levels. However, the reduced plasma dicumarol level may also be partly due to reduced absorption. Considering the low dicumarol solubility, it is possible that a more complex mechanism consisting of simultaneous increased metabolism and reduced absorption may be involved in this interaction. Aggeler and O'Reilly (19) noted that heptabarbital appears to induce dicumarol metabolism while simultaneously reducing anticoagulant absorption.

Barbiturates interact with many drugs. This effect is usually attributed to the acknowledged ability of these compounds to induce the metabolism of other drugs. Less importance, however, has been given to another possible mechanism by which barbiturate administration may alter drug disposition, *e.g.*, an increase in bile flow with a simultaneous decrease in bile salt concentration. In this work, the influence of these changes was not directly studied. However, the evidence suggests that these mechanisms could be involved in the interactions between phenobarbital and poorly soluble drugs.

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ACKNOWLEDGMENTS

Presented in part at the Research Symposium and Research Conference of the Association of Faculties of Pharmacy of Canada, Ottawa, Canada, May 20, 1974, and at the Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, San Francisco meeting, April 1975.

Supported by M.R.C. Grant MA-5358.

The analytical support of Mr. W. Campbell, Jr., of Hewlett-Packard, Ltd., is gratefully acknowledged.

Micro and Macro GLC Determination of Ethambutol in Biological Fluids

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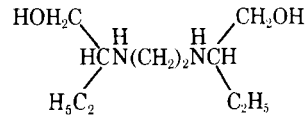
Received December 13, 1976, from the *Department of Pharmacy, School of Pharmacy, University of California, San Francisco, CA 94143.* Accepted for publication July 5, 1977. *Present address: College of Pharmacy, University of Florida, Gainesville, FL 32610.

Abstract □ Previously available GLC methods for ethambutol may only be used to measure quantities of drugs much greater than those found in biological fluids such as plasma and dialysate. A previously published GLC method for plasma samples is extended to measure ethambutol in dialysate. A second GLC method, involving derivatization with bis(trimethylsilyl)trifluoroacetamide and subsequent quantitation using a flame-ionization detector, is described for urine samples. With a dual-column and dual-detector gas–liquid chromatograph, simultaneous micro (plasma and dialysate) and macro (urine) determinations of ethambutol are possible.

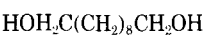
Keyphrases □ Ethambutol—GLC analysis in biological fluids □ GLC—*analysis*, ethambutol in biological fluids □ Tuberculostatic antibacterials—ethambutol, GLC analysis in biological fluids

A previous publication (1) indicated that the available GLC methods may only be used to measure quantities of ethambutol[1] (I) much greater than those found in patient samples of blood, plasma, and dialysate. Pharmacokinetic study of I requires a sensitive and specific method of measuring the unchanged compound.

In this work, the GLC method previously used for plasma is extended to the measurement of I in dialysate. Ethambutol is largely excreted unchanged into the urine (2). To avoid the tedious and possibly erroneous serial dilution of the urine necessary for the GLC assay using an electron-capture detector, another GLC method for I, involving derivatization with bis(trimethylsilyl)trifluoroacetamide and subsequent quantitation using a flame-ionization detector, is described. Decanediol[2] (II) and *d*-2,2′-(ethylenediimino)-di-1-propanol[3] (III) are used as internal standards for the urine and the dialysate assay,



I



II

[1] Myambutol, Lederle Laboratories, Pearl River, N.Y.

[2] Aldrich Chemical Co., Milwaukee, Wis.

[3] Provided by Dr. Raymond Wilkinson, Lederle Laboratories, Pearl River, N.Y.

Table I—Recovery of Ethambutol (I) from Urine Using Flame-Ionization Detection Method (n = 7)

I Added, μg	I Found[a], μg	Recovery[a], %
10	9.9 ± 0.79	99.0 ± 7.9
20	19.7 ± 1.50	98.5 ± 7.5
30	29.8 ± 1.64	99.3 ± 5.5
50	49.8 ± 2.16	99.7 ± 4.3
70	69.9 ± 2.83	99.9 ± 4.0
100	100.3 ± 3.60	100.3 ± 3.6
10–100[b]	—	99.4 ± 5.5

[a] Expressed as mean ± *SD*. [b] n = 42.

respectively. With 3% OV-17, the trifluoroacyl and trimethylsilyl derivatives can be chromatographed under the same temperature conditions. Thus, with a dual-column and dual-detector gas–liquid chromatograph, the simultaneous micro (plasma and dialysate) and macro (urine) determinations of I become possible.

EXPERIMENTAL

Reagents—Bis(trimethylsilyl)trifluoroacetamide[4], trifluoroacetic anhydride[5], potassium hydroxide[6], and chloroform[6] were analytical reagent grade. Pyridine[5], benzene[6], and methylene chloride[6] were nanograde quality. Spectroquality chloroform[7] was used as a solvent in the derivatization of ethambutol by bis(trimethylsilyl)trifluoroacetamide.

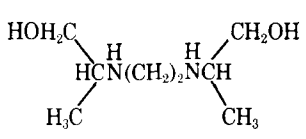
Instrumentation—GLC analyses were carried out on a dual-column instrument[8] equipped with a flame-ionization detector and a scandium tritide electron-capture detector[9]. The columns were coiled, 1.8 m × 3 mm i.d., silanized glass, packed with 3% OV-17 on 100–120-mesh Chrom W A/W DMCS[10] (leading to the flame-ionization detector) and with 3% OV-17 on 100–120-mesh Gas Chrom Q[11] (leading to the electron-capture detector).

Nitrogen was used as the carrier gas at flow rates of 66.6 and 20 ml/min, measured at the flame-ionization detector base and the electron-capture detector base, respectively. The air flow rate was 300 ml/min, and the hydrogen flow rate was 27 ml/min. The temperature settings were: oven, 157°; injection ports, 210°; and detectors, 230°.

Mass Spectrometry—Chemical-ionization mass spectra were obtained using a quadrupole mass spectrometer[12]. Source pressures were maintained at 0.5 torr (isobutane). The source temperature was 150–170°. Samples (1 ± 0.5 μg) were placed into a glass capillary by a direct insertion probe, which was then gradually heated above 200° to effect volatilization.

Preparation of Standard Solutions—Ten milligrams of the hydrochloride salt of I was dissolved in distilled water to yield a stock solution of 1 mg/ml. Working standard solutions of 2, 4, 6, 10, 14, and 20 μg of I/ml were prepared and used to establish a GLC calibration curve and for addition to dialysate and urine for the determination of percent recovery. A stock solution of III containing 1 mg of its hydrochloride salt/ml was diluted 10-fold to give the working standard solution. Because of the sparing solubility of II in water, 1 mg of II was first dissolved in 250 μl of 1-propanol and then diluted with distilled water to a concentration of 100 μg/ml.

Measurement of Ethambutol in Biological Samples—Appropriate aliquots of urine, to which 10 μg of II had been added, were extracted with 8 ml of chloroform for 10 min under alkaline conditions. Portions of the chloroform extract were transferred to another tube and evaporated to



III

[4] Regis, Chicago, Ill.
[5] Pierce, Rockford, Ill.
[6] Mallinckrodt, St. Louis, Mo.
[7] MCB, Norwood, Ohio.
[8] Varian Aerograph model 2700.
[9] Tritium content: 1 Ci adsorbed on scandium.
[10] Varian Aerograph, Walnut Creek, Calif.
[11] Applied Science Laboratories, State College, Pa.
[12] Model 3200, Finnigan Corp., Sunnyvale, Calif.

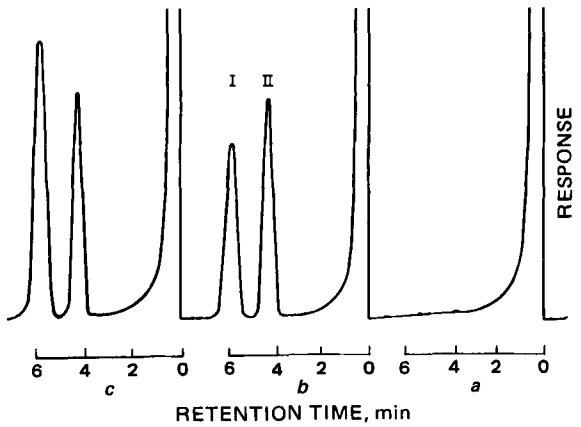


Figure 1—*Gas chromatograms of control human urine* (a), *control to which I (50 μg) and II (10 μg) were added* (b), *and human urine obtained 1 hr after dosing* (c).

dryness under nitrogen. Residues were dissolved in 0.1 ml of spectroquality chloroform. Derivatization was initiated by adding 10 μl of bis-(trimethylsilyl)trifluoroacetamide and was complete in 30 min.

For the dialysate assay, the same extraction procedures were followed as previously described, except that 2 ml of dialysate and 12.5 μg of III were used. Derivatization was initiated by adding 20 μl of trifluoroacetic anhydride and was completed in 2 hr. Aliquots of the reaction mixtures were injected into the gas chromatograph.

Standard urine and dialysate curves were constructed by plotting the peak height ratios (I to II and I to III) *versus* weight content of I. The peak height ratio of an unknown sample was then used to determine the amount of I present. The standard samples were run concurrently with the unknown samples as previously described.

Biological Studies—Six healthy adult volunteers received I intravenously and orally (tablet and solution) at a dose of 15 mg/kg within 6 months. Urine specimens were collected at the following intervals: 0–1, 1–2, 2–3, 3–4, 4–6, 6–8, 8–12, 12–24, 24–48, and 48–72 hr after drug administration.

One hemodialysis patient, regularly on 400 mg of I, was studied. Dialysates were collected at hourly intervals over a 4-hr dialysis period.

RESULTS AND DISCUSSION

Figure 1 presents gas chromatograms of the following samples using the flame-ionization detector: control human urine, control to which I and II were added, and human urine obtained 1 hr after dosing. Figure 2 presents gas chromatograms (electron-capture detection) of control dialysate, I and III added to control dialysate, and dialysate collected 1 hr after initiation of hemodialysis.

The reversed-time axis in Fig. 1 reflects the use of a dual-pen recorder where urine and dialysate samples were measured simultaneously. As indicated in Figs. 1*a* and 2*a*, no interfering peaks were found in the control urine or dialysate specimens. The trimethylsilyl derivative of I exhibited a retention time of 6 min, and the corresponding internal standard had a retention time of 4 min. The trifluoroacyl derivative of I exhibited a retention time of 4 min, and the corresponding internal standard had a retention time of 2.5 min.

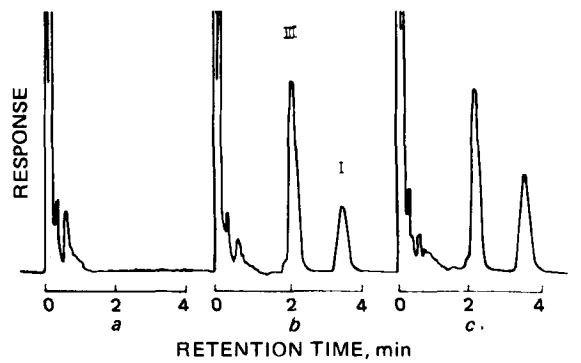


Figure 2—*Gas chromatograms of control dialysate* (a), *control to which I (0.3 μg) and III (12.5 μg) were added* (b), *and dialysate collected 1 hr after initiation of hemodialysis* (c).

Table II—Recovery of Ethambutol (I) from Dialysate Using Electron-Capture Detection Method (n = 6)

I Added, ng	I Found, ng	Recovery[a], %
100	102.2 ± 12.3	102.2 ± 12.3
200	200.3 ± 18.9	100.2 ± 9.5
300	297.1 ± 16.5	99.0 ± 5.5
500	491.6 ± 22.9	98.3 ± 4.6
700	692.8 ± 33.0	99.0 ± 4.7
1000	991.6 ± 37.6	99.2 ± 3.8
100–1000[b]	—	99.7 ± 6.7

[a] Expressed as mean ± *SD*. [b] n = 36.

Table III—Removal Rate of Ethambutol from a Hemodialysis Patient during a 4-hr Period

Hours	Dialysate Volume, liters	Concentration, μg/ml	Removal Rate, mg/hr
1	30.2	0.25	7.55
2	30.6	0.25	7.51
3	29.6	0.21	6.08
4	31.0	0.17	5.28

The results obtained following analysis of various amounts of I in control human urine are summarized in Table I. In the 10–100-μg range, the mean recovery of I from control urine was 99.4 ± 5.5% (n = 42). An excretion rate *versus* time plot in one subject following intravenous and oral dosing of ethambutol is shown in Fig. 3. Seventy-two-hour urinary excretion of unchanged drug in the six subjects accounted for 79.2 ± 3.3 (mean percent of dose ± *SD*), 63.4 ± 2.6, and 61.1 ± 3.8 for the intravenous, oral solution, and oral tablet doses, respectively. This result indicates that the availability of ethambutol from oral solution and tablet forms averaged 80 and 77%, respectively. The availability of the drug from the tablet was not significantly different from that for the solution.

Table II summarizes the results obtained following electron-capture analysis of various amount of I (10–1000 ng) added to dialysate. The mean recovery of I from control dialysate in the 10–1000-ng range was 99.7 ± 6.7% (n = 36). Removal of I by hemodialysis in one patient during 4 hr is seen in Table III.

When comparing the flame-ionization response and the electron-capture response (Figs. 1 and 2), an approximate 100-fold difference in detector sensitivity is evident. The electron-capture method of detection is required for samples such as blood, plasma, and, in particular, dialysate in which low levels of I are anticipated.

Drug removal by dialysis is sometimes hard to determine because of the large dialysate volume collected from the patient during the dialytic process (20–30-liters/hr interval). Concentration procedures (*e.g.*, rotary evaporation or extraction of the large dialysate volume with an organic solvent and subsequent evaporation of this solvent) are almost always required to bring the dialysate sample within the assay detection limits. With the highly sensitive electron-capture GLC method described here, only 2 ml of dialysate is needed for extraction.

The flame-ionization method is suitable for urine samples or for dosage form analysis in which high levels of I are anticipated. The flame-ionization method avoids the tedious and possibly erroneous dilution of the samples necessary for the electron-capture GLC assay.

Decanediol (II) was chosen as the internal standard for the urine assay because of its commercial availability at low cost. Since high concentrations of drug are measured in this assay, relatively large amounts of internal standard are required. However, II is not a good internal standard in the dialysate assay when trifluoroacetic anhydride is used as the derivatizing agent because of peak overlap.

Richard *et al.* (3) pointed out that only trimethylsilylimidazole derivatized I immediately and completely and that other trimethylsilylating agents required mild heating at 50° to complete the derivatization. In the present study, bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane satisfactorily derivatized I at room temperature, and less solvent interference was obtained than when trimethylsilylimidazole was used. The use of an internal standard presents an improvement over the other published techniques (3, 4).

The isobutane chemical-ionization mass spectra of I, the trimethylsilyl derivative of I, and the trifluoroacyl derivative of I can be seen in Figs. 4, 5, and 6, respectively. The major molecular ion at m/e 349 in Fig. 5 corresponds to the ditrimethylsilylated derivative of I. No peaks were

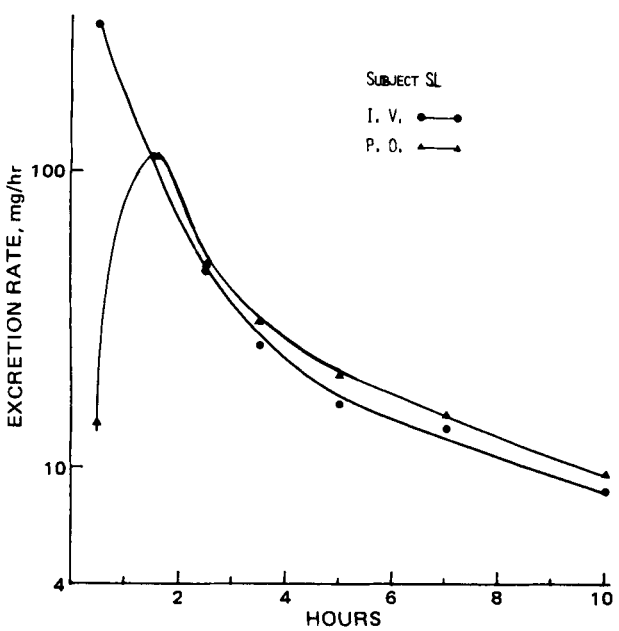


Figure 3—*Excretion rate* versus *time plot following intravenous and oral administrations of ethambutol in one subject.*

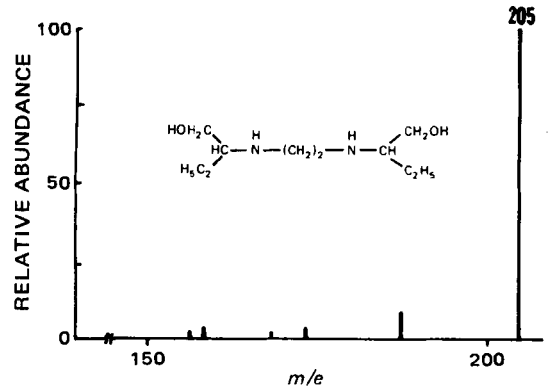


Figure 4—*Chemical-ionization mass spectrum of ethambutol using isobutane as the reagent gas.*

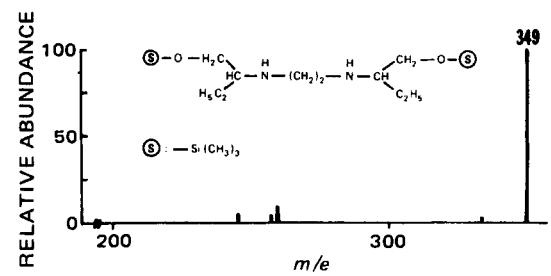


Figure 5—*Chemical-ionization mass spectrum of trimethylsilylated ethambutol using isobutane as the reagent gas.*

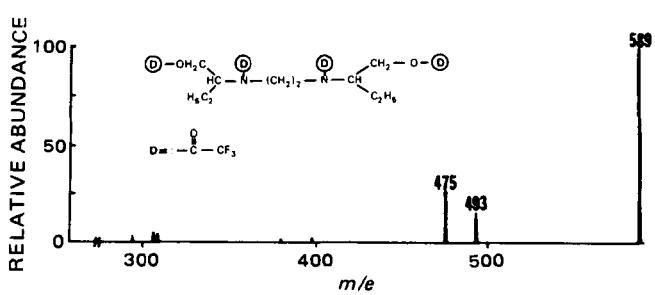


Figure 6—*Chemical-ionization mass spectrum of trifluoroacylated ethambutol using isobutane as the reagent gas.*

found beyond *m/e* 349, indicating that trimethylsilylation occurred at only two of the four possible reacting sites (*i.e.*, the two hydroxyl groups and the two secondary amines in I). The major molecular ions at *m/e* 589 and 493 in Fig. 6 correspond to the tetra- and tritrifluoroacylated derivatives of I, respectively, with relative abundance of approximately 100:15. The peak at *m/e* 475 (M^+) represents a breakdown product of the protonated molecular ion (MH^+) at *m/e* 589.

About 20% of ethambutol is metabolized to the carboxylic acid derivative and excreted in the urine (5). This highly polar compound is very poorly extracted into the organic phase in the procedure described here and, therefore, does not interfere with the assay of unchanged drug.

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ACKNOWLEDGMENTS

Presented at the APhA Academy of Pharmaceutical Sciences, New York meeting, May 1977.

Supported in part by Program Project Grant GM 16496 from the Institute of General Medical Sciences, National Institutes of Health, and by the Patent Fund from the Graduate Division, University of California, San Francisco. During this work, Dr. Lee received support as a National Institutes of Health Predoctoral Fellow through Training Grant GM 00728.

Hofmann Elimination with Diazomethane on Curare Bases and Selected Quaternary Tetrahydroisoquinoline Alkaloids

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Received June 17, 1977, from the *Department of Medicinal Chemistry, College of Pharmacy, University of Minnesota, Minneapolis, MN 55455.* Accepted for publication July 11, 1977.

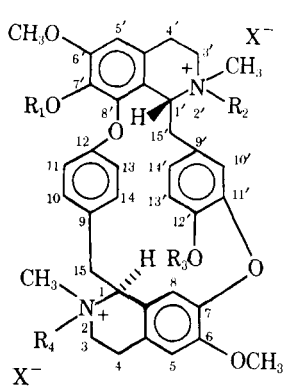
Abstract □ Use of a large excess of alkali-free diazomethane resulted in a Hofmann elimination with selected curare bases and some other quaternary tetrahydroisoquinoline alkaloids. (+)-Tubocurarine chloride provided a monostilbene methine, *O,O*-dimethyltubocurinemethine, and a monostilbene–monostyrene compound, *O,O*-dimethyltubocurinedimethine. The major elimination products of (+)-isotubocurarine chloride and (+)-carnegine methiodide were monostyrene methines, *O,O*-dimethyltubocurineisomethine and carneginemethine, respectively. Treatment of (+)-laudanosine methiodide with potassium hydroxide, under the conditions of Hofmann degradation, or alkali-free diazomethane solution provided the same stilbene compound, laudanosinemethine. The structures of the elimination compounds were further confirmed by catalytic reduction and quaternization with methyl iodide.

Keyphrases □ Curare bases, various—Hofmann elimination reaction with diazomethane □ Tetrahydroisoquinoline alkaloids, various—Hofmann elimination reaction with diazomethane □ Elimination reactions, Hofmann—various curare bases and tetrahydroisoquinoline alkaloids with diazomethane □ Alkaloids, various quaternary—Hofmann elimination reaction with diazomethane

(+)-Tubocurarine chloride (I) has been the traditional standard against which the neuromuscular junction blocking potency of many compounds has been compared. Numerous studies have dealt with the structural requirements for neuromuscular junction blockers (1–3). Methylation of the phenolic groups in I is regarded as one means of enhancing its blocking activity. Hence, the potency of *O,O*-dimethyl-(+)-tubocurarine chloride (II) was reported to be four to nine times that of I (4–6). However, the II utilized in these pharmacological evaluations was obtained through the methylation of I under alkaline conditions using methyl iodide. These conditions would undoubtedly result in the quaternization of the tertiary amino group of I.

Using NMR spectroscopy and electrometric titration, Bick and Mcleod (7) revealed the diquaternary nature of a commercial sample of II. Therefore, the reported activity probably represents that of the diquaternary species, *O,O*-dimethyl-(+)-chondocurarine iodide (V), rather than the monoquaternary–monotertiary II. In fact, following the disclosure that I is actually a monoquaternary–monotertiary compound (8), there has not yet been any report on the actual activity of true II.

The stereochemical requirements for nondepolarizing neuromuscular junction blockers have been studied in these laboratories (9–12). The conclusion that moderately enhanced activity is associated with monoquaternary neuromuscular junction blockers with an *S*-configuration



I: $R_1 = R_3 = R_4 = H$, $R_2 = CH_3$, X = Cl
II: $R_1 = R_2 = R_3 = CH_3$, $R_4 = H$, X = Cl
III: $R_1 = R_2 = R_3 = H$, $R_4 = CH_3$, X = Cl
IV: $R_1 = R_3 = H$, $R_2 = R_4 = CH_3$, X = Cl
V: $R_1 = R_2 = R_3 = R_4 = CH_3$, X = I