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Note

Gas–liquid chromatographic determination of ethambutol in plasma and urine of man and monkey

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Ethambutol (EMB) is a valuable and effective drug used in the treatment of tuberculosis. In previous plasma and urine assays for EMB microbiological[1], chemical[2], and radiological[3] methods have been utilized. Although several gas chromatographic (GC) procedures have been previously reported[4,5], these methods may only be used to measure quantities of drug much greater than those found in biological samples. The pharmacokinetic study of EMB requires a sensitive and specific method of measuring the unchanged compound in plasma and urine. A gas–liquid chromatographic (GLC) method has been developed for EMB involving derivatization with trifluoroacetic anhydride (TFAA) and subsequent quantitation using an electron capture detector. Because of a close similarity to EMB, dextro-2,2'-(ethylenediimino)-di-1-propanol (MEMB) was chosen as internal standard.

EXPERIMENTAL

Materials

EMB (Myambutol®) and MEMB were graciously supplied by Lederle Labs. (Pearl River, N.Y. 10962, U.S.A.); potassium hydroxide, chloroform (A.R.), benzene and methylene chloride (nanograde) by Mallinckrodt (St. Louis, Mo., U.S.A.); pyridine and TFAA (sequanal grade) by Pierce (Rockford, Ill., U.S.A.).

Procedure

Samples of 0.2 ml of plasma or urine, to which 5 μg of internal standard had been added, were extracted with 8 ml of chloroform for 10 min under alkaline conditions. Portions of the chloroform were transferred to another tube and evaporated to dryness under nitrogen. Methylene chloride (0.5 ml) was added and evaporated to dryness to ensure the azeotropic removal of water. Residues were dissolved in 1 ml of benzene and made alkaline with diluted pyridine (1:4 in benzene). Derivatization was initiated by adding TFAA and was complete in 2 h. Excessive derivatizing agent was washed into the aqueous phase using 0.01 *M* HCl. An appropriate aliquot of the benzene layer was injected into the gas chromatograph. A Varian-Aerograph Model 2700 equipped with a scandium tritide electron capture detector was used under the following conditions: glass column, 6 ft. × 1/8 in., 3% OV-17 on Gas-Chrom Q,

100–120 mesh; carrier gas (nitrogen) flow-rate, 20 ml/min; injector temperature, 210°; oven temperature, 157°; detector temperature, 230°.

RESULTS AND DISCUSSION

Under the chromatographic conditions described above, MEMB and EMB have retention times of 2.5 and 4 min, respectively (Fig. 1). A typical calibration curve prepared by extracting human plasma samples containing different concentrations of EMB and 5 μg of MEMB is shown in Fig. 2. The ECD response is linear between 0.1 and 2 μg, but the calibration curve was constructed between 0.1 and 1 μg, where therapeutic concentrations can be conveniently interpolated. Table I contains peak height ratios obtained from calibration curves prepared in human plasma, human

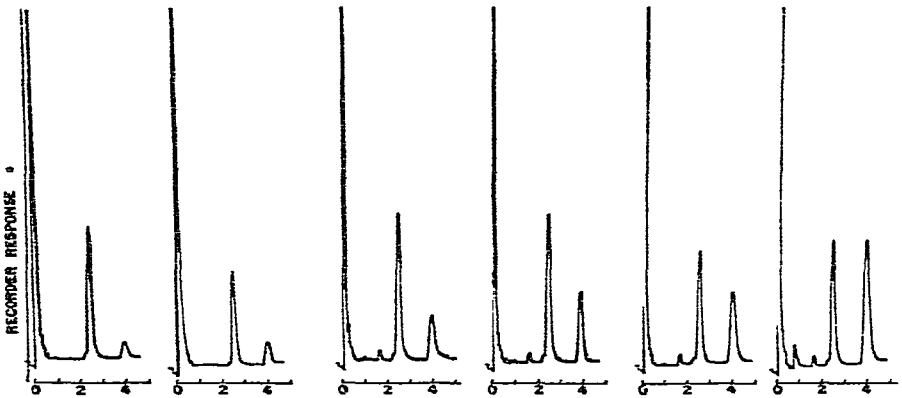

Fig. 1. Typical chromatogram of EMB (0.1 ~ 1.0 μg) and MEMB (5 μg) derivatives. Retention time, 2.5 min for MEMB and 4 min for EMB.

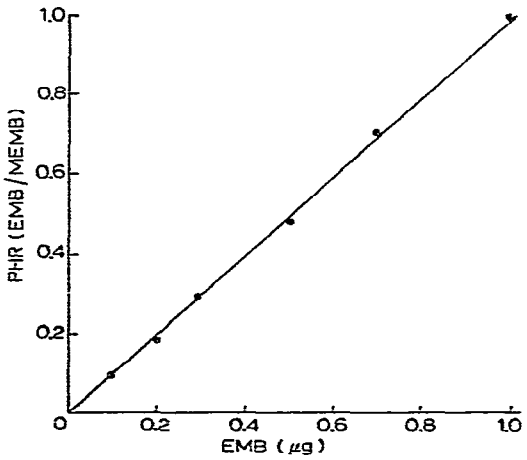

Fig. 2. Calibration curve relating the peak height ratios (PHR) to the amount of EMB present in the human plasma samples.

TABLE I

PEAK HEIGHT RATIOS FOR THE FOUR SETS OF CALIBRATION CURVES

Each number represents the average of three samples per concentration.

EMB (μg)	*Peak height ratio (PHR)*			
	Human plasma	*Human urine*	*Monkey plasma*	*Monkey urine*
0.1	0.10	0.10	0.11	0.09
0.2	0.21	0.18	0.21	0.19
0.3	0.30	0.30	0.28	0.28
0.5	0.51	0.47	0.47	0.48
0.7	0.67	0.67	0.69	0.70
1.0	0.97	0.89	0.90	0.89

urine, monkey plasma, and monkey urine. Each value represents the average of three samples at each concentration.

Arguments have been raised regarding the advantage of using peak area over peak height[6,7]. A theoretical discussion on this was presented by Janik[8]. For comparison purposes, the peak heights were measured using a Type A-25 Varian recorder and the peak areas integrated using a 3370B Hewlett-Packard integrator. Fig. 3 contrasts the calibration curves prepared from peak height ratio and peak area ratio (assay done on Varian 1400, oven, 168°, other conditions the same). Linear regression shows that both curves are equally good with $r^2 = 0.96$ for the former and $r^2 = 0.95$ for the latter. There is no essential difference between choosing peak height or peak area as a measure of the detector response.

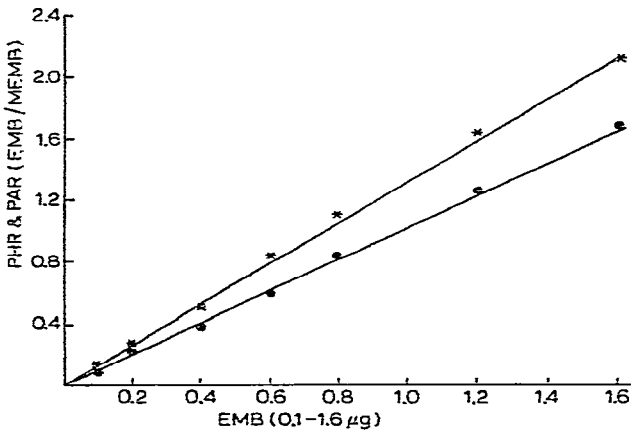

Fig. 3. Calibration curves contrasting the use of peak height ratio (PHR) and peak area ratio (PAR) as a measure of detector response. ●, PHR; *, PAR.

Several column packings were tested and 3% OV-25 on Gas-Chrom Q was found useful. However, since OV-17 is a universal liquid phase which has multiple utility, our assays were carried out exclusively on OV-17.

Heptafluorobutyric anhydride was also found useful as derivatizing agent for EMB, but minor peaks eluted with a longer retention time tend to slow down the assay

process. TFAA does not give these minor peaks and should be considered as first choice.

Another internal standard, 1,10-decanediol (Aldrich, Milwaukee, Wisc., U.S.A.) was used before the methyl analog of EMB had been supplied by Lederle Labs. Decanediol serves as a good internal standard with a retention time of 2 min. However, since the compound is insoluble in water, aliquots must be prepared in organic solvents, which then may cause difficulties in obtaining accurate volumes when pipetting procedures are used.

Preliminary one-week stability tests were performed by dissolving 0.1 and 0.5 μg of EMB in 0.2 ml of plasma, which was then frozen for periods of two, four, and seven days. The results (Table II) show that EMB is stable in plasma on freezing for at least one week.

The assay of EMB from whole blood was also attempted. Red blood cells were naturally hemolyzed by freezing. The same assay procedures were followed as described for the plasma assay. No interference from red blood cell constituents was found. The calibration curve is similar to that prepared from plasma, indicating that EMB and the internal standard may be extracted equally well from blood and plasma.

TABLE II

THE EFFECT OF STORAGE ON PEAK HEIGHT RATIO

EMB (μg)	*Time (days)*			
	0	*2*	*4*	*7*
0.1	0.104	0.106	0.111	0.107
0.5	0.495	0.506	0.511	0.508

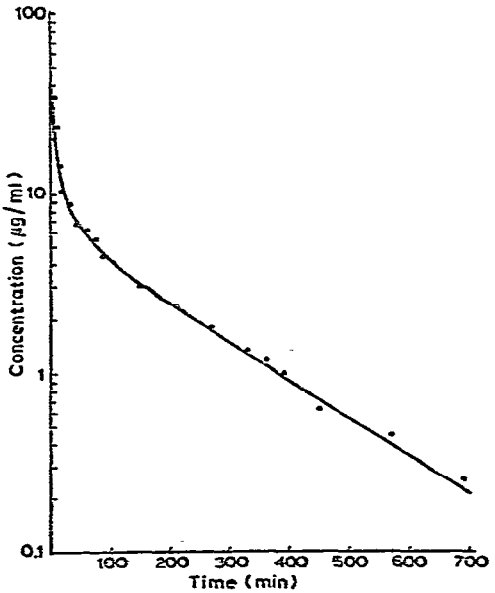

Fig. 4. A typical plasma concentration time curve for an 100-mg intravenous injection of EMB in a rhesus monkey.

The TFAA derivatives of EMB and MEMB are stable in benzene solution for several weeks. Nevertheless, the peak height ratio may change if the tubes are not tightly capped and vaporization occurs.

The assay procedures described here are well suited to measure therapeutic levels of EMB in patients. Experiments utilizing this procedure in both animals and humans are presently in process in our laboratory. A typical plasma concentration time curve for an 100-mg intravenous injection of EMB in a rhesus monkey is depicted in Fig. 4.

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