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GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC DETERMINATION OF ETHAMBUTOL IN HUMAN PLASMA*

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SUMMARY

A quantitative gas chromatographic—mass spectrometric assay has been developed for the determination of ethambutol (EMB) in human plasma. Plasma samples were taken from a patient after oral administration of EMB (with proven tuberculosis infection). Deuterated EMB and a non-deuterated analogue of EMB were synthesized and used as internal standards in this procedure; both gave excellent agreement in the analysis. The derivatizing agent used was trifluoroacetic anhydride (TFAA) and quantitative derivatization was complete in one hour, forming EMB-$(TFA)_4$. Selective ion monitoring was utilized to monitor the gas chromatographic effluent. Ions were generated by electron impact at 70 eV. The limit of detection was 36 ng EMB per ml plasma. This method is compared with the electron-capture gas chromatographic procedure of Lee and Benet.

INTRODUCTION

Tuberculosis (TB) is a prevalent disease in the U.S.A. with a reported incidence in 1979 of 12.6 per 100,000 population (27,669 newly diagnosed cases in one year) [1]. It is estimated that there are more than 80,000 patients with TB at any one time. Ethambutol (EMB), (+)-2,2'-(ethylenediimino)-di-1-butanol, is an effective drug used in the treatment of TB. It is usually given in combination with other anti-TB drugs for it suppresses the growth of resistant tubercle bacilli which develop after exposure to these drugs. Approximately 75% of the human type strains of *Mycobacterium tuberculosis* are sensitive to 1 μg/ml of EMB and resistance to EMB develops slowly and with difficulty in vitro [2, 3].

A number of chromatographic methods have been developed for the anal-

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ysis of EMB. Richard et al. [4] reported a flame ionization gas chromatographic method. The drug was derivatized forming a trimethylsilyl (TMS) derivative; however, this procedure was found not to be sensitive enough for measurement of EMB in concentrations found in human plasma after therapeutic doses. Lee and Benet [5] developed an electron-capture gas chromatographic (ECGC) method which could be used effectively for measurement of small quantities of EMB in human plasma and urine. The derivatizing agent used was trifluoroacetic anhydride (TFAA) and the internal standard for quantitation was (+)-2,2'-(ethylenediimino)-di-1-propanol (MEMB). Blair et al. [6] developed a chemical ionization gas chromatographic—mass spectrometric (CI-GC—MS) method using deuterated EMB as internal standard; derivatization with TMS was carried out by the procedure of Richard et al. [4].

In this paper is described the development of a new GC—MS procedure for the analysis of EMB in human plasma. Electron impact (EI) ionization was used at 70 eV and the derivatizing agent was TFAA. [$^2H_4$]EMB and MEMB were synthesized and used as internal standards in this procedure. Methane CI was also studied to see if greater sensitivity could be obtained over EI ionization. A comparison of sensitivities and reproducibilities is made between this GC—MS procedure and the ECGC method of Lee and Benet [5].

EXPERIMENTAL

Materials

Nanograde chloroform and pyridine were obtained from Mallinckrodt (St. Louis, MO, U.S.A.). Trifluoroacetic anhydride was obtained from Pierce (Rockford, IL, U.S.A.). Ethambutol was obtained from Lederle Labs. (Pearl River, NY, U.S.A.). (+)-2-Amino-1-butanol, (+)-2-amino-1-propanol and 1,2-dibromoethane were obtained from Aldrich (Milwaukee, WI, U.S.A.). 1,2-Dibromo-[$^2H_4$]ethane was obtained from Merck & Co. (Rahway, NJ, U.S.A.).

Synthesis of internal standards

The procedure of Blair et al. [6] was not found to be workable in our laboratory for the synthesis of [$^2H_4$]EMB. Therefore, the original procedure of Wilkinson et al. [7] was used for the synthesis of [$^2H_4$]EMB and MEMB with slight modification. (+)-2,2'-(Ethylene-[$^2H_4$]diimino)-di-1-butanol was synthesized by heating a stirred solution of 4.5 g (0.050 mole) of (+)-2-amino-1-butanol with 0.95 g (0.005 mole) of 1,2-dibromo-[$^2H_4$]ethane under reflux for 25 min at 100—115°C. The reaction mixture was allowed to cool (30—40°C) before the addition of 0.65 g potassium hydroxide in 5.0 ml hot *n*-propanol (which precipitated potassium bromide). After cooling in a dry ice bath, the reaction mixture was filtered to remove potassium bromide. The filtrate was concentrated (under reduced pressure), and the residue, an orange oil, was dissolved in 5.0 ml of acetone—*n*-propanol (1:1, v/v), and cooled again (in a dry ice bath) and filtered to effectively remove all potassium bromide. The filtrate was diluted with 4.0 ml of 7.8 *N* ethanolic hydrochloric acid. The solution was concentrated under reduced pressure and the residue (a red gum) was dissolved in a minimum amount of cold absolute ethanol.

If precipitation of white crystals did not occur, then cold acetone was added to affect precipitation. The mixture was cooled in a dry ice bath for 30 min before filtration and the solid residue was washed with cold acetone. The material was recystallized twice from hot ethanol or benzene which yielded 0.56 g of white crystals (45% yield) of m.p. 189—190°C.

MEMB was synthesized by the procedure described above: a mixture of 3.75 g of (+)-2-amino-1-propanol (0.050 mole) and 0.95 g (0.005 mole) of 1,2-dibromoethane was heated under reflux for 1 h. The product, 70 mg (8% yield) of m.p. 170—171°C was isolated as above.

Instrumentation

MS was performed with a Finnigan 4000 GC—MS quadrupole mass analyzer spectrometer with a Model 6000 automated data system. GC was accomplished on a 1.8 m × 2 mm I.D. glass column of 3% OV-17 on Gas-Chrom Q (100—200 mesh). The column temperature was maintained at 160°C (helium flow-rate of 20 ml/min), injection port at 190°C, jet separator at 200°C and the ion source at 250°C. The ionization potential was 70 eV. When MEMB was used as the internal standard for quantitation of EMB, the ions monitored were *m/e* 280 (MEMB) and *m/e* 307 (EMB). For quantitation of EMB with [$^2H_4$]EMB as internal standard, the ions monitored were *m/e* 307 (EMB) and *m/e* 310 ([$^2H_4$]EMB). In experiments using CI, methane was used as the reagent gas.

ECGC was performed on a Varian 3700 gas chromatograph with ^{63}Ni detector with a Varian CDS-111 microprocessor. Samples were analyzed on a 1.8 m × 2 mm I.D. glass column with 3% SE-30 on Gas Chrom Q (100—120 mesh). The column temperature was maintained at 170°C (nitrogen flow-rate of 30 ml/min), injection port at 190°C and detector temperature at 250°C. A 10% retention index window was imposed on EMB and MEMB. Only MEMB could be used as the internal standard for this procedure.

Sample collection and preparation

Informed consent was obtained from a patient who exhibited proven infection with tubercle bacilli but in otherwise good clinical status. The patient was not taking other medications at the time of the study. A heparin lock was placed in a superficial arm vein for a 24-h sampling period. EMB at a dose of 800 mg (as tablets and 300 mg isoniazid) was given daily and 3 ml of blood were withdrawn at 0, 1, 2, 4, 6, 12, 24 h after dosing. Plasma was obtained by centrifugation for 10 min at 500 *g*. The extraction procedure was similar to that of Lee and Benet [5]. Plasma (200 μl) was diluted with 800 μl of water containing 3.0 μg of MEMB for ECGC analysis or 3.0 μg [$^2H_4$]EMB or MEMB for GC—MS analysis. After addition of 10 ml chloroform and 1 ml of 4 *N* sodium hydroxide, the solution was shaken for 10 min and centrifuged for 10 min at 500 *g*. The organic layer (8 ml) was transferred into a clean conical centrifuge tube (15 ml) and evaporated under a stream of air at room temperature. The residue was dissolved in 100 μl of benzene—pyridine (7:1, v/v) and 25 μl of TFAA were added. Following derivatization at 4°C for 1 h, the reaction mixture was washed with 400 μl of 0.1 *N* hydrochloric acid to remove excess TFAA (shaking for 2 min and centrifuga-

tion for 10 min at 500 *g*). The sample tubes were kept refrigerated until analyzed. All samples were analyzed (by ECGC or GC—MS) within 4 h after completion of derivatization.

RESULTS AND DISCUSSION

The EI mass spectra of EMB, MEMB, and [$^2H_4$]EMB are presented in Figs. 1—3, respectively. The ions monitored at 70 eV were *m/e* 280 (MEMB), *m/e* 307 (EMB), and *m/e* 310 ([$^2H_4$]EMB). The M^+ ions were not observable by EI. The relative abundances of *m/e* 280, 307, and 310 were 100%, 35.3%, and 29.8%, respectively. These compounds did not have any interfering contribution to the above selected ions from the presence of the other two compounds. The signal intensities of the above mentioned ions were 50% less at 30 eV than at 70 eV.

To test if greater sensitivity could be achieved, spectra of pure standards of each compound were obtained using CI with methane as reagent gas. The relative abundances of the $(MH)^+$ ions of MEMB (*m/e* 561), EMB (*m/e* 589), and [$^2H_4$]EMB (*m/e* 593) were 0.12%, 1.02%, and 2.87%, respectively. The relative abundances of ions *m/e* 280, 307, and 310 were 80.0%, 48.6%, and 37.5%, respectively. It was found that the CI signal intensities at 70 eV of these ions (*m/e* 280, 307, and 310) were three times less than the EI signal intensities at 70 eV. Since there were no other characteristic ions of sufficient

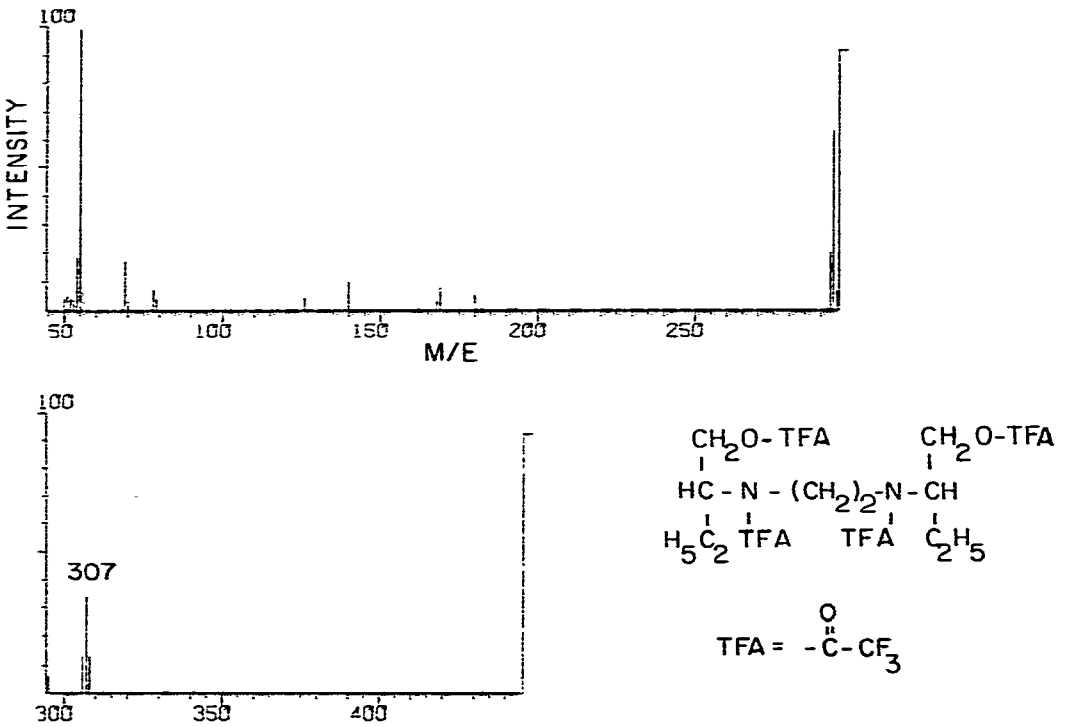

Fig. 1. EI mass spectrum of EMB-(TFA)$_4$.

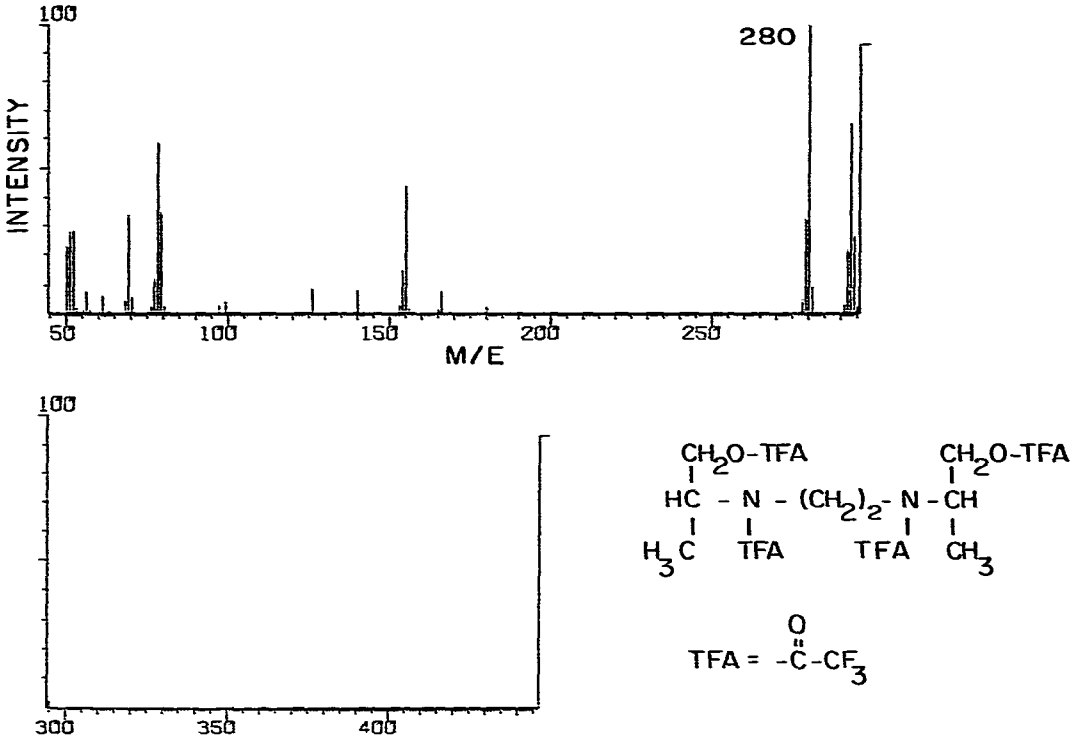

Fig. 2. EI mass spectrum of MEMB-(TFA)$_4$.

intensity generated by Cl that could be employed for quantitative analysis, EI at 70 eV was used for measurement.

The minimum detectable amount (with a 2:1 signal-to-noise ratio) of pure EMB was 1.5 ng (*m/e* 307) by GC—MS. The high sensitivity of the present method enables quantitative analysis (2:1 signal-to-noise ratio) with a detection limit of 10 ng EMB per ml plasma.

Calibration curves were constructed in which known amounts of EMB (0—10 μg) were added to water or plasma containing a fixed amount of [$^2H_4$]-EMB (3 μg) or MEMB (3 μg) and carried through the extraction and derivatization procedure. Samples containing EMB—[$^2H_4$]EMB or EMB—MEMB were analyzed by GC—MS while those containing only EMB—MEMB were run by ECGC. The calibration curves were constructed by a least-squares fit of area ratio versus weight ratio of EMB to internal standard used and the slopes, intercepts and correlation coefficients are given in Table I. As can be seen by the data in Table I, excellent agreement is found in the standard curves regardless of the method chosen or internal standard used.

In Fig. 4 is presented a mass fragmentogram of EMB (with [$^2H_4$]EMB as internal standard) extracted from human plasma following oral dosing. The chromatographic retention time of 0.80 min of the isolated EMB was identical to that of its deuterated analogue; MEMB had a chromatographic retention time of 0.70 min (3% OV-17 column). In Fig. 5 is presented an

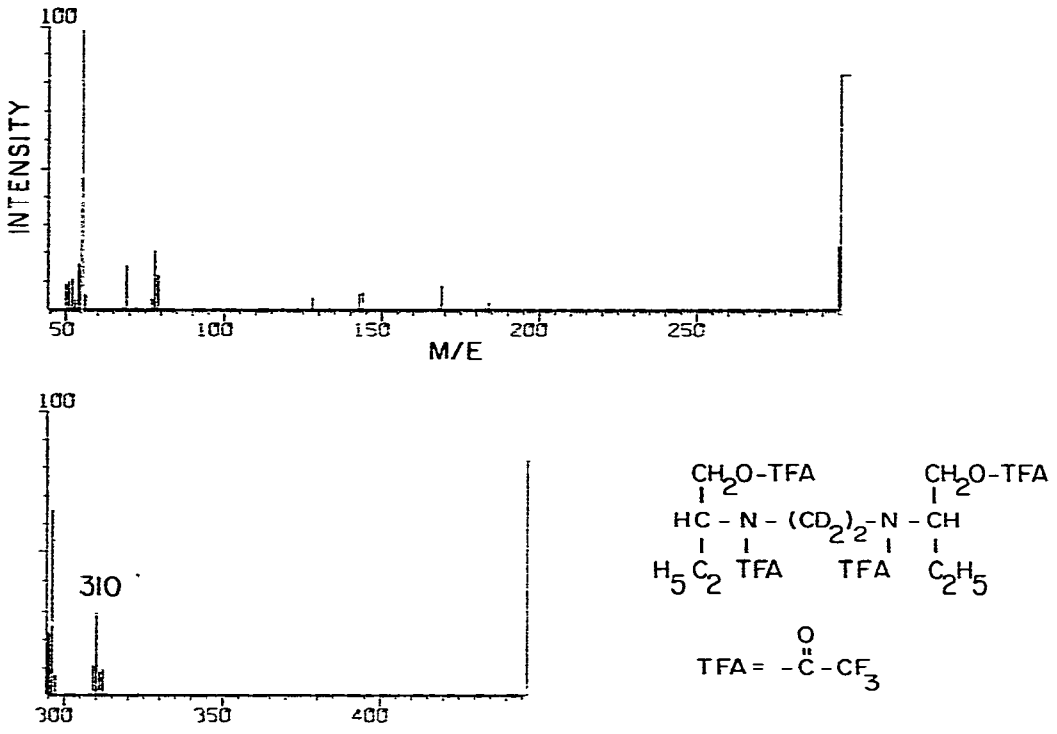

Fig. 3. EI mass spectrum of [$^2H_4$]EMB-(TFA)$_4$.

TABLE I

STANDARD CURVE DATA FOR ETHAMBUTOL BY GC—MS AND ECGS

Internal standard	GC—MS			ECGC		
	Slope	Intercept with *y*-axis	Corr. coeff.	Slope	Intercept with *y*-axis	Corr. coeff.
MEMB	1.140*	—0.009	0.999	1.140**	—0.002	0.999
[$^2H_4$]EMB	1.145***	—0.005	0.999	—	—	—

*Area ratio *m/e* 307/280.
**Area ratio EMB/MEMB.
***Area ratio *m/e* 307/310.

ECGC chromatogram of EMB and MEMB; retention times for the two compounds were 2.73 and 4.31 min, respectively (3% SE-30 column). The total time required for ECGC analysis is 7 min whereas the run time required for GC—MS is 2.5 min. The GC—MS selective ion monitoring is relatively free of interfering peaks.

In Table II are given the levels of EMB after an oral dose of 800 mg EMB

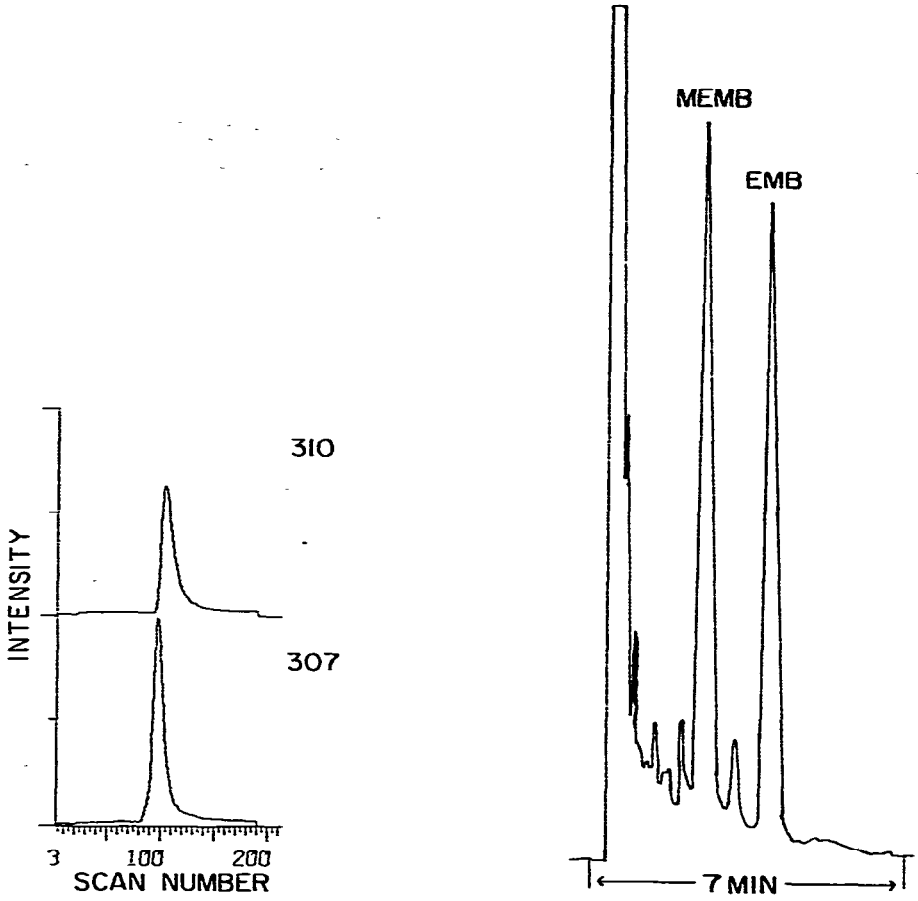

Fig. 4. Selected ion monitoring of EMB (*m/e* 307) with [$^2H_4$]EMB (*m/e* 310) as internal standard from plasma sample.

Fig. 5. ECGC chromatogram of EMB with MEMB as internal standard.

TABLE II

LEVELS OF ETHAMBUTOL IN HUMAN PLASMA DETERMINED BY THE GC–MS AND ECGC PROCEDURES

Levels expressed as μg EMB per ml plasma (average of three determinations) ± standard deviations. Either [$^2H_4$]EMB or MEMB was used as internal standard.

Time* (h)	GC–MS		ECGC
	[$^2H_4$]EMB	MEMB	MEMB
0	0.30 ± 0.10	0.33 ± 0.07	0.31 ± 0.11
1	2.66 ± 0.27	2.58 ± 0.20	2.78 ± 0.29
2	3.36 ± 0.39	3.66 ± 0.44	3.71 ± 0.40
4	1.68 ± 0.30	1.71 ± 0.29	1.81 ± 0.35
6	1.15 ± 0.31	1.17 ± 0.38	1.19 ± 0.28
12	0.80 ± 0.20	0.80 ± 0.19	0.86 ± 0.17
24	0.48 ± 0.09	0.42 ± 0.07	0 52 ± 0.12

*Time after an oral dose of 800 mg EMB and 300 mg isoniazid.

(in the presence of a 300-mg dose of isoniazid). The concentration (μg/ml) in plasma of EMB was determined by GC—MS with either [$^2H_4$]EMB or MEMB as internal standards. These data are compared with values obtained by ECGC using MEMB as internal standard. As can be seen by the data, good agreement was obtained by either method regardless of the internal standard used for quantitation for GC—MS.

CONCLUSION

The GC—MS procedure presented in this paper is useful for the determination of EMB in human plasma. EI at 70 eV was used and the derivatizing agent was TFAA. The total time required per analysis was 2.5 min for GC—MS. [$^2H_4$]EMB and MEMB were used as internal standards for GC—MS and gave comparable results. This GC—MS procedure was compared with the ECGC method of Lee and Benet [5] for measuring EMB in plasma following oral dosing with the drug and both methods gave similar results. The GC—MS procedure was more rapid and specific than the ECGC procedure used.

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