Journal of Chromatography, 221 (1980) 293—299
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 666

DETERMINATION OF ETHAMBUTOL IN PLASMA USING SELECTED ION MONITORING

KAZUMI OHYA, SHOZO SHINTANI and MITSUJI SANO*

Drug Metabolism Research Center, Research Institute, Daiichi Seiyaku Co. Ltd., Kitakasai 1-16-13, Edogawa-ku, Tokyo 132 (Japan)

(First received January 29th, 1980; revised manuscript received July 17th, 1980)

SUMMARY

The determination of ethambutol in plasma is described. Using ethambutol-d_4 as an internal standard, ethambutol and the internal standard were extracted with chloroform under alkaline conditions, and converted into their trifluoroacetyl derivatives with trifluoroacetic anhydride in benzene—pyridine (4:1). Selected ion monitoring was carried out by monitoring the peaks at *m/z* 294 and 296 corresponding to the fragment ion $[M/2]^+$ of the derivatives. Ethambutol was determined by use of the peak height ratio of the peak at *m/z* 294 against that at *m/z* 296.

The method was utilized successfully for studying the bioavailability and pharmacokinetics of the drug.

INTRODUCTION

An anti-tuberculosis drug, ethambutol dihydrochloride (EMB·2HCl), *d*-2,2′-(ethylenediimino)-di-1-butanol dihydrochloride, has been widely used in the treatment of tuberculosis. The absorption, metabolism and excretion of the drug have been examined in detail by use of ^{14}C-labelled EMB [1—5]. It is well-known that the therapeutic and toxic responses to EMB relate closely to the plasma levels of the drug, and that plasma levels of 3—5 μg/ml are required for treatment. Therefore it might be significant to determine the characteristics of EMB preparations by measuring the drug in plasma.

Several colorimetric methods [6—8] have been reported for the determination of EMB in biological fluids; however, they were not necessarily sufficiently sensitive or selective to apply to plasma samples. Recently an electron-capture gas chromatographic method [9,10] and selected ion monitoring (SIM) using

0378-4347/80/0000—0000/$02.25

the chemical ionization mode [5,11] were presented to this end.

In order to examine the bioavailability of EMB preparations, a more convenient method was required for the routine determination of EMB in plasma. As a result of some modifications of existing methods [5,9—11], we devised a convenient SIM method for the determination of EMB in plasma using EMB-d_4 as an internal standard. This paper describes the method and the pharmacokinetics of EMB in beagle dogs.

EXPERIMENTAL

Chemicals and reagents

EMB·2HCl was of J.P.IX grade (Lederle Labs., New York, NY, U.S.A.). EMB-d_4 · 2HCl was synthesized from *dl*-2-aminobutanol (reagent grade; Tokyo-Kasei Kogyo, Tokyo, Japan) and 1,2-dibromoethane-d_4 (isotope purity 99%; Merck Sharp & Dohme, Dorval, Canada) by the method of Wilkinson et al. [5,11,14]. Trifluoroacetic anhydride (TFAA) was purchased from Tokyo-Kasei Kogyo. All other chemicals were of reagent grade (Kishida Chemicals, Tokyo, Japan) and used without further purification. A stock EMB solution was prepared by dissolving 13.5 mg of EMB·2HCl in 100 ml of water; it was stored at 5°C protected from the light. Standard samples were prepared by spiking blank plasma with the stock EMB solution at a concentration of 0.1—5 μg of EMB per ml of plasma; these were stored at −20°C until analyzed. An internal standard solution was prepared by dissolving 13.5 mg of EMB-d_4 · 2HCl in 100 ml of water; this was stored at 5°C. A 1 *M* HCl in methanol solution was prepared by diluting concentrated HCl with methanol.

Plasma samples

Plasma samples were taken at seven intervals during 10 h after a single administration of 125 mg or 250 mg of EMB·2HCl (commercial tablet) to four male beagle dogs together with 10 ml of water. Each dog was fasted for 16 h before and for 3 h after the drug administration. Blood was collected in heparinized syringes by venipuncture, and centrifuged in the usual manner to separate the plasma. The plasma samples were stored at −20°C until analyzed.

Analytical procedure

Sample preparation. To plasma samples of 0.5—1.0 ml corresponding to 0.1—5 μg EMB, in a 12-ml glass-stoppered centrifuge tube were added exactly 0.1 ml of the internal standard solution, 0.5 ml of 4 *N* NaOH and 6 ml of chloroform. The tube was shaken vigorously for 10 min and centrifuged at 2000 *g* for 10 min. The aqueous layer was removed by aspiration. To the organic layer were added 3 drops of 1 *M* HCl in methanol, and then the solvent was evaporated to dryness in vacuo. The residue was dissolved with 50 μl of benzene—pyridine (4:1), and reacted with 50 μl of TFAA for 2 h at room temperature to give sample solutions. The sample solutions were analyzed by SIM.

Conditions for selected ion monitoring. A Hitachi gas chromatograph—mass spectrometer equipped with multi-ion monitor, Model RMU-6MG, was used. The column was a 1 m × 3 mm I.D. glass tube packed with 2% OV-17 coated on 80—100 mesh Gas-Chrom Q. The column was kept isothermally at 150°C,

the injection port and the separator were held at 180°C and 250°C, respectively. Helium was used as a carrier gas at a flow-rate of 40 ml/min. The ionization, acceleration and ion multiplier voltage were set at 30 eV, 3.2 kV and 1.8 kV, respectively. The ionization chamber was held at 160°C. Both the exit and collector slits were set at 0.2 mm. The ions at *m/z* 294 and *m/z* 296 were used for monitoring. The recorder attenuation was chosen in the range 0.05—2.0 V in accordance with the concentration of EMB in the sample solutions. The sample size was 1—3 μl.

Calculations. The concentration of EMB in plasma samples was determined from a calibration curve prepared by using the peak height ratio of *m/z* 294 against *m/z* 296. The calibration curve was obtained with the standard samples treated in the same manner as the plasma samples.

Gas chromatography—mass spectrometry

About 5 ml of the stock EMB solution (0.5 mg EMB) or of the internal standard solution (0.5 mg EMB-d_4) were taken into a glass-stoppered tube, and evaporated to dryness in vacuo. The residue was reacted with TFAA in a manner similar to that described in *Sample preparation* to prepare the injection solution for gas chromatography—mass spectrometry. Mass spectra were obtained by electrographical recording under conditions similar to those described for SIM.

RESULTS AND DISCUSSION

Mass spectra

In order to determine the EMB derivative for SIM analysis, we examined both the trifluoroacetyl (TFA) and the trimethylsilyl (TMS) derivatives. Both TFA EMB and TMS EMB eluted as a single, sharp and symmetric peak as reported by several authors [5,9—13]. After examining in detail the comparative merits of both derivatives, we chose TFA EMB in view of its stability. The stability of TFA EMB is superior to that of TMS EMB as suggested by the referees of this journal.

Fig. 1 shows the mass spectra of TFA EMB and TFA EMB-d_4 together with the assignment of the main peaks. The spectra did not involve the molecular ion peaks $[M]^{\pm}$ (*m/z* 588 for TFA EMB and *m/z* 592 for TFA EMB-d_4); however, the peaks at *m/z* 570 of TFA EMB and at *m/z* 574 of TFA EMB-d_4 indicated the respective tetratrifluoroacetyl derivatives. The base peak consisted of the peak corresponding to the fragment ion $[M/2]^+$ (*m/z* 294 for TFA EMB and *m/z* 296 for TFA EMB-d_4). In addition, the fragment ion $[M/2]^+$ had neither the cluster peak at *m/z* 296 arising from TFA EMB nor that at *m/z* 294 arising from TFA EMB-d_4. Taking into account the mass spectral feature of the TFA derivatives, the fragment ion $[M/2]^+$ was preferred for the SIM analysis.

Selected ion monitoring profiles

Fig. 2 shows the SIM profiles of EMB in plasma obtained using the analytical procedure. TFA EMB and TFA EMB-d_4 elute at 2.3 min and 2.0 min, respec-

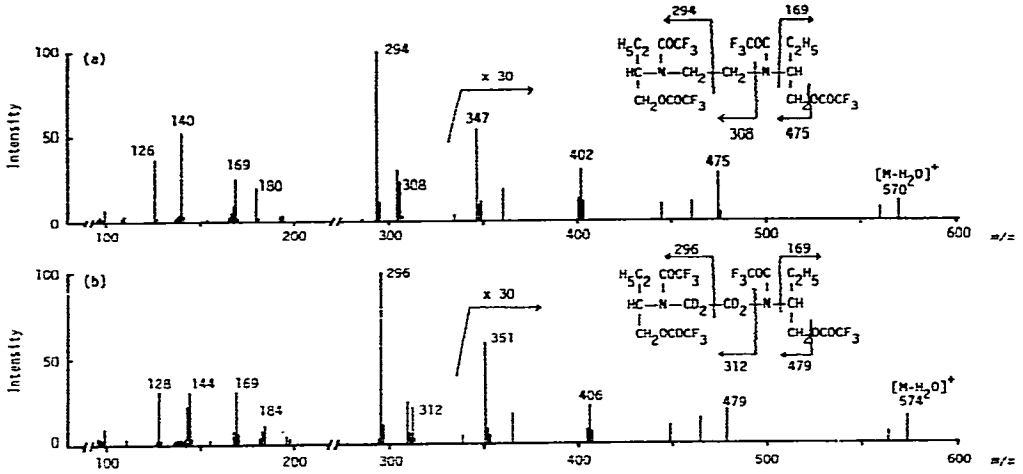

Fig. 1. Mass spectra of TFA derivatives of ethambutol (a) and ethambutol-d_4 (b) obtained by means of GC—EI—MS.

tively, as sharp and symmetric peaks. The difference in their retention times may be due to an isotopic effect.

There were no interferences from plasma components and no overlapping caused by the cluster ions of the monitoring ions (*m/z* 294 and *m/z* 296). The TFA derivatives remained unchanged for several days at room temperature when protected from moisture; the SIM profiles were scarcely changed 3 days after trifluoroacetylation.

Calibration curve and precision

The calibration curve showed good linearity ($r \geqslant 0.998$) between the peak height ratio and EMB concentrations in the range 0.1—5 μg/ml of plasma. The precision decreased somewhat with decreasing EMB concentrations, as seen in Table I; however, EMB could be determined within ± 9% of the coefficient of variation (C.V.) at 0.1 μg/ml of plasma or above. It was considered that such

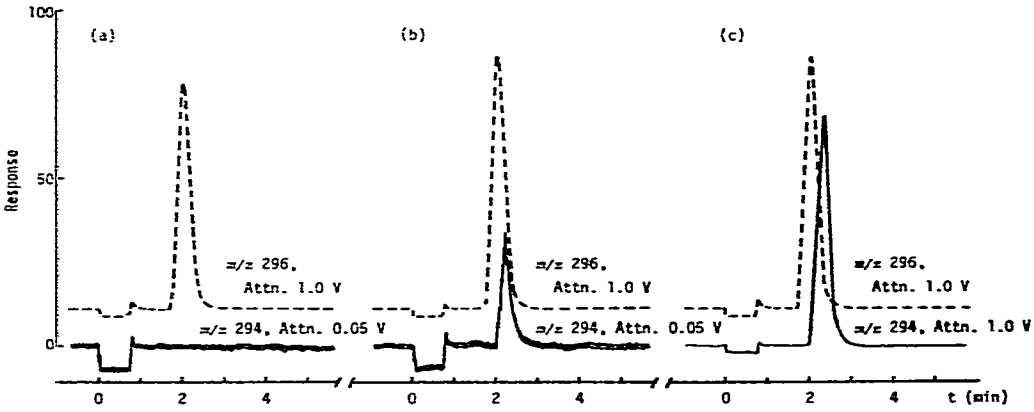

Fig. 2. Selected ion monitoring profiles of blank plasma (a) and standard samples; 0.3 μg ethambutol per ml of plasma (b) and 5.0 μg ethambutol per ml of plasma (c).

TABLE I

PRECISION OF THE SIM METHOD FOR THE DETERMINATION OF ETHAMBUTOL (EMB) IN PLASMA

$n = 7$.

EMB added (μg/ml)	EMB found (μg/ml) Mean ± σ_n	C.V. (%)	Recovery (%)
0.105	0.102 ± 0.0087	8.53	97.1
0.527	0.520 ± 0.0291	5.60	98.7
5.27	5.31 ± 0.116	2.18	100.8

sensitivity and precision would be acceptable for the evaluation of the bioavailability of EMB preparations and the pharmacokinetics of the drug.

The addition of hydrogen chloride to the chloroform extract was necessary to prevent the loss of EMB and the internal standard (EMB-d_4) in the concentration process. When HCl was not added, no quantitative results could be obtained.

On the use of the TMS derivatives

The TMS derivatives of EMB and EMB-d_4 were also applicable to SIM by monitoring the fragment ion $[M—CH_2OSi(CH_3)_3]^+$ (*m/z* 245 of TMS EMB, *m/z* 249 of TMS EMB-d_4) using the same clean-up method and analytical conditions as described in *analytical procedure*. Trimethylsilylation proceeded quantitatively to completion with N-trimethylsilylimidazole in acetonitrile within 10 min at room temperature. The derivatives were stable for 24 h at room temperature; however, they decomposed gradually giving no peaks 3 days after derivatization. We did not use TMS EMB because its stability was inferior to that of TFA EMB.

Application to the bioavailability and pharmacokinetics of EMB

The SIM method was utilized for studying the bioavailability of EMB preparations in dogs. Fig. 3 summarizes the time course of EMB in the plasma of beagle dogs after a single oral administration of 125 mg and/or 250 mg of EMB·2HCl tablets prepared in our laboratory.

There were individual differences in the plasma profiles of EMB, however, the mean values showed good dose-dependence. EMB was absorbed rapidly from the gastrointestinal tract to reach a maximum plasma level at 1 h after oral administration; it was eliminated smoothly, the plasma level becoming one-tenth of the maximum level at 7 h. In addition, the elimination process could be interpreted in terms of a two-compartment open model.

The distribution rate constants, k_{12} and k_{21}, between the central compartment (C_1) and the tissue compartment (C_2), and the elimination rate constant K_{el}, were calculated by computer fitting to give 0.36 h^{-1}, 0.34 h^{-1} and 0.83 h^{-1}, respectively. It was also estimated that EMB in the plasma was distributed

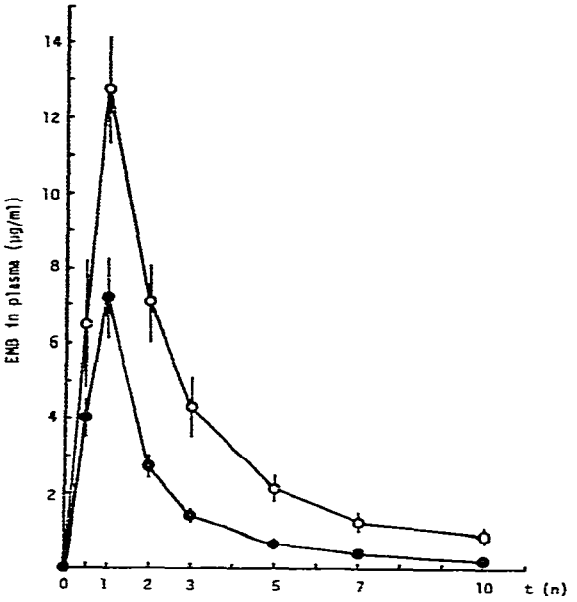

Fig. 3. Time course of ethambutol (EMB) in beagle dogs after the oral administration of 125 mg of EMB·2HCl per dog (•) and of 250 mg of EMB·2HCl per dog (○) in the form of commercial tablets. Each value is the mean ± standard error (n = 4).

into the tissue compartment with a half-life of 0.6 h, and that it was eliminated with a disposition (β) half-life of 3.6 h. It is of interest that the pharmacokinetic pattern of EMB in man [5] is similar to that in dog, and different from that in rhesus monkey [9].

The SIM method was also applicable to urine samples. We believe that this SIM method can be utilized conveniently for the routine analysis of EMB in biological fluids.

REFERENCES

1 E.A. Peets and D.A. Buyske, Biochem. Pharmacol., 13 (1964) 1403.
2 E.A. Peets, W.M. Sweeney, V.A. Place and D.A. Buyske, Amer. Rev. Resp. Dis., 91 (1965) 51.
3 V.A. Place, E.A. Peets, D.A. Buyske and R.R. Little, Ann. N.Y. Acad. Sci., 135 (1966) 775.
4 J.A. Burton and L.S. Schanker, Xenobiotica, 4 (1974) 291.
5 A.D. Blair, A.W. Forrey, T.G. Christopher, B. Maxwell and R.E. Cutler, in E.R. Klein and P.D. Klein (Editors), Proc. 2nd Int. Conf. on Stable Isotopes, October 20—23, 1975, Oak Brook, IL, p. 208.
6 I. Strauss and F. Erhardt, Chemotherapy, 15 (1964) 1403.
7 U. Gundert-Remy, U. Klett and E. Weber, Eur. J. Clin. Pharmacol., 6 (1973) 133.
8 N.U. Tatarinova, Probl. Tuberk., 3 (1974) 86; C.A., 81 (1974) 72445u.
9 C.S. Lee and L.Z. Benet, J. Chromatogr., 128 (1976) 188.
10 C.S. Lee and L.Z. Benet, J. Pharm. Sci., 67 (1978) 470.

11 A.D. Blair, A.W. Forrey, T.G. Christopher, B. Maxwell and R.E. Cutler, in E. Reid (Editor), Methodological Developments in Biochemistry, Vol. V, North-Holland Publishing Company, Amsterdam, 1976, p. 231.
12 A. Calò, C. Cardini and V. Quercia, J. Chromatogr., 37 (1968) 194.
13 B.M. Richard, J.E. Manno and B.R. Manno, J. Chromatogr., 89 (1974) 80.
14 R.G. Wilkinson, R.G. Shepherd, J.P. Thomas and C. Baughn, J. Amer. Chem. Soc., 83 (1961) 2212.