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Short communication

Determination of ethambutol in plasma by high-performance liquid chromatography after pre-column derivatization

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Abstract

A new HPLC assay using UV detection (200 nm) was developed to determine ethambutol (EMB) concentrations in plasma. Following extraction (0.1 ml plasma) with chloroform, EMB and octylamine (used as internal standard) were derivatized with phenylethylisocyanate. Quantitation in plasma was achieved at 200 nm. There were no interferences from endogenous compounds. Intra- and inter-day variabilities were lower than 5.2 and 7.6%, respectively. The limit of quantitation of the method was 0.2 μg/ml. In plasma, ethambutol was found to be stable for at least one month when samples were stored at −20°C. This assay was applied to the therapeutic monitoring of EMB concentrations in 19 patients suffering from tuberculosis. © 1998 Elsevier Science B.V.

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1. Introduction

Tuberculosis remains one of the most common infectious diseases [1]. Its treatment, lasting from six to nine months, is based on combination therapy, to avoid the development of resistance [2]. Toxicity and compliance problems may arise [3,4]. Therapeutic monitoring of antituberculosis agents allows the clinicians to ensure compliance and to determine effective doses while reducing the risks of toxicity [5,6].

Ethambutol, D-(*R*,*R*)-*N*,*N*′-ethylenebis(2-aminobutan-1-ol) dihydrochloride, is a synthetic antimycobacterial agent with substantial activity against *Mycobacterium tuberculosis*, *Mycobacterium bovis*, and most strains of *Mycobacterium kansasii*. Ethambutol (EMB) is effective adjunct therapy for the treatment of tuberculosis but is not indicated as monotherapy. In contrast to rifampicin, isonazide or pyrazinamide which are essentially eliminated by the liver, EMB is renally excreted, with two-thirds of the administered dose being recovered unchanged in urine [7]. Renal failure is associated with significantly higher risks of toxicity, notably with retro bulbous optical nevritis.

Several methods, i.e., microbiological [8], isotopic [9], colorimetric [10–12] and chromatographic [13–19], have been proposed to determine EMB concentrations. Only chromatographic methods combined the necessary specificity and sensitivity to be applicable to a clinical setting. The goal of our work was to develop a new high-performance liquid

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chromatography (HPLC) method to measure EMB concentrations in a clinical setting.

Since HPLC with UV detection has been associated with insufficient detection limits, it has seldom been used to measure EMB concentrations in clinical samples. Lacroix et al. [21] attempted to circumvent these difficulties by measuring the 270 nm absorbance of an EMB–copper sulfate complex. However, their technique required the use of two analytical columns and the synthesis of the internal standard (I.S.). Gamberini and Ferioli [22] proposed the use of chemical derivatization with phenylethylisocyanate (PEIC) and the subsequent measurement of the 254 nm absorbance. Their assay has been used for the quality control of raw material. It could not be applied to the analysis of biological samples because of insufficient sensitivity.

We optimized the PEIC derivatization to obtain sufficient sensitivity. We also investigated the use of a more appropriate I.S., i.e., a molecule which could also be derivatized by PEIC and would be commercially available. Octylamine (OA) satisfied all the criteria.

Following validation, the assay was applied to the determination of EMB concentrations in patients suffering from tuberculosis and treated with EMB-containing combination therapy.

2. Experimental

2.1. Chemicals and reagents

EMB dihydrochloride and PEIC were purchased from Sigma (St. Louis, MO, USA). OA was obtained from Fluka (Buchs, Switzerland). Solvents and reagents were of HPLC analytical grade and obtained from Merck (Darmstadt, Germany). Ultra-pure water was prepared with an inverse osmosis system (Milli-Q Reagent Water System, Millipore, St. Quentin en Yvelines, France).

2.2. Solutions

Stock solutions of OA (80 μg/l) and PEIC (2000 μg/l) were prepared in acetonitrile. The EMB stock solution (1000 mg base/l) was prepared in acetonitrile containing 2% triethylamine (to allow its solubilisation [22]). Working solutions were obtained by diluting stock solutions in acetonitrile (1:10 and 1:5 for OA and PEIC, respectively). Plasma controls were prepared by spiking blank plasma with known amounts of EMB.

2.3. HPLC system

The HPLC system (Shimadzu, Touzart et Matignon, Les Ulis, France) consisted of a LC-6A pump, a SIL-6A autosampler (with a 20-μl injection loop), a SPD-6AV UV detector and a CR3A integrator. Analytes were separated at room temperature on a C_{18} column, (Hypersil, 150 mm×4.6 mm I.D., 3 μm, Interchim, Montluçon, France) and a C_{18} guard column (30 mm×4.6 mm I.D., Interchim). The mobile phase was methanol–water (70:30, v/v) and pumped at a flow-rate of 1 ml/min.

2.4. Selected wavelength

In spite of published data, the 254 nm absorption of EMB–PEIC was too low to allow its quantitation in plasma samples. To determine the optimal wavelength of analysis, an absorption spectrum was carried out from 195 to 400 nm using a HPLC system connected to a diode array detector (HPLC Integral 4000, Perkin-Elmer, St. Quentin en Yvelines, France). The UV spectrum presented a peak of absorption at 200 nm and this wavelength was therefore selected for the analyses.

2.5. Sample preparation

Plasma samples (100 μl) were spiked with the I.S. (100 μl), alkalinised with 4 *M* NaOH (100 μl) and extracted with 4 ml of chloroform as previously described by Lee and Benet [16]. Following a 10-min agitation and a 5-min centrifugation at 3000 rpm, the aqueous phase was discarded. The organic layer was spiked with 100 μl of PEIC, mixed 1 min and evaporated to dryness under a stream of nitrogen. The dry residue was reconstituted with 100 μl of mobile phase and 20 μl were injected onto the HPLC system.

2.6. Assay validation

2.6.1. Specificity

Putative interferences with other antituberculosis agents and with common anti-infectious and antiviral drugs were investigated. Plasma samples were spiked with the selected drugs and EMB, and extracted as previously described.

2.6.2. Linearity

Standard curves were constructed with plasma controls spiked with 1, 2.5, 5, 10, 15 and 20 μg/ml of EMB. Plasma concentrations were derived from linear regression analysis of the peak area ratios (analyte/I.S.) vs. concentration curves. Standard curve linearity was verified statistically using standard methods: (1) correlation coefficient (r) estimation, (2) comparison of the y-axis intercept with zero (Student's test), (3) significance of the slope (Fisher test). In all cases, concentrations were assumed to be normally distributed and variances were tested for homogeneity (Cochran test) [23]. Alpha was fixed to 5%.

2.6.3. Precision and accuracy

Precision was determined on sexplicate measurements of three EMB concentrations (2, 8 and 16 μg/ml). All samples were spiked with EMB on day 1, and extracted and analyzed on day 1 (intra-day, $n=6$) and on various days (inter-day, $n=6$). Accuracy was assessed at the same concentrations ($n=6$) and expressed as the percent deviation from the theoretical value. The confidence interval of the mean recovery was calculated.

2.6.4. Limit of quantitation (LOQ) and limit of detection (LOD)

These values were estimated mathematically from the standard curve equations. The LOD was equal to 3.3-times the standard deviation (S.D.) of the y-axis intercepts. The LOQ was obtained by multiplying the S.D. of the y-axis intercepts by 10 [24].

2.7. Recovery

The recovery yield was assessed by comparing the peak areas of EMB and of the I.S. between extracted plasma controls spiked with 2, 8 and 16 μg/ml of EMB ($n=6$ for each concentration) and non-extracted acetonitrile solutions spiked with the same amounts of EMB ($n=6$ for each concentration).

2.8. Stability

The stability of stock solutions and plasma samples (10 μg/ml of EMB) was evaluated at +20°C, +4°C and −20°C. For plasma samples, EMB stability was also assessed after sample heating at 56°C for 45 min, as is routinely done for decontamination purposes [25]. The stability of PEIC-derivatized EMB and octylamine was evaluated after 24 h at 20°C. Peak areas were compared to those obtained with a freshly prepared solution/sample of EMB using an analysis of variance.

2.9. Clinical study

The goal of our work was to set up an analytical method allowing the therapeutic monitoring of EMB concentrations. Patients suffering from tuberculosis and treated with oral EMB (20 mg/kg/day), alone or in combination, were included in the study. Patients (seven females and twelve males) were aged from 31 to 57 years. Blood samples were withdrawn before dosing and after 3 h, to reflect trough and peak EMB concentrations. Samples were collected in heparinized test tubes and immediately centrifuged at 1000 g for 5 min. Plasma was frozen at −20°C until analysis.

3. Results

3.1. Chromatography

Chromatograms representing a blank plasma sample and plasma spiked with 2.5 and 10 μg/ml of EMB and octylamine are presented in Fig. 1. Retention times of derivatized EMB and octylamine were 8.9 min (intra-day C.V.: 0.1%) and 13.7 min (intra-day C.V.: 0.15%), respectively. Both compounds were well separated with a resolution factor of 1.6. There was no interfering peak in blank plasma. Rifampicin, isoniazide, pyrazinamide, rifabutine, itraconazole, fluconazole, amphotericin B,

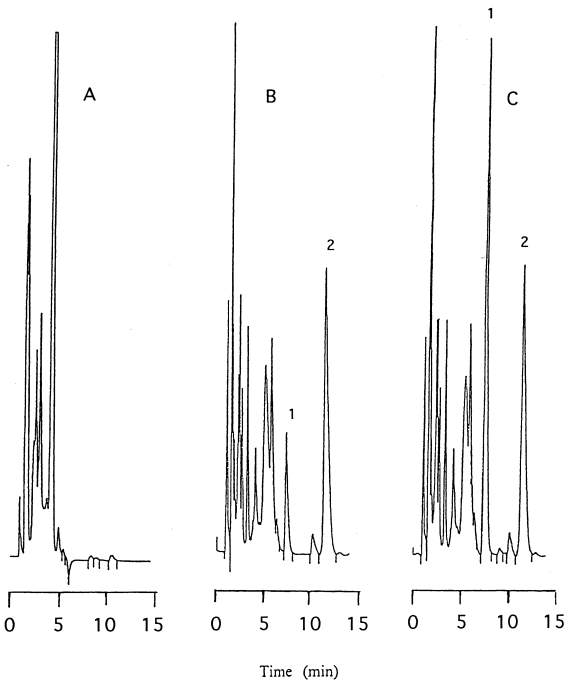

Fig. 1. Chromatogram of blank plasma derivatized (A); chromatogram of blank plasma spiked with 2.5 μg/ml of EMB (B); chromatogram of blank plasma spiked with 10 μg/ml of EMB (C). Peaks: 1=ethambutol; 2=octylamine.

pyrimethamine, dapsone, sparfloxacin, zidovudine and didanosine did not interfere with EMB analysis.

3.2. Linearity

At the selected concentrations, EMB standard curves were found to be linear, as illustrated by correlation coefficients greater than 0.998. Standard curves prepared and analyzed on the same day or on different days were found to have similar slopes and *y*-axis intercepts (Table 1).

3.3. Precision and accuracy

The intra- and inter-day precision of the method is presented in Table 2. Coefficients of variation (C.V.s) were lower than 10% at the studied concentration range.

Table 1
Intra- and inter-day linearity of ethambutol

	Ethambutol linearity (mean±S.D.)
Intra-assay	
Slope	0.118±0.006
Intercept	−0.023±0.011
r	0.9997±0.0004
Inter-assay	
Slope	0.136±0.015
Intercept	−0.045±0.020
r	0.998±0.003

3.4. LOQ and LOD

The LOD and the LOQ were 0.07 and 0.2 μg/ml, respectively.

3.5. Recovery

The recovery of EMB from plasma varied from 96 to 107% at the three concentrations tested. Recovery averaged 99% (n=18).

3.6. Stability studies

Under our conservation conditions, no degradation could be evidenced. In acetonitrile, EMB was stable for at least two months when solutions were stocked at +4 or −20°C. Plasma samples were stable for at least two days at +20°C, 15 days at +4°C and one month at −20°C. PEIC-derivatized EMB and I.S. were stable for at least 24 h at +20°C. When plasma samples were heated for 45 min at 56°C, EMB concentrations decreased by approximately 30%.

3.7. Clinical study

In 19 patients, peak EMB concentrations (3 h after oral treatment) averaged 2.3±1.1 μg/ml and ranged from 1.1 to 4.6 μg/ml.

In 16 out of 19 patients, trough EMB concentrations were found to be below the quantitation limit. In three patients, trough EMB levels varied from 0.5 to 1.3 μg/ml.

Table 2
Precision and accuracy of the HPLC method

Theoretical concentration (μg/ml)	Intra-day variability measured concentration		Inter-day variability measured concentration		Accuracy (%)
	Mean (mg/l)	C.V. (%)	Mean (mg/l)	C.V. (%)	
2	2.01	5.2	2.02	6.8	101
8	8.02	2.7	7.86	7.6	98
16	15.92	1.7	15.88	4.5	99

4. Discussion and conclusion

Several methods have been described to measure EMB levels but only chromatographic assays allow routine plasma concentration measurements. Gas chromatography, when coupled with electron capture detection or mass spectrometry [14,16,17], is sensitive enough, but is not adapted to therapeutic drug monitoring. Recently, a HPLC method with fluorescence detection was developed for the determination of EMB in human plasma and urine [20]. EMB was extracted and derivatized with 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole. This method is also too long and complex for therapeutic drug monitoring. Lacroix et al. [21] attempted to circumvent these difficulties by measuring the 270 nm absorbance of an EMB–copper sulfate complex. However, their technique required the use of two analytical columns and the synthesis of the internal standard. The present method has significant advantages, being simple and rapid. The analytical equipment is fairly basic (HPLC with UV detection) and part of almost any analytical laboratory. Chromatographic run times are short (15 min), as is the sample preparation step. The derivatization is carried out during the organic solvent evaporation, without the need for extended waiting periods characteristic of chemical derivatizations. In contrast to published methods [13–19], the I.S. is a commercially available compound, leading to its easier supplying and to a guarantee of its quality, indispensable elements to routine analyses. At 200 nm, there were no interfering peaks and absorbance was 600-fold higher than at 254 nm allowing the use of smaller plasma sample volumes (100 μl) with a limit of quantitation of 0.2 μg/ml. This is comparable to the volume needed for GC methods (10–200 μl) [14,16,17], and considerably lower than the volume typically used in HPLC assays (1 ml) [20,21]. A smaller plasma volume allows the use of smaller amounts of organic solvents but is mostly advantageous in a clinical setting. Plasma volumes are often limited, especially in children or in patients receiving multiple treatments and requiring the therapeutic monitoring of several molecules.

Plasma concentrations measured in patients receiving multiple antituberculosis agents showed the absence of interfering drugs or metabolites. Peak plasma concentrations were found to be comparable in patients and in healthy volunteers (3.2 to 5.4 μg/ml), ranging within the 3–5 μg/l therapeutic window [3,25].

Thus this method is helpful to detect compliance problems and to optimize EMB efficacy.

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