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RAPID LC/MS^2 DETERMINATION OF ETHAMBUTOL IN HUMAN PLASMA FOR THERAPEUTIC DRUG MONITORING

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ABSTRACT. A rapid and sensitive liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) method for the quantification of ethambutol in human plasma was developed and validated. The separation was performed on a Gemini C18 column under isocratic conditions using a mixture of methanol and 0.1% ammonia 65:35 (v/v) as mobile phase, at 40 ºC with a flow rate of 0.6 mL/min. The detection of ethambutol was performed in multiple reaction monitoring mode using an ion trap mass spectrometer with atmospheric pressure chemical ionization, operating in positive MS/MS mode. The human plasma samples (0.1 mL) were deproteinized with methanol and aliquots of 2 µL from supernatants obtained after centrifugation were directly injected into the chromatographic system. The method shows a good linearity ($r > 0.994$), precision (CV < 8.8%) and accuracy (bias < 12.3%) over the range of 0.36-17.18 µg/mL plasma. The lower limit of quantification (LLOQ) was 0.36 µg/mL and the recovery was between 93-111%. The developed and validated method is simple, rapid and specific for the determination of ethambutol in human plasma and was successfully applied in therapeutic drug monitoring of ethambutol in patients with tuberculosis.

Keywords: *ethambutol, LC/MS/MS, human plasma*

INTRODUCTION

Ethambutol, d-N,N'-bis (1-hydroxymethylpropyl) ethylenediamine (Fig. 1) is a synthetic oral antibiotic derivative of ethylenediamine which is used for the treatment of tuberculosis in conjunction with at least one other antituberculosis drug.

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Although the majority of tuberculosis patients respond to a standardized short course of therapy, it has been shown that among a small group of patients with a poor response to therapy, some have low serum drug concentrations, suggesting that these low concentrations may contribute to the 2–5% of patients with adverse outcomes such as clinical failure or relapse. Most of the patients who had low drug concentrations did not respond to standard therapy but they were successfully treated by dose adjustment after drug monitoring. These findings demonstrate the need for an efficient means of measuring standard antituberculosis drug concentrations to facilitate early screening of therapeutic failure [1].

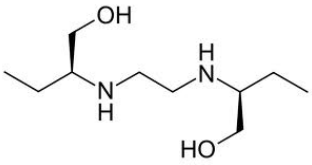

Figure 1. Chemical structure of ethambutol

Several methods involving high-performance liquid-chromatography (HPLC) with fluorescence (FL) [2] or with mass spectrometry (MS) [1, 3-6] detection have been reported for determination of ethambutol in biological matrix.

The HPLC-FL method requires derivatization of ethambutol after liquid-liquid extraction (LLE) in order to transform it in a fluorescent molecule [2]. However, both extraction and derivatization are time-consuming steps, increasing the cost of assay and can affect the recovery. The LC/MS or LC/MS/MS methods offer considerable advantages by their powerful performances: speed, selectivity, sensitivity and robustness. However, the sample preparation procedure by liquid–liquid extraction [4], precipitation-evaporation [3] or double precipitation [1] may complicate the analysis in terms of speed and recovery.

The aim of this work was to develop and validate a new rapid, simple, specific and efficient LC/MS/MS assay for the quantification of ethambutol in human plasma for application in therapeutic drug monitoring.

RESULTS AND DISCUSSION

Sample preparation

In LC/MS assays the sensitivity depends on MS detection mode, but the method involved in sample preparation may also influence the chromatographic background level and can generate matrix suppression effects. Usually an extraction step of analyte from matrix prior to analysis (SPE or LLE) has two main advantages: sample purification and sample pre-concentration. As stated before, the extraction step (either SPE or LLE) is laborious, time consuming

and usually needs an internal standard to compensate the extraction variability. The protein precipitation (PP) as sample processing method is desirable when one needs a high-throughput analysis and low sample-to-sample extraction variability. However, the two main advantage of SPE or LLE extraction mentioned before becomes drawbacks in case of PP: first - the sample is not really purified so matrix interferences or high background noise may appear; and second - the sample is physically diluted during precipitation, lowering the method sensitivity. Thus, the working parameters in developing an analytical method are related to the performance needed: the sample preparation time and costs, the method speed and sensitivity (Table 1).

Table 1. Analytical characteristics of reported LC/MS and LC/MS/MS methods for the determination of ethambutol in biological matrix:

Matrix	Pre-treatment/ extraction[a]	Mobile phase constituents[b]	Detection mode[c]	LOQ[d] (ng/mL)	Run time (min)	Reference
Human plasma	Double PP	Formic acid in MeOH and water	ESI+MS/MS, MRM (m/z 205→116)	50	4	**1**
Human plasma	PP-evaporation	TFA in MeOH and water	APCI+MS/MS MRM (m/z 205→116)	10	3.8	**3**
Human plasma	LLE	MeOH- water-formic acid	APCI+MS/MS, SRM (m/z 205→116)	10	3	**4**
Human plasma	PP	N/A	MS/MS	50	2.8	**5**
Human plasma	PP	N/A	MS/MS	200	N/A	**6**
Human plasma	PP	MeOH-ammonia in water	APCI+MS/MS MRM (m/z 205→116)	360	2	**Current report**

[a]LLE, liquid–liquid extraction, PP, protein precipitation; [b]ACN, acetonitrile; MeOH, methanol, TFA, trifluoracetic acid; [c]ESI, electrospray ionization; APCI, atmospheric pressure chemical ionization; SIM, selected ion monitoring; SRM, selected reaction monitoring, MRM, multiple reaction monitoring; [d]LOQ, limit of quantification, N/A not available

Following a single oral dose of 25 mg/kg of body weight, ethambutol attains a serum level of 2 to 5 μg/mL after 2 to 4 hours from administration [2,4]. Therefore the calibration curve range was adapted to the concentration range of the analyzed samples, namely 0.36-17.18 μg/mL. Because the current assay does not require a high sensitivity as in human pharmacokinetic studies, the PP extraction method becomes an attracting alternative to SPE or LLE due to the high speed and to the high reproducibility of the extraction.

In this method there were analyzed volumes of only 0.1 mL plasma by PP with methanol (0.3 mL) and direct injection into the chromatographic system from supernatant after centrifugation. A good sensitivity (LLOQ of 0.36 μg/mL) and absolute recoveries between 93-111% were obtained corresponding to the needs.

LC-MS assay

The analyte detection was optimized in order to achieve maximum sensitivity and specificity, by tuning the mass spectrometer in order to improve the ion transmission, stabilization and fragmentation in the ion trap. The ethambutol is ionized in APCI source by proton addition, giving a pseudo-molecular ion with m/z 205. After fragmentation, the protonated ethambutol is converted to ion with m/z 116 (Fig. 2).

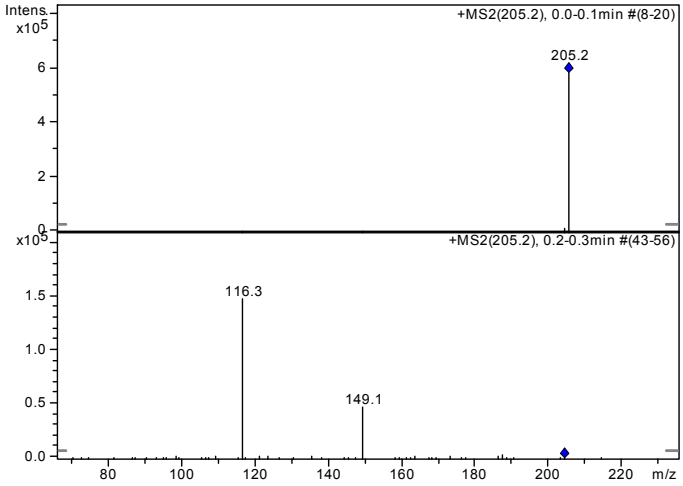

Figure 2. MS spectra of ethambutol – MS spectra (upper image), MS/MS spectra (lower image)

In order to obtain the needed specificity of analysis and a maximum signal-to-noise ratio (S/N) of analyte, we used the capability of Ion Trap mass spectrometer to do multiple stages isolation-fragmentation processes, that means MS^n analysis. This feature is specific to Ion Trap MS analyzers; other MS systems (single quadrupole, triple quadrupole, time of flight) do not have that capability. Thus, in the MS/MS stage, the m/z 205 ion obtained in the first MS was further fragmented and the obtained mass spectra was recorded (Fig. 2). The obtained m/z 116 ion is specific to ethambutol and was used for quantification. By using MS/MS detection mode, due to high specificity, the overall method sensitivity is increased significantly in comparison with MS detection.

The detection of ethambutol was carried out in multiple reaction monitoring (MRM). The extracted ion chromatogram (EIC) of m/z 116 from m/z 205 was analyzed. In the selected chromatographic conditions the retention time of ethambutol was 1.2 min.

Assay validation

The method was validated in accordance with international guidelines regarding bio-analytical method validation procedure [7,8,11]. A representative chromatogram of a blank sample, a human plasma spiked with ethambutol at LLOQ and a plasma sample from a patient treated with 1600 mg ethambutol/daily for 7 days, sampled after 2h from administration is shown in Fig. 3. No interfering peaks from the endogenous plasma components were observed at the retention time of ethambutol.

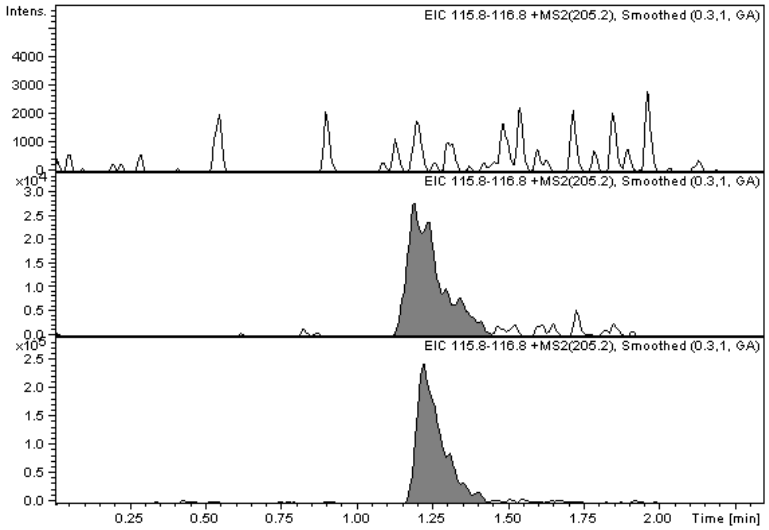

Figure 3. Representative chromatograms of a blank plasma (upper image); plasma spiked with ethambutol (R_T = 1.2 min) at lower limit of quantification (0.36 μg/mL), (middle image); and a plasma sample from a patient treated with ethambutol (lower image).

The calibration curves were linear over the concentration range of 0.36 –17.18 μg/mL in human plasma, with a good correlation coefficient (0.994). The LLOQ was 0.36 μg/mL. The obtained values for intra-day, inter-day precision and accuracy are shown in Tables 2 and 3, respectively. All values for accuracy and precision were within guidelines recommended limits (<15%) [7,8]. The absolute recovery values were between 93-111%.

Table 2. Intra-day precision, accuracy and recovery (n = 5) for ethambutol

$c_{nominal}$ µg/mL	Mean c_{found} µg/mL (± S.D.)	CV %	Bias %	Recovery % (± S.D.)
0.36	0.34±0.01	4.0	-4.2	110.4±4.9
1.07	1.02±0.07	6.6	-5.4	105.7±4.0
2.15	1.88±0.12	6.3	-12.3	111.2±7.1
7.16	7.01±0.44	6.3	-2.1	98.8±6.2

Table 3. Inter-day precision, accuracy and recovery (n = 5) for ethambutol

$c_{nominal}$ µg/mL	Mean c_{found} µg/mL (± S.D.)	CV %	Bias %	Recovery % (± S.D.)
0.36	0.38±0.03	8.8	6.4	104.0±15.7
1.07	1.00±0.02	1.8	-7.0	101.5±7.9
2.15	1.95±0.14	7.4	-9.3	99.2±3.9
7.16	7.12±0.56	7.9	-0.6	93.1±3.8

Method application

The validated method for determination of ethambutol in human plasma was successfully applied in therapeutic drug monitoring of ethambutol in patients with tuberculosis. Overlapping plasma profiles of ethambutol are presented in Fig. 4 (spaghetti plot representation). Although the dosing regimen was similar between patients, one can observe a great inter-individual variability of ethambutol plasma levels, this being the main reason for the necessity of therapeutic drug monitoring and pharmacokinetic studies for this drug.

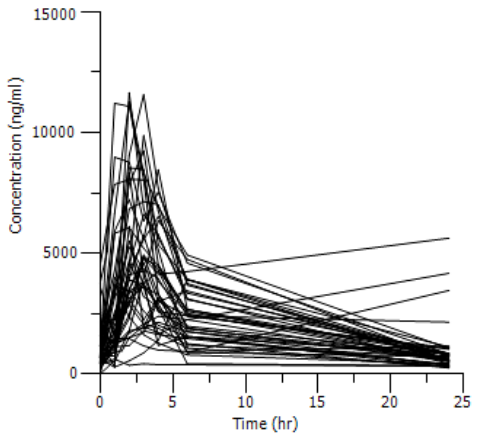

Figure 4. Overlapped plasma profiles of ethambutol administrated to patients with tuberculosis

CONCLUSION

Antimicrobial therapeutic drug monitoring could be an important tool in clinical practice if compliance is poor. Therefore, quantification of drug in plasma is an important issue in clinical practice to enhance efficacy and to reduce toxicity.

The developed LC/MS/MS assay is specific, accurate and not expensive. In terms of analysis time (throughput), this is fastest analytical method published to the date for analysis of ethambutol in biological matrix using a simple protein precipitation as plasma processing method. This new fast and specific method was successfully applied in therapeutic drug monitoring of ethambutol in patients with tuberculosis.

EXPERIMENTAL SECTION

Reagents

Ethambutol was reference standard from USP (Rockville, MD, USA). Methanol of gradient grade for liquid chromatography and 30% ammonia solution of analytical-reagent grade were purchased from Merck KGaA (Darmstadt, Germany). Bidistilled, deionised water pro injections was purchased from Infusion Solution Laboratory of University of Medicine and Pharmacy Cluj-Napoca (Romania). The human blank plasma was from healthy volunteers.

Apparatus

The following apparatus were used: 204 Sigma Centrifuge (Osterode am Harz, Germany); Analytical Plus and Precision Standard Balance (Mettler-Toledo, Switzerland); Vortex Genie 2 mixer (Scientific Industries, New York, USA); Ultrasonic bath Elma Transsonic 700/H (Singen, Germany). The HPLC system used was an 1100 series Agilent Technologies model (Darmstadt, Germany) consisting of one G1312A binary pump, an in-line G1379A degasser, an G1329A autosampler, a G1316A column oven and an Agilent Ion Trap Detector 1100 SL.

Chromatographic and spectrometric conditions

Chromatographic separation was performed on a Gemini (100 mm x 3.0 mm i.d., 3.0 μm) column (Phenomenex) under isocratic conditions using a mixture of methanol and 0.1% ammonia in water, 65:35 (v/v) as mobile phase at 40 ºC with a flow rate of 0.6 mL/min. In order to maintain the ESI source clean, the column effluent was diverted to waste for the first 0.8 minutes after injection. The detection of ethambutol was performed in multiple reaction monitoring (MRM) mode using an ion trap mass spectrometer with atmospheric pressure chemical ionization (APCI) source, positive ionization

(capillary 4000 V, nebulizer 50 psi (nitrogen), heater 400 ºC, dry gas nitrogen at 7 L/min, dry gas temperature 350ºC). The extracted ion chromatogram (EIC) of m/z 116 from m/z 205 was analyzed (MS/MS mode).

Standard solutions

A stock solution of ethambutol (1.193 mg/mL) was prepared by dissolving an appropriate quantity of ethambutol in methanol. A working solution (35.79 μg/mL) was prepared by appropriate dilution in human blank plasma. This solution was used to prepare seven plasma calibration standards with the concentrations between 0.36 and 17.18 μg/mL, respectively. Quality control (QC) samples of 1.07 μg/mL (lower), 2.15 μg/mL (medium) and 7.16 μg/mL (higher) were prepared by adding the appropriate volumes of working solutions to human blank plasma.

Sample preparation

Standards and plasma samples (0.1 mL) were deproteinized with methanol (0.3 mL). After vortex-mixture (10 s) and centrifugation (3 min at 12000 rpm), the supernatants (0.15 mL) were transferred in autosampler vials and 2 μL were injected into the HPLC system.

Method validation

The specificity of the method was evaluated by comparing the chromatograms obtained from the plasma samples containing ethambutol with those obtained from plasma blank samples.

The concentration of ethambutol was determined automatically by the instrument data system using peak areas and the external standard method. The calibration curve model was determined by the least squares analysis: $y = b + ax$, weighted ($1/y^2$) linear regression, where y - peak area of the analyte and x - concentration of the analyte (μg/mL).

The intra-day precision (expressed as coefficient of variation, CV %) and accuracy (expressed as relative difference between obtained and theoretical concentration, bias %) were determined by analysis of five different samples (n = 5) from each QC standards (at lower, medium and higher levels) on the same day. The inter-day precision and accuracy were determined by analysis on five different days (n = 5) of one sample from each QC standards (at lower, medium and higher levels). The lower limit of quantification (LLOQ) was established as the lowest calibration standard concentration with an accuracy and precision less than 20%. The relative recoveries (at LLOQ, lower, medium and higher levels) were determined by comparing the response of the spiked plasma with the response of standard solutions with the same concentration of ethambutol as plasma (n = 5) [7-10].

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