UFLC/MS method for the estimation of ethambutol in human plasma and its application in tuberculosis patients

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ABSTRACT :

Ultra Fast liquid chromatography – mass spectrometry method (UFLC-MS), for detection and quantification of the anti-tuberculosis drug, ethambutol (EMB), in human plasma is described here. The method uses a single Quadrapole mass spectrometer equipped with positive electrospray ionization detector, linked with Shimadzu 20AD ultrafast LC system. Ethambutol was extracted from human plasma (100 µl) through a single step, simple and straight forward acetonitrile deproteinization method, achieving 98 - 102 % analyte recovery. Short C_{18} column (50 mm x 2.1 mm) was used with isocratic mobile phase system consisting of 80% aqueous acetonitrile and 0.5% formic acid as additive. Selective ion monitoring (SIM) mode was selected with EMB m/z 205.3 in positive ion mode (MH⁺). This method achieved wide range of detection from microgram to nanograms of EMB (Linearity R value = 0.9999), with great precision and accuracy. The limits of quantification and detection were achieved upto 19.18 ng/ml and 5.78 ng/ml respectively. This method was applied successfully to determine EMB in plasma samples from TB patients who were receiving EMB along with other anti-TB drugs.

Keywords: Ethambutol–LCMS method – Plasma-Tuberculosis.

INTRODUCTION:

Ethambutol (EMB) is one of the four drugs in the first line anti- tuberculosis (TB) treatment regimen. It is used to protect against rifampin resistance in the event of preexisting resistance to isoniazid [1]. Ethambutol is a bacteriostatic agent with a mechanism of action that has been suggested to occur by inhibition of mycobacterial cell wall synthesis. Ethambutol has been reported to have an oral bioavailability of approximately 80 % and concomitant food intake decreases the rate of absorption but not the extent of absorption [2, 3]. The systemic concentrations of EMB in TB patients have been described previously [4-9]. Also, adverse effects like optic neuritis, arthralgia, hyperuricaemia, vertical nystagmus, and peripheral neuropathy were associated with use/misuse of EMB. Thus, accurate monitoring of plasma EMB levels in TB patients is mandatory for better clinical management. In this regard, several analytical methods by HPLC and LCMS have been developed for the detection of EMB in biological fluids [10 – 14]. These methods facilitate the detection of etham butol either alone or in combination with other anti-TB drugs. All these methods have their own advantages and disadvantages such as cost effectiveness, analysis time, sensitivity limits, accuracy and area of suitability. In the present paper, we have described a method for estimation of EMB with high accuracy and precision, cost effective extraction, high-throughput compatible analysis time, and good detection range, suitable for application in both adult and pediatric populations. The novelty of this method is, it require smaller volume of plasma samples, shorter run time which facilitate high throughput sample analysis, using isocratic chromatographic conditions, and with no ion suppression agents as LCMS solvent additives.

EXPERIMENTAL:

Instrumentation and Reagents

All solvents and chemicals were of HPLC and LCMS grade (Acetonitrile and formic acid) and were purchased from Merck (India). Ethambutol pure powder was purchased from Sigma (USA).

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Conflict of interest: Authors reported none

Mass spectrometry grade ultrapure water was obtained from in-home MilliQ water purification system. All solvents were filtered through 0.22 μm nylon membrane filters and degassed with ultrasonicator prior to the analysis. Pooled human plasma was obtained from the blood bank, Chennai, India.

Chromatographic conditions

The Liquid chromatography system used was Shimadzu UFLC 2020 series equipped with binary LC20AD pumps, Shimadzu SIL-HTc autosampler with PDA-visible detector. Phenomenex Gemini C18 column (diameter of 50 mm x 2.0 ID x 5 μm pore size) with particle size of 110 A, equipped with compatible guard column (Phenomenex) was used for the separations. The isocratic mobile phase used for the analysis was acetonitrile: water (80:20) with 0.5% formic acid (pH 2.4) with the flow rate of 0.75 ml/min. The mass spectrometer used for the detection was Shimadzu 2010EV equipped with electrospray ionization source (ESI Detector) and a nitrogen generator (Peak Scientific, Japan). ESI detector voltage was set to 1.5 kV (positive mode) and nebulizer gas flow was set to 1.5 ml/min. Mode of detection was set to selective ion monitoring (SIM) at m/z 205.

Method

All EMB standards were prepared in pure LC grade milliQ water. Initially, a stock solution of 1 mg/ml EMB was dissolved in LC grade milli-Q water. Calibration curve standards of concentrations 0.15625, 0.3125, 0.625, 1.25, 2.5, and 5.0 μg/ml were prepared by diluting the stock using acetonitrile. Calibration curves in human plasma were prepared by adding appropriate volumes of EMB stock solution to 100 μl blank human plasma and serially diluted with blank plasma to yield EMB concentrations of 0.3125, 0.625, 1.25, 2.5, 5, μg/mL. Another set of standards of lower concentrations, namely, 40, 80, 160, 320 and 640 ng/ml were also prepared similarly in plasma.

Sample preparation

Ethambutol was extracted from plasma by protein precipitation using acetonitrile. 100 μl of plasma was mixed with 50 μl of acetonitrile and vortexed for 4 minutes, followed by centrifugation at 8000 rpm for 10 minutes. Further, 50 μl supernatant was transferred into a clear vial and 50 μl of acetonitrile was added to it and vortexed for 2 minutes, followed by centrifugation. The supernatant was loaded in micro-inserts for UFLC injection. An aliquot of 3 μl was injected through auto-sampler for analysis.

METHOD VALIDATION

Linearity

Two sets of calibration curves to test the linearity of assay were constructed using EMB standards prepared in plasma ranging from 0.3125 – 5 μg/ml (set 1) and 40 -640 ng/ml (set 2). Five calibration curves were assessed for their repeatability on separate days. Evaluation of linearity was done by plotting the actual concentration vs concentration of response. Linear regression analysis (linear equation y = m X+C) of calibration data was applied to calculate the slope (m), intercept (c), correlation coefficient (r >=0.999) and coefficient of determinants (R2 >=0.99).

Accuracy and precision

Accuracy and precision were evaluated at three different concentration levels of EMB namely, (low (0.625 μg/ml), medium (1.25 μg/ml) and high (5 μg/ml)) in triplicate on same day (intra-day) and on different days (inter-day). Precision was expressed as % CV. A total of 5 intra- and inter day runs were performed.

Specificity

Interference from endogenous compounds was investigated by analysing blank plasma samples obtained from different batches. Interference from certain anti-tuberculosis drugs such as rifampicin (RMP), isoniazid (INH), pyrazinamide (PZA), and streptomycin (SM) at high concentrations (10 μg /ml) were also evaluated.

Extraction Recovery

The recovery of EMB from human plasma was estimated by spiking known low (0.625 μg/ml), medium (1.25 μg/ml) and high (5 μg/ml) concentrations were subjected to extraction according to the method described above. The same concentrations were spiked in pure solvent. Extraction recovery was estimated using the formula; Percentage recovery = (peak area of EMB concentration extracted from spiked plasma/peak area of EMB concentration directly added to extraction solvent) ×100)

Limit of Detection and quantitation

The limit of detection (LOD) and lower limit of quantitation (LLOQ) were calculated using slope analysis of 40 – 640 ng/ml of EMB.

Stability

Plasma samples spiked with EMB was kept at room temperature and values were evaluated within 24 hours at 25° C for short term stability. Long term stability was tested by analyzing EMB spiked plasma samples after storage for 10 days at -10° C. The stability of the sample after preparation loading into the auto sampler (4° C) was also tested. The stability was expressed as change in peak area between each analysis (% change bias).

Pharmacokinetic applications

Plasma samples were collected from eighteen adult TB infected patients, who were receiving EMB (1200 mg) as part of anti-TB treatment regimen for a minimum period of 15 days. These patients were participating in an ongoing pharmacokinetic study. Blood samples

were drawn at pre - dosing and at 2, 4, 6, and 8 hours after directly observed drug administration in heparin vacutainers. The blood samples were centrifuged immediately and plasma stored at -20ºC until assay. The plasma samples were subjected to LCMS analysis for estimation of EMB, as described previously.

RESULTS AND DISCUSSION:
Optimization of chromatographic conditions

Different chromatographic conditions, columns and mobile phases were tested for optimizing chromatographic conditions. The C_{18} columns such as Shim-pack VP-ODS 250 x 2, Shim-pack VP-ODS 150 x 2, and Gemini 50 x 2 were used, in which Gemini C_{18} (50 X 2) showed good peak shape and short retention time with good resolution without any peak tailing. Further, we tried different mobile phases such as formic acid (in varying percentages from 0.1 – 0.6%), Ammonium acetate and ammonium formate. Formic acid at 0.5% showed satisfactory peak shape, good protonation in ESI detector, and retention time within one minute. Also, at different isocratic organic modifier percentages, 80% acetonitrile in water showed quick retention of EMB without losing peak resolution. Among different flow rates (0.1 ml/min to 1 ml/min), 0.750 ml/min showed no peak tailing with short retention time to allow high sample throughput analysis. Although, EMB was eluted at an average retention time of 0.7 minutes, total run time was set to three minutes to allow complete column re-equilibrium between successive injections. The representative total ion chromatogram (TIC) of EMB is given in Figure 1. The analysis was run in selective ion monitoring mode (SIM), the EMB peak was found well resolved with baseline. The co-spiked anti-TB drugs, PZA, INH, RMP and SM did not interfere in the assay.
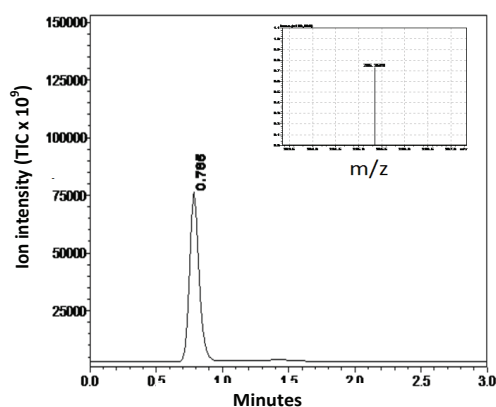

Fig 1: Representative total ion chromatogram (TIC) of EMB detected through positive electrospray ionization mass spectrometery

Two different ranges of EMB concentrations in plasma were tested for linearity. Good linearity was achieved at both lower concentrations of EMB (40 - 640 ng/ml as well as higher concentrations (0.3125 - 5 µg/ml plasma). The accuracy, precision and linearity of EMB are summarized in Table I.

	Spiked Conc (ng/ml)	Observed Conc (Mean) (ng/ml)	Accuracy (%)	Precision (%RSD)
Linearity in Low concentrations	40	39.1168126	97.792	4.073
	80	78.860	98.575	2.632
	160	160.570	100.356	0.550
	320	322.793	100.873	2.205
	640	638.656	99.790	1.316
	Line (mx+c)	y = 1x + 0.0001		
	r value	0.99997		
	R²	0.9999		
	Spiked Conc (µg/ml)	Observed Conc (Mean) (µg/ml)	Accuracy	Precision (%RSD)
Linearity in High concentrations	0.3125	0.305	97.636	6.247
	0.625	0.635	101.641	1.341
	1.25	1.262	101.016	0.373
	2.5	2.476	99.072	0.556
	5	5.007	100.151	0.157
	Line (mx+c)	y = 1x + 1E-07		
	R²	0.9999		
	r value	0.99996		

Table I: Intermediate precision, accuracy and linear regression parameters of EMB determination in human plasma by UFLC-MS.

At low concentrations (40-640 ng/ml), the accuracy ranged from 98 to 101% while precision ranged from 0.5 to 4%; The correlation coefficient (R2) value was 0.9999 and the coefficient of determinants (r) was 0.9999. At higher standard concentrations (0.3125 -5.0 µg/ml plasma), accuracy ranged from 97.6 to 101.6%, while precision ranged from 0.15 - 6.24%. As observed in low concentrations, both correlation coefficient (R2) and Coefficient of determinants (r) were observed as 0.9999. The representative graphs showing both lower and higher concentrations indicating linear regression analysis were given in Figure 2 and 3.
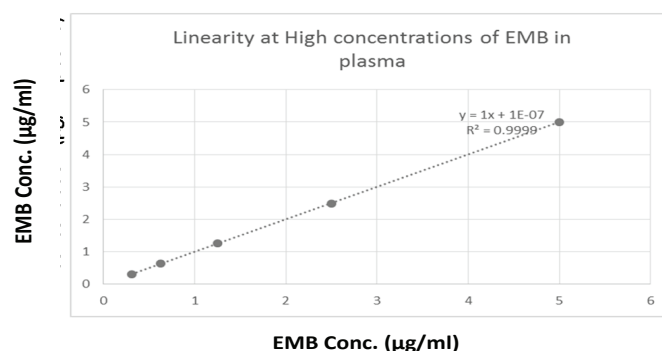

Fig 2: Linear response of spiked plasma EMB at lower concentrations ranging from 40 – 640 ng/ml
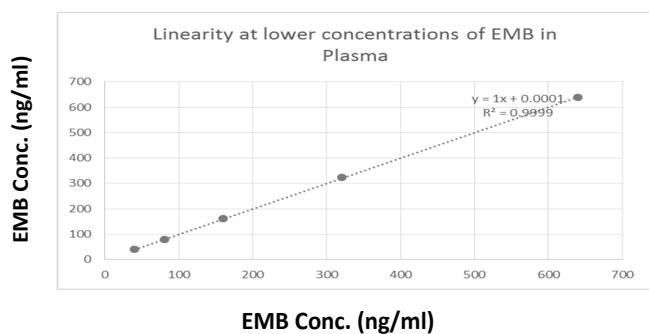
Fig 3: Linear response of spiked plasma EMB at higher concentrations ranging from 0.3125 – 5.0 µg/ml

Stability of EMB spiked in plasma was evaluated in three different conditions viz., short term stability at 25º C for 24 hours, long term stability (at -10º C for 10 days) and auto-sampler stability (for 48 hours at 4º C).

The plasma EMB concentrations didn't get affected when kept at different storage conditions and % CV ranged 0.27 to 5.32. As per the ICH guidelines, the stability in three different conditions were tested for EMB to validate any compound loss during chromatographic processes. It was observed that, no significant variation (in terms of % Change bias) was observed between three groups. At short duration storage in room temperature, stability values of EMB in plasma were at acceptable levels of < 10% change bias. Similar trend was observed in autosampler stored samples and plasma samples stored at deep freezer conditions (-10 ^{0}C). In all these studies, peak shape and retention time of EMB does not show any variations with different storage conditions. The results of stability data are given in Table.

EMB concentration in plasma	Intra-day				Inter-day			
	Mean (n=5)	SD	Accuracy	Precision (%RSD)	Mean (n=5)	SD	Accuracy	Precision (%RSD)
Low (0.625 µg/ml)	0.633	0.003	101.439	0.304	0.632	0.001	101.266	0.240
Medium (1.25 µg/ml)	1.191	0.021	95.283	2.108	1.183	0.010	94.687	0.890
High (5 µg/ml)	4.977	0.012	99.545	1.283	4.972	0.006	99.454	0.129

Table II: Intra-day and inter-day accuracy and precision

The EMB spiked plasma samples were extracted and quantified to check intra- and inter-day accuracy and precision. Three representative concentrations (low, medium and high) of EMB were analyzed on a single day and between batches analysed at different days of interval showed good accuracy and precision. % RSD values of intra-day analysis of EMB at three concentrations (0.625, 1.25 and 5 µg/ml) are 0.304, 2.108 and 1.283 respectively. The % RSD of precision between days (inter-day) were 0.2406, 0.8908 and 0.1290 for the corresponding three concentrations The results are given in Table II.

EMB Conc	Short duration (at 25 0C for 24 hours			Long duration (at -100C for 10 days)			Auto-sampler (at 4 0C for 48 hours)		
	Observed (Mean)	%CV	% Change bias	Observed (Mean)	%CV	% Change bias	Observed (Mean)	%CV	% Change bias
Low (0.625 µg/ml)	0.636	4.48	-6.345	0.638	1.46	2.076	0.638	0.563	o.797039
Medium (1.25 µg/ml)	1.306	0.54	0.773	1.222	5.32	7.529	1.183	2.733	-3.86534
High (5 µg/ml)	4.960	0.43	-0.615	4.983	0.42	0.606	4.976	0.270	-0.38283

Table III: Stability of EMB during different assay conditions

The mean extraction recovery percentages of EMB spiked in plasma at three levels of concentrations, low (0.625 µg/ml), medium (1.25 µg/ml) and high (5 µg/ml), were 102.0, 99.0 and 99.5 respectively. This method requires small volume of sample (100 µl of plasma) and is highly suitable for pediatric samples. The recovery data is given in Table IV.

EMB Conc in plasma	Mean Recovery (µg/ml)	%CV	Average Recovery %
Low (0.625 µg/ml)	0.637	1.02	102.04
Medium (1.25 µg/ml)	1.237	0.98	98.97
High (5 µg/ml)	4.973	0.99	99.47

Table IV: Extraction recovery of EMB

Both LOD and LLOQ were calculated using slope analysis of linearity data. The LOD and LLOQ were 5.755 ng/ml and 19.183 ng/ml respectively.

The present method was applied to estimate EMB concentrations in the pharmacokinetic study conducted in TB patients.
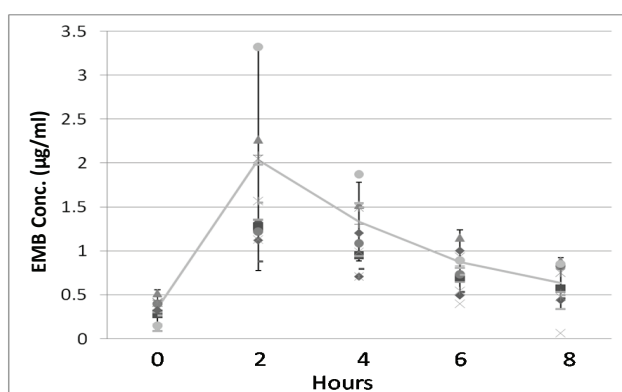

Fig 4: Pharmacokinetic profile of EMB concentrations in patient samples (Line indicates the median values of the measured concentrations among patients).

The peak plasma EMB concentration was attained at fourth hour after drug administration, ranging from 0.884 - 3.882 µg/ml with a average of 2.038±1.259 µg/ml. Since this method has good range of reliable quantification with high sensitivity, it can be applied to both pediatric and adult samples. Although, similar works on the quantification of EMB in human plasma were reported previously, in this method, we have used no ion pairing additives such as trifluoroacetic acid which may hinder the sensitivity of the mass spectrometry instrument through ion suppressing.

CONCLUSION

Mass spectrometry has advantages of being specific and accurate for estimation of EMB in plasma. Several methods have been reported for the detection of EMB in human plasma. Our method has advantages such as retention time of 0.7 mins for EMB and uses micro volume sample (100 µl). The method described has the advantage of accurate detection in short run time, thereby facilitates high throughput analysis of patient samples in short duration. Also, the extraction procedure is quite simple, with high level of extraction efficiency. This can be applied in any laboratory with minimum sample preparation facilities. Also, this method has an advantage of free from ion suppression agents, which dramatically reduce the specificity of the instrument during continuous use. The minimum sample requirement makes this method highly suitable for pharmacokinetic studies both in adult and children.

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