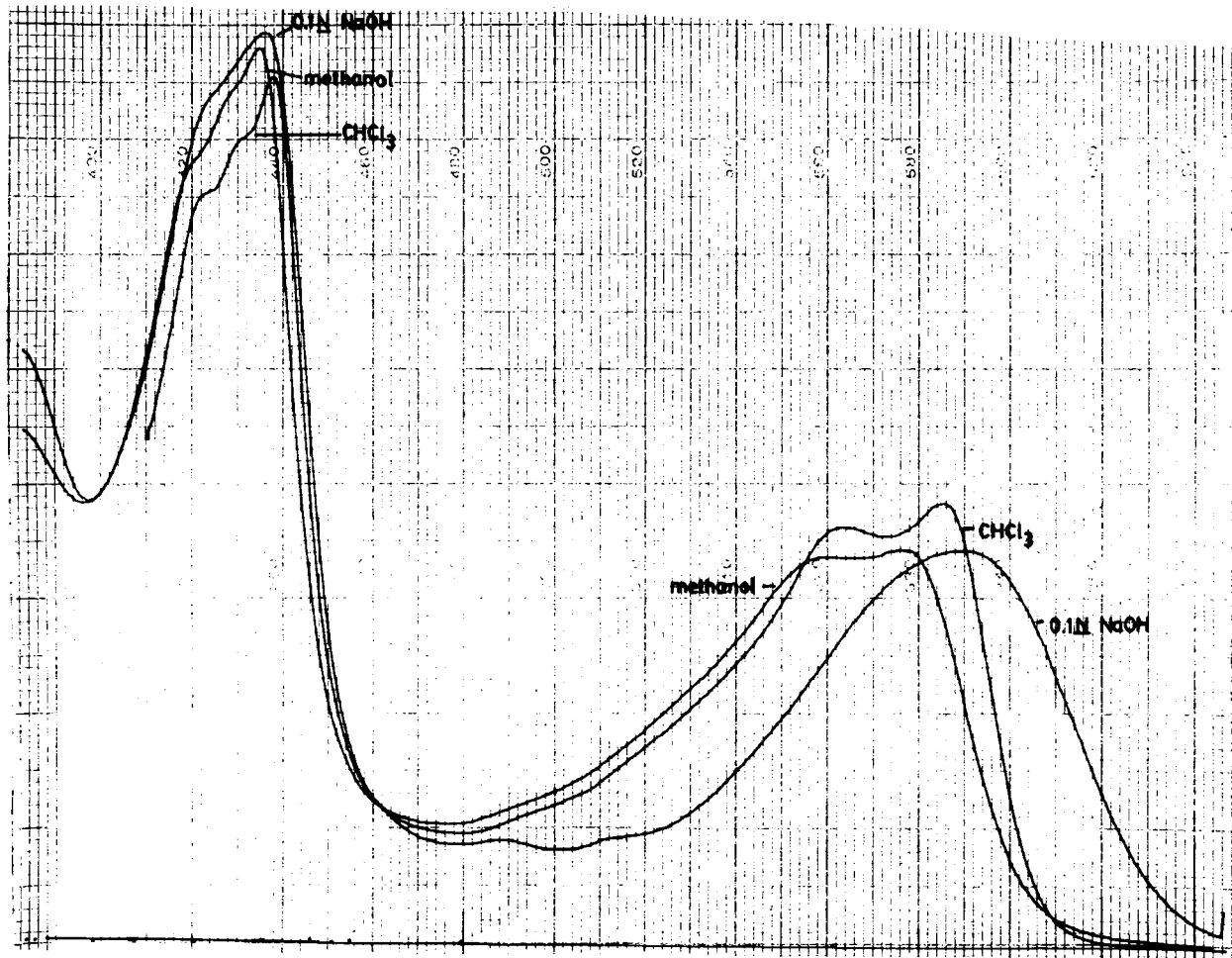
Figure 4

Figure 5.
ULTRAVIOLET SPECTRA
OF NALIDIXIC ACID

2.15 Fluorescence

Nalidixic acid exhibits strong fluorescence
in acidic solutions. McChesney and co-workers
used 0.1 $\underline{N}$ H_2SO_4 [8] with an excitation wavelength
of 330 nm, measuring emission at 375 nm.
Browning[9] used 21.5 $\underline{N}$ H_2SO_4 with excitation at
325 nm and emission at 408 nm. Figure 6 shows the
fluorescence excitation and emission spectra of
nalidixic acid in 0.5 $\underline{N}$ H_2SO_4 determined on an
Aminco Bowman Spectrophotofluorimeter.

2.2 Crystal Properties

2.21 Crystallinity and X-ray Diffraction

Prismatic crystals of nalidixic acid elongated
along the c axis were grown in ethanol/water solu-
tion by Achari and Neidle.[10] The crystals were
found to be monoclinic and of the space group
$P2_1/C$. The x-ray diffraction pattern of the single
crystal was solved for the structure of the mole-
cule. The bond lengths and angles of all non-
hydrogen atoms were determined. The values found
compare favorably with those reported for 1,8-
naphthyridine and 3-ethoxycarbonyl-4-oxo-6-methyl
homopyrimidazole. Nalidixic acid is calculated to
be slightly non-planar; each ring is planar but the
ring fusion has induced a slight buckling of the
ten-membered ring. The above-mentioned compounds
also have similar buckling. An intramolecular
hydrogen bond is found between the carbonyl oxygen
and the hydrogen of the carboxylic acid.

The parameters of the monoclinic crystal are

a = 8.913 $\mathring{A}$

b =13.133 $\mathring{A}$

c = 9.31 $\mathring{A}$

β =99.75 $\mathring{A}$

The measured density is 1.41, the calculated
density is 1.425 g/cm^3 for Z=4. The refractive
indices of crystals of nalidixic acid have been
reported as α=1.510, β=1.800, and γ =1.880.[4][11]

Figure 6

FLUORESCENCE SPECTRUM
of Nalidixic Acid

Excitation maximum 330 nm

Emission maximum 365 nm

(uncorrected spectrum)

$0.5\underline{N}\ H_2SO_4$

nm
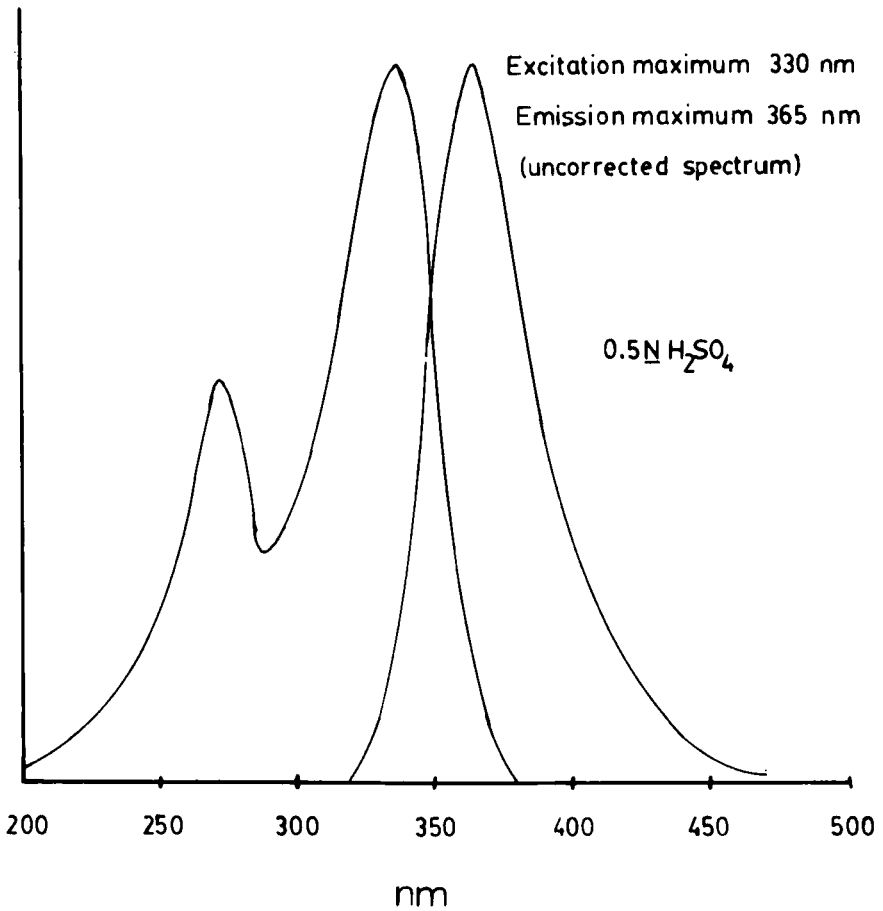
2.22 Solubility

The solubilities of nalidixic acid in various solvents at 23° are listed below.

Solvent	Solubility (mg/ml)
chloroform	35
toluene	1.6
ethyl acetate	.8
methanol	1.3
ethanol	.9
isopropanol	.4
water (distilled)	.1
ethyl ether	.1

The partition coefficients between water and various organic solvents have been reported by Sulkowska and Staroscik.[12]

The pH-solubility profile has been studied by these workers and also by Takasugi and co-workers.[13]

2.23 pKa

The pKa of the protonation of the nitrogen in position 8 has been reported as 6.02 and the pKa for the carboxylate anion formulation has been reported as -0.94. These were determined by Staroscik and Sulkowska by a spectrophotometric method.[14] Further study by the same workers on the partition equilibria of nalidixic acid between water and various organic solvents led to calculations of the pKa values of 5.99 + 0.03 for N-protonation and -0.86 + 0.07 for carboxylate anion formation.[12] Takasugi and co-workers reported the apparent pKa of nalidixic acid to be 5.9 at 28° by a spectrophotometric method.[13]

2.24 Melting Range

The melting range of nalidixic acid is reported to be 225-231°, determined as a class 1 compound.[1][2]

2.25 <u>Differential Scanning Calorimetry</u>

A DSC scan for nalidixic acid was performed
by Houghtaling.[9] The instrument used was a
Perkin-Elmer DSC-1B at a scan rate of 1°/min. The
curve is presented in Figure 7. A sharp peak at
229-230.5° (corr) represents the sample melting.

3.   <u>Synthesis</u>

The synthesis of nalidixic acid was reported by Lesher
and Gruett.[15][16] It may be prepared by the procedure
shown in Figure 8.

4.   <u>Stability and Degradation</u>

Nalidixic acid is stable up to five years under reason-
able conditions of temperature and humidity. Pawelczyk and
Plotkowiakowa[17] subjected sodium nalidixate solutions to
accelerated aging, but were unable to identify decomposition
products. Detzer and Huber[18] studied the photolysis and
thermolysis of nalidixic acid in the presence of oxygen.
Photolysis produced de-carboxylated nalidixic acid, structure
A, and a diketone product, structure B, as well as carbon
dioxide and ethylamine.
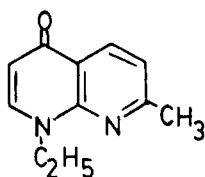
Structure A   Structure B
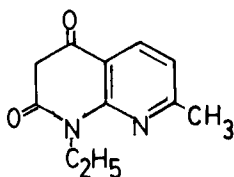
Thermolysis also produced the decarbonylation product
plus a dimer, structure C.

Structure C

Figure 7

DSC Scan
of Nalidixic Acid

2.5°/min
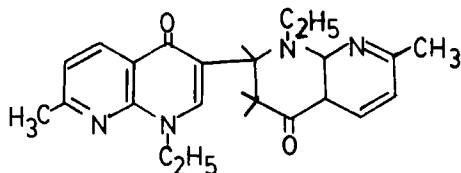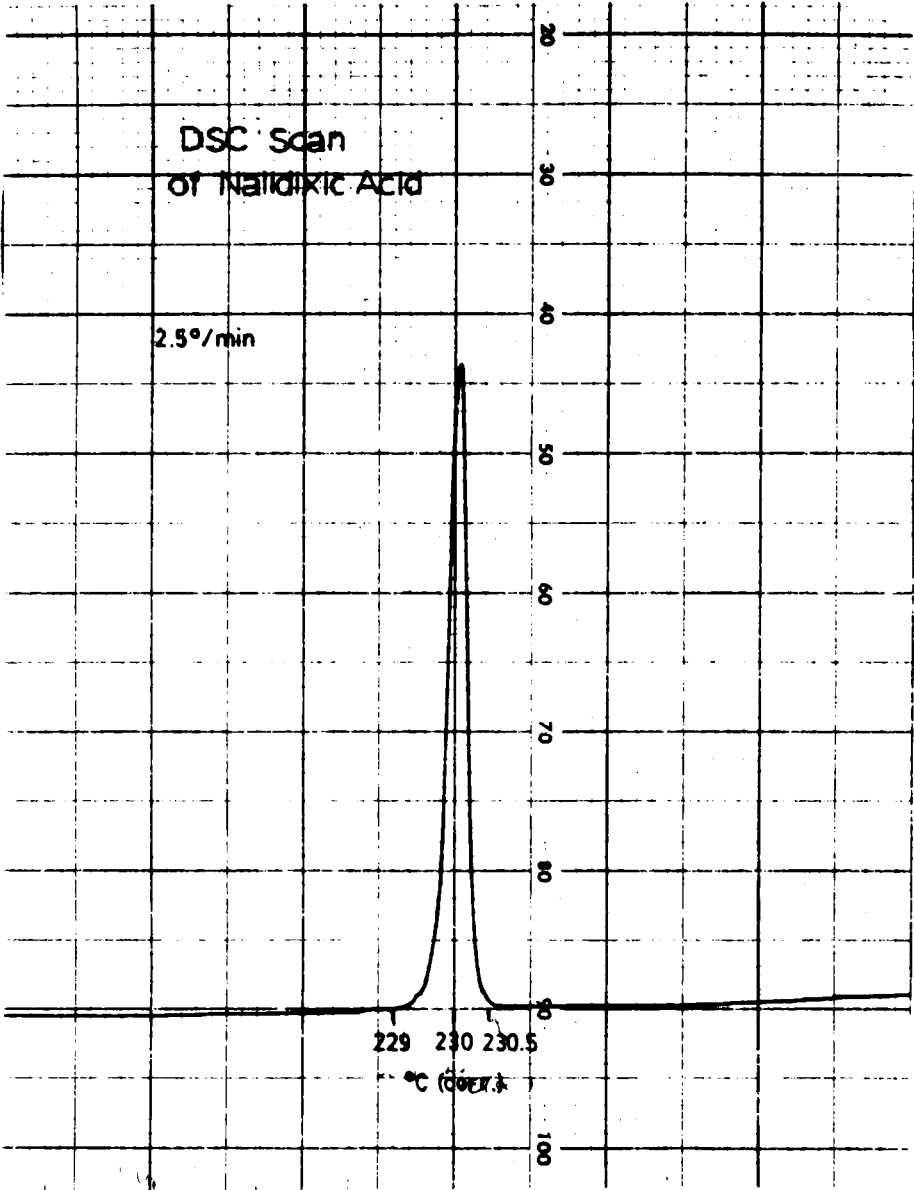
Figure 8

SYNTHESIS of NALIDIXIC ACID
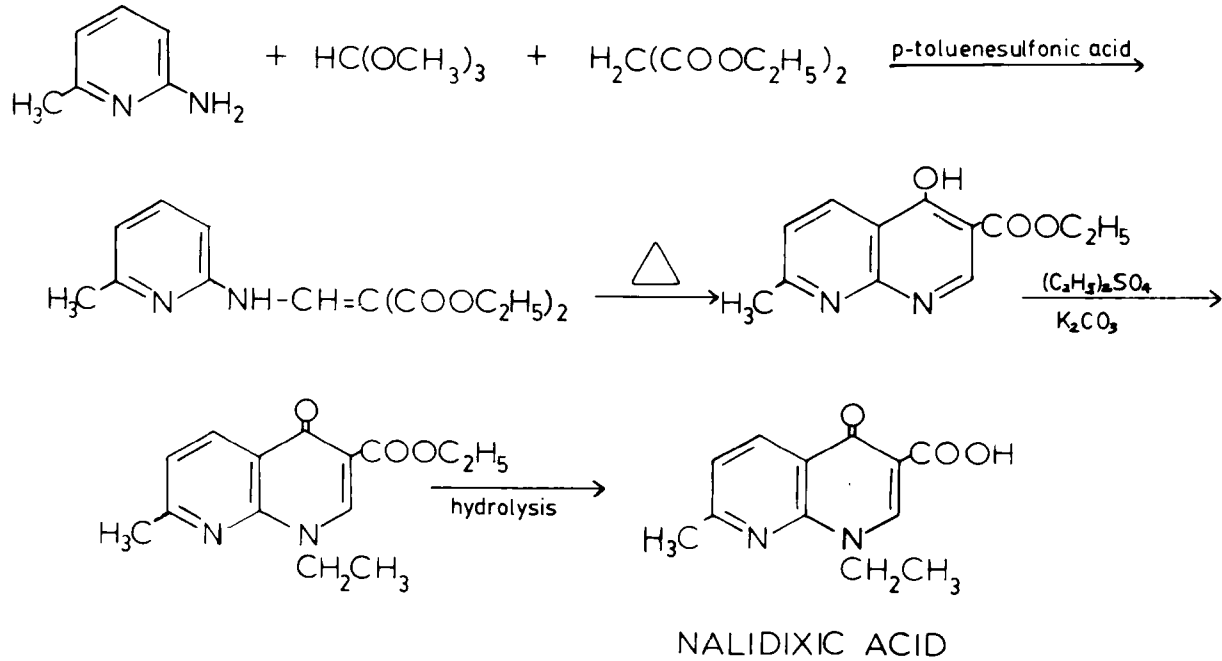
NALIDIXIC ACID

5.   Drug Metabolic Products and Pharmacokinetics

5.1   Metabolic Products

Nalidixic acid is a synthetic antibacterial compound
that is used in the treatment of urinary tract infec-
tions. It is more active against gram-negative than
gram-positive organisms.[4] The compound is rapidly
absorbed as the free acid; it is excreted in the urine
by man in several forms.[8] A small amount is excreted
unchanged, but a much larger fraction is conjugated as
the monoglucuronide, The most important metabolite is
1-ethyl-1,4-dihydro-7(hydroxymethyl)-4-oxo-1,8-
naphthyridine-3-carboxylic acid, referred to as hydro-
xynalidixic acid. This metabolite has shown the same
order of microbiological activity in vitro as nalidixic
acid. The monoglucuronide conjugate of 7-hydroxynali-
dixic acid has also been found, as well as the 3,7-
dicarboxylic acid product. No antibacterial activity
has been found for the glucuronides or for the 3,7-
dicarboxylic acid. In addition, no glucuronide of the
3,7-dicarboxylic acid has been reported.

Similar metabolites were found produced by
man[19][20][21], monkeys[8], dogs[8], chickens[22],
calves[23] and microorganisms[4]. The ratios of these
metabolites, however, were found to vary with the
individual. The overall conversion of nalidixic acid
to hydroxynalidixic acid had been reported by
McChesney[8] to be normally about 32%; bicarbonate sup-
plementation increased this to about 40%. Bicarbonate
supplementation also increased the amount of total
naphthyridine excreted in the biologically active form.

Two separate studies have reported on the ratios
of the various metabolic products excreted in the
urine by man. In addition, Portmann and co-workers
calculated theoretical amounts based on their rate
equations.[24]

Compound	Calc.amts. (mg per 1g dose)	Found[24] (mg per 1g dose)	Found[21] %
Nalidixic acid	9	8 ± 3	0.5-5 %
Hydroxy-nalidixic acid	105	129 ± 8	2.5-6 %
NA-glucuronide	517	537 ± 49	24-80%
1-HNA-glucur-onide	221	229 ± 32	11-12%
3,7-dicarbo-xylic acid	74	43 ± 6	not reported

In glucuronides were not found as metabolic prod-
ucts by Hamilton[4] in studies of various fungi. The
major metabolite of the microorganisms was hydroxy-
nalidixic acid. The dicarboxylic acid was also found
as a product in a number of microorganisms.

The mechanism of action of nalidixic acid against
E. coli. was found to be inhibition of DNA synthesis.[25]
No selective effect on purines or pyrimidines was found
and no inhibition of initiator synthesis was demonstra-
ted. In addition the nalidixic acid could be removed
from cultures after exposures up to 75 minutes by
rinsings and cells would recover from the block.

5.2   Pharmacokinetics

The pharmacokinetics of nalidixic and hydroxy-
nalidixic acids have been studied by several different
groups. Takasugi et al studied in-situ and in-vitro
absorption of nalidixic acid from the gastrointestinal
tracts of rats as a function of pH. They reported that
the absorption of non-ionized nalidixic acid was faster
than the ionized form, with the maximum absorption
rate constant found when the drug was administered from
a pH=3 buffer solution. The absorption in-situ was
found to be ten times the rate in-vitro, but this
was dependent on several factors.[13]

Moore and co-workers[19] described a simplified
model for nalidixic acid metabolism by combining the

two microbiologically active forms, nalidixic and
hydroxynalidixic acid, and the two conjugated
products of the active forms. This model is shown
below in Figure 9.

Figure 9

Simplified Model for Kinetic pathways of
Nalidixic acid in Man

Nalidixic acid $\xrightarrow{\text{lag time}}$ A $\xrightarrow{k_A}$ B

(dosage form) k_M

                                    U $\xleftarrow{k_E}$ CB

                                    k_U

                                                     CU

A = Nalidixic acid in GI tract
B = total active drug in body
CB = conjugated drug in body
U = total active drug excreted
CU = conjugated drug excreted
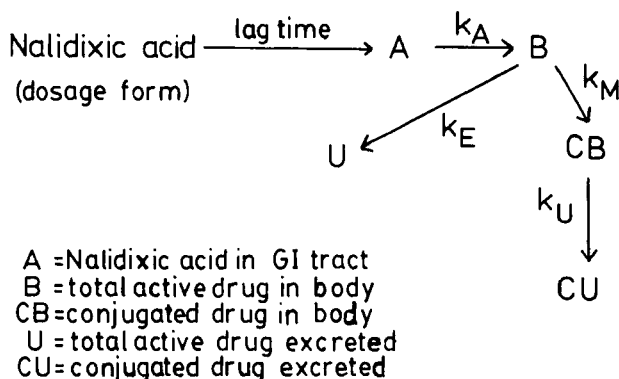
        The rate constants for metabolism and excre-
tion were further combined to a rate constant for
the disappearance of the drug, k_d. Four dosage
forms were studied and it was found that k_d was
constant for all forms except for a coarse,
slowly dissolving powder.

        Portmann and co-workers then studied the
kinetic pathways in man for hydroxynalidixic acid,
the active primary metabolite.[26] The rate
constants for glucuronide formation, oxidation to
the dicarboxylic acid and excretion of hydroxy-
nalidixic acid were calculated. Essentially total
absorption of hydroxynalidixic acid was found in
every case. Good agreement between experimental
and theoretical plasma levels, based on the first
order rate approximations used for the model, was
found. Again, the disappearance rate constant,
k_{d_2}, was found to be very similar for each subject,
although the individual excretion and metabolic
rate constants varied widely. The disappearance
rate constant, k_{d_2}, was defined as the sum of the
excretion rate constant, k_{E_2}, and the metabolic
rate constants to the glucuronide and dicarboxylic
acid, k_{M_3} and k_{M_4}, respectively.

This data was then used in another study by Portmann and co-workers[24] of nalidixic acid metabolism in man in which a more elaborate model was developed and various rate constants were reported (Figure 10). This model was based on the oral administration of 1 g of nalidixic acid. Theoretical curves for plasma levels of nalidixic and hydroxynalidixic acid vs. time agreed with experimental values.

McChesney and co-workers[27] then studied the effect of repeated oral dosage of nalidixic acid and found some carry-over of nalidixic acid from day to day but reported no important change in the metabolism due to multiple dosings.

The half-life of nalidixic acid in plasma in man was found to be between 85 and 100 minutes by McChesney and co-workers[8] and was reported as about 100 minutes by Bruehl and co-workers[27].

Another study by McChesney and co-workers on the metabolism of nalidixic acid in the immature calf[23] demonstrated a pattern of metabolism very different than in man. The half-life of nalidixic acid was found to be about 24 hours and a large amount was excreted into the feces. The immature calves were also unable to excrete nalidixic acid into the urine at concentrations greater than found in the plasma and conjugated drug was present at low levels only. Calves seven months old had metabolic patterns much closer to man; the plasma half-life was about 1.5 hours, the concentration of excretion into the urine was at least ten times that in plasma and the extent of conjugation was increased. The inability to metabolize nalidixic acid by the immature calf was considered to be due to incomplete development of its metabolic system. A similar effect was seen in human infants.[29]

6.   Methods of Analysis

6.1   Elemental Analysis

The molecular formula is $C_{12}H_{12}N_2O_3$.

Figure 10

METABOLIC PATHWAYS
of Nalidixic Acid in Man

Nalidixic Acid
in GI tract

$\downarrow k_A$

Nalidixic Acid $\xrightarrow{k_{M_2}}$ Nalidixic Acid
in body   Glucuronide
in body

k_{E_1}

Nalidixic Acid
in urine

k_{M_1} k_{U_2}

Nalidixic Acid
Glucuronide
in urine

k_{E_2}

Hydroxynalidixic Acid
in body

Hydroxynalidixic Acid
in urine

k_{M_3} k_{M_4}

Dicarboxylic Acid
in body

Hydroxynalidixic Acid
Glucuronide   in body

k_{U_1} k_{U_3}

Hydroxynalidixic Acid
Glucuronide   in urine

Dicarboxylic Acid
In urine

$k_A = 1.8 \times 10^{-2}\,min^{-1}$

$k_{E_1} = 1.0 \times 10^{-4}\,min^{-1}$

$k_{E_2} = 1.93 \times 10^{-3}\,min^{-1}$

$k_{M_1} = 4.53 \times 10^{-3}\,min^{-1}$

$k_{M_2} = 5.77 \times 10^{-3}\,min^{-1}$

$k_{M_3} = 4.05 \times 10^{-3}\,min^{-1}$

$k_{M_4} = 1.37 \times 10^{-3}\,min^{-1}$

Element	Theory	Found for standards[30]	
C	62.07%	62.11	61.98
H	5.21%	5.21	5.20
N	12.07%	12.00	12.32

6.2 Non-aqueous Titration

The titration of nalidixic acid in DMF with lithium methoxide has been reported[1][2] with thymolphthalein as the indicator. It has also been titrated with sodium methoxide in ethylene-diamine or DMF:methanol 1:2 with thymol blue indicator.[31] An error for this titration was reported as + 0.7%. A titration with sodium borohydride followed potentiometrically or with thymol blue indicator has also been reported by Bachrata and co-workers. The standard deviation was reported as + 0.60%.[32]

6.3 Spectrophotometric

The ultraviolet absorption spectrum of nalidixic acid in methanol or chloroform has an absorption maximum at about 258 nm and a broad double peak at 324 to 333 nm. In 0.1 $\underline{N}$ NaOH the band at 324 nm is shifted to a single peak at about 332 nm. The a of the band at about 258 nm is approximately 110 but varies with the sol-vent.[2][5][6][7]

6.4 Colorimetric

Nalidixic acid and sodium nalidixate form a strong colored complex with iron III. The maximum of absorbance of the complex is 410 nm and Beer's Law is obeyed from 10 to 250 μg of nalidixic acid per ml[33], and from 0.43 to 17.05 mg iron III per ml.[34] Nalidixic acid complexes through the oxo-group on C-4 and the carboxylic acid group on C-3. Three moles of nalidixic acid complex with one mole of iron III. The instability constant of the complex was calculated to be 2.11×10^{-8} by Dick and Murgu.[34]

6.5 Polarographic

The polarographic behavior of nalidixic acid
has been studied by Staroscik and co-workers.[35]
The pH range of -2.9 to 11 in 20% DMF was investi-
gated in the concentration range of 5×10^{-4}m and
three stages of reduction were found. The poten-
tials were found to vary linearly with pH for the
first two reduction stages, while the third was
constant and appeared at pH >8. The carbonyl on
C-4 was shown to be reduced to the 4-hydroxy
product. Nalidixic acid was reduced with sodium
borohydride and the product was demonstrated to be
the same as that in the polarographic reduction
by TLC.

6.6 Chromatographic

6.61 Thin-layer and paper chromatography

Several thin-layer and paper chromato-
graphic systems for nalidixic acid are listed
below.

Chromatographic Systems for Nalidixic Acid

Adsorbent	Mobile Phase	R_f	Ref.
paper	toluene:dioxane(9:1) descending	.6	8
paper	butanol:acetic acid: H_2O (4:1:1)	--	36
paper	propanol:ethanol:water (6:1:3)	--	36
paper impreg. with 0.1 M Na and HPO_4	iso-butanol:ethanol:ethyl acetate:water:acetone (8.2:1:5:2) ascending	.73	17
silica gel	methanol:water:ammonia (100:12:16)	.88	35
silica gel	chloroform:methanol:formic acid (90:7:3)	.54	37
silica gel	ethyl acetate:methanol: isopropylamine (76:20:4)	.18	37
silica gel	benzene in ethanol:acetic acid (80:10:10)	.5	23

6.62 Liquid chromatography

A high-performance liquid chromatographic
method for nalidixic acid on a strong anion-
exchange resin column has been reported, using
a mobile phase of 0.01 $\underline{M}$ sodium tetraborate
at pH 9.2 and 0.003 $\underline{M}$ sodium sulfate. The
relative retention time for nalidixic acid in
the system reported by Sondach and Koch was
0.86 with sulfanilic acid as the standard at
1.00.[38]

6.63 Gas chromatography

A gas chromatographic procedure for
nalidixic acid after esterification has been
reported for biological samples.[39]

6.7 Spectrofluorimetric

Nalidixic acid has a strong fluorescence
spectrum which has been used for its determination
in biological fluids.[8][9][24][26][40]

7. Identification and Determination in Biological Fluids

The first spectrofluorimetric methods reported for the
determination of nalidixic acid and its metabolites in
biological fluids did not differentiate between nalidixic
acid and hydroxynalidixic acid. The determination of free
nalidixic acid and the hydroxy-metabolite in human urine
plasma and feces was performed by extraction by toluene from
acidified biological fluid and subsequent fluorimetric
measurement at 325/375 nm of sample re-extracted into aqueous
solution.[8] Conjugated nalidixic and hydroxynalidixic acids
were determined by acid hydrolysis and then toluene extraction
for fluorimetric measurement of the total drug. The conju-
gated nalidixic acid was then determined by difference.

A refined method involving extraction of two aliquots of
biological material, buffered at two different pH's, by
toluene, and re-extraction into aqueous solution allowed the
simultaneous determination of nalidixic and hydroxynalidixic
acid[24][26] by their differential extractabilities and
fluorescent intensities. This method was extended to the
differential determination of the conjugated forms of
nalidixic and hydroxynalidixic acid.

The 3,7-dicarboxylic acid metabolite was also measured fluorimetrically by extraction with ethyl acetate:chloroform 1:1 at pH=1 and re-extraction into aqueous solution. The fluorescence was determined at 350/435 nm; there was no cross interference with the nalidixic acid determination.[8]

Extraction of nalidixic acid with chloroform from urine has also been reported.[40] Another fluorimetric method for chicken liver and muscle containing not less than 100 ppb nalidixic acid was reported by Browning[9] using an ethyl-acetate extraction and alumina column to retain the nalidixic acid. The fluorescence was measured at 325/408 nm.

Spectrophotometric measurements of nalidixic acid have been reported. Gafari et al[41] used an extraction with ultraviolet measurement at 255 or 327 nm for blood and tissue samples. Takasugi and co-workers extracted buffered tissue homogenate with chloroform and measured the optical density at 334 nm.[13]

Several chromatographic procedures have been used for nalidixic acid and metabolites in biological fluids. Urine samples were extracted with chloroform and chromatographed on filter paper using the system toluene dioxane (9:1) descending chromatography. The known spots were eluted off the paper with methanol and quantitatively measured spectro-photometrically.[8] Other chromatographic systems reported include butanol:acetic acid:water (4:1:1) and propanol:ethanol:water (6:1:3) for paper chromatography. The glu-curonides were also separated without prior hydrolysis using isopropanol:ethyl acetate:water (6:1:3) or propanol:ethyl acetate:acetic acid:water (5:5:1:3).[36]

A high performance liquid chromatographic method was developed by Shargel and co-workers for the assay of nalidixic and hydroxynalidixic acids in human plasma and urine.[42] Extraction into chloroform and re-extraction into aqueous solution was used as the sample treatment. The column used was a 0.5 M stainless steel (2.1 mm i.d.) packed with Zipax SAX strong anion exchange resin. The column pressure was 600 psi with a flow rate of 0.8 ml/min. A modi-fication of this procedure, using a 1 m column with a column pressure of 900 psi and a flow rate of 0.7 ml/min. and an internal standard of 1-ethyl-1,4-dihydro-4-oxo-7-(4-pyridiyl)-3-quinolinecarboxylic acid, was used by Goehl and co-workers[43] for improved separation. A separate liquid chro-matographic procedure for the dicarboxylic acid was used by Lee[44] et al. The column used was a 25 cm x 4.6 mm i.d.

Partisil PXS 10/25 PAC pre-packed Magnum 9 column with a
mobile phase of methanol:pH 3 0.1 M citrate buffer 85:15
with a flow rate of 1.6 ml/min. The retention time of
the dicarboxylic acid was 12 minutes.

A thin-layer gas chromatographic system was devised by
Pittman and Shekosky[39] for chicken tissue and feces. A
TLC system of benzene:methanol:acetic acid (9:1:1) was used
for prior separation; the spots were then removed and
esterified in 14% BF_3 in methanol. A 4 ft. 3% OV-17 column
at 240° was used. Retention times of about 7 minutes for
nalidixic acid, 10 minutes for hydroxynalidixic acid and
17 minutes for the dicarboxylic acid were reported.

A pulse polarographic system for detection of the di-
carboxylic acid in the presence of nalidixic and hydroxy-
nalidixic acids was devised by Koss and Warner.[45] The
reduction potential in the system used was -.54V vs. SCE.

Microbiological assay procedures for nalidixic acid
have also been used for biological samples. Since nalidixic
and hydroxynalidixic acids have the same order of anti-
bacterial activity in-vitro, then cannot be determined sepa-
rately.

Organism	Reference
P. boviseptica	8
B. pumilus	46
E. coli	25,47,
	48,49

8.   Identification and Determination in Dosage Forms

Nalidixic acid has been determined spectrophotometri-
cally in tablets after chloroform extraction at a wavelength
maximum of 258 nm.[1][2] Another source reported 259 nm as
the maximum for chloroform and 258 nm for 0.1 N NaOH.[7] The
infrared spectrum has also been used to identify nalidixic
acid in tablets.[7]

Non-aqueous titration of tablet extracts with sodium
methoxide[27] or lithium methoxide[1][2] has been reported.
A polarographic determination of nalidixic acid in tablets at
-0.6V vs. SCE in 20% aq. DMF with 0.1 N HCl has also been
used.[35]

High performance liquid chromatography was used by Sondack and Koch[38] to assay nalidixic acid in aluminum hydroxide gel suspension.

A microbiological detection has been described for use for pharmaceutical preparations of nalidixic acid. A diffusimetric determination using E. coli (Bruxelles) was reported by Monciu.[48]

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NEOMYCIN

William F. Heyes

1.   Description.

1.1.   Composition, Name, Formula, Molecular Weight

Composition

Commercial neomycin is a complex mixture of aminoglycoside antibiotics originally isolated from a culture of *Streptomyces fradiae* by Waksman and his co-workers in 1949. The principle components of the mixture are neomycin B(I) and neomycin C(II) together with a small quantity of neamine[1], a degradation product of neomycin formerly known as neomycin A. Table 1 shows the content variability of neomycin B and C and neamine in commercial samples of neomycin as reported in the literature. Neomycins LP-A, LP-B and LP-C which chemically are the mono N-acetyl derivatives of neomycins A, B and C[2] may also be present in small amounts. Several other minor components have recently been identified as paromamine, paromomycin I and paromomycin II[3]. (Also known as neomycins D, E and F respectively).

Table 1

Content Variability of Neomycin

Source	Neomycin B Content %	Neomycin C Content %	Neamine Content %	Reference
Canada	69.2 to 97.5	30.8 to 4.0	Nil	5
U.K.	80.5 to 92.0	17.5 to 8.0	O to 2.5	6
U.S.S.R.	63.7 to 91.6	40.0 to 3.4	Nil	7,8

neamine....

I   R1 = H   R2 = CH$_2$NH$_2$;   neomycin B
II R1 = CH$_2$NH$_2$ R2 = H   ;   neomycin C
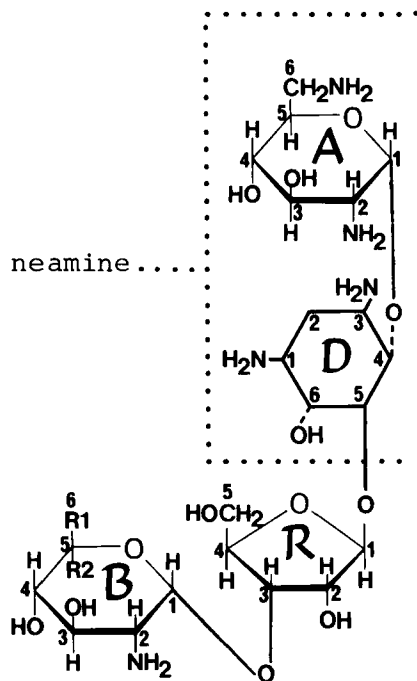
Figure 1. Structure of neomycin and neamine

Names

    The following are alternative names for the neomycin complex, all of which have been used by Chemical Abstracts:-

Colimycin, Colistin(not to be confused with Polymixin), Dextromycin,Flavomycin,Fradiomycin, Framycetin, Mycerin, Mycifradin, Neomix, Soframycin, Streptothricin BI, BII.

Chemical Names[4]

Neomycin B	O-2,6-diamino-2,6-◦dideoxy-α-D-glucopyranosyl-(1→4)-O-[O-◦2,6-diamino-2,6-dideoxy-β-L-◦idopyranosyl-(1→3)-β-D-ribo-furanosyl-◦(1→5)]-2-deoxy-D-streptamine.
Neomycin C	O-2,6-diamino-2,6-◦dideoxy-α-D-glucopyranosyl-(1→4)-O-[O-◦2,6-diamino-2,6-dideoxy-α-D-◦glucopyranosyl-(1→3)-β-D-ribo-furanosyl-◦(1→5)]-2-deoxy-D-streptamine.
Neamine (formerly Neomycin A)	2-deoxy-4-O-(2,6-◦diamino 2,6-dideoxy-α-D-glucopyranosyl)-D-streptamine.

Chemical Abstracts Registry No.

Neomycin B	119-04-O
Neomycin C	66-86-4
Neamine	3947-65-7

Formula

Neomycin B,C	$C_{23}H_{46}N_6O_{13}$
Neamine	$C_{12}H_{26}N_4O_6$

Molecular Weight

Neomycin B,C	614.67
Neamine	322.36

1.2. Appearance, Colour, Odour, Taste

The sulphate salt of neomycin (the usual commercial form) is an amorphous, white odourless, powder which is practically tasteless.

1.3. Definition of International Standard

The International Reference Standard for neomycin[9] is defined as containing 680 units of activity per mg or alternatively 1 unit of activity is contained in 0.00147mg of standard material.

Similarly, the International Reference Standard for neomycin B is defined as containing 670 units per mg, or alternatively 1 unit of activity is contained in 0.001492 mg of standard material.

2. Physical Properties

2.1. Spectra

2.11. Infra-Red Spectrum

The solid state infrared spectra of neomycins B & C sulphate have been recorded as a dispersion in potassium bromide and are illustrated in Fig. 2 and 3.

The infrared spectrum of neamine sulphate (formerly called neomycin A) also as a potassium bromide dispersion is illustrated in Fig. 4. All spectra are of authentic material supplied by The Upjohn Company, Kalamazoo.

Sammul et al[10] have published the infrared spectrum of neomycin undecylenate.

2.12. Ultraviolet Spectrum

No absorption maxima in the ultraviolet region 200-340nm is observed with solutions of neomycin.

2.13. NMR Spectrum

The proton NMR spectrum of neomycins B and C has been used by Rinehart and co-workers[1] to elucidate the structure and
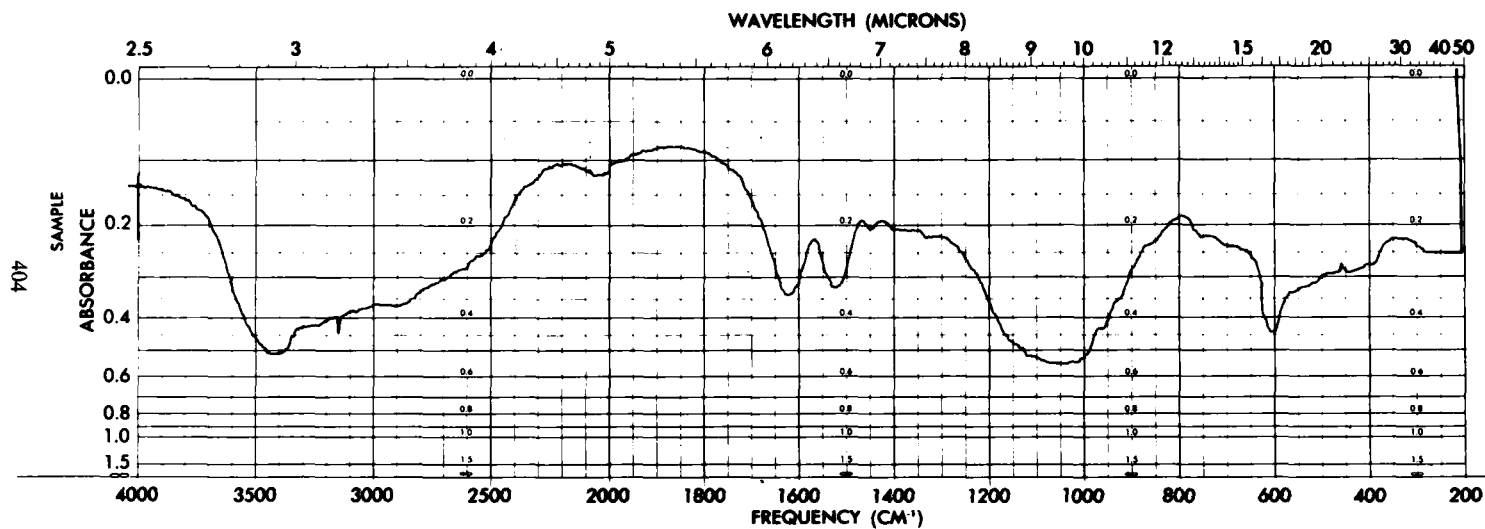
Figure 2.   Infrared Spectrum of Neomycin B
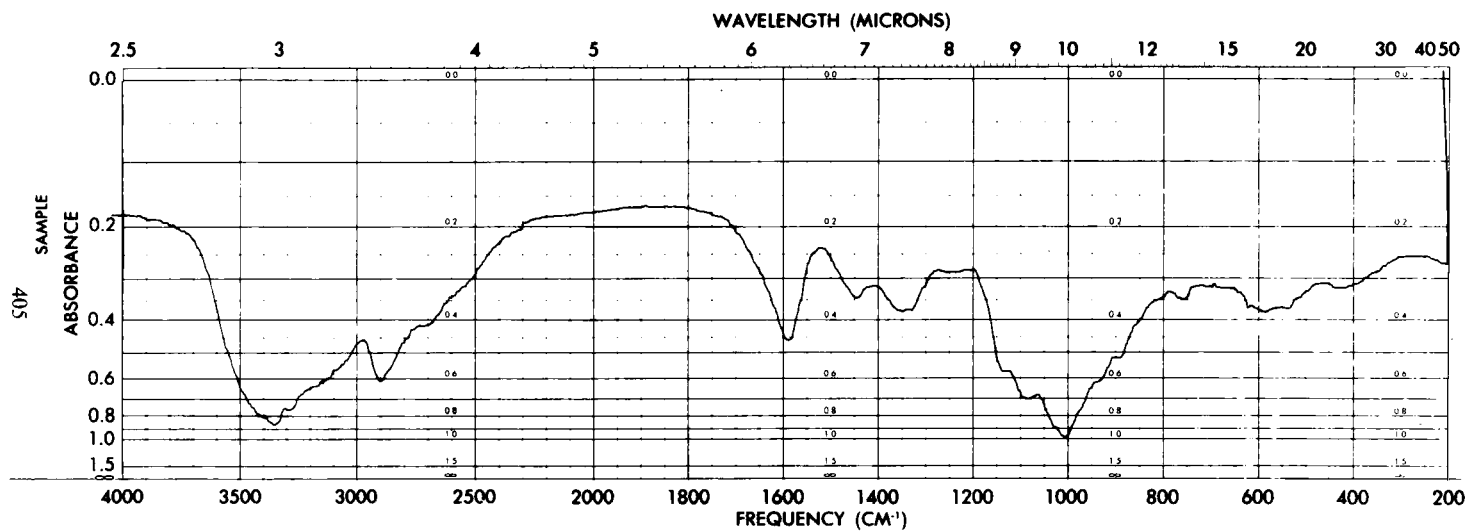
Figure 3.   Infrared Spectrum of Neomycin C
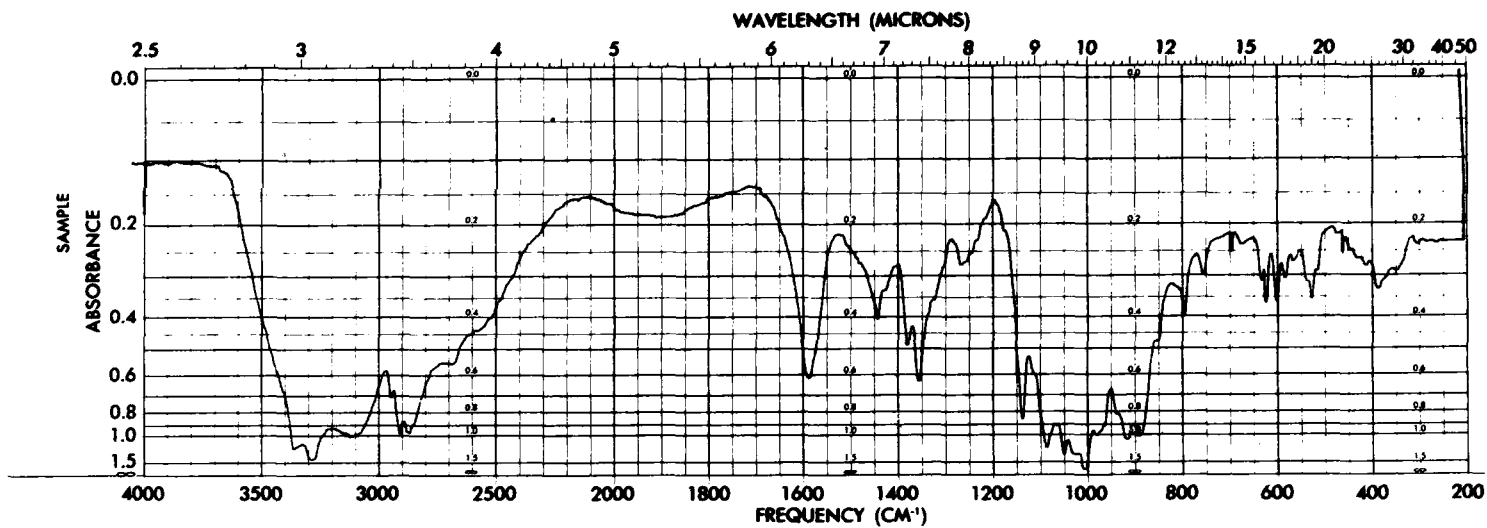
Figure 4.   Infrared Spectrum of Neamine

stereochemical configuration of these antibiotics.

Truitt[12] has extensively studied the ^{13}C NMR of the neomycins and demonstrated the pH dependance of the spectrum of neomycins B and C. The spectra of the hexa N-acetyl derivatives however, were shown to be independant of pH thus allowing assignment of the observed chemical shifts as given in Table 2.

2.14. Mass Spectrum

Neomycin is insufficiently volatile for direct mass spectrometric analysis. To overcome this problem Inouye[14] prepared the volatile N-salicylidene Schiff's base, the M.S. of which, however, did not exhibit a peak for the molecular ion. To observe the molecular ion it was necessary to use the o-trimethylsilyl ether of the N-salicylidene Schiff's base. The spectrum of N-salicylidene neomycin was found to be dependant on the ion-chamber temperature indicating that thermal decomposition plays a significant part in the fragmentation process.

The M.S. examination of neomycin trimethylsilyl ether following GLC has been reported by Murata et al[15]. The fragmentation pattern obtained by these workers is somewhat different to that observed by Inouye[14].

It is interesting to note that the molecular ion corresponding to neamine(a major product of neomycin hydrolysis) was not observed in the mass spectrum of either the N-salicylidene or the T.M.S. derivative.

In an attempt to avoid the need for derivatisation Rinehart and Cook[16] applied the technique of Field Desorption Mass Spectrometry to neomycin and successfully observed an intense response for the molecular ion at m/e 615(M + H) together with very small peaks at m/e 455, 307 and 206 representing the loss of sugar fragments.

Table 2

^{13}C NMR Absorptions of hexa-N-acetyl-neomycin[12]

Carbon atom (see Fig.1)	δ ppm (from TMS)	
	Hexa-N-acetyl neomycin B	Hexa-N-acetyl neomycin C
A1	97.0	96.9
A2	54.2	54.1
A3	71.4	71.4
A4	71.4	71.4
A5	71.4	71.4
A6	40.8	40.8
D1	50.4	50.4
D2	33.2	33.1
D3	48.9	48.9
D4	76.7	76.8
D5	86.0	86.0
D6	74.5	74.5
R1	109.3	110.0
R2	74.5	73.5
R3	77.2	75.2
R4	82.4	81.8
R5	62.2	62.9
B1	98.8	97.3
B2	51.8	54.3
B3	70.3	71.7
B4	68.5	72.3
B5	74.0	71.4
B6	40.8	40.6
CH_3	23.0(x 3) 22.9(x 3)	23.2 22.8(x 5)
CO	175.2 174.9 174.8 174.7 174.5 174.1	175.2 175.1 174.9(x 2) 174.6 174.1

2.2. Physical Properties of the Solid

2.21. Hygroscopic Nature

Hirtz et al[17] demonstrated the hygroscopic nature of neomycin sulphate using a thermogravimetric technique. In a more recent study Russian workers[18] determined the adsorption of water by neomycin sulphate at temperatures of 23°, 90° & 100°C. Table 3 gives the results obtained at 23°C.

Table 3

Adsorption of Moisture by Neomycin Sulphate

Humidity %	Moisture adsorbed % (350 hours storage)
0	0
10	4.15
15	5.25
25	6.90
40	10.20
60	16.70
80	38.90

2.22. Solubility

The solubilities of eleven salts of neomycin in some twenty six solvents have been reported.[19,20,21] The values for the most commonly encountered salts are listed in Table 4.

2.23. Specific Surface Area

Using a method based on the filtration of air through a compressed layer of powder Ezerskii et al[22] determined the visible specific surface area of neomycin sulphate to be $2.6\ m^2 g^{-1}$.

Table 4

Solubility of Neomycin Salts(mg/ml,corrected for solvent blank)

Solvent	Neomycin B hydrochloride	Neomycin sulphate	Neomycin undecylenate	Neomcyin oleate	Neomycin stearate
Water	14.98*	6.28	0.93*	0.318	0.478
0.1N sodium hydroxide				>20	
0.1N hydrochloric acid				11.300	6.825
Methanol	5.685*	0.21	>20*	>20	17.410
Ethanol	0.61	0.055	>20*	>20	9.994
Isopropanol	0.05	0.082	>20*	>20	7.564
Isoamyl alcohol	0.285	0.202	>20	>20	11.322
Ethylene glycol	3.838*	0.033			
Propylene glycol				3.852	2.132
2-methoxy ethanol			>20*		
Benzyl alcohol	1.0*	12.35*	>20*		
Cyclohexane	0.055	0.075		>20	0.542
Cyclohexene			1.903		
Benzene	0.005	0.025	4.73	>20	0.580
Toluene	0.0	0.0	3.42		

All values $\pm$ 0.025mg except * $\pm$ 0.05mg.

Table 4 (Contd..)

Solubility of Neomycin Salts (mg/ml, corrected for solvent blank)

Solvent	Neomycin B hydrochloride	Neomycin sulphate	Neomycin undecylenate	Neomycin oleate	Neomycin stearate
Petroleum ether	0.0	0.005	2.62	>20	0.188
Isooctane	0.03		0.342	15.852	0.0
Ethyl acetate	0.03	0.028	4.70*	6.885	1.030
Isoamyl acetate	0.06	0.06	7.36*	14.080	7.014
Acetone	0.04	0.15	16.00*	9.260	2.083
Methyl ethyl ketone	0.058	C.005	5.795*	6.578	1.742
Diethyl ether	1.485*	0.07	7.989	11.288	7.328
Ethylene chloride	0.005		0.77*	3.257	0.217
Chloroform	0.06	0.0	>20*	>20	9.463
Carbon tetrachloride		0.075	7.938	>20	1.046
1,4 dioxane	0.14*	0.14*	6.485	13.038	4.500
Carbon disulphide			9.40*	>20	3.918
Pyridine	0.323*	0.923*	>20*	>20	>20
Formamide	0.45*	0.35*	>20	1.365	0.217
Dimethylsulphoxide				10.580	3.862

All values $\pm$ 0.025mg except * $\pm$ 0.05mg.

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2.3. Physical Properties of Solutions

2.31. Density

Kigel et al[18] have reported the density of 6 & 18% aqueous neomycin sulphate solutions over the temperature range 20° to 80°C. The published values are given in Table 5.

Table 5

Densities of Neomycin Sulphate Solutions

Neomycin Concentration %	Density			
	20°C	40°C	60°C	80°C
6	1.025	1.021	1.014	1.007
18	1.086	1.081	1.076	1.068

2.32. Viscosity

Table 6 shows the reported[18] viscosities for 6 and 18% aqueous neomycin sulphate solutions over the temperature range 20°C-80°C.

Table 6

Viscosity of Neomycin Sulphate Solutions

Neomycin Concentration %	Viscosity (cps)			
	20°C	40°C	60°C	80°C
6	1.25	0.86	0.64	0.623
18	1.99	1.30	0.95	0.74

2.33. Surface Tension

Kigel et al[18] have reported the surface tension values of 6 & 18% aqueous neomycin sulphate solutions (Table 7).

<div align="center">

Table 7

Surface Tension Values for Neomycin
Sulphate Solutions

</div>

Neomycin Concentration %	Surface Tension (dynes/cm)			
	20°C	40°C	60°C	80°C
6	69.81	66.00	63.30	60.30
18	72.01	68.12	65.45	62.36

2.34. Adsorption by Clays, Soils & Minerals

The adsorption of antibiotics from aqueous solution by clays and minerals has been reported by a number of authors. Pinch et al[23] concluded that, for strongly basic antibiotics such as neomycin, adsorption by clays such as montmorillonite distorted the crystal lattice of the clay 4.4Å, corresponding to monolayer adsorption. The same authors also suggested the bonding in the monolayers to be strongly electrostatic. Attempts to release the antibiotic by the addition of buffers has been demonstrated to be only partially successful[24]. Phosphate buffers released approx. 50% of the adsorbed antibiotic whereas citrate, glycine and universal buffers were completely ineffective. In a recent study McGinity and Hill[25] have shown divalent magnesium ions to be more efficient than monovalent sodium ions in displacing neomycin from the negatively charged clays attapulgite and montmorillonite.

From these studies the limiting adsorptive capcities for three clays were calculated using the Langmuir equation.

Wayman et al[26] studied the binding of neomycin to the filtration materials celite, cellulose powder and Seitz filters. Neomycin was found to be adsorbed on all three materials. Acid-washing the cellulose powder failed to desorb all of the neomycin.

2.35. Adsorption by Ion Exchange Resins

During a study of the physico-chemical properties of some aminoglycoside anti-biotics Kuzyaeva[11] determined the static and dynamic exchange capacities of neomycin on the cation exchange resin KB-4P-2(a phenoxyacetic acid-formaldehyde resin) using the resin in the Na^+ form.

Kil'fin et al[27] have reported values for the exchange enthalpy of neomycin for three ion-exchange resins. The values of ΔH were calculated by application of the Gibbs-Helmholtz equation; the published results are tabulated below:-

Table 8
Some Exchange Enthalpies for Neomycin

Ion exchange resin	Type	ΔH cal.equiv^{-1}
KFU,Na^+form	methacrylic acid-divinylbenzene	-40
KMDM6,Na^+form	methacrylic acid-hexamethylenedimethyl acrylamide	+340
KB4P2,Na^+form	phenoxyacetic acid-formaldehyde	+570

In a further publication Kil'fin and Samsonov[28] reported investigations into the variation of the Selectivity Coefficient (K) with the amount of neomycin adsorbed for fourteen different ion-exchange resins. In all cases the selectivity coefficient increased with the amount of adsorbed neomycin(expressed as the % surface-coverage of the ion exchange resin), though different types of ion exchangers resulted in different responses. The degree of cross-linking of the ion exchange resin was also report-ed to affected the value of the selectivity coefficient. The authors concluded that poly-condensation-type ion exchange resins adsorb

neomycin more selectively than the polymerisation -
type resins and that variation of the components
of either type of resin had little effect on the
adsorption of neomycin.

Klyueva and Gel'perin[29] have
compared the equilibrium distribution curves for
the adsorption of neomycin on ion-exchange resins
from both pure solutions and typical fermentation
broths.

3.   Salts, Derivatives & Complexes

3.1. Salts

Many attempts to alter the physical
properties of neomycin by the formation of various
salts have been described. Thus the neomycin
salts of the higher fatty acids, such as the
stearate, palmitate and myristate[30,31] were pre-
pared and being water-insoluble were formulated in
ointment bases. Similarly, the undecylenate salt
has been prepared and processes for its production
patented[32,33,34]. The undecylenate and caprylate
salts have been described as being particularly
suitable as antimitotic compounds[35].

Various carboxylic acid salts have also
been reported. Gallardo[36] produced the maleate
salt of neomycin which, it was claimed, improved
the aqueous stability of the antibiotic. A prac-
tically tasteless compound, the citrate salt,has
been described by Szyszka[38]. Neomycin mandelate
has been claimed to be particularly useful in the
treatment of urogenital infections[39] while the di-
hydroxy-dinaphthylmethane-dicarboxylate[40] and the
pamoate salts[41,67,68] have a low intestinal ab-
sorption and are thus effective treatments for
intestinal infections.

Fisher and Hall reported the propion-
ate salt to be unsuitable for use in ophthalmic
preparations[37]. Other salts described in the
literature are neomycin glucuronate[42], succi-
nate[43,44], lactate[45], ascorbate[45], phthalate[44]and
the sulphosuccinate[47,48] which has the property
of improved skin penetration.

Amongst the organic sulpho acids used to prepare neomycin salts, the p-toluene sulphonate has been reported and incorporated in an aerosol spray formulation[49]. Salts of neomycin with halogen substituted 8-hydroxyquinoline 5-sulphonic acids have been described as possessing low toxicity, antiseptic and antiamebic besides antimicrobial properties[50,51]. Stankovics et al[52] and Veciana[53] precipitated a complex salt of neomycin, by mixing aqueous solutions of neomycin sulphate and sodium lauryl sulphate, which had improved activity against *Staphylococcus aureus*. In a more recent investigation of this reaction, Leucuta et al[113] found that the precipitated complex dissolved on addition of excess sodium lauryl sulphate. I.R. studies of the complex have indicated that amide groups, coupling reactions and H-bonding are involved in the formation of the compound. Michele and Valette have reported an enhanced antibiotic activity and a reduced toxicity for the eucalyptol sulphonate[54]. Preparation of the dodecylbenzene-sulphonate[55] and the cyclohexylsulphonate[56]has also been described.

The pantothenate salt of neomycin, first described by Keller et al[57], also exhibits the property of a lower toxicity than the parent antibiotic. A further paper by the same authors[58] attributed the lower toxicity of the pantothenate to be due to a reduction in the calcium-binding ability of the antibiotic when in the form of the pantothenate salt. A similar effect has been noted by Weitnauer et al[59] with an equimolar mixture of neomycin and calcium gluconate. Numerous procedures for the preparation of the pantothenate salt have been patented[60,61,62,63]. Abbou[64] reported the preparation of a complex salt of neomycin with a mixture of glutamic and pantothenic acids and described the compound as possessing better organoleptic properties than other neomycin salts.

Salts with the amino acids N-methyl-stearoylglycine[65] and glutamic acid[66], and the vitamins ascorbic acid[63] and nicotinic acid[63] have also been evaluated.

Chlorophenols are of a sufficiently acidic nature to be able to form salts with neomycin and have been reported to possess both antibacterial and mycocidal properties[69,70,71]. Shaw[70] has described the topical use of such a compound, neomycin hexakispentachlorophenate, in the treatment of dental root infection and periodontic cavities.

A salt formed by reacting neomycin with p-amino benzene sulphoacetamide was described by two groups of workers[72,73]. The salt combined the chemotherapeutic properties of the components. Another unusual salt of neomycin described in the literature is that with m-sulphonylbenzaldehyde isonicotinoylhydrazone[78,79]. The compound is reputed to be less toxic than neomycin and to be more active against tercule bacilli than the individual constituents.

Salts of neomycin with the inorganic acids have also been described. The sulphate salt is the usual commercial form in use today but neomycin borate has been used in ophthalmic preparations[46].

Not all neomycin salts, however, possess advantages such as a reduced toxicity. In fact, the orotic acid(1,2,3,6-tetrahydro-2,6-dioxo-4-pyrimidine carboxylic acid) salt was reported to have a greater toxicity than neomycin itself[74,75]. Some uncertainty of this fact is apparent as other authors have reported the two compounds to be of a similar toxicity[76]. The salt of neomycin with usnic acid(an antibacterial substance found in lichens) has been reported[77]to have less antibacterial activity than the neomycin used in the preparation of the salt.

3.2. Derivatives

The neomycin molecule contains both free amino and free hydroxy groups. Many workers have exploited the possibility of chemical derivatisation at these positions in attempts to reduce the toxicity of the parent antibiotic. Substitution of methane sulphonate groups at the amino nitrogen has been reported by Umezawa et al[83,84] and by Boissier et al[85]. Both groups of workers described the derivative to be less toxic

than the parent antibiotic. Boissier claimed that
the 'in vitro' and 'in vivo' antibacterial
activity of the neomycin is preserved following
derivatisation. However, Umezawa, working with
fradiomycin, described the N-methyl sulphonate
derivative as having half the activity of fradio-
mycin when measured against gram-positive bacteria
and only one tenth the activity when measured
against gram-negative bacteria. Russian workers[89]
have reported the reduction in antibacterial
activity to be proportional to the degree of N-
substitution. Alternative procedures for the
preparation of N-methyl sulphonyl neomycin have
been reported by a number of authors[86,87,88] and
the pharmacology of the compound has been extens-
ively investigated by Di Marco and Bertazzoi[90].
The preparation of the following sulpho deriva-
tives has also been described:

     N-methylsulphino neomycin[87,91]

     N-phenylsulphonyl neomycin[93]

     N-acetamidophenylsulphonyl neomycin[93]

     N-p-aminophenylsulphonyl neomycin[93]

     N-trichloromethylthio neomycin[92]

     N-ethylthiocarbonyl neomycin[93]

     and neomycin N-sulphonate[94]

       Vanderhaeghe[80] has studied the
alkylation of neomycin. A reduction in toxicity
was noted following alkylation but this was
coupled with a complete loss of antibacterial
properties. The resulting compound, however, was
found to possess good hypocholesteremic activity.
The effect of N-alkylation, O-alkylation, N-
acylation and O-acylation has been investigated
by Magyar et al[81]. These workers reported the
loss of biological activity to be proportional to
the number of groups substituted. Further investi-
gations by these workers demonstrated that con-
version of amino groups into quaternary ammonium
groups resulted in the production of compounds
having a mild tuberculostatic effect. Penasse et
al[82] have described an alternative procedure for
the preparation of N-alkyl derivatives.

3.3. Complexes

The formation of a zinc-neomycin complex has been reported by Chornock[95] and by Keller and Cosar[96]. Electrophoresis was used to demonstrate the formation of a complex which was described as possessing a similar biological activity to neomycin but a decreased toxicity. More recently Agrawal, Harmalker and Vijayawargiya[97] have described the formation of a copper-neomycin complex. With a stoichiometry of 1:1, the blue coloured complex has been made the basis of a spectrophotometric assay method for neomycin(See Section 6.26).

The complexation of neomycin with a number of biochemically important compounds has been reported. Interaction of the antibiotic with heparin was first investigated by Higginbotham and Dougherty[98]. Using a spectrophotometric procedure, which measured the amount of dye released from a toluidine blue-heparin complex on addition of neomycin solution, these authors studied a number of antibiotics and their reaction with heparin. Gubernieva and Silaev[99] employed electrophoresis to investigate the nature of the neomycin-heparin complex and suggested the compound may be more than a simple cation-anion interaction. The possibility of additional H-bonding following the initial ionic interaction was postulated. A more detailed study of the complexation of neomycin with heparin, DNA,RNA and polyphloretin phosphate has been described by Hein[100]. The complexes were prepared by mixing aqueous solutions of neomycin sulphate and the complexing agent under investigation. The precipitate which formed redissolved on addition of sodium hydroxide solution or aqueous solutions of silver nitrate, manganese sulphate and cobalt chloride. Subjection of the precipitated complex to an agar diffusion experiment demonstrated the compound to be biologically active.

Using an agarose gel system Kunin and Tupasi[101] observed the formation of a precipitation band between zones of dextran sulphate and neomycin. The authors attributed the precipitation to be a result of complex formation

and not the result of an antigen-antibody inter-
action which the system is usually employed to
detect.

Harris[102] has studied the complexa-
tion of neomycin with pectin and demonstrated the
inhibition of complex formation in the presence of
an electrolyte. Potentiometric measurements in-
dicate the mechanism of the reaction to be a
cation-anion interaction. H-bonding between the
hydroxy groups of pectin and sugar moieties of
neomycin has been suggested and would further
stabilise the compound.

The complexation of neomycin with
anionic dyes such as amaranth (F.D. & C. Red No.2)
is well known[100,102] and has been made the basis
of a quantitative assay for neomycin[102,25]. These
complexes are again of the cation/anion type and
their formation is dependant on the ionic strength
of the solution.

Inorganic condensed phosphates have
been reported to complex with neomycin and in a
study of these compounds, Singh[103] demonstrated the
strength of the complex to be dependant on the
degree of polymerisation of the phosphate. By in-
creasing the number of phosphate units in the
polymer over the range 1 to 16 units an increasing
strength of complexation was observed. Beyond this
upper limit, however, the strength of the complex
remained constant. Relative complex-strengths
were assessed by titrimetry with potassium chloride
solution.

A number of workers have utilised
electrophoresis to establish the formation of a
complex between neomycin and the proteins in blood-
serum and plasma[104,105,106,107,108,109,110,111].
However, the nature of the complex is still un-
known. Geitman has extensively studied the pheno-
mena of protein-binding of antibiotics[111,112].
In a paper published jointly with Kivman[112],
Geitman reported that neomycin did not bind to any
of the protein fractions when added to serum but
bound to the isolated albumin and globulin
fractions. An earlier paper[111] described the
globulin-neomycin complex to be of greater stabil-

ity than the albumin-neomycin complex and estab-
lished lmg of serum protein to bind 0.06 units of
neomycin.

4.   Synthesis and Production

4.1. Commercial Biosynthesis

Waksman   et al[286], in 1949, first re-
ported the production of neomycin by fermentation
of a culture of *S.fradiae*(3535). The same organism
subsequently formed the basis of an industrial
fermentation process for the biosynthesis of
neomycin[287,288]. Isolation of the antibiotic from
the fermentation media is accomplished by use of
ion-exchange resins, such as Amberlite
IRC 50[225,250,251].

Following the original report by
Waksman, a number of other authors have described
S.fradiae to yield antibiotics on fermentation.
The resulting substances were named streptothricin
BI[289], mycerin[290] and colimycin[291], but have since
been shown to be identical to the neomycin com-
plex.

4.2. Synthesis of Radio-Labelled Neomycin

The biosynthetic preparation of ^{14}C
labelled neomycin was first described by Sebek[267]
who studied a number of substrates for this pur-
pose but concluded ^{14}C-labelled glucose was the
most satisfactory. Although 68% of the radio
nuclide was lost as respiratory $^{14}CO_2$, 19% of the
total activity added prior to fermentation was
incorporated into the neomycin sulphate. This
method of preparing^{14}C-labelled neomycin has been
extensively used to study the mechanism of neo-
mycin biosynthesis. Stroshane[13] has described
the synthesis of ^{13}C-neomycin and ^{15}N-neomycin,
again as part of an investigation of neomycin
biosynthesis. ^{13}C-glucose served as precursor
for ^{13}C-neomycin and glucosamine labelled with
^{15}N was utilised for the preparation of ^{15}N-
neomycin.

A procedure for labelling neomycin
with tritium has been described by Jackson et

at[292] and involves passing an electric discharge
across a vessel containing tritium and neomycin
sulphate. Labile tritium was removed by washing
with a polar solvent and the tritium-labelled
neomycin purified by multiple recrystallisation.

5.   Stability and Degradation

   5.1. Hydrolytic Degradation

         The degradation of neomycin has been
extensively studied by Rinehart[1] as a means of
establishing the structures of the neomycin compo-
nents. Fig. 5 illustrates the route by which com-
plete degradation of the antibiotic was achieved.
Extremely vigorous conditions are necessary for the
hydrolysis of neamine as this compound is resistant
to acid-hydrolysis. However, under these conditions
the chemical entities neosamine C(from neamine) and
ribose are not observed because vigorous hydrolysis
immediately decomposes the compounds further. By
derivatising the amino groups of the neamine and
neobiosaminide molecules Rinehart was able to lower
the resistance of these compounds to hydrolysis and
thus use mild hydrolytic conditions to successfully
isolate neosamine C and ribose:

a) neamine $\xrightarrow[\text{CH}_3\text{OH } 5^\circ\text{C}]{(\text{CH}_3\text{CO})_2\text{O}}$ tetra N-acetyl neamine

$\downarrow$ 3N HCl

$\quad$ 10hr @ 95°C

$\qquad$ deoxystreptamine + neosamine

b) methyl $\xrightarrow[\text{NaOH}]{\text{PhCOCl}}$ methyl N,N'-dibenzoyl
   neobiosaminide   neobiosaminide

$\downarrow$ 1.6N HCl

ribose $\xleftarrow{\text{0.1N H}_2\text{SO}_4}$ $\text{CH}_3\text{OC}_5\text{H}_6\text{O(OH)}_3$

         Russian workers[198] attempted to pre-
pare the degradation products in a pure form by
carrying out the hydrolysis in the presence of an
ion exchange resin which adsorbed the intermediate

Figure 5. Hydrolytic degradation of neomycin C

hydrolysis products,thereby preventing further de-
composition. The degradation products were re-
leased from the resin by gradient-elution.

5.2. Stability of Bulk Material

Japanese workers[115] have described the
stability of bulk fradiomycin (neomycin B)sulphate
which was shown to have retained 99% of its micro-
biological potency after storage for 24 months.
Neomycin sulphate powder has been reported to be
stable for at least 3 years at 20°C [298,305].

Neomycin sulphate may also be heated
at 110°C for 10 hours, during dry-heat sterilisa-
tion, without loss of potency, though some degree
of yellowing is apparent[298].

5.3. Stability in Aqueous Solution

Swart et al have demonstrated the
stability of neomycin hydrochloride in aqueous
solution over the pH range 2-9[293]. Further in-
vestigations by Simone and Popino[298] confirmed the
aqueous stability at 23°C but at 45°C potency
losses of up to 94% were noted over a period of
2 years with solutions in the pH range 4-8.

Leach et al[294] described a purified
neomycin to be stable to the action of alkali but
not to acids. Refluxing the antibiotic with barium
hydroxide for a period of eighteen hours failed to
show a loss in microbiological potency.

Japanese workers have described a stable
aqueous solution of neomycin with the incorporation
of a borate buffer (pH 6) and E.D.T.A.[295] The
presence of 1-10% of glycerol, propylene glycol or
mannitol has been claimed to improve the solution
appearance by preventing discoloration[296]. The
presence of polyols also prevented a decrease in
pH value of the solution. Discoloration may also
be prevented by addition of 0.1% sodium metabi-
sulphite at a solution pH of 6.6 to 6.8[297].

5.4. Stability in Pharmaceutical Formulations

Numerous reports concerning the stability of neomycin in various dosage forms have been published. Simone and Popino[298] studied the stability of neomycin in liquid dosage forms such as nasal drops, mouth washes and tinctures. The antibiotic was stable in all the formulations tested, except Dobells solution (a mouth wash), for at least 6 months at 20°C. Some formulations were stable for considerably longer.

Heyd[299] has studied the stability of neomycin in aqueous gels and formulated a satisfactory product by adsorbing the antibiotic on an ion exchange resin, Amberlite IRP-69M, prior to incorporation in the gel.

Reconstituted aqueous suspensions of neomycin sulphate, containing tragacanth and sugar have been shown to be stable for 10 days when stored at 4°C but some loss of potency was observed when the suspension was exposed to daylight[300]. Non-aqueous suspensions containing peanut oil and lanolin have been reported[298] which are stable for 1 year at 20°C.

Simone and Popino[298] have considered the stability of neomycin in both hydrophobic and hydrophilic ointment bases. No loss of potency over a period of 1 year at 20°C was reported for formulations containing carboxymethylcellulose, polyethylene glycol(P.E.G.) or white-soft paraffin. However, formulations containing hydrous lanolin were reported to be unstable. All materials used in the formulations were obtained from U.S. sources. Coates et al[301] investigated the use of P.E.G. from British sources and described neomycin as being incompatible with the materials tested.

Solid dosage forms of neomycin as tablets and troches have been prepared[298] and are reported to be stable at 20°C and at 56°C.

In the area of animal health nutrition a feed-premix containing neomycin and oxytetracycline has been described. With neomycin

at a level of 20g/lb the product is stable for 1
year at 20°C, though an 8% loss of potency was ob-
served on storage for 6 months at 38°C[305]. A med-
icated drinking-water containing 100 ppm of neo-
mycin and prepared with tap-water, showed no signi-
ficant loss of potency after 48 hours storage at
38°C in either glass containers or galvanised
troughs[305].

5.5. Compatibility with Excipient Materials

The reaction of neomycin with many com-
pounds has been described in Section 3, hence
numerous reports of neomycin incompatibility may be
expected. Dale and Rundman[304] have extensively re-
viewed the compatibility of neomycin with substances
that may be encountered by the formulation pharma-
cist. Kudalker et al[303] have described the incom-
patibility of the antibiotic with rancid oils, and
the incompatibility with bentonite, a montomorill-
onite clay, has been reported by Danti and Guth[306].
The incompatibility with lactose, causing a dis-
coloration of the mixture has been studied by
Hammouda and Salakawy[307]. The amount of browning
produced was shown to be dependant on the initial
pH of the solution. The rate of discoloration of
the lactose/neomycin powder was directly related
to the temperature of storage and the relative
humidity of the atmosphere. Discoloration was over-
come by addition of sodium bisulphite.

Florestano et al[308] compared the re-
lease of neomycin from P.E.G. diester ointment
bases and grease-type bases and concluded the
former to be preferred though Coates et al[301] have
described P.E.G. from British sources to be incom-
patible with neomycin.

5.6. Compatibility with Other Actives

Neomycin forms an insoluble complex when
added to aqueous solutions of corticosteroid phos-
phates. To overcome this incompatibility, McGinty
and Brown[304] incorporated disodium hydrogen phos-
phate in the opthalmic formulation. The mechanism
by which the complexation is prevented has not yet
been fully determined.

6. Methods of Analysis

6.1. Identification

Table 9 lists a number of colorimetric tests which have been used as identity tests for neomycin. No one test, however, has been demonstrated to be specific for neomycin and it is thus also advisable to establish the absence of other chemically similar antibiotics by suitable means.

Table 9

Colorimetric Identity Tests for Neomycin

Test	Chemical grouping utilised in reaction	Colour obtained	Reference
Glucosamine	glycoside	cherry	114
Molisch	pentose	purple	115
Ninhydrin	amino	purple	115,116
Furfural	ribose	pink-red	116
Phloro-glucinol	ribose	pink-red	117

Both thin-layer and paper chromatography will provide specific identification of neomycin. Numerous systems for this purpose will be found listed in section 6.34.

In addition to the above chemical tests a number of microbiological procedures have been reported. Heinmann et al[118] have described a simple test to differentiate neomycin-like molecules from tetracyclines and chloramphenicol. When added to a column of innoculated agar only the neomycin-like molecules show an area of growth-inhibition at the top of the column.

A more specific microbiological procedure has been reported by Freres and Bulland[119]. By using four test organisms (*Bacillus cereus, Bacillus subtilis, Sarcina lutea* and *Micrococcus flavus*) patterns of inhibition were determined for each of the 12 antibiotics extracted into three solvents. Comparing the inhibition pattern of an

unknown antibiotic with the standard patterns, the
authors were able to identify the presence of
individual substances in animal tissue. A similar
test has also been described by Tiecco et al[120].

6.2. Chemical Procedures

6.21. Titrimetric Assay

The determination of neomycin by
non-aqueous titration has been described by Penau
et al[121]. Neomycin base is allowed to react with
standardised perchloric acid; the excess acid is
then back-titrated with potassium hydrogen phtha-
late using crystal violet as indicator. To deter-
mine the neomycin content of the sulphate salt the
same authors precipitated the sulphate with benzi-
dine before reacting the neomycin with perchloric
acid. The amount of benzidine required to pre-
cipitate the sulphate is calculated from the sul-
phate content which is itself determined by titra-
tion with sodium hydroxide.

An alternative method for deter-
mining the sulphate content of neomycin sulphate
has been reported by Roets and Vanderhaeghe[122].
The procedure involves a titration with barium
chloride and must be carried out in a 30-40% solu-
tion of alcohol in order to obtain a sharp end-
point. As the antibiotic is insoluble in alcohol
it is necessary to remove the neomycin part of the
molecule prior to titration. This is accomplished
using a cation exchange resin such as Dowex 50-X8.

During an investigation of the
complexation properties of neomycin, Harris[102]
reported the titration of neomycin conductometri-
cally with amaranth (F.D. and C Red No. 2). The
end-point of the titration is sharp and has good
reproducibility.

6.22. Polarography

The use of differential pulse
polarography to determine neomycin sulphate in
aqueous solutions has been described by Siegerman
et al[123]. Following acid hydrolysis of neomycin,
the solution was adjusted to pH 4 and 0.1M acetate

buffer(pH 4) added as supporting electrolyte.
Reduction potentials of -0.19 and -0.36V were ob-
tained. The above authors also described the
application of this technique to the examination
of samples of skin previously treated with neo-
mycin-containing formulations.

6.23. Polarimetry

Neomycins B and C have been shown
to differ in their specific rotation values, neo-
mycin B having a specific rotation of + 80° and
neomycin C a specific rotation of + 120°[124,127].
Brooks et al[125] made this fact the basis for a
number of methods to determine the B & C content of
commercial neomycin. The specific rotation of the
test solution is determined at 25°C and total neo-
mycin determined either titrimetrically or spectro-
photometrically. By substitution of these values
in a suitable equation the concentration of neo-
mycins B and C are calculated. In a second method
the same authors determined the specific rotation
of the test-solution at temperatures of 25° and
75°C. The change in the value of specific rotation
on increasing the temperature from 25°to 75°C can
then be used to calculate the amounts of neomycin B
and C in the sample.

The use of an automatic polari-
meter with a flow-cell has been reported by
de Rossi[126],to monitor the eluate from an ion-
exchange column (Bio-Rad AGl-X2) through which a
solution of neomycin was passed. The detection of
an optically active substance was recorded electro-
nically with a suitable pen recorder. By determin-
ing the areas of the peaks recorded, the amounts of
neomycins B and C and neamine in a number of
commercial samples have been determined.

6.24. Radio-chemical Assay

To determine the relative amounts
of neomycins B, C and neamine by a radio chemical
method, Kaiser[128] separated the ^{14}C-labelled N-
acetyl derivatives by paper chromatography and
quantitated the chromatograms by liquid scintilla-
tion counting. A coefficient of variation of 3.6%
was obtained.

Seaman and Stewart[129] have de-
scribed a radio-chemical assay for determining
neomycin on cotton-fabric. Neomycin is reacted
with carbon disulphide forming a dithiocarbonate
which is then decomposed with $[^{110}Ag]$ silver
nitrate. The precipitated $[^{110}Ag]$ silver sulphide,
which is directly related to the amount of neomycin
present, is estimated by counting.

Recently an isotope dilution
method has been reported[130] for assaying neomycin
sulphate. However, it is first necessary to
prepare ^{14}C-labelled neomycin sulphate. This is
accomplished by adding ^{14}C-labelled glucose to a
small-scale fermentation of *S. fradiae*. ^{14}C-labelled
neomycin can then be extracted by solvent-extraction
or by ion-exchange chromatography.

6.25. Fluorimetric Assay

The neomycin molecule does not
exhibit fluorescence but following suitable de-
rivatisation two spectrofluorimetric determinations
have been described. Maeda et al[131] reported the
complexation of simple hexosamines with pyridoxal
and zinc ions to result in a fluorescent deriva-
tive. Applying this procedure to neomycin,
Simpson[132] demonstrated a linear response over the
concentration range 0-50 µg/ml of neomycin sulphate.

The reaction of neomycin with
fluorescamine to form a fluorescent complex has
been reported by Kusnir & Barna[133]. The fluore-
scence intensity varies with both the pH of the
solution (optimum pH range 7.5-9.5) and the amount
of fluorescamine added. This procedure may be used
to determine neomycin at very low levels, the
minimum concentration determinable being 45 ng/ml.

6.26. Spectrophotometric Assay

Spectrophotometric assays of
neomycin may be conveniently divided into two
groups:-

(a)   Direct methods, involving the
      intact neomycin molecule.
(b)   Indirect methods, involving hydrolysis
      of neomycin prior to assay.

(a) Direct Methods

O'Keefe and Russo-Alesi[134] first reported
the application of ninhydrin to the assay of neo-
mycin, the procedure being an adaptation of that
described by Moore and Stein[135] for the assay of
amino acids and involving measurement at 570nm of
the purple-coloured complex formed. Maehr and
Shaffner[136] have applied this procedure to the
determination of neomycin in chromatography -
column eluates. Gerosa and Melandri[137] attempted
to apply the Moore and Stein method to the quanti-
tative determination of neomycin in pharmaceutical
preparations but reported a linear response only
over the short concentration range of 400-600µg/ml
of neomycin. Borowiecka[138] and Thorburn-Burns et
al[139] however, reported no such limitation and
successfully applied the method to the assay of
neomycin in pharmaceutical formulations.
Pregnalatto and Sabino[200] incorporated glycerine
into the ninhydrin solution and reported an im-
proved sensitivity and reproducibility enabling
neomycin concentrations as low as 4 µg/ml to be
determined. An automated ninhydrin assay has been
described by Kaptionak, Biernacka and Pazdera[140]
based on a modified Moore and Stein method[141].
An alternative reagent which also reacts with the
primary amino groups of neomycin has recently been
described[172]. The reagent, diclone(2,3-dichloro-1,
4-naphthoquinone), reacts with neomycin base to
form an orange-coloured product when the solution
is adjusted to pH 4.

Complexation of neomycin with various
dyes was reported by Hein[100] and the aqueous in-
solubility of the complex with amaranth (F.D. &
C Red No. 2) has been utilised by Hill[25] and by
Bufton and Saddler[142] to assay neomycin in aqueous
solution. Amaranth may also be used to determine
neomycin in production samples from fermentation-
recovery[168]. The dye, Orange II, has been simi-
larly[143] described(λ max. = 484 nm). Complex for-
mation between neomycin and the dye is very de-
pendant on the ionic strength of the solution,
thus necessitating careful control of reaction
conditions to ensure complete precipitation of the
complex during the assay procedure. Reaction of
sodium 1,2-naphtho-quinone-4-sulphonate with

neomycin[144] produces an orange/yellow product, λmax 460nm in acetic acid, which has been utilised to determine the neomycin content of ophthalmic solutions giving results in good agreement with microbiological assays.

Strongly basic antibiotics may be precipitated by formation of the coloured reineckate salt which may then be determined spectrophotometrically[165]. Bickford[166] dissolved the precipitated neomycin reineckate in acetone and has successfully used this procedure to assay neomycin extracted from topical formulations. Roushdi et al[173] preferred to oxidise the precipitate with potassium permanganate and then colorimetrically estimate the chromate produced with diphenylcarbazide.

Aromatic aldehydes react with primary amines forming Schiff's bases which are often coloured. The colour may then be used to quantitate the amine. Using this principle Kocy[167] has shown neomycin to form a yellow-coloured Schiff's base with salicylaldehyde though the quantitative aspect of this procedure is, as yet, incomplete.

The complexation of neomycin with copper results in the formation of a blue-coloured compound which has also been made the basis of a colorimetric determination of neomycin in pharmaceutical formulations[97]. Maximum colour intensity was observed at a solution pH of 10. Further investigation showed the stoichiometry of the complex to be 1:1 in alkaline solution.

(b) Indirect Methods

Hydrolysis products of neomycin may be an amino-sugar, a pentose or furfural depending on the reaction conditions chosen. Each of these entities has been utilised for indirect spectrophotometric determination of neomycin.

The presence of a sugar moiety in neomycin was demonstrated by Hamre et al[145] who used carbazole and anthrone to quantitate neomycins B and C in commercial neomycin. The procedures employed were those previously applied to manosido-streptomycin[146,147]. Penau et al[151] reported poor

agreement between results obtained by the anthrone
assay and microbiological procedures and suggested
the presence of carbohydrate impurities in neo-
mycin to be the reason.

Other reagents which form coloured com-
plexes with sugars such as orcinol[148,149,150](3,5-
dihydroxytoluene) and phloroglucinol[137](1,3,5-
trihydroxybenzene) have been reported for the
spectrophotometric assay of neomycin. Hoodless
claimed the phloroglucinol procedure to be less
time-consuming than other methods[117] and applied
this procedure to neomycin sulphate raw material.
Doulakas[174] adopted a similar procedure to deter-
mine the neomycin sulphate content of ophthalmic
ointments. Interfering substances co-extracted
from the ointment were separated from neomycin by
TLC before reacting the antibiotic with phloro-
glucinol.

The orcinol procedure has been used to assay
the neomycin content of fermenter broths[148,149,150]
though it is necessary to first separate the anti-
biotic by ion-exchange chromatography. Korchegin[150]
reported good agreement between the orcinol assay
and the microbiological assay of production sam-
ples. Khromov and Starunova[175] and Korchagin et
al[169] compared the orcinol and microbiological
assays for determining the neomycin content of
polymer-films and, again, good agreement between
the two procedures was noted.

After mild hydrolysis, neomycin has been
shown to give a positive Elson-Morgan reaction[151],
a reaction characteristic of amino-sugars[152]. A
method involving this reaction has been made the
basis of a quantitative assay for neomycin[151]
which has been used to determine the neomycin con-
tent of fermentation broths[154].

Vigorous hydrolysis of neomycin results in
the formation of furfural[154] which may be quanti-
tated in a number of different ways[155,156].Dutcher
et al[157] used a UV spectrophotometric procedure,
measuring the absorbance of the solution at 280nm.
A similar procedure combined with measurement of
the optical rotation has been used by Brooks et
al[158] to determine the neomycin B and C content

of the antibiotic. Colorimetrically, the furfural
obtained from neomycin may be assayed with an anil-
ine by measuring the amount of pink/red complex
formed. Morgan et al[159] used 4-bromoaniline in this
context, the 4-bromo derivative being more resis-
tant to the formation of interfering coloured pro-
ducts than aniline itself[160]. This method has been
applied to various pharmaceutical formulations and
results show good agreement with microbiological
assays. Aniline has been used to determine the
neomycin content of fermenter broths and other pro-
duction samples[161], neomycin undecylenate and neo-
mycin caprylate[162].

Much work has been carried out to establish
optimum conditions for a reproducible conversion of
neomycin to furfural, quantitative conversion of
the pentose moiety to furfural being difficult and
the procedures tedious[163,164]. The acid strength
for optimum production of furfural must be such that
it is strong enough to hydrolyse the sugar molecule,
yet insufficiently strong to decompose the furfural
formed. A review of the literature by Ivashkiv[170]
showed 12% hydrochloric acid to be accepted as op-
timum for conversion of pentoses to furfural.
However, as neomycin must first be decomposed to the
pentose before hydrolysis of the sugar can take
place, more vigorous conditions are required.
Dutcher et al[154] used 40% sulphuric acid to produce
an acid concentration of 37% in the solution for
hydrolysis and obtained maximum formation of fur-
fural after 1½ hrs reflux. Morgan et al[159] however,
used 70% sulphuric acid to produce a 25% acid con-
centration in the hydrolysis solution and obtained
maximum formation of furfural after one hour.
Ivashkiv[170] has evaluated the use of both hydro-
chloric and sulphuric acids and found up to four
times more furfural to be produced by sulphuric
acid, the yield being extremely sensitive to the
concentration of acid employed. Continuing further
work with sulphuric acid, the formation of furfural
as a function of neomycin concentration was ex-
amined and the conclusion reached that better
yields of furfural are obtained at low concentra-
tions of the antibiotic. Heyes[171] has shown a con-
stant amount of furfural to be produced on hydro-
lysis with 27-28% sulphuric acid, refluxing for 1
hour. This makes it unnecessary to accurately

pipette concentrated solutions of sulphuric acid.

From the above discussion it would,therefore
appear necessary to establish optimum conditions
for furfural production in each individual case.

6.3. Chromatographic Procedures

6.31. Counter-Current Distribution

Swart, Lechevalier and Waksman[176]
have examined neomycin by the method of counter-
current distribution using a partition-system of
borate buffer(pH 7.6)/pentasol(synthetic amyl al-
cohol)/stearic acid. Three major components with
varying amounts of neamine were found in samples of
neomycin B from various sources. Determination of
the amount of antibiotic remaining in each tube
following partition was carried out microbiologi-
cally. In an extension of this study to the
whole group of neomycin-like molecules Schaffner[177]
however, has been unable to separate neomycin B
into the separate components reported by Swart et
al[176] using either of four partition systems. The
systems employed were:-

    Systems 1-3) 0.5M borate buffer at pH 7.3,
                 7.6 or 7.8/pentasol/stearic acid.

           4) 0.5% bicarbonate buffer/pentasol/
              stearic acid.

Both chemical and microbiological
assays were utilized to determine the distribution
of antibiotic in the tubes. In all experiments
the microbiological assay results were more erra-
tic than the corresponding chemical assays and on
this basis Schaffner explained the difference be-
tween his results and those of Swart et al[176]
who used only microbiological determinations. In
this same work Schaffner was able to demonstrate
the same distribution pattern for both framycetin
and neomycins B and C and thus tentatively identi-
fied framycetin as neomycin. Similarly, but using
a partition system of 3% p-toluene sulphonic acid
in 1N acetate buffer (pH 3.8)/butanol Baikina et
al[178] have identified mycerin and colimycin as
neomycin and Sannikov[179] has identified framicin
with neomycin.

From a preparative aspect, Peck
et al[180] have employed the counter-current distri-
bution technique with a partition system of water/
butanol/p-toluene sulphonic acid to separate and
purify the neamine (neomycin A) present in commer-
cial neomycin.

6.32 Electrophoresis

Neomycin is a readily ionisable
molecule and should thus be separable from other
antibiotics by application of an electric field
(zone electrophoresis). Various workers have
successfully applied this technique to neomycin
and Table 10 summarises the conditions reported in
the literature. A number of authors described the
qualitative separation of neomycin from other
chemical-types of antibiotics using paper-electro-
phoresis[181,185,187] while Ochab[189] described sys-
tems specifically designed to separate compounds
within the aminoglycoside group of antibiotics.
Carr et al[186] have reported the quantitative deter-
mination of neomycin in the presence of polymixin
and bacitracin. Using paper electrophoresis
quantitation of neomycin was accomplished colori-
metrically with ninhydrin.

Lightbown and DeRossi[188] substi-
tuted an agar-gel support for the more usual
chromatography-paper and successfully separated
neomycin from a variety of antibiotics but not from
paromomycin. However, quantitation of this pro-
cedure was not possible due to the elongated zone
of inhibition produced by neomycin on incubation of
the agar-gel. The same authors also reported some
anomalous behaviour of the neomycin ion in the
presence of certain types of agar. Neomycin is a
basic molecule and under normal circumstances
would be expected to behave as a cation and migrate
to the anode. However, with certain types of agar
the neomycin was observed to migrate towards the
cathode. This phenomena was repeatedly confirmed
though not explained. A system suitable for the
quantitative estimation of neomycin using an agar-
gel support has been described by Grynne[183]. To
obtain a good clear zone for neomycin it was neces-
sary to change the buffered electrolyte/agar-gel to
a phosphate system at pH 6.5. Detection of the

Table 10

Electrophoresis of Neomycin

Electrolyte	Medium	Conditions	Detecting Agent (See also Section 6.34)	Reference
1. Veronal sodium buffer, pH 8.6	paper	15.3v/cm	ninhydrin	181
0.07M barbital buffer, pH 8.6	cellulose acetate	200v for 2 hours	Ponceau S.	182
Veronal buffer, pH 8.6	paper	25v/cm	*B.subtilis* ATCC 663	183,184
2. 5% formic acid(pH 2)	paper	15.3v/cm	ninhydrin	181
3. Acetic acid/pyridine/ water(2:6:92)	paper	225v for 6 hours	ninhydrin;aniline	185
(0.25:7.5:92.25)	cellulose acetate	400v for 1 hour	ninhydrin;Ponceau S	186
(20:6:9.74)	paper	225v for 6 hours	ninhydrin	187
4. Acetic acid/formic acid/water(6:3:91)	paper	700v	ninhydrin,Ponceau S	186

Table 10(Contd...)

Electrophoresis of Neomycin

Electrolyte	Medium	Conditions	Detecting Agent (See also Section 6.34)	Reference
5. Trishydroxymethylamino-methane/maleic acid/ sodium hydroxide (0.907%:0.870%:0.002%) pH 5.6	agar-gel buffered, pH 5.6	2000v, 200 mA	B.*subtilis* NCTC 8241	188
6. 2M formic acid/0.1M toluene sulphonate/ propanol/water (50:50:5:40)pH 1.8	paper	340v	B.*subtilis* ATCC 6633	189
(40:5:5:50) pH 1.9				189
7. 2M formic acid/0.1M toluene sulphonate/ water(40:5:55)pH 1.9	paper	340v	B.*subtilis* ATCC 6633	189
8. 1M ammonium hydroxide/ 1M sodium hydroxide/ 0.1M toluene sulphonate/ propanol/water (20:0.5:10:10:69.5) pH 10.8	paper	340v	B.*subtilis* ATCC 6633	189

Table 10(Contd...)

Electrophoresis of Neomycin

	Electrolyte	Medium	Conditions	Detecting Agent (See also Section 6.34)	Reference
9.	1M ammonium hydroxide/ 1M sodium hydroxide/water (20:0.25:79.75)pH 11.5	paper	340v	*B.subtilis* ATCC 6633	189
10.	1M sodium hydroxide/ water(5:95)pH 12.2	paper	340v	*B.subtilis* ATCC 6633	189
11.	Acrylamide gel , pH 4.6	acrylamide gel	100v, 2mA	Naphthalene Black	190
12.	pH 6.5 phosphate buffer (KHPO$_4$,22g/l; KH$_2$PO$_4$,28g/l)diluted 1:40 with distilled water.	agar-gel buffered at pH 6.5	310 to 360v (current $\ngtr$ 50mA)	*Staphalococcus epidermidis* ATCC 12228	183

antibiotic was achieved bioautographically. The
organism *Staphalococcus epidermidis* ATCC 12228 was
found to be the most sensitive for this purpose.
(The description of alternative bioautographical
systems will be found in section 6.34.)

Using an acrylamide-gel as
support, Coombe[190] has demonstrated the quantita-
tive assay of neomycin with a recovery of 96% in
the presence of bacitracin and polymixin. In this
case quantitation was achieved by densitometry
after staining the gel with naphthalene black.

Electrophoresis has also been
employed to separate neomycin from analytically-
interfering substances such as proteins. Hence
Brammer and Hemson[182] have determined the neomycin
content of blood serum. Neomycin was separated
from the serum proteins by electrophoresis on
cellulose acetate and assayed colorimetrically
following elution from the support.

A modified technique consisting
of an electric field applied perpendicular to a
flowing buffer solution and using chromatography
paper as support(cross-electrophoresis) has been
exploited to study the reaction of neomycin with
heparin[99,184]. Evidence for the formation of a
neomycin-heparin complex was obtained by this means.

6.33. Column Chromatography

The column chromatographic sep-
aration of neomycin B, neomycin C and neamine on
an ion-exchange column has been described by Maehr
and Schaffner[136]. Dowex 1X2(OH form) was the ion-
exchange resin and water the eluting solvent. The
column eluate was monitored by reaction with nin-
hydrin. Using a slower elution rate and a smaller
particle size of the same ion-exchange resin Inouye
and Ogawa[191] reported improved resolution of the
peaks. The application of this procedure to the
quantitative determination of neomycin C and nea-
mine in commercial samples of raw material has
also been described[6]. When compared with TLC re-
sults on the same samples, the column chromato-
graphic method gave consistently higher results
for the neamine content. TLC examination of the

fraction of eluate containing neamine,demonstrated
the presence of three other unidentified compounds
which gave a positive ninhydrin reaction thus ex-
plaining the observed difference between the two
procedures.

Roets and Vanderhaeghe[193] have
also examined neomycin by chromatography on
Dowex 1X2 but these authors monitored the column
eluate conductometrically. Gillet et al[194] app-
lied a similar procedure to the examination of
various samples of neomycin to ascertain the ratio
of neomycin B:C.

A number of minor components
present in commercial neomycin have been separated
by column chromatography on a carboxylic cation-
exchange resin, Amberlite CG-50[199]. The components
were eluted from the resin with ammonium hydroxide
solution. T.L.C. of the eluent fractions showed
the presence of two previously unreported impuri-
ties which were then isolated on Dowex 1X2 and
tentatively identified using NMR and mass spectro-
metry.

When investigating the acid
hydrolysis of neomycin, Brazhnikova and Kudinova[198]
added a sulphocationic-type ion-exchange resin to
the acid solution of antibiotic to adsorb inter-
mediate hydrolysis products and thus prevent fur-
ther decomposition during heating. Following
elution of the resin .in a column the products of
neomycin hydrolysis were identified and shown to
be dependant on the time of hydrolysis.

An ion-exchange chromatographic
procedure utilising Dowex 1X2 has been made the
basis of an industrial process for manufacturing
neomycin B which is free from neomycin C[195].

The isolation of neamine on
Amberlite IRC 50(Na form) following the acid hydro-
lysis of neomycin has been described[196]. Neamine
is eluted from the ion-exchange column with hydro-
chloric acid. This process had been patented[197]
as a means of manufacturing neamine.

Many column chromatographic procedures have been described for the commercial separation and purification of neomycin following fermentation.

6.34. Paper and Thin-Layer Chromatography

The examination of neomycin by paper and thin-layer chromatography has been for two main purposes:-

    a) to separate and identify neomycin in the presence of other antibiotics, and

    b) to separate individual components and degradation products of neomycin.

The solvent systems employed for these purposes are summarised in Tables 12 and 13 (paper chromatography) and Tables 14 and 15 (thin-layer procedures).

Visualisation of the chromatograms has been reported using both chemical and microbiological means. Chemical spray reagents which have been described are listed in Table 16. The various organisms that have been employed for bioautography are given in Table 11.

Table 11

Visualisation of Chromatograms Bioautographically

Organism	Reference
B.subtilis	201,202,204,228 234,235
B.pumilus	224
Escherichia coli	205,206
Staphalococcus aureus ATCC 6538 strain	207,220,231,232

Table 12
Paper Chromatographic Systems Separating Neomycin
from other Antibiotics

Paper	Direction	Solvent System	Rf Value	Use	Reference
Whatman No.1	Ascending	Water-saturated butanol	0.84	)	202
Whatman No.1	Ascending	n-Butanol/acetic acid/water(2:1:1)	0.85	)))	202
Whatman No.1	Ascending	n-Butanol/pyridine/ water(1:0.6:1)	0.94	)Identifi-)cation of)neomycin in	202
Whatman No.1	Ascending	3% Aqueous ammonium chloride	0.00	)the presence)of other	202
Whatman No.1	Ascending	Benzene/acetic acid/ water(2:1:1),organic layer	0.89	)antibiotics)))	202
Whatman No.1	Ascending	n-Butanol-saturated water	0.07	)	202
	Ascending	Methanol/3% aqueous sodium chloride(5:1)	0.03	)Separation of)neomycin from	203
Toyo No.50	Descending for 9 hrs.	n-Butanol/pyridine/ acetic acid/water (15:10:3:12)	0.13	)other)streptomyces)antibiotics	204

Table 12(Contd..)

Paper Chromatographic Systems Separating Neomycin from other Antibiotics

Paper	Direction	Solvent System	Rf Value	Use	Reference
Toyo No.50	Descending for 9 hrs.	2% p-toluene sulphonic acid + 2% pyridine in wet n-butanol	0.48-0.54	)Separation of)neomycin from)other)streptomyces)antibiotics.	204
Toyo No.50	Ascending	t-Butanol/acetic acid/ water(55:6:39)	0.06	)))	204
Whatman No.1	Ascending	Water	0.98	))	205
Whatman No.1	Ascending	Water-saturated n-butanol	0.0	)Systematic)identification)of the	205
Whatman No.1	Ascending	Water-saturated ethyl acetate	0.0	)antibiotics)	205
Whatman No.1	Ascending	Water-saturated benzene	0.0	)	205
Whatman No.1	Ascending	Methanol/water(40:60)	0.21	)	205,206
Whatman No.1	Ascending	n-Propanol/water(40:60)	0.20	)	205,206
Whatman No.1	Ascending	Methanol/3% aqueous ammonium chloride(70:30)	0.11	))	205,206
Whatman No.1	Ascending	Methyl ethyl ketone/n-butanol/water(30:5:65)	0.27	))	205,206

Table 12 (Contd...)
Paper Chromatographic Systems Separating Neomycin from other Antibiotics

Paper	Direction	Solvent System	Rf Value	Use	Reference
Whatman No.1	Ascending	66.6% aqueous propanol/benzene/ethylene glycol/acetic acid(5:5:1.5:1)	0.03	Separation of neomycin from chloramphenicol, tetracycline and penicillin	207
Whatman No.1	Ascending	50% aqueous propanol/acetic acid(25:1)	0.27		207
Whatman No.1	Ascending	Water-saturated butanol/acetic acid/potassium cyanide (100:1:0.05g)	0.00		207
Whatman No.3	Ascending	n-Butanol/water/acetic acid(30:13:8)	0.02	Separation of neomycin from polymixin B and bacitracin	186,208
Whatman No.3	Ascending	n-Butanol/acetic acid/pyridine/water/ethanol (60:15:6:5:5)	0.04		186,208
Whatman No.3	Ascending	n-Butanol/water/acetic acid/pyridine/sodium chloride(30:12:7:2:0.1)	0.05		186,208

Table 13

Paper Chromatographic Systems Separating Neomycin
Components and Degradation Products

Paper	Direction	Solvent System	Rf Value			Use	Reference
			Neomycin B	C	Neamine		
-	-	Water/butanol/ methanol/methyl orange . (2:4:1:1:5)	Not available			Stability testing fradio- mycin	209
Whatman No.1	Descending	Butanol/pyridine/ water(60:30:40)	0.29	0.19		Separation of B and C as acetyl derivatives	210
-	-	Butanol/pyridine/ water(3:2:1)	Not available			Separation of B and C as acetyl derivatives	211
Toyo No.50	Descending for 9 hrs	n-Propanol/ pyridine/acetic acid/water (15:10:3:12)	0.13 - 0.15		0.28)))))	Separation of neamine from neo- mycins B and C	204

Table 13 (Contd..)

Paper Chromatographic Systems Separating Neomycin
Components and Degradation Products

Paper	Direction	Solvent System	Rf Value			Use	Reference
			Neomycin		Neamine		
			B	C			
Toyo No.50	Descending for 9 hrs	2% p-toluene sulphonic acid in water saturated n-butanol	0.11-	0.20	0.23	)Separation)of neamine)from neo-)mycins B	204
Toyo No.50	Ascending	1.5% sodium chloride in 80% methanol	0.08-	0.11	0.22	)and C)	
Grade M	Ascending	Butanol/acetic acid/water (4:1:5)	Not available			Separation of B and C as acetyl derivatives	212
Whatman No.4	Descending for 24-36 hrs , 28°C	n-Butanol/water/ piperidine (84:16:2)	0.70 (values relative to neamine)	0.35	1.00	Quantitative spectrophotometric determination of components	213

Table 13(Contd...)

Paper Chromatographic Systems Separating Neomycin
Components and Degradation Products

Paper	Direction	Solvent System	Rf Value			Use	Reference
			Neomycin		Neamine		
			B	C			
Whatman No.1	Descending for 24-36 hrs , 25-27°C	Methyl ethyl ketone/t-butanol/ methanol/6.5N ammonium hydroxide (16:3:1:6)	0.54 (values relative to neamine)	0.30	1.00	Separation as free bases Quantitation by spectro- photometry	214
Whatman No.1	Descending for 8-40 hrs , 24°C	Methyl ethyl ketone/ isopropanol/6.5N ammonium hydroxide (80:20:30)	0.57 (values relative to neamine)	0.34	1.00		215
-	Radial	Butanol/pyridine/ acetic acid/water (15:10:3:12)	Not available			Separation of degradation products	216
Whatman No.1 im- pregnat- ed with pH 8 buffer	Descending for 18 hrs	Butanol/water(1:2) containing 4% toluenesulphonic acid and 2% piperidine hydrochloride	0.5	0.6	0	Separation of neamine from neomycin B and C	228

Table 14

Thin Layer Chromatographic Systems Separating
Neomycin from Other Antibiotics

Adsorbent	Solvent System	Rf Value	Use	Reference
Silica gel G/ aluminium oxide (1:1)	n-Propanol/ethylacetate/ water/13.4 M ammonium hydroxide(50:10:30:10)	0.52	Separation of neomycin from other aminoglycoside antibiotics	217
Cellulose MN	n-Propanol/pyridine/ acetic acid/water (15:10:3:12)	0.10	Separation of neomycin from other water soluble basic antibiotics	218
Kieselgel G	Water/methanol/butanol/ butyl acetate/acetic acid(12:2.5:7.5:40:20)	0.0)	) Separation of) neomycin from) bacitracin,	219
Kieselgel G	Water/butanol/pyridine/ acetic acid.(14:30:20:6) (16:40:8:16)	0.12) 0.00)	) polymixin and) tyrothricin)	219
Kieselgel G	Water/butanol/acetic acid(39:55:6)	0.00)	)	219

Table 14(Contd...)

Thin Layer Chromatographic Systems Separating Neomycin
from Other Antibiotics

Adsorbent	Solvent System	Rf Value	Use	Reference
Kieselgel G	Methanol/17% aqueous ammonia chloroform,aqueous layer(10:10:20)	0.31	)Separation of)neomycin from)bacitracin,)polymixin and)tyrothricin	219
Sephadex G-15	pH6 phosphate buffer (0.025M)+ 0.5M sodium chloride	0.75	)))Identification)of antibiotics	220
Silica gel G	Benzene/acetone/acetic acid(4:4:2)	0.35	))	221
Kieselgel G	Butanol/acetic acid/ pyridine/water (30:22:6:38)	0.14	)Separation of)neomycin from)zinc bacitracin)and polymixin	186,208
Kieselgel G	Butanol/water/acetic acid/ethanol/pyridine (60:10:15:5:6)	0.05	))	186,208

Table 14 (Contd...)

Thin Layer Chromatographic Systems Separating
Neomycin from Other Antibiotics

Adsorbent	Solvent System	Rf Value	Use	Reference
Kieselgel G	Propanol/ethyl acetate/water/ 25% ammonium hydroxide	0.08		222
Kieselgel G + Kieselguhr G(1:1)	(100:20:60:20)	0.14		222
Kieselgel G + Kieselguhr G(1:2)	"	0.22		222
Cellulose MN-300	"	0.32	Separation of glycosidic antibiotics	222
Kieselgel G + aluminium oxide G type E(1:1)	"	0.12		222
Kieselgel G impregnated with sodium acetate	"	0.33		222

Table 14(Contd..)

Thin Layer Chromatographic Systems Separating
Neomycin from Other Antibiotics

Adsorbent	Solvent System	Rf Value	Use	Reference
Kieselgel G	Propanol/ethyl acetate/ water/25% ammonium hydroxide/	0.11	)	222
Kieselgel G + Kieselguhr G(1:1)	pyridine/3.85% aqueous ammonium acetate (100:20:60:20:10:200)	0.13	)))	222
Kieselgel G + Kieselguhr G(1:2)	"	0.16	))))	222
Kieselgel G impregnated with 3.85% aqueous ammonium acetate	"	0.08	)))Separation of)glycosidic)antibiotics)	222
Kieselgel G	Ethanol/ethylacetate/water/ 25% ammonium hydroxide/	0.08	))	222
Kieselgel G + Kieselguhr G(1:1)	pyridine/3.85% aqueous ammonium acetate (100:20:60:20:10:200)	0.11	))	222
Kieselgel G + Kieselguhr G(1:2)	"	0.12	))	222

Table 14(Contd..)

Thin Layer Chromatographic Systems Separating
Neomycin from Other Antibiotics

Adsorbent	Solvent System	Rf Value	Use	Reference
Kieselgel G + aluminium oxide G (type E)(1:1)	Ethanol/ethylacetate/water/ 25% ammonium hydroxide/ pyridine/3.85% aqueous ammonium acetate (100:20:60:20:10:200)	0.08	))))))	222
Kieselgel G	Methanol/ethyl acetate/ water/25% ammonium hydrox-	0.06	))	222
Kieselgel G + Kieselguhr G(1:1)	ide/pyridine/3.85% aqueous ammonium acetate (100:20:60:20:10:200)	0.17	)) Separation of) glycosidic	222
Kieselgel G + Kieselguhr G(1:2)	"	0.14	) antibiotics))	222
Kieselgel G + aluminium oxide G, type E(1:1)	Methanol/ethyl acetate/ water/25% ammonium hydrox- ide/pyridine/3.85% aqueous ammonium acetate (100:20:60:20:10:200)	0.11	)))))	222

Table 14 (Contd..)

Thin Layer Chromatographic Systems Separating
Neomycin from Other Antibiotics

Adsorbent	Solvent System	Rf Value	Use	Reference
Kieselgel G impregnated with 3.85% aqueous ammonium acetate	Methanol/ethyl acetate/ water/25% ammonium hydrox- ide/pyridine/3.85% aqueous ammonium acetate (100:20:60:20:10:200)	0.08	)))))))	222
Kieselgel G	Butanol/methanol/ethyl acetate/water/25% ammonium hydroxide/pyridine/3.85% aqueous ammonium acetate (100:100:20:60:20:10:200)	0.06	))	222
Kieselgel G + Kieselguhr G(1:1)		0.07	)))Separation of)glycosidic)antibiotics	222
Kieselgel G + Kieselguhr G(1:2)	"	0.13	))	222
Kieselgel G + aluminium oxide G, type E(1:1)	"	0.05	))))	222
Kieselgel G + Kieselguhr G(1:2)	25% ammonium hydroxide/ water/acetone (16:144:40)	0.36	)))	222

454

Table 14 (Contd..)

Thin Layer Chromatographic Systems Separating
Neomycin from Other Antibiotics

Adsorbent	Solvent System	Rf Value	Use	Reference
Kieselgel G + Kieselguhr G(1:2)impregnated with 2% sodium acetate	25% ammonium hydroxide/ water/acetone (16:144:40)	0.34)	))))))	222
Kieselgel G + aluminium oxide G, type E(1:1)	"	0.48)	)Separation of)glycosidic)antibiotics))	222
Kieselgel G + Kieselguhr G(1:1)impregnated with 2% sodium acetate	"	0.43)	)))))	222
Kieselgel G	Water/sodium citrate/ citric acid (100:20:5)	0.95	Classification of antibiotics	223

Table 15

Thin Layer Chromatographic Systems Separating Neomycin Components and Degradation Products

Adsorbent	Solvent System	Rf Value			Use	Reference
		Neomycin B	Neomycin C	Neamine		
Acidified carbon	Water	0.10	0.10	0.60	)Assay of)neomycin)components	224
Acidified carbon	0.5N sulphuric acid	0.21	0.43	0.61	)in commer-)cial form-)ulations	224
Untreated carbon	Water	0.00	0.00	0.00	)))	224
Untreated carbon	0.5N sulphuric acid	0.24	0.45	0.54	)	224
Carbon treated with hydro- chloric acid	0.5N hydro- chloric acid	0.37-0.45	0.53-0.74	0.63-0.92	)))Separation)of neomycins)B and C and)neamine	204
"	0.5N hydro- chloric acid/ methanol(4:1)	0.44-0.72	0.58-0.81	0.64-0.91	)))	204

Table 15 (Contd...)

Thin Layer Chromatographic Systems Separating
Neomycin Components and Degradation Products

Adsorbent	Solvent System	Rf Value			Use	Reference
		Neomycin B	Neomycin C	Neamine		
Carbon treated with water	0.5N hydro- chloric acid	0.41	0.57	0.62)		204
"	0.5N hydro- chloric acid/ methanol (4:1)	0.48	0.58	0.68)		204
Carbon/ gypsum treated with sul- phuric acid	0.5N sulphuric acid	0.20	0.59	0.80)	Separation)of neomycins)B and C and)neamine	204
"	0.5N sulphuric acid/methanol (4:1)	0.64	0.82	0.85)		204
Carbon/ gypsum treated with water	0.5N hydro- chloric acid	0.16	0.28	0.30)		204

Table 15 (Contd..)

Thin Layer Chromatographic Systems Separating Neomycin Components and Degradation Products

Adsorbent	Solvent System	Rf Value			Use	Reference
		Neomycin B	Neomycin C	Neamine		
Carbon/ gypsum treated with water	0.5N sulphuric acid	0.24	0.40	–	))))	204
"	0.5N hydro-chloric acid/ methanol(1:1)	0.36	0.36	0.46	)Separation)of neomycin)B and C and)neamine	204
"	0.5N hydro-chloric acid/ propanol(1:1)	0.48	0.48	0.52	)))	204
Kieselgel H	3.85% aqueous ammonium acetate	0.13	0.13	0.36	Assay of neamine	196,9
Kieselgel H	3.4% ammonium hydroxide	0.37	0.44	0.47	Assay of neomycin C	196,9

Table 15 (Contd..)

Thin Layer Chromatographic Systems Separating Neomycin Components and Degradation Products

Adsorbent	Solvent System	Rf Value			Use	Reference
		Neomycin B	Neomycin C	Neamine		
Cellulose MN	Methyl ethyl ketone/iso- propanol/6.5N ammonium hydr- oxide(80:20:30)	0.21	0.12	0.31	Separation of neomycin components	215
Cellulose MN-300	Methyl ethyl ketone/methanol/ isopropanol/7.9N ammonium hydr- oxide(10:8.5:3:7)	0.52	0.24	0.61		236
Cellulose MN-300	Propanol/pyridine/ acetic acid/water (100:66:20:80)	single development 0.25 double development 0.33	0.20 0.25	0.47 0.60	)Assay of)neomycins B)and C and nea-)mine in pharm-)aceutical form-	226
Silica gel G	"	0.57	0.66	0.73	)ulations	226

Table 15 (Contd..)

Thin Layer Chromatographic Systems Separating
Neomycin Components and Degradation Products

Adsorbent	Solvent System	Rf Value			Use	Reference
		Neomycin B	Neomycin C	Neamine		
Cellulose MN-300/ Kieselguhr G(1:1)	Propanol/pyridine/ acetic acid/water (100:66:20:80)	0.53	0.46	0.73)))))		226
Silica gel G	Propanol/ethyl acetate/water/25% ammonium hydroxide (100:20:60:20)	0.23	0.23	0.42))))Assay of)neomycins B		226
Cellulose MN-300/ Kieselguhr G(1:1)	Methanol/3% aqueous sodium chloride(2:1)	0.36	0.17	0.43)and C and)neamine in)pharmaceutical)formulations)		226
Silica gel G/ Kieselguhr G(1:2)	3.85% aqueous ammonium acetate	0.22	0.22	·0.39))))		226

Table 15 (Contd..)

Thin Layer Chromatographic Systems Separating
Neomycin Components and Degradation Products

Adsorbent	Solvent System	Rf Value			Use	Reference
		Neomycin B	Neomycin C	Neamine		
Silica gel G	Methyl ethyl ketone/t-butanol/methanol/ 6.5N ammonium hydroxide (160:30:10:60)	0.14	0.11	0.22)		226
Kieselguhr G purified	"	0.46	0.68	0.84)	)Assay of)neomycins B)and C and)neamine in)pharmaceutical)formulations	226
Silica gel G	Propanol/ethan-ol/ethyl acetate/ water/25% ammonium hydroxide/pyridine/ 3.85%ammonium acetate (100:100:20:60:20:	0.18	0.15	0.38)	)	
Silica gel G/	10:200)				)	
Kieselguhr G(1:2)	"	0.18	0.11	0.32)		226

<u>Table 15</u>(Contd..)

<u>Thin Layer Chromatographic Systems Separating</u>
<u>Neomycin Components and Degradation Products</u>

Adsorbent	Solvent System	Rf Value			Use	Reference
		Neomycin B	Neomycin C	Neamine		
Dowex 50X8 sodium form(Ionex 25SA)	1.5M Sodium acetate, pH 8.5 + 58.4g sodium chloride/ t-butanol(10:1)	0.15	0.15	0.28	Separation of neamine from neomycins B and C	227
Silica gel	3% Ammonium hydroxide/acetone (160:40)	0.33	0.33	–	Quantitative analysis of neomycin sulphate	229
Kieselgel G	3% Ammonium hydroxide/acetone (160:90)	Not available			Stability of neomycin after ethylene oxide sterilisation	233

Table 16
Visualisation of Chromatograms by
Chemical Means

Spray Reagent	Colour of Neomycin zone	Reference
1. Ethanolic sodium hypochlorite followed by starch/ potassium iodide solution	Dark blue	210,211
2. Chlorine/ethanol followed by starch/ potassium iodide solution	Dark blue	212
3. Chlorine/carbon tetrachloride followed by starch/ potassium iodide/ pyridine	Dark blue	213
4. t-butyl hypochlo- rite/dichloro- methane/acetic acid followed by starch/ potassium iodide solution	Dark blue	196
5. Ninhydrin	Purple	186,203,208 214,215,216 217,218,219 229,231
6. Ninhydrin/cad- mium acetate	Purple/pink	227
7. p-dimethyl amino- benzaldehyde	Pink or purple	223
8. Ninhydrin/p- dimethyl amino- benzaldehyde		226
9. Copper sulphate/ ammonium hydr- oxide	Blue	221

Quantitative analysis of neo-
mycin after chromatography utilising some of the
visualisation techniques given in Tables 11 and 16
have been reported. Majumder and Majumder[213] sep-
arated neomycins B and C as the acetyl derivatives
on paper then, following conversion to the chloro
derivatives, the colour formed by reaction with the
starch/iodine/hydrochloric acid reagent was measur-
ed at 570nm. A later paper by the same authors[214]
described the separation of neomycins B and C as the
free bases. The resulting chromatograms were de-
developed with ninhydrin then the coloured complex
eluted into methanol and the absorbance of the
solution measured at 570nm.

Thin-layer chromatography has
been used by Foppiano and Brown[229] to assay the
total neomycin B and C content of neomycin sulphate.
The neomycin zone was scraped off the plate and re-
acted with orcinol/ferric chloride reagent, the ab-
sorbance of the resulting colour being measured at
665nm. By this procedure a precision of $\pm$ 2% was
achieved.

Ninhydrin has also been used
to quantitate neomycin colorimetrically following
thin-layer chromatography. Pregnolatto and
Sabino[200] reported the addition of glycerine to the
ninhydrin solution to improve the sensitivity and
reproducibility of the procedure. An alternative
colorimetric procedure for the quantitation of neo-
mycin after chromatography has been described by
Doulakas[174]. Neomycin is eluted from the silica
gel with pH 12.5 phosphate buffer then oxidised
with sodium hypobromite. The resulting aldehyde
is complexed with phloroglucinol yielding a pink-
coloured product which is measured spectrophoto-
metrically.

The use of bioautography for
the quantitation of chromatograms has been describ-
ed by many workers. Emilianowicz-Czerska and
Herman[228] separated neamine from neomycins B and C
then quantitatively determined total neomycin B and
C by bioautography with *B.subtilis*. A linear re-
sponse for concentrations between 0.25 and 10µg
of neomycin was obtained. With a similar technique
but using a mathematical analysis of the bioauto-

grams, Simon[230] reported a quantitative determina-
tion of neomycin suitable for use with irregular-
shaped zones. Brodasky[224] employed bioautography
with *Bacillus pumilus* for the assay of neomycin and
neamine after separation by T.L.C. on a layer of
carbon. The quantitation of small amounts of neo-
mycin C however, proved difficult, the difficult-
ies being associated with the enhanced growth of the
organism in the presence of carbon(from the T.L.C.
plate) and ammonium ions(from the chromatographic
procedure). Similar difficulties were experienced
by Sokolski and Lummis[234] who attributed poor neo-
mycin zones to be the result of antagonism by the
sodium chloride present in the bioautographic sys-
tem. An extensive study by Langner[201] demonstrated
maximum sensitivity for the determination of neo-
mycin to be apparent using the organism *B.subtilis*
(ATCC 6633) together with pH 8 Tris agar. The de-
tection limit following chromatography was report-
ed to be 0.05µg.

6.35. Gas-Liquid Chromatography

Tsuji and Robertson[192] achiev-
ed the separation of neomycin B, neomycin C and
neamine as the trimethylsilyl ethers on a 6ft.
column of 0.75% OV-1 on Gas Chrom Q at a tempera-
ture of 290°C. The same conditions have also been
shown to separate neobiosamine B, neosamine and
deoxystreptamine from neomycin and neamine. Hence
the method could be used to study the stability of
neomycin or to monitor the biosynthetic production
process. Use of the procedure to assess the stab-
ility of neomycin in pharmaceutical formulations
has been demonstrated by Van Giessen and Tsuji[237]
with trilaurin as internal standard. However,these
authors recommended a 2ft. column packed with 3%
OV-1 on Gas Chrom Q as longer columns required a
higher temperature to chromatograph neomycin and
consequently had a reduced column life. A concen-
tration of 3% OV-1 was preferred, as 2% or less
resulted in increased column adsorption of neomycin.
After a further study of the procedure Margosis
and Tsuji[238] recommended a number of improvements
to optimise the analysis of neomycin by G.L.C. The
improvements to the assay included the modifica-
tion of the injection port to prevent sample de-
composition by contact of injected material with

metal or Teflon and the addition of an accurate
volume of silylating reagent to each sample and
standard to overcome inconsistent derivatisation.

A comparison of the G.L.C. and micro-
biological(agar-diffusion) assays has been re-
ported by Tsuji et al[239] who showed the neomycin
content of neomycin sulphate powders determined
by the G.L.C. method, to correlate well with values
obtained by the microbiological assay when it is
assumed neomycin C has 35% of the biological ac-
tivity of neomycin B.

Neomycin has been separated from
mixtures of other aminoglycoside antibiotics con-
taining the 2-deoxystreptamine moiety as both the
pertrimethylsilyl derivative and the N-trifluoro-
acetyl pertrimethylsilyl derivative using a column
of 0.75% OV-1 on Gas Chrom Q[240]. The procedure
may be used to estimate the number of sugar moiet-
ies bound in the antibiotic as a close relation-
ship exists between the number of rings and the
retention time.

Examination of neomycin B by a combin-
ed G.C.-M.S. procedure has been reported by Murata
et al[15]. G.C. was accomplished with the tri-
methylsilyl derivative on a 1m column of 1% OV-1 on
Chromosorb W, by which means neomycin was separat-
ed from kanamycin. The resulting mass spectra of
the neomycin derivative exhibited a minute mole-
cular ion peak at m/e 1550 indicating that all
active hydrogens of both hydroxy and amine groups
were completely silylated.

6.4. Microbiological Procedures

6.41. Turbidimetric Assay

Various organisms have been used
to assay neomycin turbidimetrically. These are
summarised in Table 17 with the working concen-
tration for the antibiotic as reported by the
respective authors.

Of the organisms tabulated over-
leaf *K.pneumoniae* is generally used for routine
assays.

Table 17
Conditions of Turbidimetric Assay

Organism	Concentration Range of Assay (Total µg neomycin base required in solution to be assayed)	Reference
S.aureus NRRLB314	2.5 - 5.0	241
" - " 209P	100-300	242
" " F1	0.016 - 0.04	243
Streptococcus faecalis ATCC 1054	120-200	248
Klebsiella pneumoniae PC1602	6-15	244
Klebsiella pneumoniae ATCC 10031	6-14	245,246, 268
E.coli ATCC 10536	1-3	247
" " ATCC 11105	1-20	253
Lactobacillus arabinozus	3-7	248
Lactobacillus casei	20-60	248
Lactobacillus fermenti	2-6	248
Lactobacillus leichmanni	4-6	248
Aerobacter aerogenes IPL 22K5118	0-6	249

Hein[243] noted an apparent decrease in the potency of neomycin when assayed turbidimetrically if the innoculum contained either sodium chloride or potassium phosphate. With S.aureus as organism, Sokolski et al[252] demonstrated the presence of KCl to antagonise the activity of neomycin C more than that of neomycin B.

A comparative study of the responce of neomycin B and neomycin C in the turbidimetric assay using K.pneumoniae(ATCC 10031) has
been reported[239]. The response-ratio neomycin B:
neomycin C was 100:38.9.

An automated turbidimetric procedure using a Technicon Autoanalyzer has also been
described (See Section 6.5).

6.42. Agar-Diffusion Assay

Table 18 lists the organisms that
have been recommended for determining the microbiological potency of neomycin.

Table 18

Recommended Organism	Working Concentration (µg/ml)	Reference
B.pumilis NCTC 8241	4-21	9
S.aureus ATCC 6538	4-20	245
S.epidermidis ATCC 12228	0.64 - 1.56	245,268

In all the above references, the
cylinder-plate assay procedure is the one described
though alternative procedures have been reported in
the literature. Thus Davis and Parke[245] reported a
linear-diffusion system in which a solution of the
antibiotic is allowed to diffuse into agar-filled
glass capilliaries. The standard deviations calculated for seven experiments were between 5 and
10%(100 results) for the assay of neomycin. The
procedure is more economic in terms of agar and
nutrients than other diffusion methods. Kuzel and
Coffey[255] modified the conventional cylinder plate
procedure by using a cylinder sealed at one end,
forming a cup. The cups are filled with the antibiotic solutions to be assayed, then a plate of inoculated agar is inverted and placed over the top of
the cups. With this method a significant reduction
in the standard deviation for the assay of neomycin

was claimed. The replacement of cylinders by paper
discs has also been reported[256,275,278]. Rossi[275]
claimed a standard deviation of $\pm$ 2% for the assay
of neomycin by this means and recommended the paper
disc procedure in preference to the cylinder method.
The type of paper used has been shown to affect the
diameter of the inhibition-zone[276] Whatman seed
test paper producing the smallest zones. When de-
termining neomycin levels in milk,Kosikowski[277]
improved the sensitivity of the procedure by sub-
stituting dried milk tablets for paper discs.

          Several studies of the factors
affecting the inhibition-zone size in the neomycin
agar diffusion assay have been reported. Pinzelik
et al[257] demonstrated that various delays during
the analytical procedure affected the size of the
inhibition zone. Refrigeration of the agar-plates
prior to incubation caused an increase in zone-
size when compared with similar plates held at 20°C.
This principle has been utilized by Siddique et
al[264] to improve sensitivity when determining the
neomycin content of milk. The presence of KCl,NaCl,
$CaCl_2$ has been shown to markedly increase neomycin
diffusion in agar[258,265,194]. Conversely the pre-
sence of phosphates[258] and of glucose/starch with
NaCl[265] decreases the size of the inhibition zone.
Incorporation of non-fat milk in the agar signifi-
cantly reduces the size of the inhibition zone be-
cause of the interaction of neomycin with the free
carboxyl group of milk protein[262]. Fedorko and
Katz[263] diluted neomycin solutions with blood serum
and reported an increase in the size of the in-
hibition zone when compared with similar solutions
diluted with pH 8 phosphate buffer. Sokolski et
al[252] and Yousef et al[259] demonstrated the physical
binding of neomycin to agar, a process which may be
reversed by addition of potassium or sodium chlor-
ide. Further studies[260,261,252] have reported the
binding to be a function of both the pH of the agar
and the manufacturer. Incorporation of O.1M tris
buffer with the agar (pH 8) has been recommend-
ed[260,261] in order to achieve the highest sensi-
tivity. Tris buffer results in a greater sensitiv-
ity than phosphate buffer[261] and does not affect
the size of the inhibition zone whereas the pre-
sence of phosphate reduces zone size[252]. Gillet et
al[194] concluded that the quality of the agar affec-

ted the inhibition zone size. A comparison of 3 agars showed the best response for a given concentration of neomycin was obtained with agarose.Combining agarose with recommendations described by other authors, such as the addition of CaCl$_2$ and the use of tris buffer, further increased the size of the inhibition zone.

The literature described above refers to the assay of the total neomycin complex i.e. neomycins B and C and neamine. To assay neomycin B in the presence of neomycin C and neamine by a microbial method Sokolski and Carpenter[265] adopted the following procedure. These authors employed a neomycin C - resistant organism(B.*subtilis* UC 564) and added KCl to the agar to totally depress diffusion of neamine and increase diffusion of neomycin B.

Neomycin B and C differ in their biological activities though this difference may vary with the conditions of the assay.Typically neomycin C has an activity of 30-50% of that of neomycin B[266,239]. The presence of potassium chloride or phosphate reduces the diffusion of neomycin C more than that of neomycin B[252]. Furthermore, this antagonistic effect of the salts has been shown to be more pronounced when using S.*aureus* as organism than when using B.*subtilis*. By altering the ionic strength of the medium a system has been developed in which the responses of neomycins B and C are identical[266].

The microbial assay of neomycin in the presence of other antibiotics can present difficulties if both antibiotics are active against the same test organisms. The assay of neomycin in the presence of dihydrostreptomycin, to which the test organism S.*aureus* is also sensitive, has been reported. Levine et al[269] rendered the dihydrostreptomycin inactive by hydrolysis with barium hydroxide prior to determining neomycin with S.*aureus*. DeNuzio[270] however, chose to overcome the same problem by cultivating a strain of S.*aureus* which was resistant to dihydrostreptomycin. When assaying neomycin in a mixture containing tetracycline, penicillin and dihydrostreptomycin, Tanguay et al[271] found it necessary to

separate tetracycline and penicillin by solvent ex-
traction before determining neomycin by the method
of DeNuzio.

The determination of neomycin in
animal feeds has received much attention. Barbiers
and Neff[272] recommended the use of a tris buffer at
pH 8 to prepare neomycin solutions and the addition
of magnesium or calcium chloride to the agar for
enhancement of the inhibition zones. Extraction of
neomycin from the feed required the use of sodium
chloride solution for 100% recovery. A collabora-
tive study of this procedure by eighteen labora-
tories resulted in an average recovery of 100.3%
with a coefficient of variation of 15.2%[279]. Modi-
fication of the previous method has been described
by Williams and Wornick[273] who incorporated the
tris buffer in the agar and deleted the addition of
calcium chloride. A three-fold increase in sensi-
tivity was reported. However, a collaborative
study of the modified procedure resulted in an av-
erage recovery of 115% with a coefficient of varia-
tion of 20.4%[274].

An investigation of the agar dif-
fusion assay of neomycin at very low levels(50ng-
1000ng) in meat products has been reported[280]. The
points on the dose-response graph covering the above
concentration range were scattered about the aver-
age straight line. However, by joining all the
points together a complex curve resulted, the equa-
tion of which was derived.

6.5. Automated Procedures

An attempt to automate the turbidi-
metric method for the determination of neomycin
with *K.pneumoniae* has been reported by Gerke et
al[281] who obtained satisfactory assays with sol-
utions containing 150-1200 µg/ml of neomycin. An
automated respirometric method, which measured the
amount of CO_2 produced by interaction of antibiotic
and the bacteria *E.coli* was also reported with a
similar sensitivity. In both methods Auto Analyzer
systems were employed.

The modification of the above respiro-
metric procedure to include continuous culture of

the test organism E.coli has been described[282]. With the organism grown in this manner the sensitivity of the assay is improved. Greely et al[283] have applied the automated respirometric method to the determination of neomycin in pharmaceutical products and compared these assays with the results obtained by the cylinder-plate procedure on the same samples. Good correlation between the two procedures was demonstrated.

An automated colorimetric assay for the quantitation of the separate components of neomycin (B, C and neamine) has also been reported[140]. The method utilizes a separation of the components on a column of carbon and Kieslguhr G (4:1). The column eluate is reacted with ninhydrin to determine the amounts of neomycin B, C and neamine.

6.6. Use as an Analytical Reagent

Turbidimetric procedures for determining ribonuclease[284] and deoxyribonuclease[285] using neomycin as the precipitant have been described. The turbidity was shown to be dependant on the relative concentrations of reactants, the molecular weight of the RNA or DNA and the ionic strength of the solution thus necessitating the careful control of assay conditions. Extraneous proteins do not interfere enabling the procedure to be applied directly to blood serum.

6.7. Determination in Body Fluids and Tissues

An agar diffusion assay, based upon the method described by Groves and Randall[246], has been utilised by the majority of workers for determining neomycin levels in clinical samples. To compensate for the protein-binding effect exhibited by neomycin, Kivman and Geitman[309] recommended the addition of serum and 3% KCl to the neomycin standard solution. Danielova[310], however, preferred to release the bound antibiotic by enzymatic hydrolysis prior to microbiological assay. An agar diffusion method requiring only 20µl of serum sample has been described by Axline et al[311].

The application of a turbidimetric
method to the examination of serum samples has
been reported by Felsenfeld[312].

More recently a laser light scattering
bioassay for determining the neomycin content of
milk, serum, urine and bile has been reported[313].
The procedure uses a helium-neon laser light-
source and has a linear dose-response graph over
the range 0.1 to 10µg/ml for serum.

An enzymic assay involving the reaction
of neomycin with aminoglycoside 4' adenyltransferase
has been described[314]. A linear response for neo-
mycin was observed over the range 2.5 to 20µg/ml
serum.

The concentration of neomycin in urine
has been determined with a fluorimetric method[315].
Interfering amines were separated by chromatography
on SE Sephadex C-25 before reacting the isolated
neomycin with fluorescamine.

Brammer and Hemson[182] employed an
electrophoretic procedure to separate neomycin
from blood-proteins before determining the neomycin
concentration colorimetrically.

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This profile attempts to cover the published
literature on neomycin up to and including
Chemical Abstracts, Volume 85.

PSEUDOEPHEDRINE HYDROCHLORIDE

Steven A. Benezra and John W. McRae

1.   Description

    1.1   Name, Formula, Molecular Weight
        d-Pseudoephedrine hydrochloride is (+)-threo-α-(1-
(methylamino)ethyl)benzyl alcohol hydrochloride.   Throughout
this analytical profile, d-pseudoephedrine will be referred
to as pseudoephedrine.
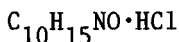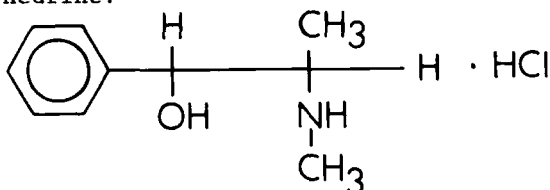
$C_{10}H_{15}NO \cdot HCl$ 201.72

    1.2   Appearance, Color, Odor
        Pseudoephedrine hydrochloride occurs as fine white
to off-white crystals or as a powder having a faint odor[1]

2.   Physical Properties

    2.1   Infrared Spectrum
        The infrared spectrum of pseudoephedrine hydro-
chloride is shown in Figure 1.   It was obtained as a 0.2%
dispersion of pseudoephedrine hydrochloride in KBr with a
Nicolet Model 7199 FT-IR spectrophotometer.[2]   Table I gives
the infrared assignments consistent with the structure of
pseudoephedrine hydrochloride.

Table I

Infrared Spectral Assignments for Pseudoephedrine
Hydrochloride

Frequency (cm^{-1})	Assignment
3270	OH stretch
3010	Asym. C-H stretch
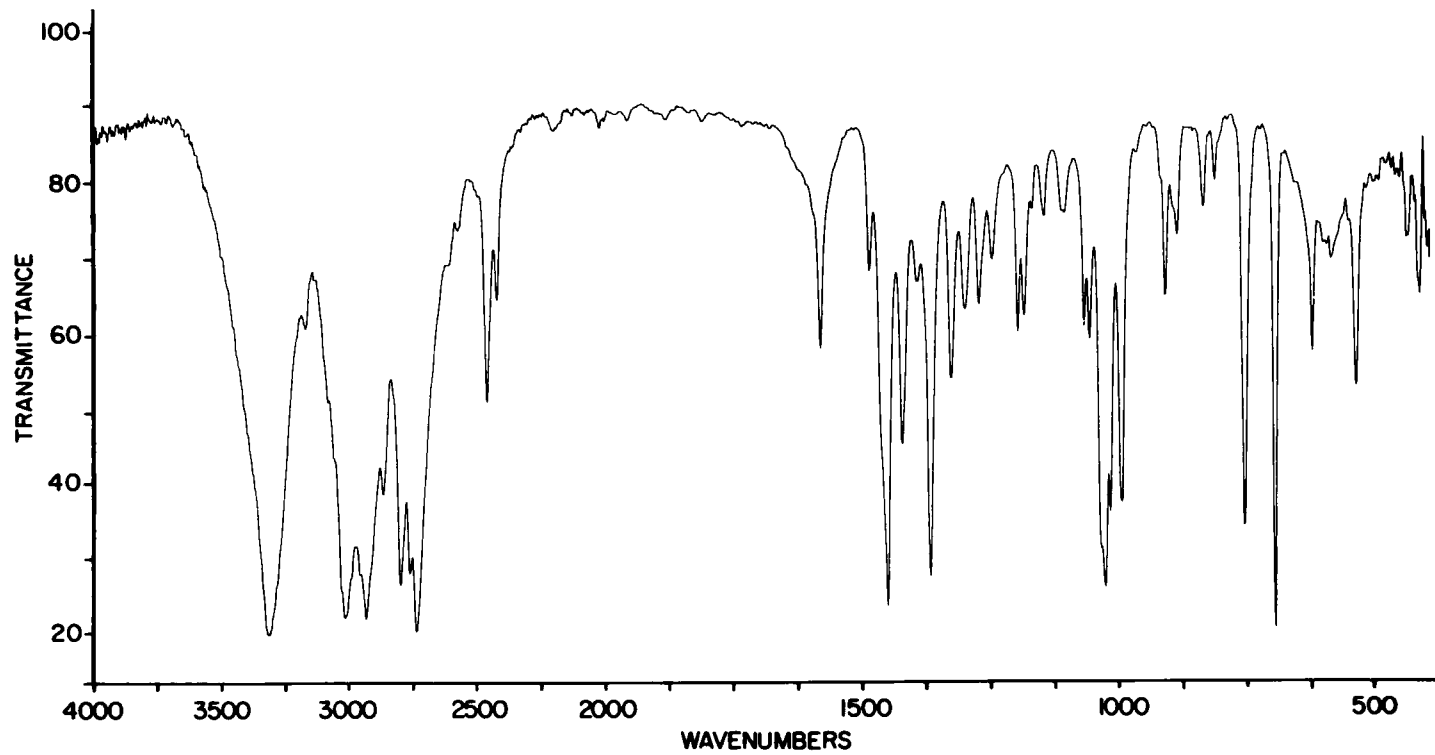
Figure 1 - Infrared Spectrum of Pseudoephedrine Hydrochloride

2930	Sym. C-H stretch
2700	$^+$NH stretch
1587, 1490	C=C aromatic stretch
1430	OH bend, secondary alcohol
762, 702	C-H bend, monosubst. benzene

2.2   Nuclear Magnetic Resonance (NMR) Spectrum

The NMR spectrum of pseudoephedrine hydrochloride is shown in Figure 2. The spectrum was obtained with a Varian model CFT-20 80 MHz NMR spectrometer. Deuterated DMSO was used as the solvent with tetramethylsilane as an internal standard. Table II gives the NMR assignments consistent with the structure of pseudoephedrine hydrochloride.[3]
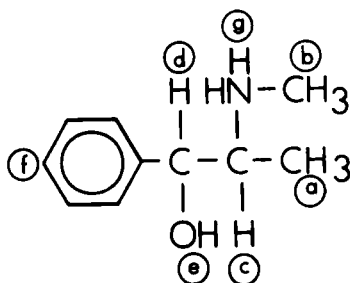
Table II

NMR Assignments for Pseudoephedrine Hydrochloride

Proton	No. of Protons	Shift (ppm)	Multiplicity
a	3	0.96	doublet
b	3	2.55	singlet
c	1	3.25	quartet (partially obscured by H_2O)
d	1	4.54	doublet of doublets
e	1	6.32	doublet
f	5	7.34	singlet
g	2	8.90	broad singlet
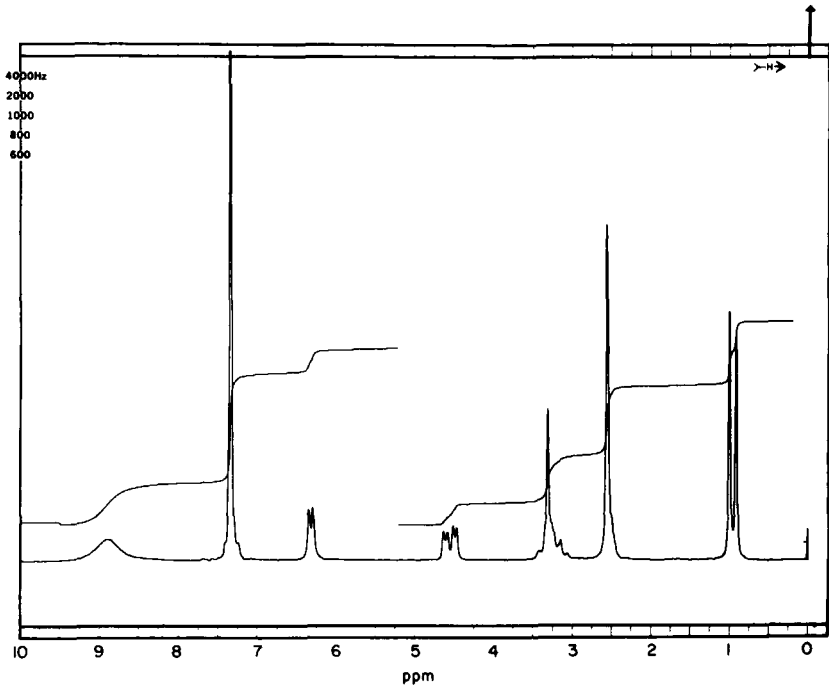
Figure 2 - NMR Spectrum of Pseudoephedrine Hydrochloride
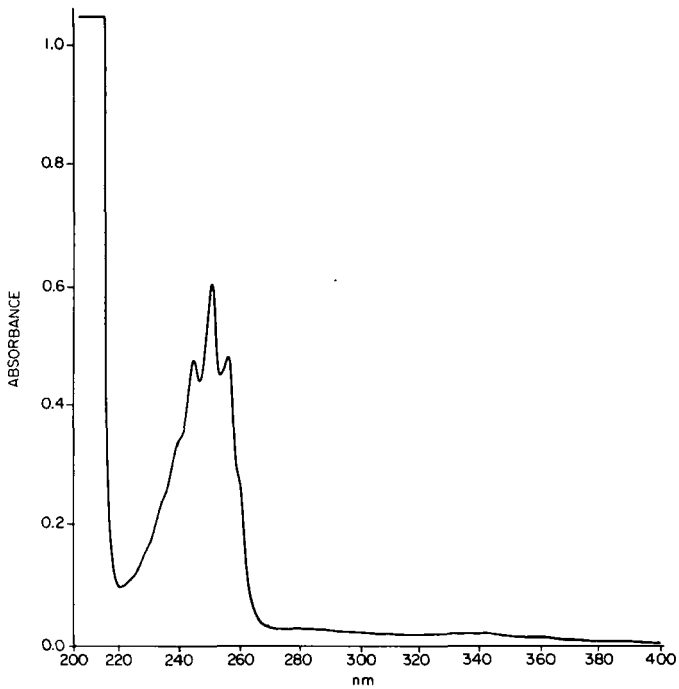
Figure 3 - Ultraviolet Spectrum of Pseudoephedrine Hydrochloride

2.3 Ultraviolet Spectrum

The ultraviolet spectrum of pseudoephedrine hydro-
chloride in ethanol was obtained with a Beckman ACTA CIII
ultraviolet spectrophotometer and is shown in Figure 3.[4]
Pseudoephedrine hydrochloride exhibits absorption maxima at
208, 251, 257, and 264 nm with extinction coefficients of
8300, 161, 201, and 161, respectively.

2.4. Mass Spectrum

The low resolution mass spectrum of pseudoephedrine
hydrochloride is shown in Figure 4.[5] It was obtained with a
Varian MAT CH5-DF mass spectrometer. Direct probe at 80°C
into the ion source was used to obtain the mass spectrum.
The electron energy was 70 eV. The assignments of the major
ions formed in the mass spectrometer are shown below. The
molecular ion is not observed.
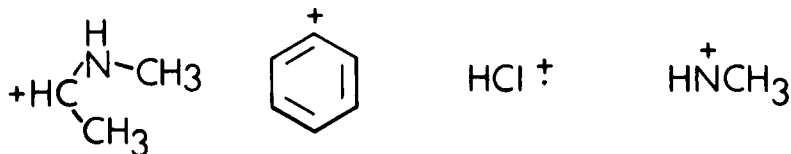
m/e 58 (100%)   m/e 77 (9%)   m/e 36 (10%)   m/e 30 (12%)

2.5 Melting Point

Pseudoephedrine hydrochloride melts between 182°
and 185°C.[1]

2.5 Specific Rotation

The specific rotation, $[\alpha]_D^{20}$, of d-pseudoephedrine
hydrochloride in water is between +61.0° and +62.5°.[1]

2.7 Solubility

The solubility of pseudoephedrine hydrochloride in
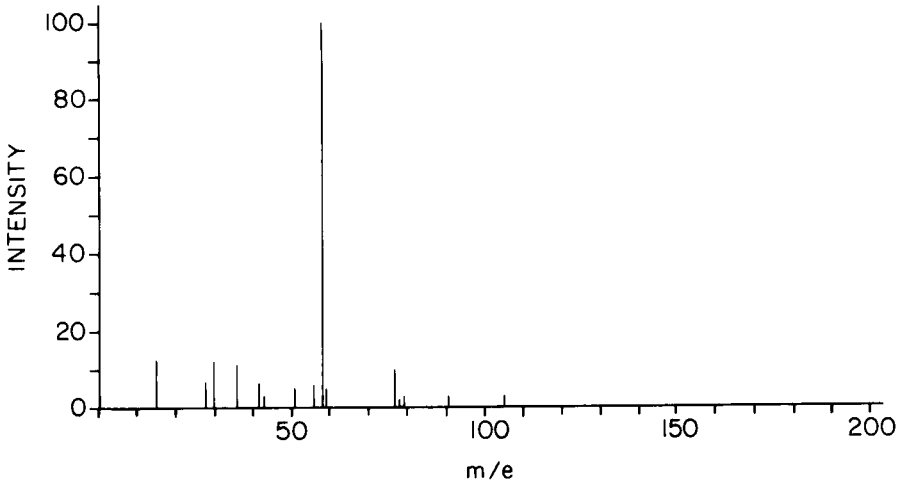
Figure 4 - Mass Spectrum of Pseudoephedrine Hydrochloride
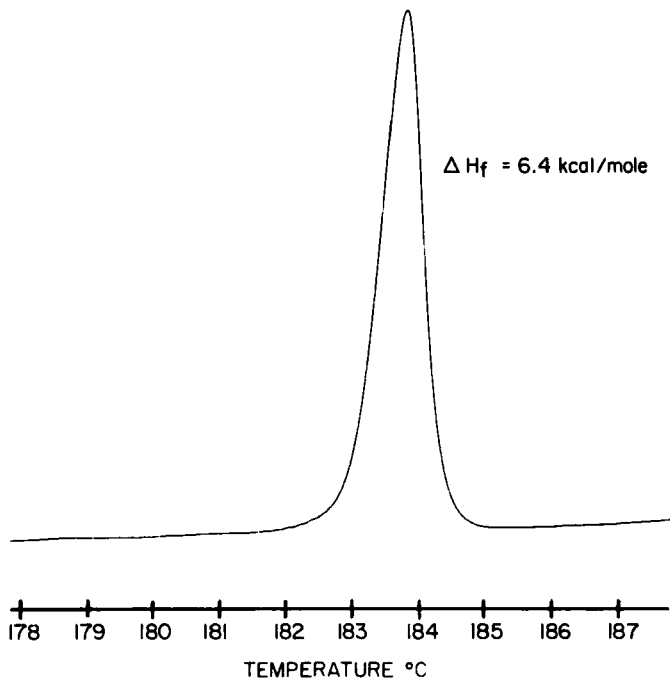
Figure 5 - DSC Curve of Pseudoephedrine Hydrochloride

various solvents at 25°C is given in Table III.[1]

<div align="center">Table III

Solubility of Pseudoephedrine Hydrochloride at 25°C</div>

Solvent	Solubility (gm/ml)
Water	2.0
Chloroform	0.011
Ethanol	0.278
Ether	1.4×10^{-4}

2.8 Partition Coefficient

The partition coefficients of pseudoephedrine hydrochloride at 25°C in n-octanol/aq. pH 1.2 and n-octanol/aq. pH 6.0 are 0.010 and 0.049 respectively.[6]

2.9 Differential Scanning Calorimetry (DSC)

The DSC curve of pseudoephedrine hydrochloride obtained with a Perkin Elmer DSC-1B differential scanning calorimeter is shown in Figure 5.[7] The heating rate was 5°C/min. The heat of fusion is 6.4 Kcal/mol. The melting point (uncorrected) is 184°C.

2.10 Crystal Structure

The crystal properties of pseudoephedrine hydrochloride were determined with a GE model XRD-6 x-ray diffractometer using Zr filtered MoK_α radiation on a crystal grown from water.[8] Pseudoephedrine hydrochloride has an orthorhombic crystal system belonging to the $P2_12_12_1$ space group. The cell dimensions are a=25.358 Å, b=6.428 Å, c=6.901 Å with each cell containing four molecules.

2.11 Dissociation Constant

The pKa of pseudoephedrine hydrochloride determined

titrimetrically in 80% aqueous methylcellosolve is 9.22.[9]

3. Synthesis

        Pseudoephedrine hydrochloride is prepared by a
Welsh rearrangement[10] of ℓ-ephedrine hydrochloride with ace-
tic anhydride followed by deacetylation with hydrochloric
acid.[11] ℓ-Ephedrine can be resolved from dℓ-ephedrine with
ℓ-mandelic acid.[12] ℓ-Ephedrine occurs naturally in certain
plants of the Ma Huang species.

4. Stability

        Pseudoephedrine hydrochloride can be considered a
stable compound in bulk and in formulations. After 4-weeks
under fluorescent lights (2400 ft. candles) and ultraviolet
light (190 $\mu w/cm^2$) no discoloration or chemical degradation
was observed. The bulk drug was stable for at least 6 months
at 37°C and 3 months at 50°C. Tablet and syrup formulations
stored at 15-30°C for 5 years showed no appreciable degra-
dation.[13]

5. Metabolism and Pharmacokinetics

        The major biotransformations of pseudoephedrine
hydrochloride are parahydroxylation, N-demethylation, and
oxidative deamination.[14] The proposed pathways for the
metabolism of pseudoephedrine are shown in Figure 6.
        In a study with human subjects, whose urine pH was
controlled with sodium bicarbonate and ammonium chloride, it
was found that 10-25% of the administered pseudoephedrine
hydrochloride was metabolized to norpseudoephedrine and the
elimination of pseudoephedrine and norpseudoephedrine was
related to urine pH. As the urine pH increased, the serum
half-life of pseudoephedrine and norpseudoephedrine increas-
ed.[15] In another similar study it was found that a decrease
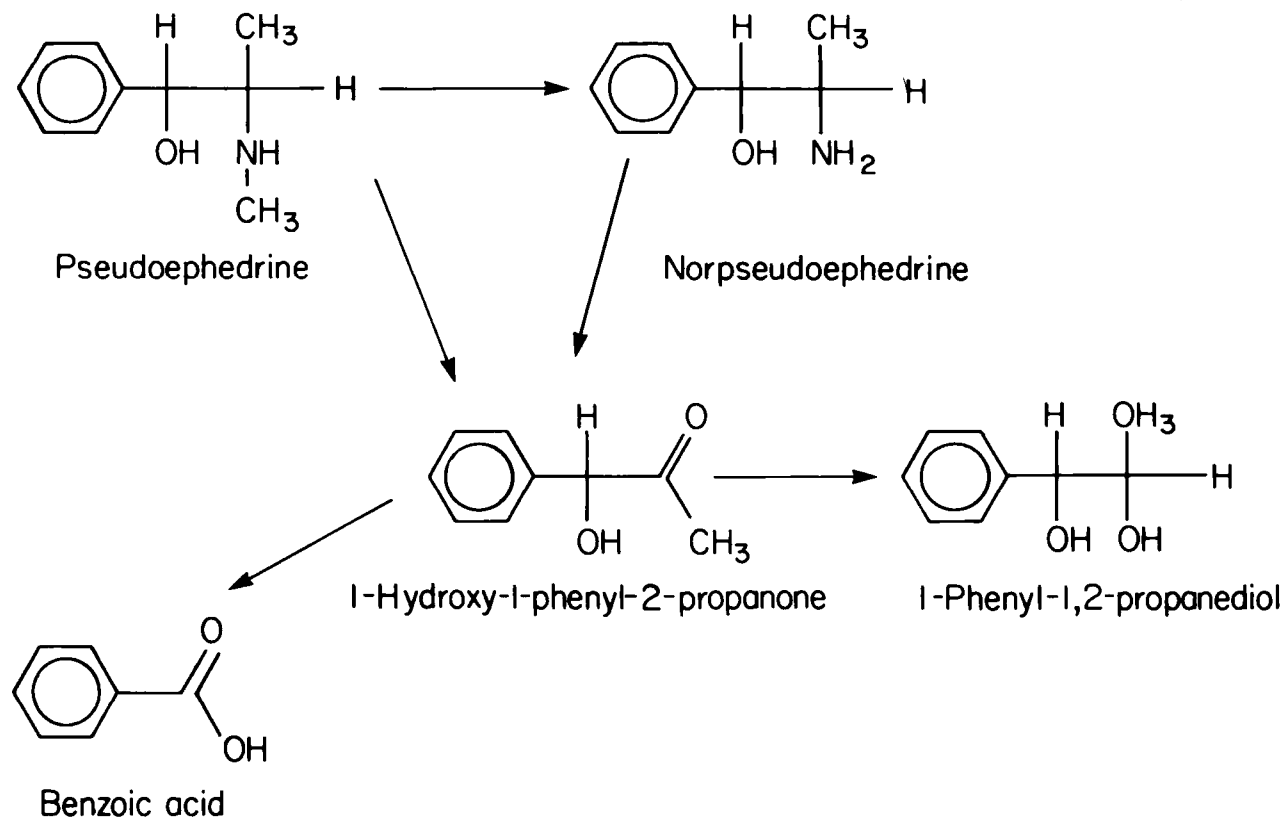
Figure 6 - Metabolism of Pseudoephedrine Hydrochloride

in urine pH caused a decrease in plasma half-life of pseudo-
ephedrine.[16] Plasma half-lives measured in normal human
subjects were 5.2-8.0 hours.[16]

In a rat study using ^{14}C-labelled dℓ-ephedrine,
85% of the i.p.-administered drug was eliminated in the first
40 hours. Two major metabolic pathways were postulated after
analysis of the metabolites. The major metabolic pathway was
ring para-hydroxylation forming para-hydroxyephedrine and
para-hydroxynorephedrine. The minor metabolic pathway was
oxidative deamination, giving acidic metabolites such as
hippuric and benzoic acids.[17]

The relative tissue distribution in mice 15 minutes
after i.v.-administered ^{14}C-ephedrine was kidney > lung,
adrenal, spleen, liver > intestines, stomach > brain,
heart > plasma.[17]

The LD_{50} in mice of pseudoephedrine administered
i.p. is 1.0 mmole/kg.[18]

6.   Methods of Analysis

6.1 Elemental Analysis

The results of the elemental analysis of pseudo-
ephedrine hydrochloride are given in Table IV.[6] The analysis
was performed on a NF Reference Standard.

Table IV
Elemental Analysis of Pseudoephedrine Hydrochloride

Element	Theory (%)	Found (%)
C	59.55	59.54
H	8.00	8.11
N	6.95	6.81

6.2   Nonaqueous Titration

Pseudoephedrine hydrochloride is dissolved in a
mixture of glacial acetic acid and mercuric acetate test
solution. A standardized solution of 0.1N perchloric acid is
used to titrate the solution to a blue-green end point with
crystal violet indicator. Each ml of 0.1N perchloric acid is
equivalent to 0.1 mmole of pseudoephedrine hydrochloride.[1]

6.3   Ultraviolet Spectrophotometric Analysis

An ultraviolet spectrophotometric analysis is used
to assay pseudoephedrine hydrochloride in tablets. A portion
of finely powdered tablets equivalent to approximately 30 mg
of pseudoephedrine hydrochloride is placed in a distilling
flask which is part of a micro-steam distillation apparatus.
Sodium chloride, water, and concentrated sodium hydroxide are
added. A minimum of 30 ml of distillate is collected in a
volumetric flask containing dilute hydrochloric acid. The
flask is made to volume with distilled water and the absorb-
ance of the solution is determined at 257 nm in 1 cm cells
and compared to a solution of known concentration of NF
Pseudoephedrine Hydrochloride Reference Standard.[1]

An ultraviolet spectrophotometric method based on
the absorbance of a periodate oxidation product of pseudo-
ephedrine hydrochloride will be the official method of analy-
sis in the USP XX.[19,20] A portion of tablets or syrup in
water is placed in a separatory funnel. Sodium bicarbonate
and sodium metaperiodate are added. After standing for 15
minutes, 1 N HCl is added. The solution is extracted with
hexane. The hexane extract is filtered and its absorbance
determined at 242 nm in 1 cm cells. The amount of the oxi-
dation product of pseudoephedrine hydrochloride is determined
by comparison of the sample absorbance against the absorbance
of a Pseudoephedrine Hydrochloride Reference Standard treated
in the same manner.

6.4 Colorimetric Analysis

Pseudoephedrine hydrochloride in syrup formulations
has been analyzed by colorimetry. Pseudoephedrine forms a
stable blue-colored chelate with cupric sulfate at pH 12.5.
The complex has a maximum absorbance at 500 nm. The complex
is extracted from an aqueous layer with 1-pentanol. Inter-
fering substances such as glycerine and sugars normally found
in syrup formulations, which form complexes with cupric sul-
fate, are not extracted into 1-pentanol.[21]

6.5 Chromatography

6.51 High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography has been
used to analyze pseudoephedrine hydrochloride and dosage
forms containing pseudoephedrine hydrochloride. Table V
gives the HPLC conditions used for separations.

Table V

HPLC Conditions for Pseudoephedrine Hydrochloride

Column	Mobile Phase	Rention Time (min)	Reference
Corasil®/Phenyl	acetonitrile:	1.8 (phenyl)	22
Corasil®/C_{18}	0.1% ammonium	1.9 (C_{18})	
	carbonate		
	(9:1) pH 8.9		
Corasil®/Phenyl	acetonitrile:	2.1 (Phenyl)	22
Corasil®/C_{18}	1% ammonium	2.2 (C_{18})	
	carbonate (6:4)		
	pH 7.4		

Zipax®/SCX	0.02 M dibasic ammonium phosphate:dioxane (64:36)	7	23
Nucleosil®/ silica gel	methanol:0.5M sodium dihydrogen phosphate: phosphoric acid (195:50:2)	6	24
Spherisorb®/ silica gel	ethanol:0.4% ammonium acetate (85:15)	8	25

6.52 Thin Layer Chromatography (TLC)

Table VI lists the various TLC systems used for pseudoephedrine hydrochloride.

Table VI

TLC Systems for Pseudoephedrine Hydrochloride

Mobile Phase	Adsorbent	Rf	Reference
ethyl acetate: cyclohexane: methanol:conc. $NH_4OH(70:15:10:5)$	silica gel	0.25	26
n-butanol:ethanol: water:acetic acid (60:30:10:0.2)	silica gel	0.46 0.18 (free base)	27

chloroform: methanol: acetone (7:3:5)	silica gel	0.33 0.10 (free base)	28
ethyl ether: benzene (1:1)	silica gel	0.35 (as 4-chloro- 7-nitro- benzo-2,1,3- oxadiazole derivative)	29
chloroform: water:acetic acid (20:75:20:1) (lower phase)	alumina	0.70 (as acetylated product)	16

6.53 Paper Chromatography

Paper chromatography has been used to separate and detect pseudoephedrine hydrochloride from other pharmacologically active amines. Whatman No. 1 paper developed in n-butanol:water:95% acetic acid (4:5:1), n-butanol:toluene:water:95% acetic acid (10:10:5:5), ethyl acetate:water:95% acetic acid (3:3:1), or chloroform:water:95% acetic acid (10:5:4) gave Rf values of 0.73, 0.35, 0.57, and 0.52 for pseudoephedrine hydrochloride respectively. Visualization of pseudoephedrine hydrochloride was done by spraying the chromatogram with 0.5% bromcresol green in methanol or 0.2% ninhydrin in acetic acid:butanol 5:95.[30]

6.54 Gas Chromatography

Pseudoephedrine hydrochloride has been separated from other amines by gas chromatography. The oxazolidine

derivative has been prepared by the reaction of pseudo-
ephedrine with anhydrous acetone. On a 1.15% SE-30,
glass column, 2.4 m x 3 mm i.d. at 104°C the oxazoli-
dine derivative has a retention time of 16.4 minutes.[31]
On a 15% PEG 6000, glass column 2 m x 4 mm i.d. at 175°C
the oxazolidine derivative had a retention time of 10.1
minutes.[32]

  The N-trifluroacetyl-L-prolylchloride derivative
of pseudoephedrine has retention time of 105 minutes on a 3%
SE-30, stainless steel column, 2 m x 3 mm i.d. at 170°C.[33]

  An on-column acetic anhydride derivatization tech-
nique has been described for pseudoephedrine hydrochloride.
Immediately after injection of a solution of pseudoephedrine
onto a 20% SE-30, 1.8 m x 7 mm i.d. glass column at 125°C, an
injection of acetic anhydride was made. The pseudoephedrine
derivative formed on column has a retention time of 55.5
minutes as compared to a retention time of 8.7 minutes for
underivatized pseudoephedrine.[34]

  A variety of methods have been used to determine
pseudoephedrine hydrochloride levels in plasma and urine by
gas chromatography. Bye and co-workers[35] extracted basefied
plasma or urine with diethyl ether. The ether extract con-
centrate was chromatographed on a 1.2 m x 2mm i.d. glass
column packed with 2% Carbowax 20 M +5% KOH. The column was
maintained at 187°C for plasma samples and 150°C for urine
samples.

  The heptafluorobutyric anhydride derivative of
pseudoephedrine and electron capture detector have been used
to enhance the sensitivity of the gas chromatographic method.
Lin and co-workers[36] and Cummins and Fourier[37] extracted
basefied urine or serum with benzene. Heptafluorobutyric
anhydride is added to the benzene extract. The heptafluoro-
ibutyric anhydride derivative extracted was chromatographed

on a 3% OV-17, 1.82 m x 2 mm i.d. glass column at 150°C[36] or a 5% ethylene glycol succinate, 1.82 m x 2 mm i.d. stainless steel column at 140°C.[37]

Pseudoephedrine in urine was analyzed by gas chromatography using a 2% polyethylene glycol 600 + 5% KOH, 2 m x 2 mm i.d. stainless steel column at 165°C. The urine was extracted with diethyl ether and then made basic with 5N NaOH. The pseudoephedrine was extracted with diethyl ether, concentrated, and injected directly into the gas chromatograph, or derivatized with acetone and then chromatographed.[38]

Pseudoephedrine was determined after acidification, precipitation, and acetic anhydride derivatization. The ester derivative was injected onto a 2.5% SE-30, 1.8 m x 4 mm i.d. column at 190°C.[39]

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TRIPROLIDINE HYDROCHLORIDE

Steven A. Benezra and Chen-Hwa Yang

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1.   Description

1.1   Name, Formula, Molecular Weight

Triprolidine hydrochloride is E-2-[3-(1-pyrroli-
dinyl)-1-p-tolylpropenyl]pyridine, monohydrochloride;
E-1-(2-pyridyl)-3-pyrrolidino-1-p-tolylprop-1-ene, monohydro-
chloride.
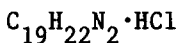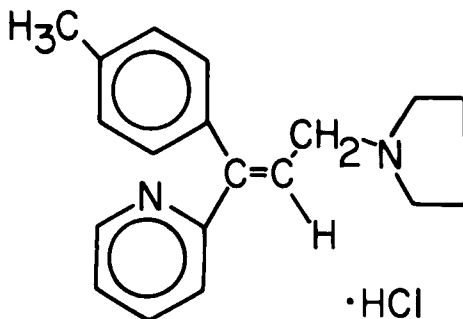
$C_{19}H_{22}N_2 \cdot HCl$ M.W. 314.85

1.2   Appearance, Color, Odor

Triprolidine hydrochloride occurs as a white,
crystalline powder, with no more than a slight unpleasant
odor.[1]

2.   Physical Properties

2.1   Infrared Spectrum (IR)

The infrared spectrum of triprolidine hydrochloride
is shown in Figure 1. The IR spectrum was recorded with a
Nicolet model 7199 FT-IR spectrophotometer as a 0.2% disper-
sion of triprolidine hydrochloride in KBr.[2] Table I gives
the infrared assignments consistent with the structure
of triprolidine hydrochloride.

Table I

Infrared Spectral Assignments for Triprolidine

Hydrochloride Monohydrate

Band (cm^{-1})	Assignment
3480	OH stretch (hydrate)
2958	CH stretch (-CH$_3$)
2690	NH$^+$ stretch
1630	C=C stretch (conj. diene)
1582	C=C stretch (aromatic)
1562	C=C stretch (pyridine)
1462	-CH$_3$ asym. bend
1386	-CH$_3$ sym. bend
1358	C-N stretch (tert. amine)
846	=C-H rock
824	para subst. benzene
776	1-subst. pyridine

2.2   Nuclear Magnetic Resonance (NMR) Spectrum

The NMR spectrum of triprolidine hydrochloride is shown in Figure 2.   It was obtained with a Varian XL-100 100 MHz NMR spectrometer.   Deuterated DMSO was used as the solvent with tetramethylsilane as an internal standard.[3] The interpretation of the NMR spectrum is given in Table II.
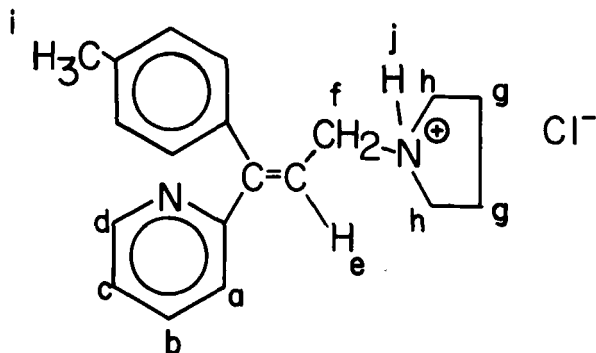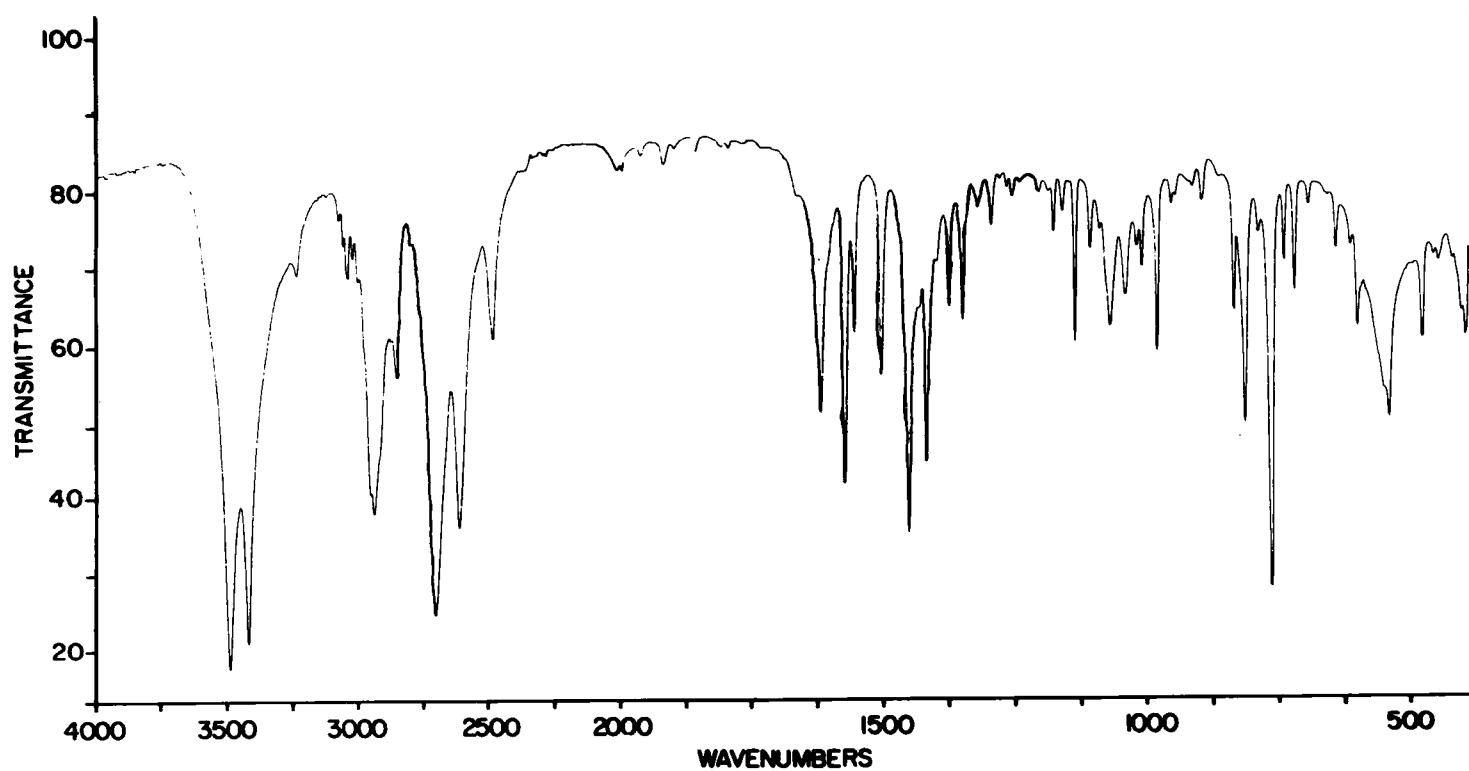
Figure 1 - Infrared Spectrum of Triprolidine Hydrochloride
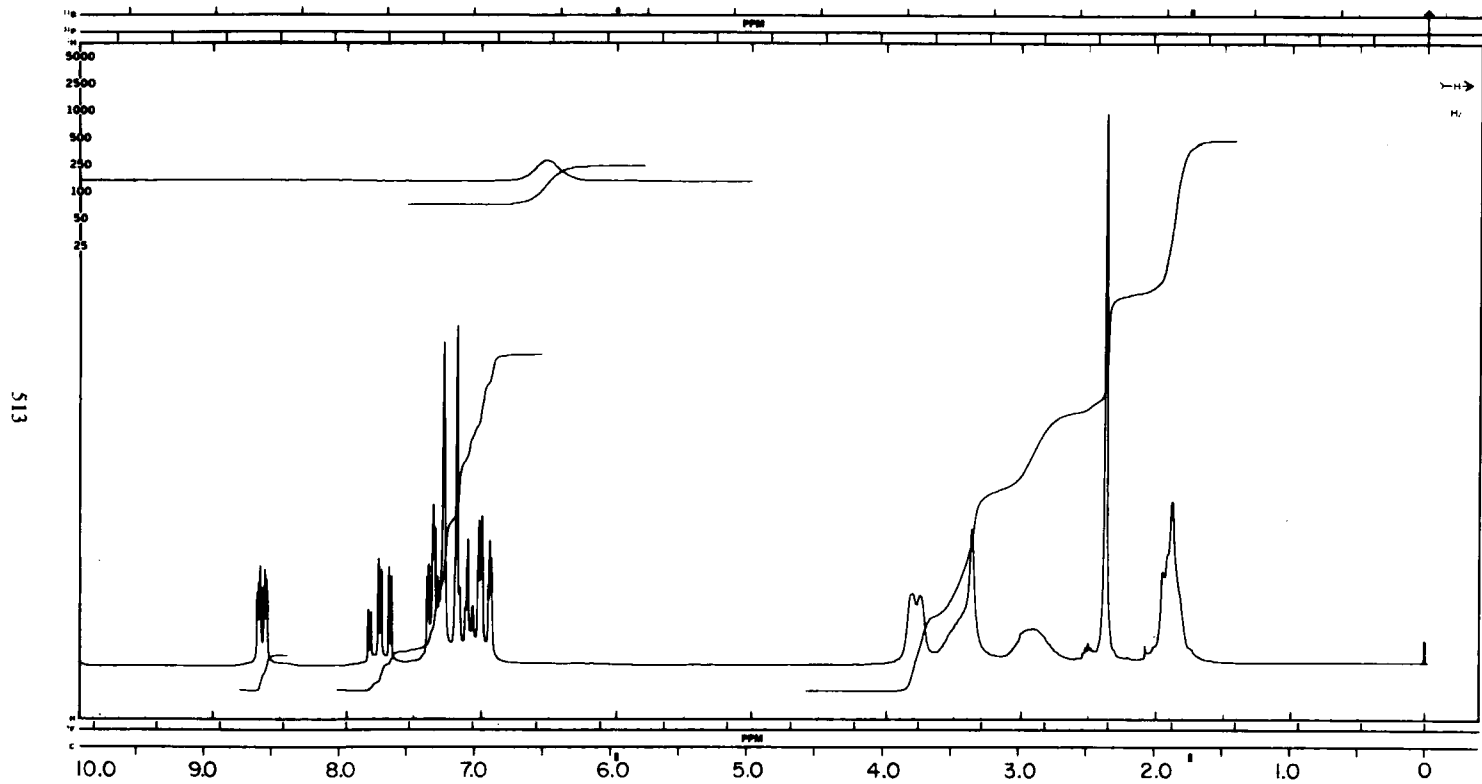
Figure 2 - Nuclear Magnetic Resonance Spectrum Triprolidine Hydrochloride

Table II

NMR Assignments for Triprolidine Hydrochloride

Chemical Shift (ppm)	No. of Protons	Multiplicity	Assignments
11.55	1	1	j
8.63	1	multiplet	a
7.74	1	6	b
7.35	1	multiplet	c
7.00	1	multiplet	d
7.12-7.38	4	4	benzene ring
6.95	1	3	e
3.78	2	2	f
3.49	2	1 (broad)	h
2.94	2	1 (broad)	h
2.37	3	1	i
1.88	4	multiplet	g

$J_{a,c}$=4.7, $J_{a,d}$=0.9, $J_{a,b}$=2.0, $J_{b,c}$=8, $J_{c,d}$=1.2, J_{af}=7

2.3 Ultraviolet (UV) Spectrum

The ultraviolet spectrum of triprolidine hydro-
chloride in 0.1N HCl was taken with a Beckman ACTA CIII UV
spectrophotometer and is shown in Figure 3.[4] Table III
gives the UV data for triprolidine hydrochloride in various
solvents.

Table III

UV Spectral Data for Triprolidine Hydrochloride

Solvent	λmax (nm)	ε_{max}
0.1N NaOH	234	17000
	275	8000
0.1N HCl	232	13000
	290	9900
Methanol	235	15000
	282	7200
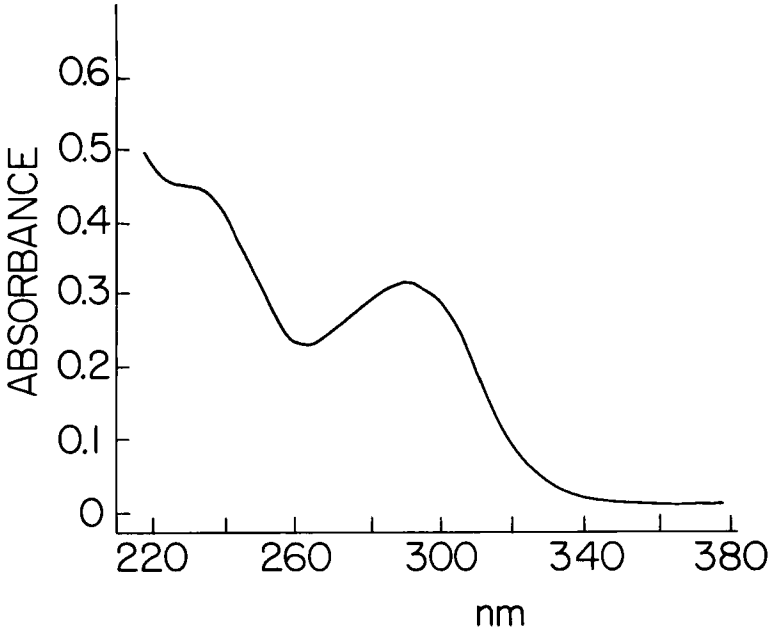
Figure 3 - Ultraviolet Spectrum of Triprolidine Hydrochloride
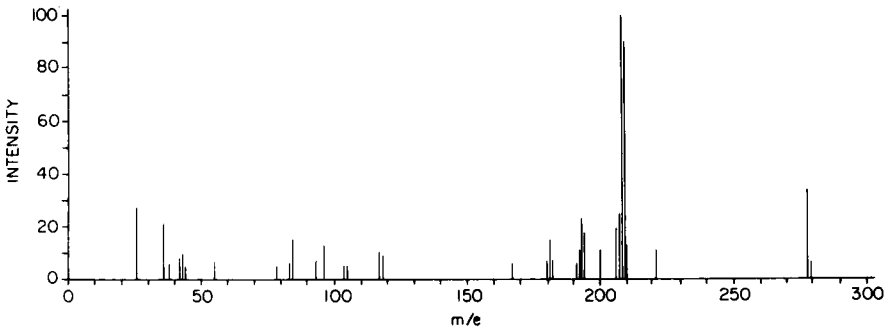
Figure 4 - Mass Spectrum of Triprolidine Hydrochloride

2.4 Mass Spectrum

The mass spectrum of triprolidine hydrochloride is shown in Figure 4. It was obtained with a Varian MAT CH5-DF mass spectrometer. The electron energy was 70 eV and the sample was introduced via direct probe at 120°C.[5] The characteristic fragments are in agreement with those found by Kuntzman and co-workers.[6] The major fragments character-istic of triprolidine are shown in Figure 5.

2.5 Fluorescence Spectrum

Triprolidine hydrochloride in 0.1M sulfuric acid has an excitation maximum at 305 nm and an emission maximum at 445 nm. The fluorescence is much diminished in hydro-chloric acid and almost non-existent in distilled water. The addition of halide ions to 0.1M sulfuric acid solutions of triprolidine hydrochloride has a quenching effect on the fluorescent intensity. The degree of quenching is $I^- > Br^-$ $>Cl^- > F^-$. A concentration of $10^{-3}M$ Cl^- has little effect on the fluorescent intensity, while 0.1M Cl^- reduces the intensity by approximately 75% of the chloride-free sulfuric acid solution.[7]

2.6 Crystal and Molecular Structure

James and Williams have determined that triproli-dine hydrochloride monohydrate crystals belong to the $P2_1/c$ space group.[8] There are 4 molecules per unit cell. The cell parameters are a = 14.777 Å, b = 9.5785 Å, c = 13.099 Å, and β = 90.48°. The 2-pyridyl ring and p-tolyl group make dihedral angles of 29.7° and 55.3° respectively with the double bond plane. The inter-aryl dihedral angle is 106.5°. The data was obtained on crystals grown from a solution of the compound in anisole. A Picker FACS-1 dif-fractometer with CuK_α radiation was used for the measurements.

Figure 5 - Major Ions in Mass Spectrum of Triprolidine Hydrochloride

2.7 Melting Point

        Triprolidine hydrochloride melts at 115-120°C in a sealed capillary tube.[9]

2.8 Solubility

        The solubility of triprolidine hydrochloride, as the monohydrate, in various solvents at 25°C is given in Table IV.[9]

Table IV

Solubility of Triprolidine Hydrochloride at 25°C

Solvent	Solubility (mg/ml)
Water	316
0.1N HCl	319
95% Ethanol	374
n-Octanol	75
Chloroform	480
Propylene Glycol	237
Diethyl Ether	<1

2.9 Partition Coefficients

        The partition coefficients of triprolidine hydrochloride in n-octanol/aqueous pH 1.2 and n-octanol/aqueous pH 7.4 are 0.041 and 7.0 respectively.[9]

2.10 Dissociation Constant

        The pKa_1 and pKa_2 of triprolidine are 3.6 and 9.3 respectively.[9]

3. Synthesis

        The synthesis of triprolidine hydrochloride is shown in Figure 6.[10] The 4-methyl-w-pyrrolidinopropio-phenone used in the first step is prepared by the Mannich

Figure 6 - Synthesis of Triprolidine Hydrochloride

reaction of 4-methylacetophenone and pyrrolidine. With
proper dehydration conditions of the carbinol, a product
almost pure in the desired E-conformation is obtained.

4.   Stability

        Ultraviolet light will convert the E-form of
triprolidine hydrochloride to the Z-form. After 2 years at
37°C, triprolidine hydrochloride in a syrup formulation does
not decompose more than 10%. After 3 years at 37°C tripro-
lidine hydrochloride in tablet formulations does not decom-
pose more than 10%. The formulations are kept in light re-
sistant containers since triprolidine hydrochloride dis-
colors on exposure to light.[9]

5.   Drug Metabolic Products and Pharmacokinetics

        Figure 7 shows the major metabolic pathway of
triprolidine hydrochloride as determined in a study of [14]C
labelled triprolidine hydrochloride in guinea pigs.[6] Tri-
prolidine is converted to metabolite I by liver microsomes.
Metabolite I is converted to metabolite II which is the
major metabolite found in guinea pig urine.

        The plasma levels of triprolidine hydrochloride
were determined in 16 normal male subjects.[12] When admin-
istered orally at a concentration of 3.75 mg triprolidine
hydrochloride in 15 ml of syrup, peak plasma levels of
8.2 ng/ml were achieved in 2 hours with a drug half-life of
5 hours. The low plasma levels found indicate a large
volume of tissue distribution which was consistent with data
obtained from rat studies.

6.   Methods of Analysis

    6.1 Elemental Analysis

        The results of the elemental analysis of tripro-
lidine hydrochloride are given in Table V.[3] The theoretical
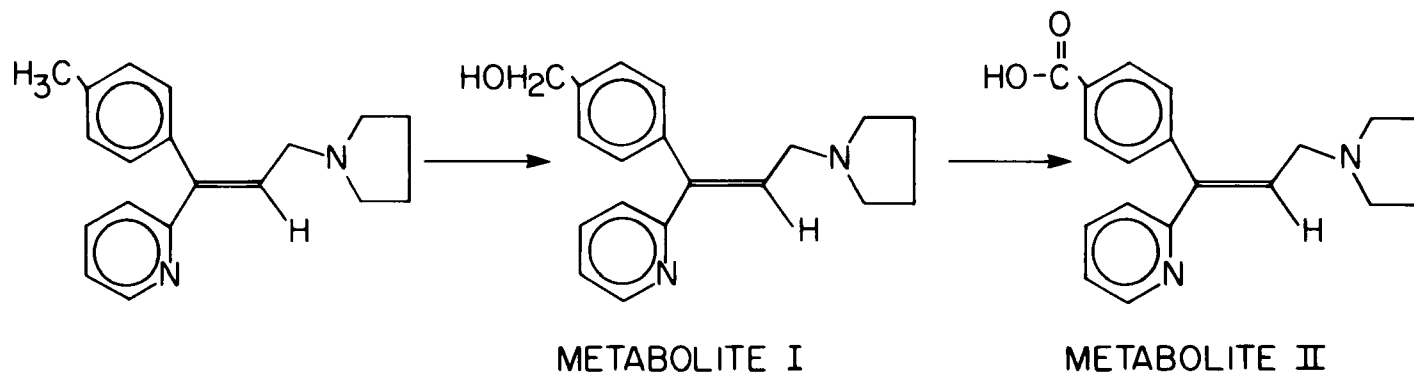
Figure 7 - Metabolites of Triprolidine Hydrochloride

figures are based on the monohydrate.

<div align="center">

Table V

Elemental Analysis of Triprolidine Hydrochloride

Element	Theory (%)	Found (%)*
C	58.50	58.59
H	7.51	7.58
N	8.41	8.39

</div>

*N.F. Reference Standard

6.2 Nonaqueous Titration

Triprolidine hydrochloride is dissolved, with warming if necessary, in glacial acetic acid. Mercuric acetate test solution is added and the solution titrated with 0.1N perchloric acid, the end-point being determined potentiometrically. Each ml. of 0.1N perchloric acid is equivalent to 0.05 mmole of triprolidine hydrochloride.[1]

6.3 Ultraviolet Spectrophotometric Analysis

An ultraviolet spectrophotometric analysis is used to determine content uniformity of triprolidine hydrochloride in tablet formulations.[1] The triprolidine hydrochloride is extracted from finely powdered tablets with dilute hydrochloric acid. The solution is filtered and diluted to a concentration of approximately 10 μg triprolidine hydrochloride per ml. The absorbance at 290 nm of the extracted triprolidine hydrochloride solution in 1 cm cells is compared against NF Triprolidine Hydrochloride Reference Standard prepared in dilute hydrochloric acid at the 10 μg/ml level.

6.4 Quantitative Infrared Analysis

Quantitative infrared analysis is used as an assay

procedure for triprolidine hydrochloride in syrup and
tablets.[1] A portion of syrup or tablets equivalent to 20 mg
and 12.5 mg triprolidine hydrochloride, respectively, is
placed in a separatory funnel or glass-stoppered test tube.
Distilled water is added to dilute the sample followed by
concentrated NaOH solution to liberate triprolidine free
base. The triprolidine free base is extracted into cyclo-
hexane. The infrared absorbance of the cyclohexane extract
is determined at 824 cm^{-1} in 1 mm thick cells. A base line
is drawn between 840 cm^{-1} and 806 cm^{-1}. The absorbance of
the sample is compared against the absorbance of a NF Tri-
prolidine Hydrochloride Reference Standard treated in the
same manner as the sample.

   6.5 Chromatography

   6.51 High Performance Liquid Chromatography (HPLC)
         Separation of the E and Z isomers of triprolidine
hydrochloride has been performed with HPLC. A DuPont SAX
column (1m x 2 mm i.d.) with a mobile phase of 0.04M NaNO$_3$
and 0.01M Na$_2$HPO$_4$ at a pressure of 1500 psi gave retention
times of 4 minutes and 9 minutes for the E and Z isomers
respectively.[13] A 0.45m x 4 mm i.d. Partisil® silica gel
column with a mobile phase of acetonitrile:concentrated
NH$_4$OH:H$_2$O (1000:5:45) at 2 ml/min gave retention times of 15
and 22 minutes for E and Z isomers respectively.[14] A mixture
of triprolidine hydrochloride with other drugs normally found
in combination with triprolidine hydrochloride was separated
with a 1.22m x 2.3 mm i.d. column containing a chemically
bonded diphenyldichlorosilane pellicular packing. A mobile
phase consisting of 60:40 acetonitrile:1% aqueous ammonium
acetate (pH 7.4) with a flow rate of 1.4 ml/min gave a
retention time of 310 seconds for triprolidine.[15] Syrup and

tablet formulations of triprolidine hydrochloride can be
analyzed by HPLC using a Spherisorb® 10μ C_{18} reversed phase
column eluted with ethanol:0.5% aqueous ammonium acetate
(4:1) at 2 ml/min. In this system triprolidine hydrochloride
has a retention time of 22 minutes.[16]

    6.52 Thin Layer Chromatography (TLC)
        Various TLC systems for triprolidine hydrochloride
are given in Table VI.

                        Table VI

    Thin Layer Chromatography Systems for Triprolidine
                    Hydrochloride

Mobile Phase	Adsorbant	Rf	Reference
chloroform:methanol:ammonia 80:20:1	Silica gel F	0.60	17
chloroform:diethylamine 95:5	Silica gel F	0.44	17
2-butanone:dimethylformamide 1:1	Silica gel F	0.60	17
n-butylacetate:acetone: n-butanol:methanol:10% aq. ammonia 4:2:2:1:1	Silica gel F	0.60	17
2-butanone:toluene: methanol:diethylamine 60:40:7:3	Silica gel F	0.40	17
cyclohexane:benzene: diethylamine 75:15:10	Silica gel G	0.41	18
methanol	Silica gel G pre-coated with	0.40	18

	0.1M NaOH		
acetone	Silica gel G	0.13	19
	pre-coated with		
	0.1M NaOH		
methanol	Silica gel G	0.17	18
	pre-coated		
	with 0.1M KHSO$_4$		
95% ethanol	Silica gel G	0.02	19
	pre-coated with		
	0.1M KHSO$_4$		
benzene:dioxane:ammonia 60:35:35	Silica gel G	0.40	20
ethanol:acetic acid:water 5:3:2	Silica gel G	0.48	20
methanol:n-butanol 6:4	Silica gel G	0.26	20
n-butanol:n-butyl ether: acetic acid 4:8:1	Alumina	0.81	20

6.53 Quantitative Thin Layer Chromatography

Quantitative TLC has been used to determine tri-prolidine hydrochloride in human plasma.[12] Plasma was extracted with dichloroethane at physiological pH (7.4). The organic phase was evaporated and the residue dissolved in chloroform. A portion of the chloroform solution was spotted on silica gel plates which were then developed in chloroform:methanol:ammonia (89:10:1). After development, the plate was air dried and sprayed with a 2M aqueous solution of ammonium bisulfate. After the plate was air dried for 1 hour, the fluorescent spot representing triprolidine was quantitated with a spectrodensitometer in the reflectance mode with 300 nm excitation and emission above 405 nm. With

this technique it was possible to quantitate as little as
0.4 ng triprolidine spotted on the plate.

6.54 Gas Chromatography

Triprolidine hydrochloride has been separated from
other antihistamines with a 1.52m x 2.4 mm i.d. column packed
with 2% carbowax 20M + 10% KOH on 60/80 mesh Chromosorb W.
Retention time of 32 minutes was obtained with a column
temperature of 190°C. Triprolidine hydrochloride was probab-
ly eluted as the free base.[21]

6.6 Fluorimetric Analysis

Triprolidine hydrochloride in syrups and tablets
can be analyzed by fluorimetry. A portion of the tablets or
syrup is made basic with 1N NaOH and extracted with ethylene
chloride. The organic phase is then extracted with 0.1N
H_2SO_4. The fluorescence of the acid extract is determined
with a fluorometer using a UG11 filter for excitation and a
Wratten 2A filter for emission. The fluorescence of the
sample preparation is compared against a Reference Standard
prepared in the same manner[22].

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SODIUM VALPROATE AND VALPROIC ACID

Zui L. Chang

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        1.12 Trade Names
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    2.2 Nuclear Magnetic Resonance Spectra
    2.3 Mass Spectrum
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    2.9 Hygroscopic Behavior
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        6.32 Gas–Liquid Chromatography
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7. Determination of Valproic Acid and Its Metabolites in Biological Fluids
8. References
9. Acknowledgments

1. Description

1.1 Nomenclature

1.11 Chemical Names

Sodium valproate is sodium 2-propylpentanoate.
It is also known as sodium dipropylacetic
acid, sodium 2-propylvalerate, sodium dipro-
pylacetate, sodium di-n-propylacetate, and
by many slight variations of the particular
nomenclature.

Valproic acid is 2-propylpentanoic acid. It
is also known as n-dipropylacetic acid, 2-
propylvaleric acid, DPA, and by many slight
variations of the particular nomenclature.

1.12 Trade Names

Depakene®, Depakine®, Eurekene®, Epilim®,
Ergenyl®, and Labazene®.

1.2 Formulas and Molecular Weights

$$CH_3CH_2CH_2 \diagdown$$
$$\qquad\qquad CHCOONa$$
$$CH_3CH_2CH_2 \diagup$$

$C_8H_{15}O_2Na$ M.W.   166.19

$$CH_3CH_2CH_2 \diagdown$$
$$\qquad\qquad CHCOOH$$
$$CH_3CH_2CH_2 \diagup$$
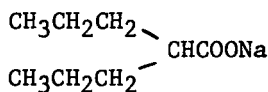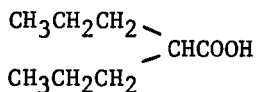
$C_8H_{16}O_2$ M.W.   144.21

1.3 Appearance, Color, Odor

Sodium valproate is a white crystalline powder with
a very slight characteristic odor of valproic acid.

Valproic acid is a colorless slightly viscous liq-
uid, and it has a characteristic odor of valproic
acid.

2. Physical Properties

2.1 Infrared Spectra

The infrared spectrum of sodium valproate is pre-
sented in Figure 1. The spectrum was measured in
the solid state as a potassium bromide dispersion.
The following bands (cm^{-1}) have been assigned for
Figure 1 (1).

a. 3000-2800 cm^{-1} Complex of strong bands due to
                          the overlapping C-H stretching
                          vibrations of the various
                          methyl and methylene groups.

b. 1560 and   Strong bands due to the anti-
    1412 cm^{-1} symmetrical and symmetrical
                          stretching vibrations of the
                          COO$^-$ grouping.

The infrared spectrum of valproic acid is presented
in Figure 2. The spectrum was measured using the
capillary method. The following bands (cm^{-1}) have
been assigned for Figure 2 (1).

a. 3400-2300 cm^{-1} Broad and diffuse absorption
                          due to the OH stretching vi-
                          bration of the carboxylic acid.

b. 3000-2800 cm^{-1} Complex of strong bands due to
                          the overlapping C-H stretching
                          vibrations of the various
                          methyl and methylene groups.

c. 1700 cm^{-1} Strong band due to the C=O
                          stretching vibration of the
                          carboxylic acid.

d. 930 cm^{-1} Broad band due to the OH bend-
                          ing vibration of the carboxylic
                          acid.

2.2 Nuclear Magnetic Resonance Spectra (NMR)

The nuclear magnetic resonance spectrum of sodium
valproate as shown in Figure 3 was obtained on a
Varian Associates T-60 NMR Spectrometer in deuterium
oxide containing tetramethylsilane as the internal
standard. The spectral peak assignments (2) are
presented in Table I.

FIGURE 1 - INFRARED SPECTRUM OF SODIUM VALPROATE

FIGURE 2 - INFRARED SPECTRUM OF VALPROIC ACID

WAVELENGTH (MICRONS)

FREQUENCY (CM⁻¹)
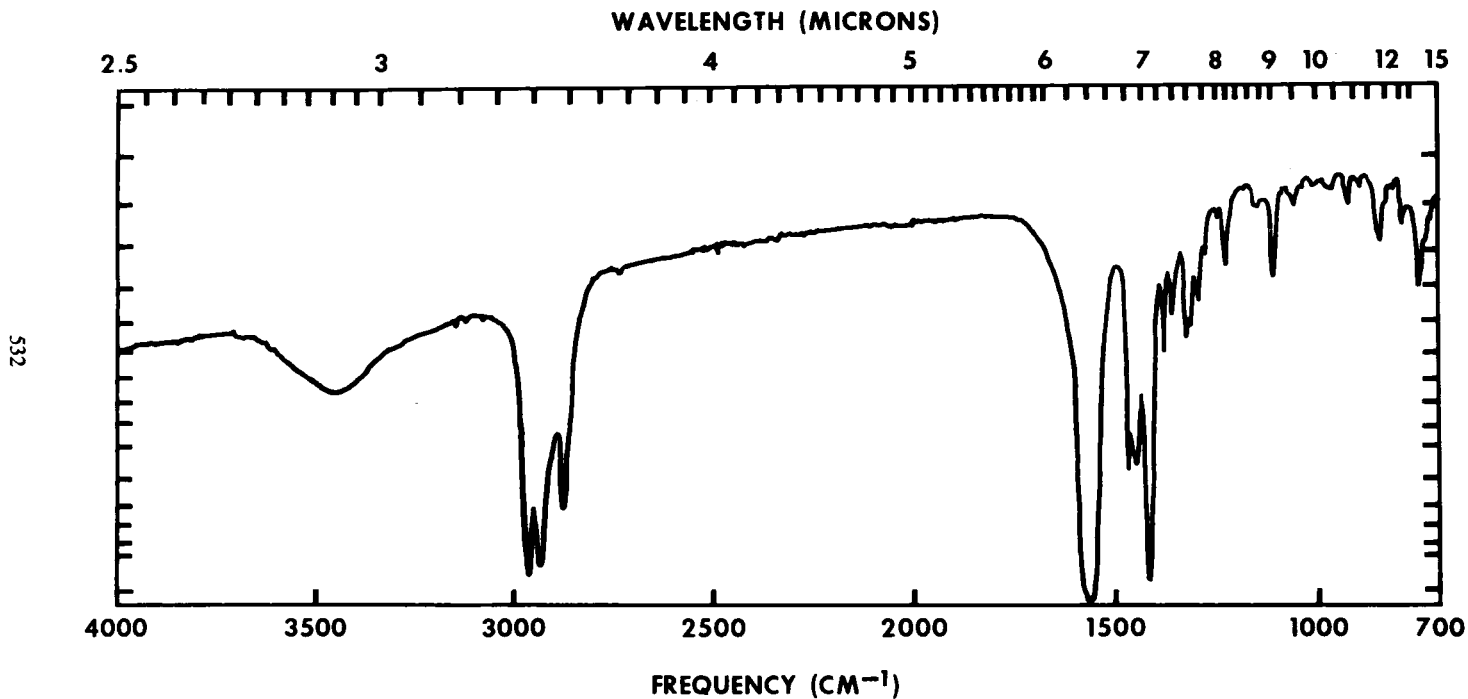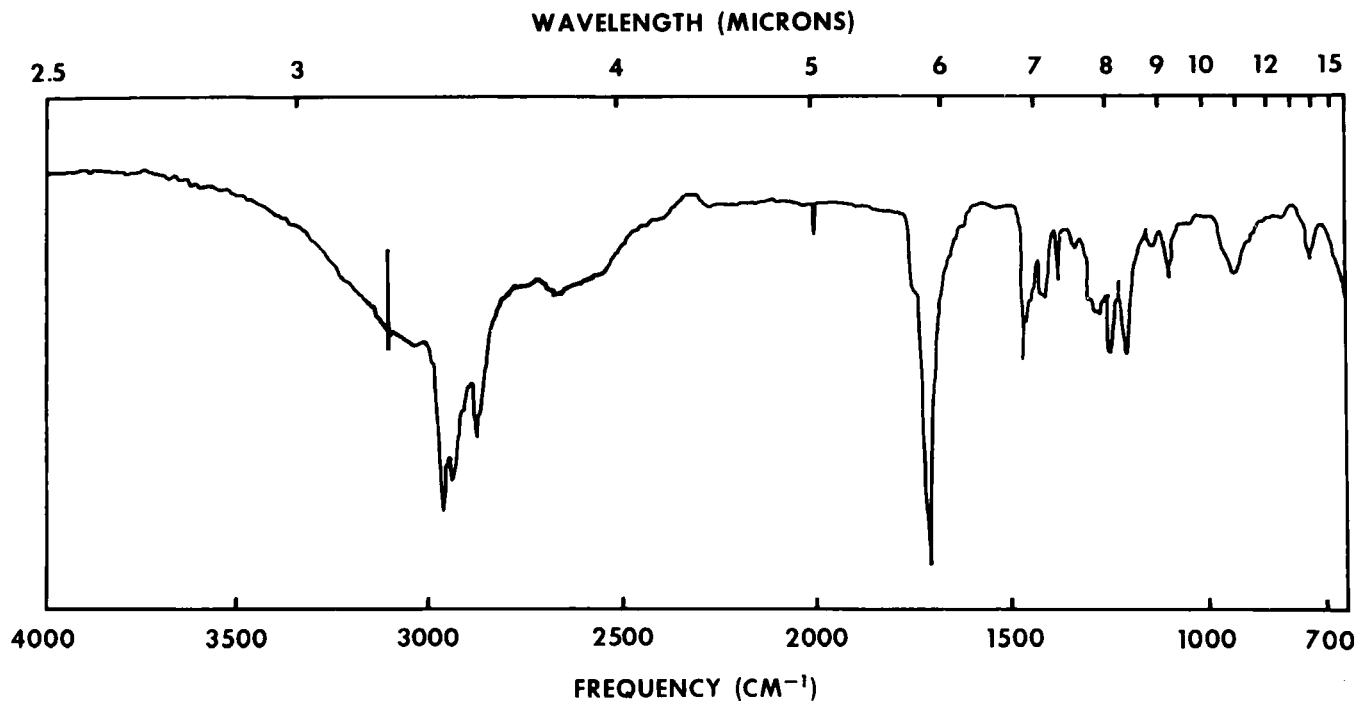
FIGURE 3 - NUCLEAR MAGNETIC RESONANCE SPECTRUM
OF SODIUM VALPROATE

PPM (δ)
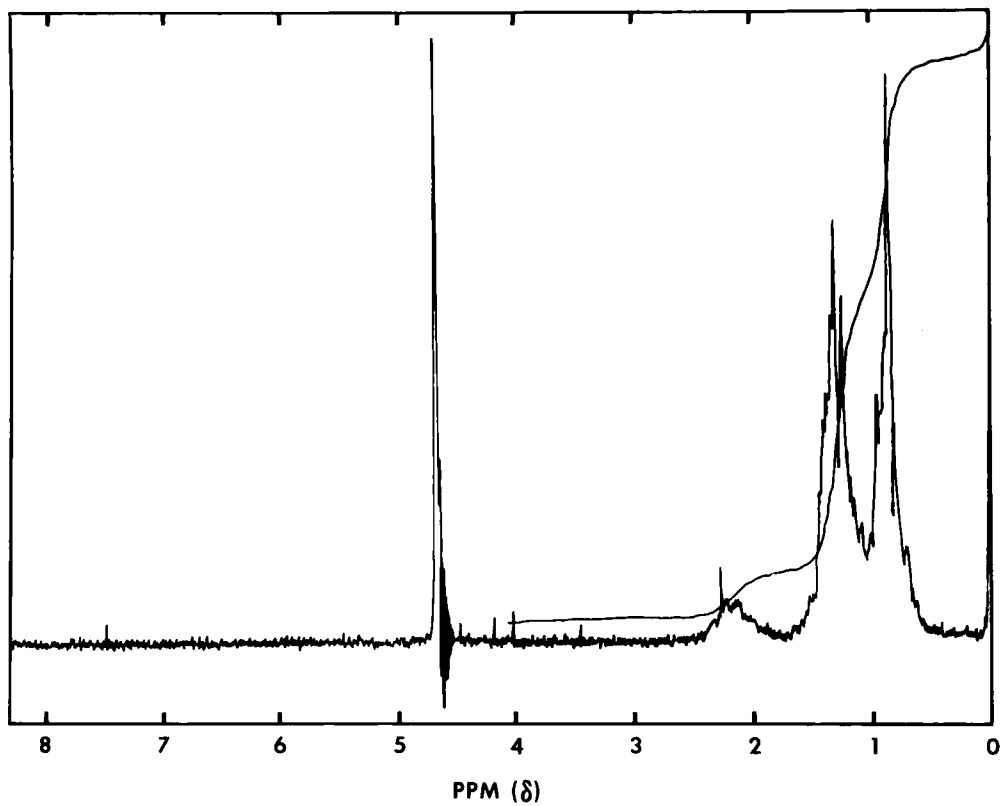
Table I

NMR Spectral Assignments for Sodium Valproate

Proton Assignment	Chemical Shift (ppm)	Multiplicity
$>$CH–CO	1.9–2.5	Multiplet
–CH$_2$CH$_2$ $\diagdown$ C $\diagup$ –CH$_2$CH$_2$	1.1–1.8	Complex
CH$_3$–	0.5–1.1	Complex

The nuclear magnetic resonance spectrum of valproic acid as shown in Figure 4 was obtained on a Varian Associates T-60 NMR Spectrometer as a 10% w/v solution in a solvent of deuterated chloroform. The spectral peak assignments (2) are presented in Table II.

Table II

NMR Spectral Assignments for Valproic Acid

Proton Assignment	Chemical Shift (ppm)	Multiplicity
CO$_2$H	11.3–11.6	Singlet
CHCO	1.9–2.5	Multiplet
–CH$_2$CH$_2$ $\diagdown$ C $\diagup$ –CH$_2$CH$_2$	1.1–1.8	Complex
CH$_3$–	0.5–1.1	Complex

2.3 Mass Spectrum

Sodium valproate was not sufficiently volatile for mass spectral analysis. The mass spectrum of valproic acid as shown in Figure 5 was obtained using an Associated Electrical Industries Model MS-902 Mass Spectrometer with the ionization electron beam energy at 70 eV. High resolution data were compiled and tabulated with the aid of an on-line PDP-11 Computer.

Valproic acid was quite volatile and vaporized as
soon as it was admitted to the source of the mass
spectrometer. Only a very weak ion was detectable
in the molecular ion region at m/e 145. This would
correspond to $(M+H)^+$, but exact mass measurement
was not possible because of the peak's small size
and the short lifetime of the sample in the mass
spectrometer.

The mass spectrum assignments of the prominent ions
and subsequent fragments are shown in Table III and
Figure 6 (3).

Table III

High Resolution Mass Spectrum of Valproic Acid

Measured Mass (m/e)	Calculated Mass	Formula
126.1044	126.1045	$C_8H_{14}O$
102.0690	102.0681	$C_5H_{10}O_2$
73.0295	73.0290	$C_3H_5O_2$

2.4 Raman Spectra

The Raman spectrum of sodium valproate as shown in
Figure 7 was obtained in the solid state on a Cary
Model 83 Spectrometer. The following bands (cm^{-1})
have been assigned for Figure 7 (1).

a. 3000–2800 cm^{-1} Complex of strong bands due to
                         the overlapping C-H stretching
                         vibrations of the various
                         methyl and methylene groups.

b. 1450 cm^{-1} Due to the superimposing C-H
                         bending vibrations of the vari-
                         ous methyl and methylene
                         groups.

The Raman spectrum of valproic acid as shown in
Figure 8, was obtained in the undiluted liquid
state on a Cary Model 83 Spectrometer. The fol-
lowing bands (cm^{-1}) have been assigned for Figure
8 (1).

FIGURE 4 - NUCLEAR MAGNETIC RESONANCE SPECTRUM OF VALPROIC ACID

PPM (δ)
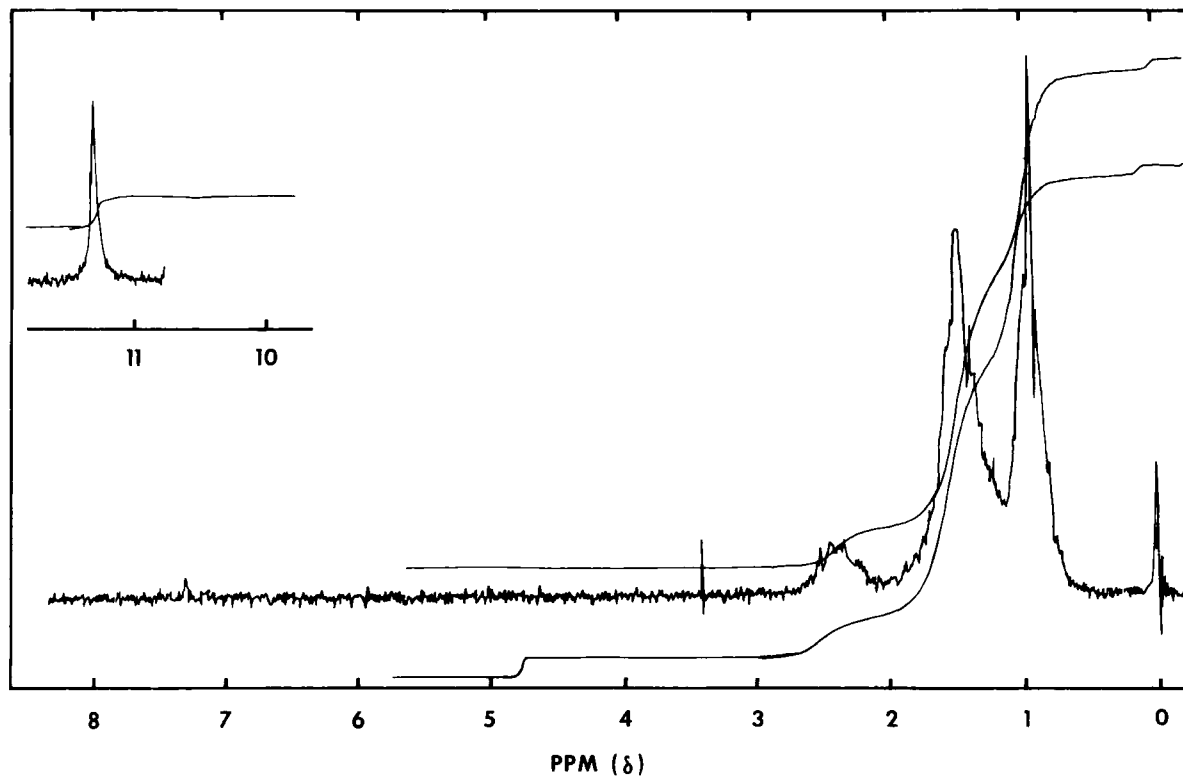
FIGURE 5 - MASS SPECTRUM OF VALPROIC ACID
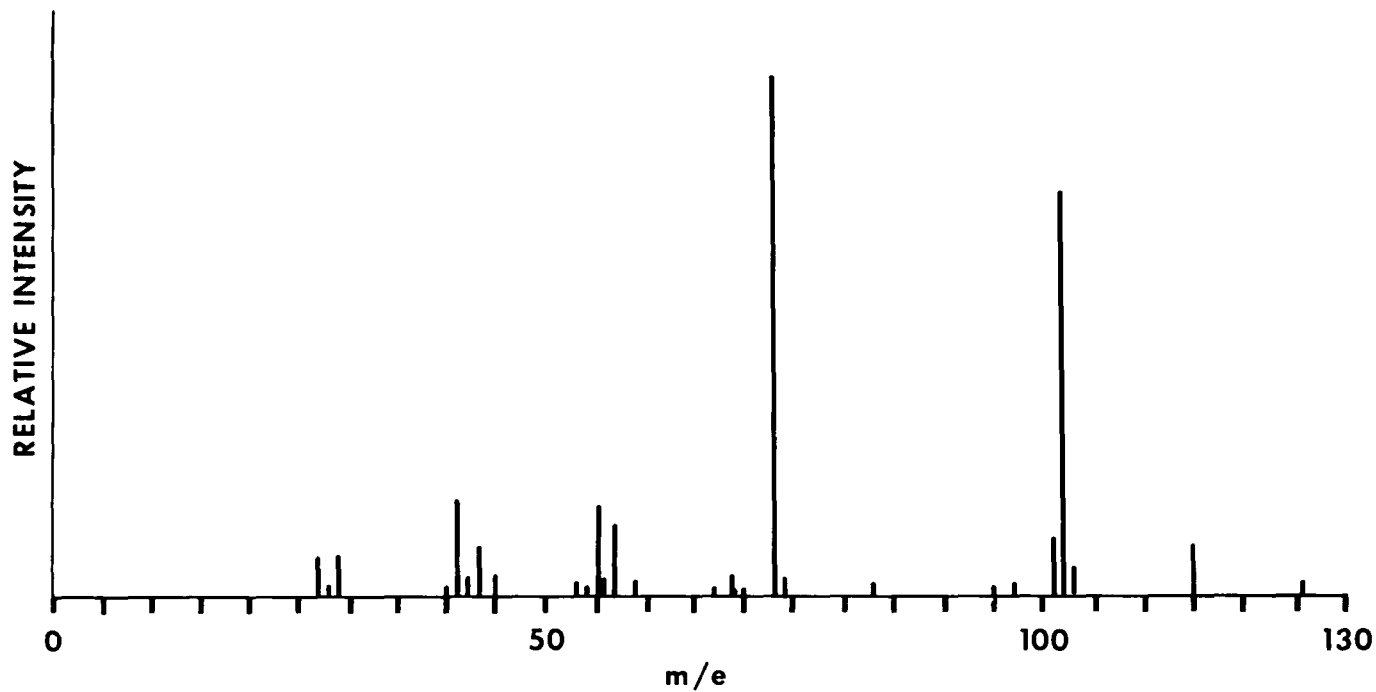
FIGURE 6 - FRAGMENTATION PATHWAYS OF VALPROIC ACID
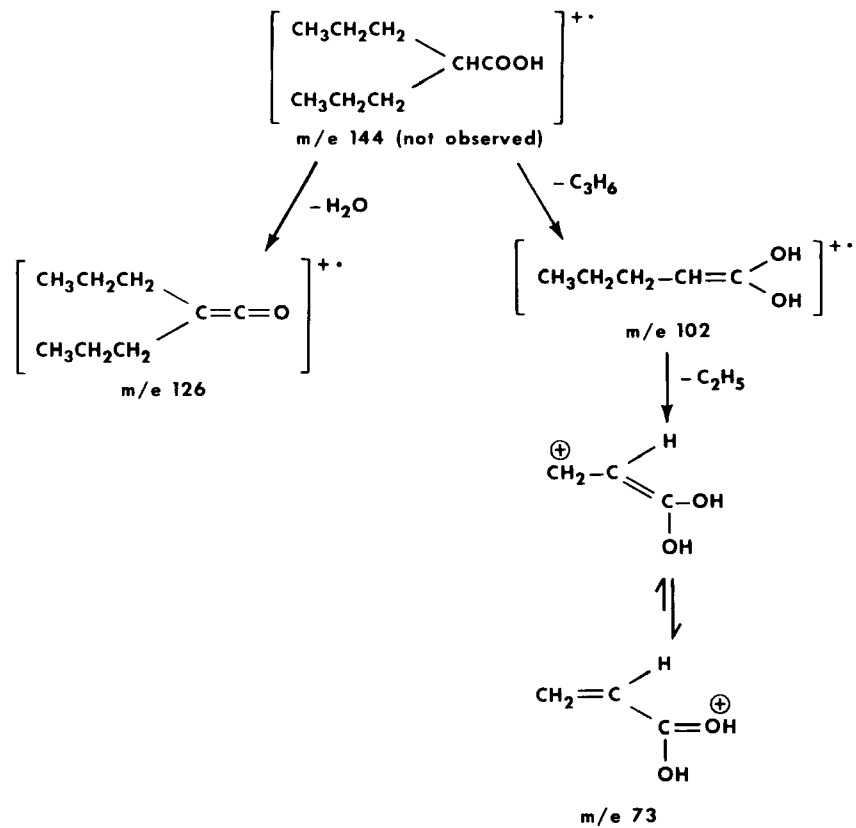
FIGURE 7 - RAMAN SPECTRUM OF SODIUM VALPROATE
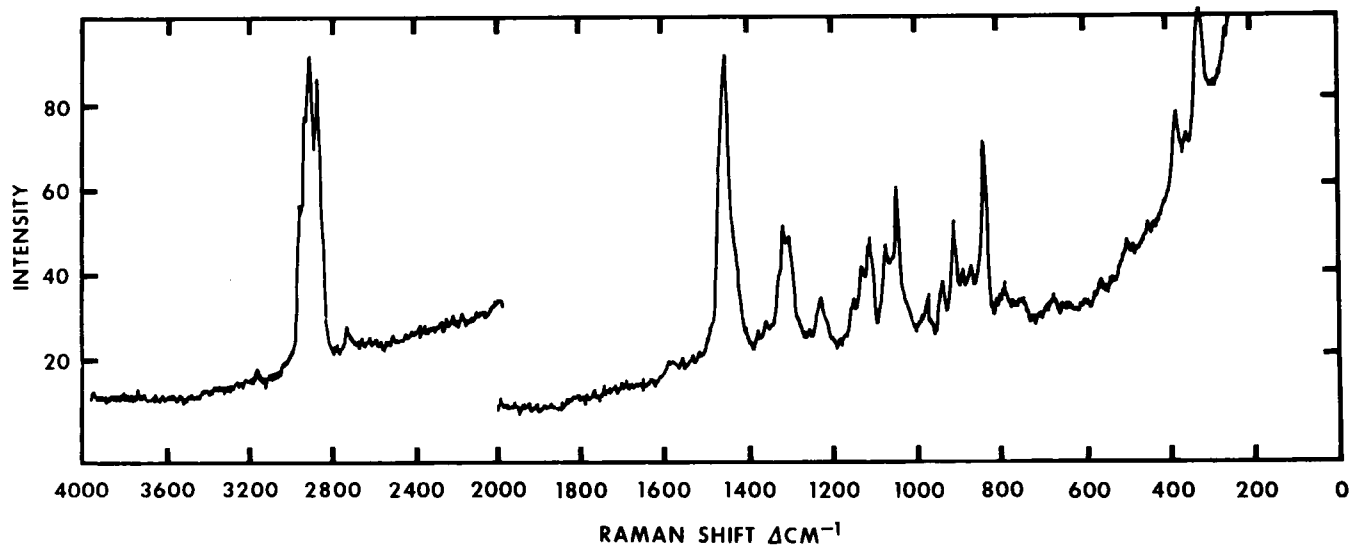
FIGURE 8 - RAMAN SPECTRUM OF VALPROIC ACID
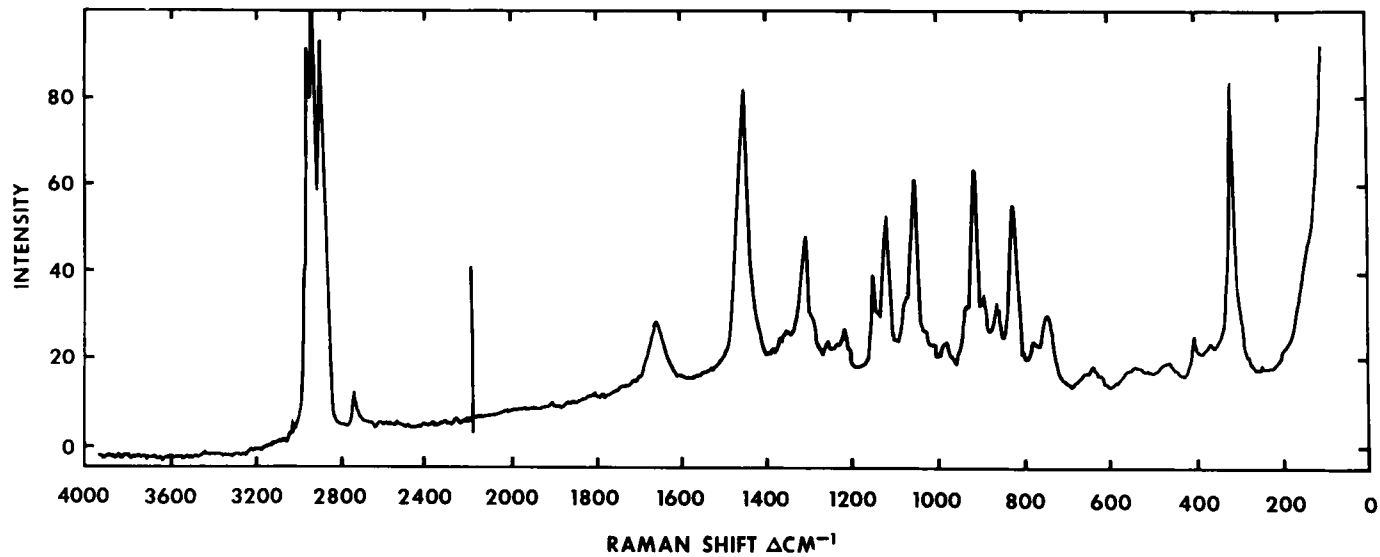
FIGURE 9 - ULTRAVIOLET SPECTRUM
OF VALPROIC ACID
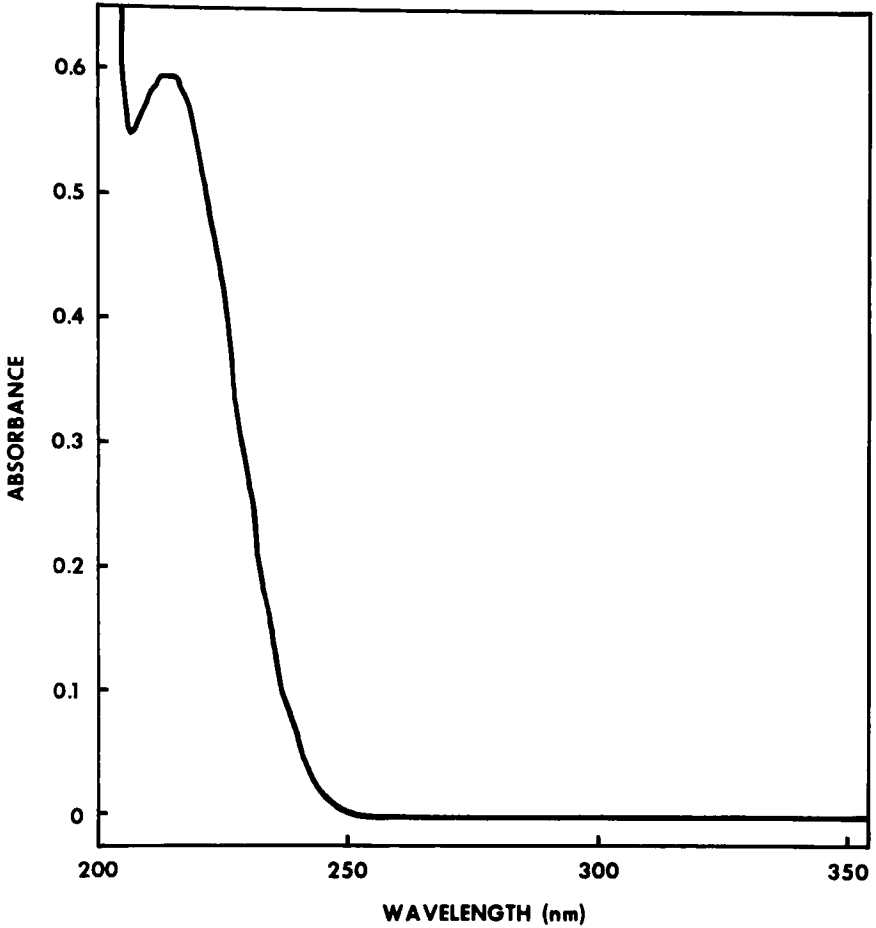
a. 3000-2800 cm^{-1} Complex of strong bands due to
                            the overlapping C-H stretching
                            vibrations of the various
                            methyl and methylene groups.

b. 1660 cm^{-1} Weak band due to C=O stretch-
                            ing vibration of the carboxylic
                            acid.

c. 1450 cm^{-1} Due to the superimposing C-H
                            bending vibrations of the vari-
                            ous methyl and methylene
                            groups.

2.5 Ultraviolet Spectrum (UV)

Sodium valproate in methanol solution has no UV
maximum between 400 and 205 nm. When the UV spec-
trum of 0.1% solution of valproic acid in methanol
solution was scanned from 400 to 205 nm, one maxi-
mum at 213 nm (ε = 86) was observed (Figure 9).
The spectrum was obtained with a Beckman Acta V
Spectrophotometer.

2.6 Solubility

Approximate solubility data have been determined for
sodium valproate at room temperature.

One gram of sodium valproate is soluble in 0.4 ml
of water and also in 1.5 ml of ethanol. It is
freely soluble in methanol (1 in 5). It is prac-
tically insoluble in common organic solvents such
as ether, chloroform, benzene, n-heptane, etc.

The following solubility data have been determined
for valproic acid at room temperature:

n-Heptane	Greater than 10%, v/v
Chloroform	Greater than 10%, v/v
Ethyl Acetate	Greater than 10%, v/v
Methanol	Greater than 10%, v/v
100% Ethanol	Greater than 10%, v/v
Acetone	Greater than 10%, v/v
Diethyl Ether	Greater than 10%, v/v
Benzene	Greater than 10%, v/v
1 N Aqueous NaOH	Greater than 10%, v/v
0.1 N Aqueous HCl	1.15 mg/ml
Water	1.27 mg/ml

2.7 Crystal Properties

The X-ray powder diffraction pattern of sodium val-
proate was determined by visual observation of a
film obtained with a 143.2 mm Debye-Scherrer Powder
Camera (Table IV). An Enraf-Nonius Difractis 601
Generator; 38 KV and 18 MA with nikel filtered cop-
per radiation; λ = 1.5418, was employed (4).

Table IV

X-Ray Powder Diffraction Pattern
d-Spacings and Intensities

dA	I/I_1	dA	I/I_1
16.0	60	2.80	5
13.4	100	2.66	2
7.7	50	2.61	1
6.7	3	2.57	1
5.8	5	2.46	1
5.25	20	2.42	1
4.9	5	2.37	2
4.77	2	2.23	2
4.42	5	2.20	2
4.21	30	2.06	2
4.09	35	2.01	1
3.95	5	1.97	1
3.85	1	1.93	2
3.64	15	1.90	1
3.40	20	1.86	1
3.20	1	1.81	5
3.13	3	1.75	1
3.02	4	1.69	1
2.87	5	1.66	1
2.84	5		

2.8 Dissociation Constants

Sodium valproate exhibits basic properties. Titra-
tion of an aqueous solution of sodium valproate
with aqueous hydrochloric acid gave a pKa value of
4.8 (proton gained).

Titration of valproic acid with aqueous sodium hy-
droxide using acetone-water as the sample solvent
and extrapolated to pure water gave a pKa value of
4.6 (proton lost).

2.9 Hygroscopic Behavior

Sodium valproate is hygroscopic. The rate of mois-
ture absorption was tested in a humidity chamber
using a Cahn Electro Balance and the results are
shown in Table V (5).

Table V

Rate of Moisture Absorption
of Sodium Valproate

Relative Humidity	Time, min.						
	10	20	30	40	50	60	Overnight
12, 22 33, 43%			No gain				
53%	1.76	3.17	4.39	5.61	6.78	7.80	42.9%*

*Sample was completely liquified. Expressed as precent
 weight gain.

2.10 Sublimation

Sodium valproate did not sublime when it was stored
at 105°C for 10 days.

2.11 Melting Range

Sodium valproate does not melt, decompose, or physi-
cally change form in the normal working range of the
Thomas-Hoover Capillary Melting Point apparatus.

2.12 Boiling Point

The following boiling points have been reported for
valproic acid: bp_{14} 120-121°C (6), bp_{20} 128-130°C
(6) and bp_{760} 221-222°C (7).

2.13 Differential Thermal Analysis

Differential thermal analysis of sodium valproate
shows a large endotherm beginning at 100°C and end-
ing at 118°C which is possible due to the loss of
water. A sharp endothermic peak at 450°C is indi-
cative of the melting point of sodium valproate.

Differential thermal analysis of valproic acid shows
a sharp endothermic response at 225°C indicative of
the boiling point of valproic acid.

FIGURE 10 - DIFFERENTIAL THERMAL ANALYSIS CURVE
OF SODIUM VALPROATE
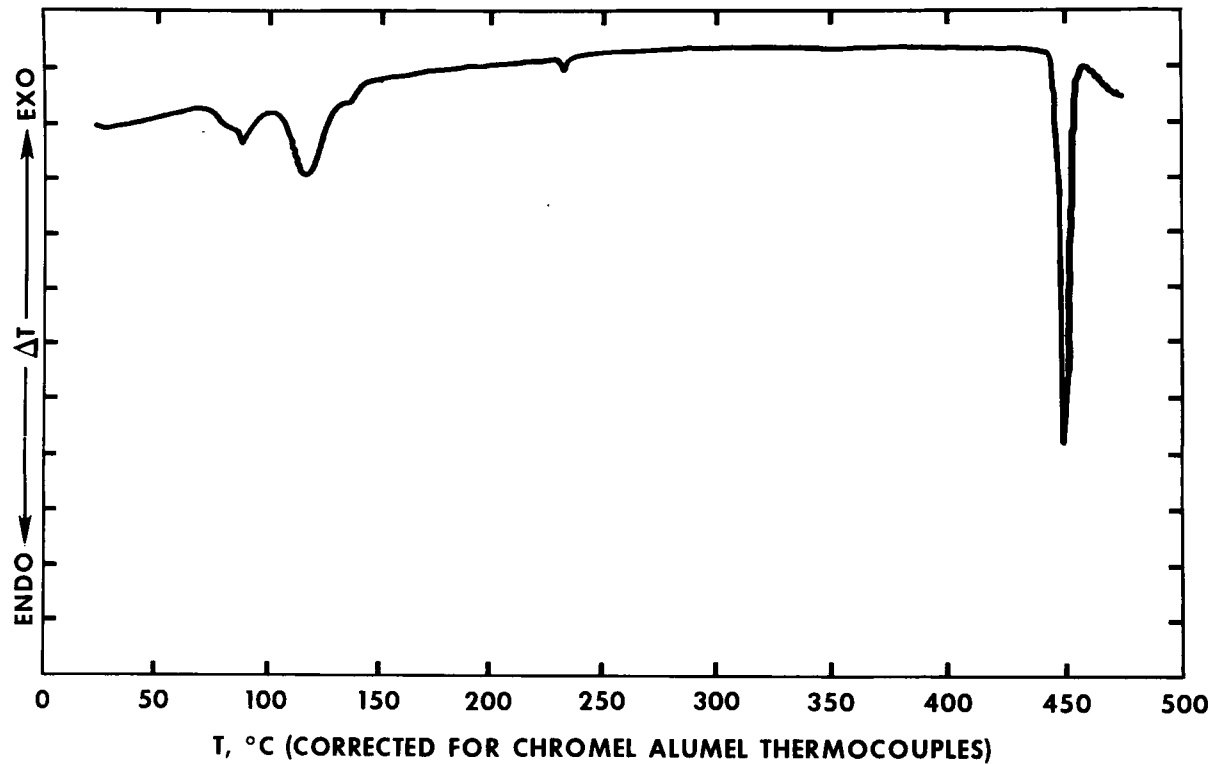
FIGURE 11 - DIFFERENTIAL THERMAL ANALYSIS CURVE
OF VALPROIC ACID

547
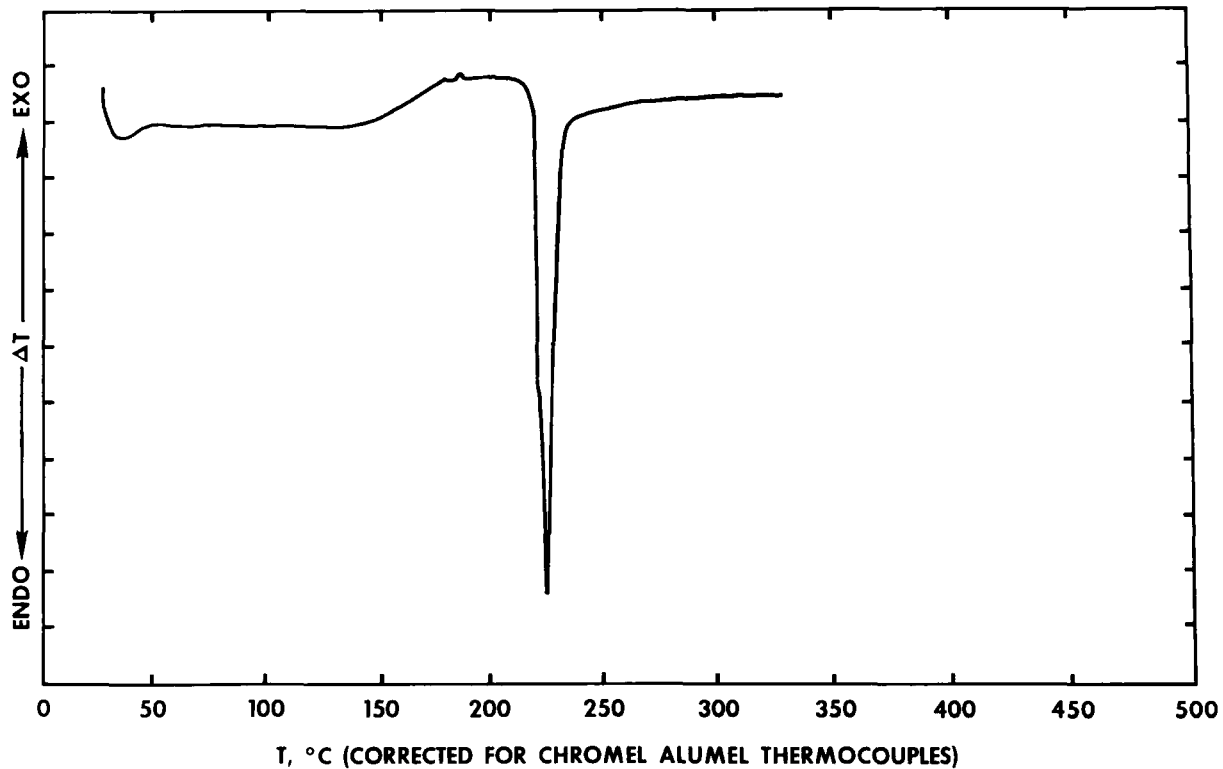
2.14 Specific Gravity

The specific gravity of valproic acid was determined
in a calibrated 25 ml pycnometer at 25°C. It has a
value of 0.904 g/ml (8).

2.15 Refractive Index

Valproic acid has a refractive index of $N_D^{24.5}$
1.425 (6).

3. Synthesis

The synthesis of valproic acid was first described in the
literature by Oberreit (9) in 1896.

The sodium valproate is preferably formed from valproic
acid by the interaction of sodium hydroxide in an aqueous
solution. The synthetic pathways are shown in Figure 12.

4. Stability-Degradation

Sodium valproate was found to be extremely stable when
refluxed in water, 1.0 N hydrochloric acid, or 1.0 N
sodium hydroxide for 3 hours. Also, it was very stable
when it was subjected to heat at 110°C for 10 days and
to natural sunlight for 30 days in the dry state.

Valproic acid is a very stable compound. No degradation
has been observed by the action of heat, light, and
strong aqueous alkali, or acid.

5. Drug Metabolism and Pharmacokinetics

In 1971, Eymard et. al. (10) studied the distribution
and the absorption of carbon-14 labeled sodium valproate
in rats by oral administration.

The metabolites and metabolic pathway of a new anticon-
vulsant drug, sodium valproate, in rats were investigated
using carbon-14 labeled sodium valproate. Most of the
metabolites in urine and bile were a glucuronide conju-
gate of valproic acid. Free sodium valproate was as
little as one-seventh of the total metabolites. In
feces, only free sodium valproate was detected, and the
possibility of enterohepatic circulation of sodium val-
proate was presumed. A part of dosed sodium valproate
was excreted in expired air in the form of CO_2. This
degradative reaction took place in liter mitochondria
and required CoA and oxygen. It was stimulated by ATP

FIGURE 12 – SYNTHETIC PATHWAYS OF VALPROIC ACID AND SODIUM VALPROATE

Diethyl Malonate   Allyl Chloride   Esters of Diallyl Malonic Acid
R=CH_3 and/or C_2H_5

Esters of Dipropyl Malonic Acid   Dipropyl Malonic Acid

2–Propylpentanoic Acid
(Valproic Acid)

Sodium 2–propylpentanoate
(Sodium Valproate)
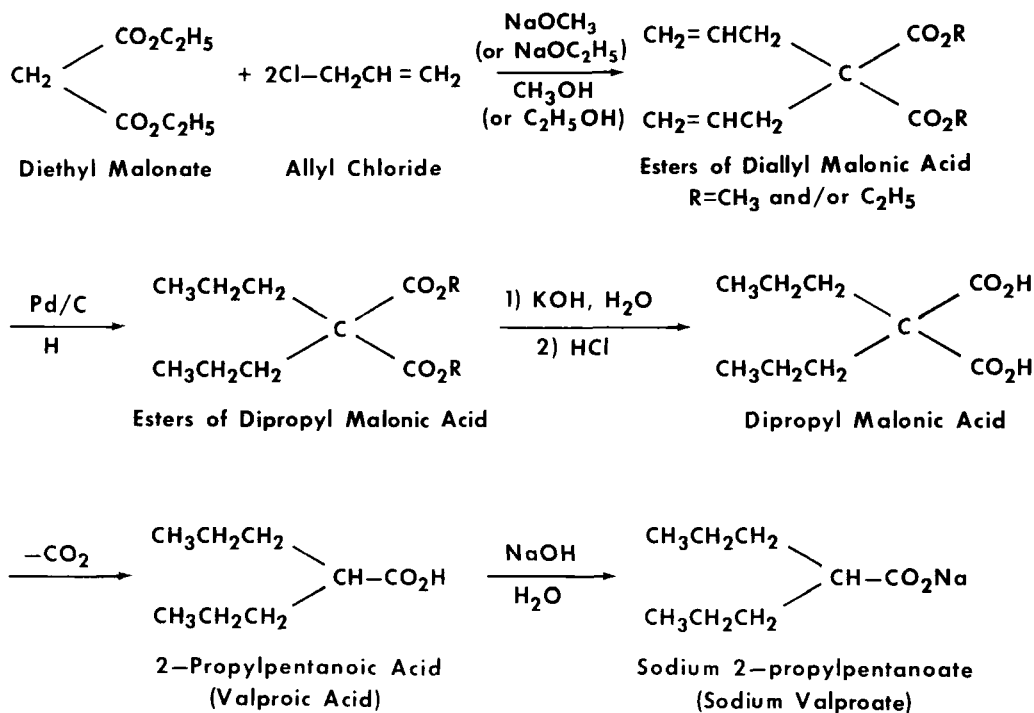
and EDTA, and inhibited by various enzyme reaction inhibitors such as malonate, Antimycin-A, chloropromazine, p-chloromercuribenzoate (PCMB) and 2,4-dinitrophenol. Therefore, this degradation is not a one-step reaction, decarboxylation, but must be β-oxidation of a fatty acid. Thin-layer chromatography and gas-liquid chromatography were used for assay of metabolites (11).

The pharmacokinetics of distribution and elimination of sodium valproate in mice and dogs has been reported by Schobben and van der Kleijn (12).

The omega-oxidation of sodium valproate in rats has been reported by Kuhara, et. al. (13).

The absorption, excretion, and biotransformation of valproic acid were studied by Kukino and Matsumoto (14).

A preliminary pharmacokinetic profile of sodium valproate in monkey has been written by Levig, et. al. (15).

The pharmacokinetics of sodium valproate have been studied in 7 patients by Schobben, et. al. (16). The plasma concentrations were determined by gas-liquid chromatography during and following subchronic treatment. Elimination of the drug appeared to follow a monophasic exponential course; biological half lives were 8 to 15 hours. The drug appeared to have a relatively restricted distribution: calculated relative distribution volumes ranged from 0.15 to 0.40 l/kg. There were large interindividual differences in clearance rate. The therapeutic range was considered to be between 50 and 100 mg/l of plasma.

In 1976, Matsumoto, et. al. (17, 18) discovered several new metabolites of sodium valproate in rat urine, which support the hypothesis that the drug is also metabolized by a β-oxidation mechanism. One of the metabolites, 2-n-propyl 3-oxo-pentanoic acid, was recently found by Gompertz, et. al. (19) and Kochen, et. al. (20) to be a major constituent in urine of children who were receiving sodium valproate. The urinary 3-oxo derivative of valproate was reported to account for 20% of the administered dose.

6. Methods of Analysis

    6.1 Identification

        The presence of sodium cation may be identified by

a flame test. A positive test for sodium is pro-
duced in a non-luminous flame imparting a yellow
color.

The presence of carboxylic anion may be identified
by the following two tests:

A. A 5% aqueous solution gives a violet precipitate
   with a 5% aqueous solution of cobalt nitrate.

B. A 5% aqueous solution gives a violet precipitate
   with potassium iodobismuthite.

6.2 Elemental Analysis

A typical elemental analysis of a sample of sodium
valproate is present in Table VI (21).

Table VI

Elemental Analysis of Sodium Valproate

Element	% Theory	% Found
C	57.82	57.82
H	9.10	9.38
O	19.25	-----*
Na	13.83	-----*

*The oxygen value cannot be determined
 due to presence of sodium.

A typical elemental analysis of a sample of valproic
acid is present in Table VII (22).

Table VII

Elemental Analysis of Valproic Acid

Element	% Theory	% Found
C	66.63	66.39
H	11.18	11.32
O	22.19	22.43

6.3 Chromatographic Analysis

6.31 Thin-Layer Chromatography

A number of thin-layer chromatographic systems

on silica gel have been found to induce the
migration of the compound. However, no sys-
tem has been found which gives resolution
equivalent to the gas-liquid chromatographic
system detailed in section 6.32. Thin-layer
chromatography is not the preferred method for
determining the impurities. The following
systems have been studied:

1. Chloroform:Methanol:Glacial Acetic Acid
    (17:2:1)

2. Ethyl Acetate:Formic Acid:Water (5:1:1)

3. Benzene:Methanol:Acetone:Ammonium Hydrox-
    ide (2:2:5:1)

4. Chloroform:Methanol:Ammonium Hydroxide
    (20:15:3)

5. n-Butanol Saturated with 10% Ammonium Hy-
    droxide Solution (Aqueous)

6.32 Gas-Liquid Chromatography

The author has found the following GLC proce-
dure to be suitable to determine the purity
of sodium valproate and valproic acid. Pre-
paratory to chromatography, the sodium val-
proate was acidified with a strong aqueous
hydrochloric acid solution, and the valproic
acid which is practically insoluble in water
was separated. The separated free acid was
then analyzed.

The following is the typical chromatographic
condition for the GLC determination of val-
proic acid:

Column: 10% DEGS-PS on Supelcoport 80/100
         mesh, Two 6 ft x 1/4 in o.d. stain-
         less steel.

Detection: Thermal Conductivity Detector.

Temperature: Inj. Port   225°C
              Column   160°C
              Detector   300°C

Flow Rate: ~20 ml/min helium

```
                Attenuation:  2 x 4

                Slope Sensitivity:  0.03 or adjust for proper
                                    sensitivity

                Sample Size:  2 µl

                Impurities as low as 0.01% could be detected
                using the above conditions.
```

6.4 Titrimetry

Sodium valproate exhibits basic properties. It can
be titrated with 0.1 N hydrochloric acid.

In addition, sodium valproate can be potentiometri-
cally titrated with standardized 0.1 N perchloric
acid using a modified glass-calomel electrode sys-
tem, in which 0.1 N lithium perchlorate in acetic
acid has been substituted for potassium chloride,
and employing glacial acetic acid as the sample sol-
vent.

Valproic acid can be potentiometrically titrated
with standardized 0.1 N tetra-n-butylammonium hy-
droxide in chlorobenzene using a modified glass-
calomel electrode system, in which 1.0 M aqueous
tetra-n-butylammonium chloride has been substituted
for potassium chloride, and employing acetone as the
sample solvent.

7. Determination of Valproic Acid and Its Metabolites in Biological Fluids

Many gas-liquid chromatographic methods for determination
of valproic acid in biological fluids have been reported.

Early methods required derivatization of valproic acid
(23, 24, 25, 26).

Although Meijer and Hessing-Brand in 1973 (27) developed
a micro diffusion method without derivatization, it re-
quired special equipment.

Recently, most of the methods which have been used for
the analysis of valproic acid in plasma, serum, cerebral
spinal fluid, saliva, breast milk, and urine involve
acidification of the biological sample, extraction into
an organic solvent, and direct injection onto a gas-
liquid chromatographic column (28, 29, 16, 30, 31, 32,

33, 34, 35, 36, 37, 38, 39).

Most of the methods employ an internal standard, and all
are reported as accurate and reproducible. However,
Schmidt, et. al. (40) recently reported great interlabo-
ratory variation in the analysis of such biological sam-
ples.

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