ELSEVIER

Journal of Chromatography A, 729 (1996) 301–307

JOURNAL OF CHROMATOGRAPHY A

Determination of ethambutol in human plasma and urine by high-performance liquid chromatography with fluorescence detection

M. Breda*, P. Marrari, E. Pianezzola, M. Strolin Benedetti

Department of Pharmacokinetics and Metabolism, Pharmacia SpA, Via per Pogliano, 20014 Nerviano, Milan, Italy

Abstract

A sensitive and selective HPLC method for the determination of ethambutol in human plasma and urine was developed. Ethambutol was extracted from basified plasma samples (0.2 ml) with diethyl ether, back-extracted into 0.01 *M* phosphoric acid and derivatized with 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole. After 30 min at 80°C and elimination of the reactive excess, the compound was determined by reversed-phase liquid chromatography. Urine was analysed for ethambutol after dilution 1:200 with distilled water and derivatization as described for plasma. Quantification in plasma and urine was achieved by fluorescence detection of the eluate. The linearity, precision and accuracy of the method were evaluated. No interference from the constituents of human plasma and urine was observed. The limit of quantification was 10 ng/ml in plasma and 10 μg/ml in urine. The suitability of the method for in vivo samples was checked by analysis of plasma and urine samples drawn from healthy volunteers who had received a 1200-mg oral dose of the test compound.

Keywords: Sample preparation; Derivatization, LC; Ethambutol

1. Introduction

Ethambutol (EMB, Fig. 1), *d*-(*R*,*R*)-N,N′-ethylenebis(2-aminobutan-1-ol) dihydrochloride, is a tuberculostatic agent widely used in the treatment of tuberculosis. Nearly all strains of *Mycobacterium tuberculosis* and *M. kansasii* and a number of strains of *M. avium* complex are sensitive to EMB [1]. Although the precise mechanism of action of EMB is unknown, the drug has been shown to inhibit the incorporation of mycolic acid into the mycobacterial cell wall [1]. In adult patients, EMB is usually administered at an oral daily dose of about 15 mg/kg, which gives peak plasma concentrations of ca. 2–5 μg/ml. Twenty-four hours after dosing, mean EMB plasma concentrations of about 0.5 μg/ml were reported [2,3].

A number of methods based on gas chromatography (GC) have been described for the determination of EMB. Richard et al. [4] reported a GC method with flame ionization detection. The drug was derivatized to form a trimethylsilyl (TMS) derivative; however, this procedure was found not to be sensitive enough for the measurement of EMB at the concentrations found in human plasma after therapeutic

* Corresponding author.

0021-9673/96/$15.00
SSDI 0021-9673(95)01093-9

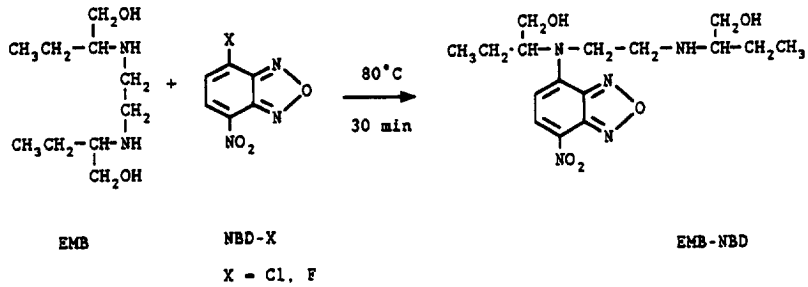

Fig. 1. Derivatization reaction.

doses. Lee and Benet [5] developed a GC method with electron-capture detection with adequate sensitivity for the measurement of small amounts of EMB in human plasma and urine after derivatization with trifluoroacetic anhydride (TFAA). This method was suitably modified by Lee and Wang [6] to obtain a higher sensitivity. Blair et al. [7] developed a method based on GC–mass spectrometry (GC–MS) with chemical ionization using the derivatization procedure previously reported by Richard et al. [4]. Holdiness et al. [3] developed a GC–MS method with electron impact ionization using TFAA as derivatizing agent. As far as we could ascertain, HPLC methods for the determination of EMB in biological fluids have not been developed. EMB shows no significant UV absorption, therefore UV detection appears unsuitable for its determination. On the other hand, the presence in the EMB molecule of functional groups which could react with selected reagents to produce derivatives more easily detectable by the more common detection techniques (UV absorption and fluorimetry) prompted us to explore this possibility.

A method based on precolumn derivatization of EMB followed by HPLC with fluorimetric detection of derivatized EMB was developed for the determination of the test compound in human plasma and urine. This method was validated and used for the quantification of EMB in human plasma and urine following an oral intake of 1200 mg.

2. Experimental

2.1. Chemicals and solutions

EMB was purchased from Sigma (St. Louis, MO, USA). 4-Fluoro-7-nitrobenzo-2-oxa-1,3-diazole [NBD-F (Fig. 1)] was obtained from Fluka (Buchs, Switzerland) and 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole [NBD-Cl (Fig. 1)] from Aldrich (Milwaukee, WI, USA). All other reagents were of analytical-reagent grade from Carlo Erba (Milan, Italy).

A stock standard solution of EMB was prepared by dissolving a weighed amount of this compound in distilled water. From this solution, working standard solutions were prepared by dilution with distilled water. NBD-F and NBD-Cl solutions were prepared daily by dissolving 4 mg of the reagent in 1 ml of acetonitrile. These solutions were kept at 4°C in the dark.

2.2. Equipment

The HPLC system consisted of an Isochrom isocratic pump [Thermo Separation Products (TSP), Fremont, CA, USA], a Waters (Milford, MA, USA) Model 717 Plus autosampler and a Jasco (Tokyo, Japan) Model 821 FP fluorescence detector equipped with a 17-μl flow cell and with a Hamamatsu (Shiwoka, Japan) R928 photomultiplier. Chromatograms were recorded with a TSP SP4270 integrator connected to an IBM computer equipped with Labnet software (TSP).

2.3. Chromatographic conditions

The chromatographic separation was performed using a 250 × 4.6 mm I.D. Spherisorb CN column, particle size 5 μm, with a Survival precolumn packed with Pellicular ODS (particle size 37–53 μm) (Whatman, Clifton, NJ, USA). The mobile phase was acetonitrile–0.01 *M* H_3PO_4 adjusted to pH 2.5 with a few drops of 10 *M* KOH (30:70, v/v). The flow-rate was 1 ml/min. The fluorimetric detector was set at an excitation wavelength of 490 nm and an emission wavelength of 540 nm. The gain was set at ×100. A 1-V signal was sent from the detector to the integrator.

2.4. Sample preparation

Plasma

The plasma sample (0.2 ml) was placed in a conical centrifuge tube and mixed with 0.3 ml of 5 *M* NaOH; after the addition of 5 ml of diethyl ether, the tube was capped, vortex mixed for 1 min and centrifuged at 1200 *g* for 3 min in order to separate the two phases clearly. The upper organic phase was aspirated and transferred into another tube and the extraction step was repeated. The combined organic phases were extracted with 0.2 ml of 0.01 *M* phosphoric acid by vortex mixing for 1 min. After centrifugation as above, the organic phase was discarded and a 0.3-ml aliquot of 0.2 *M* borate buffer (pH 7.5) was added to the aqueous phase. After brief vortex mixing (10 s), 40 μl of NBD-F solution (4 mg/ml in acetonitrile) were added. The tubes were capped and incubated in a multi-block heater for 30 min at 80°C. The reaction was stopped by addition of 50 μl of 1 *M* H_3PO_4 followed by rapid cooling in an acetone bath kept at −40 to −50°C for 1 min. Ethyl acetate (2 ml) was added and the mixture was vortex mixed for 1 min, then centrifuged at 1200 *g* for 3 min. The supernatant was aspirated and discarded and 50 μl of 5 *M* NaOH were added to the lower aqueous phase. The derivatized compound(s) was extracted with 2 ml of ethyl acetate–methanol (9:1, v/v) after mixing for 1 min and centrifugation at 1200 *g* for 3 min. The upper organic phase was aspirated and dried under a stream of nitrogen at 37°C. The residue was dissolved in 0.25 ml of 0.01 *M* phosphoric acid (pH 2.5) and 200 μl were injected into the HPLC system.

Urine

A 0.1-ml aliquot of urine was diluted to 20 ml with distilled water. To 0.2 ml of this diluted solution, 0.3 ml of 0.2 *M* borate buffer (pH 7.5) were added, followed by 40 μl of NBD-F solution (4 mg/ml in acetonitrile). The mixture was treated in the same way as described above for plasma. The residue was dissolved in 1 ml of 0.01 *M* phosphoric acid (pH 2.5) and 200 μl were injected into the HPLC system.

2.5. Determination of calibration and quality control samples

Analyses of blank human plasma and urine spiked with known amounts of EMB were carried out applying the above-described procedure. Linearity was evaluated from four calibration graphs prepared and run on four different days in the concentration range 10–1500 ng/ml for plasma and 10–500 μg/ml for urine. The precision and accuracy were evaluated by repeated analyses of EMB at three different concentrations (about 30, 200 and 1000 ng/ml for plasma and 30, 150 and 400 μg/ml for urine) in three replicate samples analysed on four different days. All chromatograms obtained were evaluated by peak-area measurement. The quality control and unknown samples were calculated with the calibration graph generated on each day by least-squares linear regression of the analyte peak area against its concentration. A weighting factor ($1/y$) was applied. To evaluate the extraction recovery, the peak area of an extracted derivatized plasma sample was compared with that obtained with unextracted derivatized standard solution.

2.6. Suitability test of the chromatographic system

On each day, before the analysis of unknown

and/or calibration samples, the performance of the chromatographic system was checked in order to ensure that controlled conditions were used in the assay. Column efficiency, expressed as the number of theoretical plates (N) of the column, and the peak symmetry (Sf) were used to define the suitability of the chromatographic system [8]. Accepted limits of these two parameters were $N > 8000$ and $Sf \leq 1.5$.

3. Results and discussion

Both NBD-Cl and NBD-F can react with primary and secondary amino groups, giving the same fluorescent compound (Fig. 1), therefore the derivatization of EMB was studied with both reagents. After reaction, the NBD derivative of EMB was easily determined in the reaction mixture by HPLC coupled with fluorimetric detection at the typical wavelengths reported for these derivatives [9]. Under the same reaction conditions, NBD-F was found to be more reactive than NBD-Cl, and the latter was consequently abandoned; similar results have been reported by Imai and co-workers [10,11]. The time course of the EMB reaction with NBD-F was then investigated as a function of pH, composition of the reaction medium, time and temperature. The optimal pH for the reaction was found to be 7.5 in an aqueous medium containing about 10% acetonitrile. Maximum reaction yield was achieved after 30 min at 80°C.

Changes in the molar ratio between the reagent and EMB were found to have a strong influence on the reaction yield; this effect was minimized when a large excess of NBD-F was added to the reaction mixture. This excess, however, had to be eliminated before the chromatographic analysis because, although the reagent is almost non-fluorescent, it nevertheless could interfere with fluorescence measurement, giving a large unretained peak in the chromatogram. Extraction of the reaction mixture with ethyl acetate at pH 3 was employed to eliminate selectively the reagent excess. The NBD-EMB derivative was poorly extracted at acidic pH by ethyl acetate and on average less than 7% of the compound of interest was lost during this step.

Under the above conditions, EMB-NBD gave only one peak in the chromatogram (Fig. 2). Collection of this peak after the chromatographic separation and isolation of the corresponding compound, which was then submitted to mass spectrometric analysis (electron impact, 70 eV), allowed this compound to be identified as the mono-NBD derivative of EMB.

Optimization of the chromatographic conditions for the determination of NBD-EMB was investigated. This compound was retained by both CN and C_{18} stationary phases, from which it could be eluted with mobile phases containing different percentages of acetonitrile and dilute phosphoric acid. The primary retention mechanism probably involves polar-type interactions with free silanol groups of silica, in addition to other minor hydrophobic-type interactions which would occur mainly with ODS-silica. This hy-

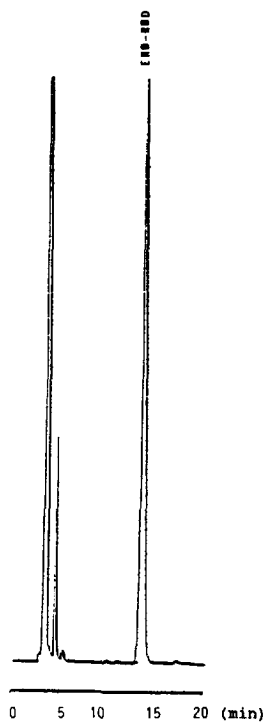

Fig. 2. Chromatogram of unextracted standard (26 ng of EMB) injected after derivatization.

pothesis was mainly based on the observation that even minor modifications of the mobile phase pH led to striking changes in the retention time of NBD-EMB with both stationary phases, but in contrast changes in the percentage of the organic modifier were found to influence the retention of the derivative to only a minor extent. Since sharper and more symmetrical peaks were obtained with CN-modified silica, the use of C_{18} columns was abandoned.

Several procedures for the selective extraction of EMB from the plasma matrix were explored; plasma protein precipitation with acetonitrile, solid-phase extraction (using cartridges with different packing materials) and liquid–liquid extraction (using chloroform as organic solvent as reported in other studies [2–5]) showed, in our hands, low recovery and reproducibility. A double extraction step with diethyl ether followed by back-extraction in an acidic aqueous solution was found to be a more effective procedure than those reported. Reproducible extraction of the analyte was indeed a key step in the development of our method, since no internal standard was employed here; a high extraction recovery and suitable reproducibility were obtained with the adopted procedure. The mean recovery from plasma samples calculated at three different concentrations (about 30, 200 and 1000 ng/ml) ranged from 76.1 to 80.9% with R.S.D. $<9\%$ ($n = 12$).

Since 50–70% of the EMB dose is excreted unchanged in urine [2,12], urine samples could be assayed by direct derivatization of suitably diluted samples.

Chromatograms of blank plasma and urine samples processed as described showed no interfering peak at the retention time of the compound of interest (Fig. 3).

The linearity of the HPLC assay was evaluated from four separate calibration graphs obtained on different days in the range 10–1500 ng/ml for plasma and 10–500 μg/ml for urine. The mean slope was 1524 (R.S.D. = 5.4%) and 4401 (R.S.D. = 5.5%) in plasma and urine, respectively. Back-calculated concentrations of spiked samples exhibited R.S.D. <11.1%. The correlation coefficients ranged from 0.9991 to 0.9997 in plasma and from 0.9974 to 0.9997 in urine. The inter-day precision, expressed as R.S.D., ranged from 9.4 to 13.7% for plasma (Table 1) and from 4.3 to 6.3% for urine (Table 2) over the range of concentrations tested. At the same concentrations, the intra-day precision for both biological fluids was better than 14.3%. The accuracy evaluated with the same samples and expressed as the percentage ratio of found to added amount, ranged from 90.4 to 116.9% for plasma (Table 1) and from 93.7 to 105.8% for urine (Table 2).

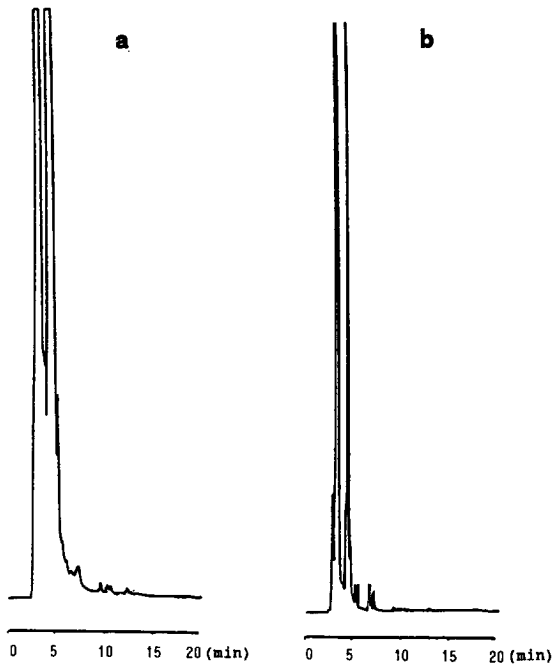

Fig. 3. Chromatograms of (a) blank human plasma and (b) urine assayed as described.

The instrumental limit of detection, defined as the mass of analyte on-column able to produce a signal-to-noise ratio (S/N) of 3, was 0.5 ng. The limit of quantification was 10 ng/ml for plasma ($S/N > 5$, Fig. 4) and 10 μg/ml for urine ($S/N > 5$, Fig. 4).

The method was used to determine EMB concentrations in plasma and urine samples from healthy human volunteers who had received a single oral dose of 1200 mg of EMB. Typical chromatograms are shown in Fig. 5.

Table 1
Accuracy and precision of EMB determination in human plasma

Concentration added (ng/ml)	Day	Concentration found (mean ± S.D., $n = 3$) (ng/ml)	Intra-day R.S.D. ($n = 3$) (%)	Inter-day R.S.D. ($n = 12$) (%)	Accuracy (mean found/added) (%)
31.17	1	36.43 ± 4.08	11.2		116.9
	2	35.61 ± 5.09	14.3		114.3
	3	35.79 ± 2.02	5.8		114.8
	4	28.18 ± 1.89	6.7	13.7	90.1
207.8	1	214.26 ± 21.43	10.0		103.1
	2	194.70 ± 18.11	9.3		93.7
	3	222.46 ± 18.24	8.2		107.1
	4	198.78 ± 14.51	7.3	9.4	95.7
1039	1	1114.88 ± 25.64	2.3		107.3
	2	1191.21 ± 54.80	4.6		114.7
	3	1080.90 ± 81.07	7.5		104.0
	4	949.46 ± 94.00	9.9	10.0	91.4

4. Conclusion

The method described is selective for the determination of EMB in human plasma and urine. It is linear, precise and capable of accurately determining this drug in the 10–1500 ng/ml concentration range in plasma and in the 10–500 μg/ml concentration range in urine. The procedure is well suited to measuring therapeutic levels of EMB in patients.

Acknowledgements

The authors thank Dr. C. Allievi and Dr. P. Zugnoni for mass spectrometric analyses and

Table 2
Accuracy and precision of EMB determination in human urine

Concentration added (μg/ml)	Day	Concentration found (mean ± S.D., $n = 3$) (μg/ml)	Intra-day R.S.D. ($n = 3$) (%)	Inter-day R.S.D. ($n = 12$) (%)	Accuracy (mean found/added) (%)
31.02	1	31.73 ± 2.17	6.8		102.3
	2	31.46 ± 0.91	2.9		101.4
	3	32.58 ± 1.71	5.3		105.0
	4	31.52 ± 0.74	2.3	4.3	101.6
155.1	1	163.82 ± 4.23	2.6		105.6
	2	156.70 ± 11.47	7.3		101.0
	3	147.62 ± 9.11	6.2		95.2
	4	154.16 ± 4.45	2.9	5.8	99.4
403.26	1	425.47 ± 21.57	5.1		105.5
	2	396.05 ± 26.74	6.7		98.2
	3	378.03 ± 9.92	2.6		93.7
	4	400.89 ± 23.01	5.7	6.3	99.4

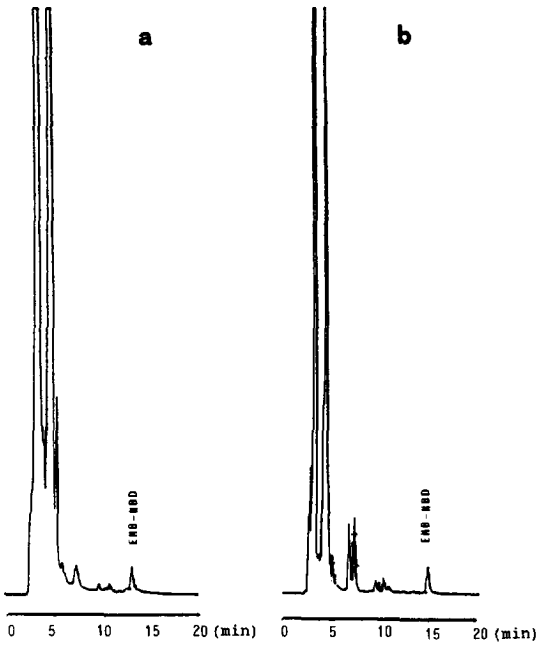

Fig. 4. Chromatograms of (a) human plasma spiked with 10 ng/ml of EMB and (b) human urine spiked with 10 μg/ml of EMB assayed as described.

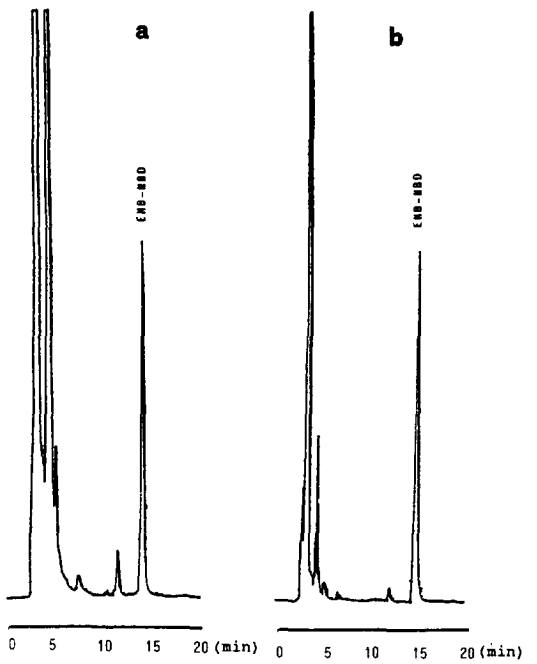

Fig. 5. Typical chromatograms of samples obtained from a healthy volunteer (a) 9 h (plasma) and (b) 6–12 h (urine) after a 1200-mg oral dose of EMB.

spectrum interpretation and Mrs. S. Cozzi for typing the manuscript.

References

[1] G.L. Mandell and M.A. Sande, in A.G. Gilman, T.W. Rall, A.S. Nies and P. Taylor (Editors), The Pharmacological Basis of Therapeutics, Macmillan, New York, 8th ed., 1990, p. 1146.
[2] C. Dollery (Editor), Therapeutic Drugs, Vol. 1, Churchill Livingstone, Edinburgh, 1991, p. E55.
[3] M.R. Holdiness, Z.H. Israili and J.B. Justice, J. Chromatogr., 224 (1981) 415.
[4] B.M. Richard, J.E. Manno and B.R. Manno, J. Chromatogr., 89 (1974) 80.
[5] C.S. Lee and L.Z. Benet, J. Pharm. Sci. 67 (1978) 470.
[6] C.S. Lee and L.H. Wang, J. Pharm. Sci. 69 (1980) 362.
[7] A.D. Blair, A.W. Forrey, T.G. Christopher, B. Maxwell and R.E. Cutler, in E. Reid (Editor), Methodological Development in Biochemistry, Vol. 5, North-Holland, Amsterdam, 1976, p. 231.
[8] US Pharmacopoeia XXII Revision, US Pharmacopoeial Convention, Rockville, MD, 1990, p. 1566.
[9] N. Seiler and L. Demisch, in K. Blau and G.S. King (Editors), Handbook of Derivatives for Chromatography, Heyden, London, 1977, p. 367.
[10] K. Imai and Y. Watanabe, Anal. Chim. Acta, 120 (1981) 377.
[11] T. Toyo'Oka, Y. Watanabe and K. Imai, Anal. Chim. Acta, 149 (1983) 305.
[12] M.R. Holdiness, Clin. Pharmacokinet., 9 (1984) 511.