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Development and Validation of an UPLC Method for Quantification of Ethambutol in Rat Plasma

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- 1. Introduction**
- 2. Materials and Methods**
 - 2.1. Reagents
 - 2.2. Apparatus
 - 2.3. Chromatographic conditions
 - 2.4. Plasma samples
 - 2.5. Preparation of standards and samples
 - 2.5.1. *Stock solutions*
 - 2.5.2. *Procedure for derivatization of EMB*
 - 2.5.3. *Preparation of the calibration curve in plasma*
 - 2.5.4. *Preparation of the quality control samples*
 - 2.6. Extraction of EMB from plasma and its derivatization
 - 2.7. Assay validation
 - 2.7.1. *System suitability*
 - 2.7.2. *System Selectivity*
 - 2.7.3. *Calibration curve, LOQ, and LOD*
 - 2.7.4. *Intraday and inter-day precision and accuracy*
 - 2.7.5. *Recovery*
 - 2.8. Stability at various storage conditions
 - 2.8.1. *Processed sample stability*
 - 2.8.2. *Autoinjector stability*
 - 2.9. Pharmacokinetic Studies
 - 2.10. Data Analysis
- 3. Results**
 - 3.1. Assay validation
 - 3.1.1. *System suitability*
 - 3.1.2. *System Selectivity*
 - 3.1.3. *Calibration curve, LOQ, and LOD*
 - 3.1.4. *Intraday and inter-day precision and accuracy*
 - 3.1.5. *Recovery*
 - 3.2. Stability at various storage conditions
 - 3.3. Pharmacokinetic Study
- 4. Discussion**
- 5. Conclusions**

ABSTRACT

Background: Effective monitoring of antitubercular drugs including ethambutol (EMB) is important for control of multiple drug resistance. Development of fast, easy and inexpensive methods for detection of low concentrations of EMB, especially near its minimum inhibitory concentration, is important. **Results:** UPLC method for estimating EMB in rat plasma is developed and validated. The method uses small injection volume (1 μ l) and elutes EMB at 3.18 min with 0.070 μ g/ml and 0.250 μ g/ml as limits of detection and quantification, respectively. The intra and inter-day accuracy was 106.01-108.82% and 107.80-109.00%, respectively with precision of <5% in both the cases. Mean extraction recovery of EMB from rat plasma was >97% at three concentration levels, establishing consistency and reproducibility (n=6) of the method. EMB was found to be stable in plasma under different storage and processing conditions. **Conclusion:** Study reports a highly sensitive and specific isocratic UPLC method for rapid quantization of EMB for use in pharmacokinetics and therapeutic drug monitoring.

1. Introduction

Tuberculosis (TB) is a dreaded bacterial infection which has been known to mankind since ancient age and remains the largest health problem, worldwide. World Health Organization recommended the fixed-dose combinations for the treatment of tuberculosis, in consideration of resistance effects with monotherapy. Four antitubercular drugs (ATDs) rifampicin, isoniazid, pyrazinamide and ethambutol (EMB) hydrochloride are recommended for treatment of TB, to overcome development of drug resistant with monotherapy. EMB, D-(*R, R*)-*N, N'*-ethylenebis (2-amino-butan-1-ol) dihydrochloride, (Figure 1) is a synthetic oral chemotherapeutic agent with significant activity against actively growing, intracellular and extracellular microorganisms of the genus *Mycobacterium* and is a valuable adjunct for the management of TB.

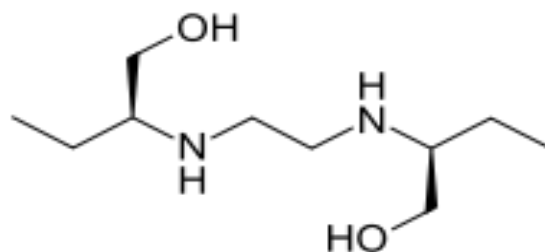


Fig. 1 Chemical Structure of Ethambutol

In contrast to rifampicin, isoniazid or pyrazinamide which are basically eliminated by the liver, EMB is renally excreted, with approximately 70% of the administered dose being recovered unchanged in urine⁽¹⁾. It may be because of this reason that in contrast to the other ATDs, it is not hepatotoxic but ocular toxicity is often associated with its use⁽²⁾. Though not recommended usually as a monotherapy, it is a drug of choice for those patients on ATDs therapy, who start showing signs of hepatic failure. A non-hepatotoxic regimen consisting of streptomycin, ethambutol and a fluoroquinolone is especially suggested for 18–24 months with or without 1 or 2 hepatotoxic ATDs^(3, 4) in such patients.

Monitoring EMB concentration and dose adjustment is helpful in patients that show poor response to treatment. At a time when many scientists have reached separation barriers with conventional HPLC, UPLC presents the possibility to extend and expand the utility of chromatography. In this technique, sub-micron particles are used as the stationary phase, and it operates at elevated mobile phase velocities with improved resolution, sensitivity and speed of analysis^(5, 6). We have recently reported on an efficient, selective and a highly sensitive UPLC

method for simultaneous determination of all-trans retinoic acid and cholecalciferol in plasma samples after oral and subcutaneous administration⁽⁷⁾.

A detailed survey of literature for methods used for quantification of EMB revealed microbiological assay⁽⁸⁾, HPLC/UV⁽⁹⁻¹⁵⁾, liquid chromatography/ mass spectrometry (LC/MS) and liquid chromatography/ mass spectrometry/ mass spectrometry (LC-MS/MS)⁽¹⁶⁻²¹⁾ as the methods of choice in pharmaceutical dosage forms alone or in combination with other ATDs and in plasma samples. Gas liquid chromatographic methods report use of capillary columns involving a series of sample preparation steps and the need for an internal standard⁽²²⁾.

The reported HPLC method, using HPLC coupled with mass spectrometry detection has high sensitivity but the process involves solid-phase extraction (which is a long tedious and costly process) while others using fluorescence or UV detection have low sensitivity or long analysis time. However we did not find any UPLC-PDA detection method for its quantification in plasma samples.

The aim of this study was to develop and validate a sensitive, specific and reproducible UPLC method for EMB quantification in rat plasma, using phenethyl isocyanate (PEIC) as a derivatizing agent, as EMB does not possess any chromophore. The method should have a low run time involving simple sample preparation and extraction procedure. At the same time, it is important that the method is efficient for analyzing large number of plasma samples obtained for pharmacokinetic or therapeutic monitoring studies.

2. Materials and Methods

2.1. Reagents

EMB dihydrochloride was a gift sample obtained from Panacea Biotech, Lalru, Punjab, India and Lupin Pharma, India. PEIC was purchased from Sigma-Aldrich, Germany. All other solvents and reagents were of HPLC analytical grade and were obtained from Merck (Darmstadt, Germany). Milli-Q Reagent Water was used in the study. Acetonitrile and methanol HPLC grade manufactured by Merck-Schuchardt, Hohenbrunn, Germany, was purchased from local vendors. HPLC grade water was produced by a Milli-DI system by Millipore (Billerica, Massachusetts, USA). Syringe filters were purchased from Waters India Pvt Ltd.

2.2. Apparatus

The instrument used was an ACQUITY UPLCTM H-Class system (Waters, Milford, MA, USA) composed by quaternary solvent manager, sample injector, column heater, and photo-diode array

(PDA) detector. Analytes were separated at 25°C using Empower 2[®] Pro software to analyze the results.

2.3. Chromatographic conditions

Chromatographic separation was performed on a UPLC BEH RP 18 (2.1mm x 50mm, 1.7 µm) column with a C18 guard and protected by an in-line filter. The optimized chromatographic conditions consisted of methanol and water in the ratio of 70:30 as mobile phase at a flow rate of 0.1 ml/min with total run time of 5 minutes. Most importantly the volume of injection is set at 1 µL. To establish the optimal wavelength of analysis, an absorption spectrum was carried out from 195 to 400 nm using a UPLC system connected to a diode array detector. The UV spectrum exhibited a peak of absorption at 205 nm and the analysis was thus performed at λ_{max} of 205.

2.4. Plasma samples

Blood samples were obtained from the rats in heparinized tubes and were centrifuged at 5000 rpm for 10 min at 4°C, to separate plasma, which was then aliquoted in 1.5 ml tubes and stored at -20°C until further analysis.

2.5. Preparation of standards and samples

2.5.1. Stock solutions

Concentrated stock solution of 1 mg/ml of EMB was prepared in acetonitrile containing 3% triethylamine (to allow its solubilisation) and was further diluted 10 times to give a working solution of 100 µg/ml of EMB. The stock solution of PEIC was prepared by dissolving 2 mg of PEIC per milliliter of acetonitrile. The working solutions were obtained by diluting PEIC stock solutions in the ratio of 1:4 with acetonitrile to give a concentration of 400 µg/ml. Plasma controls were prepared by spiking plasma with known quantity of EMB working solution.

2.5.2. Procedure for derivatization of EMB

To 400 µL volume of EMB working solution, 100 µL of PEIC working stock was added. The two solutions were mixed with gentle vortexing for 5 minute at room temperature. In case of plasma samples, volume of all supernatants was made to 400 µL with acetonitrile to which was added 100 µL PEIC reagent making the final volume of all samples to 500 µL. Latter was filtered through 0.22 µm filter and injected into the UPLC system⁽¹⁴⁾.

2.5.3. Preparation of the calibration curve in plasma

A six point calibration curve (0.25-30.00 µg/ml) was prepared by spiking blank plasma samples with working solution of EMB. Plasma (without EMB) was used as blank, to confirm the

absence of any interfering peaks at times corresponding to the retention time of EMB. The areas under the curve for various concentrations were used to construct a calibration curve, following linear regression of the data. Although it is recommended that the volume of spiking solution should not exceed 5% of the sample volume however presently, the spiking volume exceeded this limit for higher concentrations, as the calibration curve was constructed for a very wide range.

2.5.4. Preparation of the quality control samples

High quality control (HQC), medium quality control (MQC) and low quality control (LQC) samples were prepared by diluting the working solution of EMB to result in 2, 5, and 10 µg/ml of EMB. Solutions were prepared and used the same day.

2.6. Extraction of EMB from plasma and its derivatization

For the extraction of EMB from plasma, methanol was used as a protein precipitating agent. A 200 µl of methanol was added to 100 µl^(19, 23) of i) standard solution (Free EMB), ii) quality control samples, iii) blank rat plasma, iv) EMB spiked rat plasma samples, and v) pharmacokinetic plasma samples after oral administration of EMB to rats. Supernatants, of the samples vortexed at slow speed for 5 min and centrifuged at 10,000 rpm for 10 min at 4°C, were separated in 2.5 mL tubes. Volume of the supernatant was made to 400 µL with acetonitrile and it was then derivatized with 100 µl of PEIC solution following the procedure detailed above in section 2.5.2. The resulting mixture was filtered through 0.22 µm nylon filter and injected into UPLC system.

2.7. Assay validation

For assay validation following parameters were evaluated: suitability, selectivity, calibration curve, limit of quantification (LOQ), limit of detection (LOD), intra and inter-day accuracy and precision, recovery, and stability.

2.7.1. System suitability

System suitability was performed by determining the AUC for the MQC sample injected into the UPLC before the start of each analytical run and its comparison with the average AUC value obtained for the MQC, upon repetitive injections (n=6).

2.7.2. System selectivity

Interferences from endogenous compounds were investigated by analyzing six different blank plasma samples. Blank plasma samples (n=6) were prepared according to the sample preparation

procedure described above and screened for the presence of any interfering peaks corresponding to the retention time of EMB.

2.7.3. Calibration curve, LOQ, and LOD

The calibration curves were obtained using concentration levels ranging from 0.25 to 30 µg/ml for spiked plasma samples and plotting the peak areas of EMB versus concentrations. These values (LOD and LOQ) 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.

2.7.4. Intraday and inter-day precision and accuracy

Intraday precision and accuracy were obtained by analyzing three replicates of QC samples on the same day. Inter-day precision and accuracy were assayed using three determinations for each QC concentration, on three different days. Accuracy was calculated as deviation percent of the mean from the true value. Precision was expressed as the relative standard deviation (R.S.D. %) at each QC concentration.

2.7.5. Recovery

The recovery was assessed by comparing the peak area obtained for different QC samples with those obtained for corresponding spiked plasma samples extracted suitably (n=6).

2.8 Stability at various storage conditions

2.8.1 Processed sample stability

In assessing the processed sample stability, the LQC and HQC samples were quantified 6 hours after they were first analyzed (0 time reading). Samples were stored at 10°C during this period, considering that the drug may not undergo any enzymatic degradation or chemical change, when stored at room temperature.

2.8.2. Autoinjector stability

The processed replicates of LQC and HQC samples were stored in the autoinjector at 4°C after analysis and were re-injected after 24 hours. Stability of QC samples was determined in percentage by comparing the peak area values obtained at 24 hours with the values obtained for the same samples when injected fresh (zero time).

2.9. Pharmacokinetic studies

For *in vivo* pharmacokinetic studies, wistar rats weighing 160–180 g were used. The protocol was duly approved by the Institutional Animal Ethics Committee (IAEC) of Panjab University,

Chandigarh, India (vide letter no. IAEC/156, dated 25/8/2011). The animals ($n = 6$) were administered 125 mg/kg BW of EMB as a solution in water. The blood samples (0.5 ml) were withdrawn from retro-orbital plexus and collected into heparinized microcentrifuge tubes (containing heparin equivalent to 50 μ L per ml blood), at different time intervals. Plasma was separated by centrifuging the blood samples at 10,000 rpm for 10 min at 4°C. After centrifugation, the obtained plasma was stored at -20°C until analysis as described under the extraction procedure (section 2.6). All animals were sacrificed at the end of the study.

2.10 Data analysis

The pharmacokinetic parameters were calculated based on a non-compartmental model. The area under the concentration–time curve from time zero to time t (AUC_{0-t}) was calculated using the trapezoidal method. Peak concentration (C_{max}) and time of peak concentration (T_{max}) were obtained directly from the individual plasma concentration–time profiles. The area under the total plasma concentration–time curve from time zero to infinity was calculated by: $AUC_{0-\infty} = AUC_{0-t} + C_t/K_e$, where C_t is the drug concentrations observed at the last time and K_e is the apparent elimination rate constant obtained from the terminal slope of the plasma concentration–time curve after logarithmic transformation of the plasma concentration values and application of linear regression. The data obtained from pharmacokinetic parameters were analyzed statistically using Win-Nonlin software.

3. Results

3.1 Assay validation

3.1.1 System suitability

The system was found to be suitable for the determination of EMB under the optimized chromatographic conditions. Average peak area per injection was determined for HQC and standard deviation was found to be $\leq 2.7\%$. As per USFDA bio-analytical method validation guidelines, 2001, the precision for the analytical procedure should be high and the value must not exceed 5%⁽²⁴⁾.

3.1.2. System Selectivity

High specificity of the developed method was confirmed by the absence of any interfering peaks at the retention time of EMB. Further, chromatograms obtained from spiked plasma samples were found to be specific for EMB as shown in Figure 2 and 3. The retention time was 1.85 min for PEIC and 3.12 for EMB in plasma samples.

3.1.3. Calibration curve, LOQ, and LOD

Linear standard curve, as illustrated by correlation coefficients greater than 0.999 was obtained at the concentration range between 0.25-30 $\mu\text{g/ml}$ with an equation of line: $y = 21,645x - 4761.1$. Standard curve prepared and analyzed on the same day or on different days were found to have similar slopes and y-axis intercepts.

The LOD and the LOQ for plasma analysis were found to be 0.07 and 0.25 $\mu\text{g/ml}$, respectively.

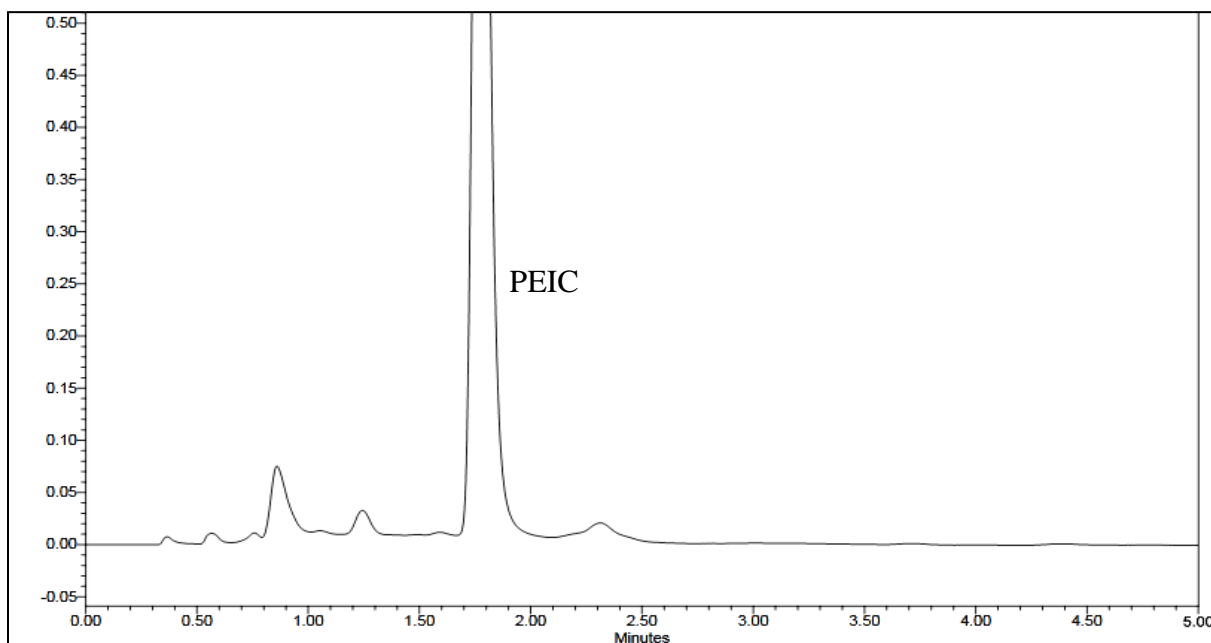


Figure 2a: Chromatogram for blank plasma sample with PEIC

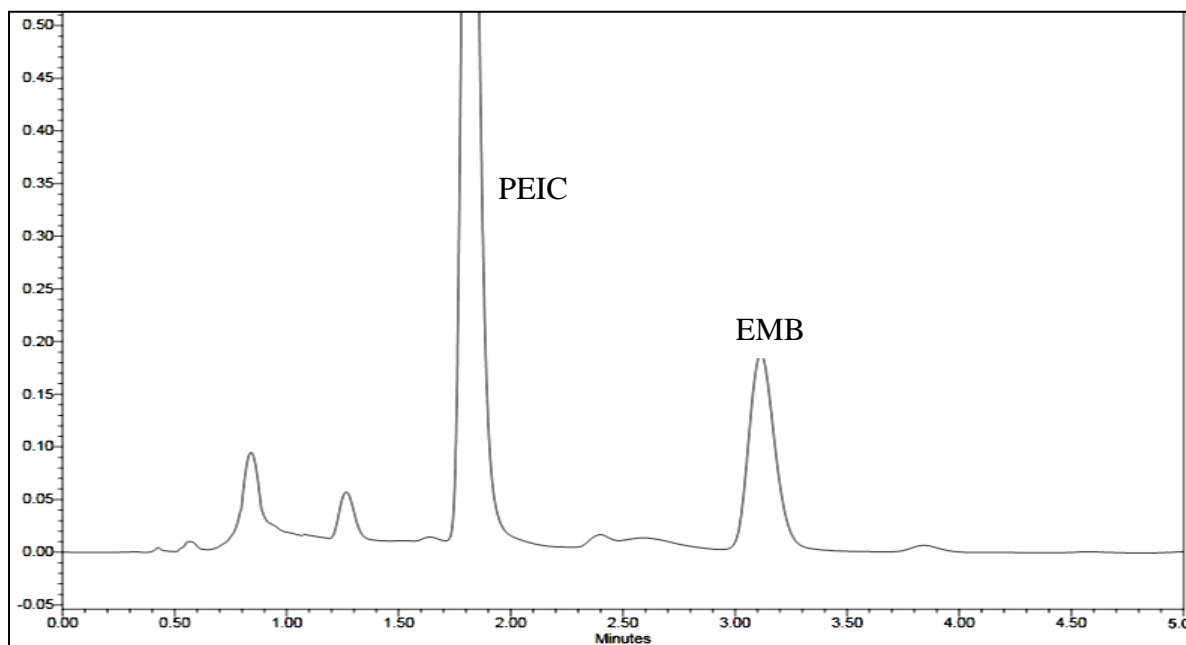


Figure 2b: Chromatogram for plasma sample spiked with EMB (20 $\mu\text{g/mL}$)

3.1.4 Intraday and inter-day precision and accuracy

The intra and inter-day precision and accuracy of the method are presented in Table 1. Coefficients of variation (CVs) were lower than 10% at the evaluated concentration range.

Table 1. Intraday and inter-day precision and accuracy of Ethambutol in rat plasma

Nominal Conc. (µg/ml)	Obtained Conc. (µg/ml) (mean ±SD)	Precision (%)	Accuracy (%)
Intraday			
2.00	2.12±0.08	3.77	106.00
5.00	5.67±0.28	4.85	113.41
10.00	10.88±0.39	3.58	108.83
Inter-day			
2.00	2.18±0.09	4.13	109.00
5.00	5.29±0.25	4.73	105.86
10.00	10.78±0.31	2.88	107.88

3.1.5. Recovery

Recovery (n=6) for EMB was found to be 94.5± 2.4 %, 101.1± 1.4 % and 96.8± 2.4% for LQC, MQC, HQC samples, respectively.

3.2 Stability at various storage conditions

Solutions of EMB were found to be stable for up to 7 days at -20°C, with their mean % stability ranging between 97.4% - 99.6%. The acceptance criterion for % accuracy for stability samples is ±15%, thus the values were sufficiently within the limits. Solutions of EMB were again found to be stable for up to 24 hours in the autosampler at 4°C and at 10°C for 6h (table 2).

Table 2: Stability of EMB under various storage conditions (n=6)

Parameters	LQC (2 µg/ml)	HQC (10 µg/ml)
	% Accuracy	% Accuracy
Long term stability at -20°C for 7 days	96.2± 1.6	98.4 ± 1.8
Processed sample stability for 6 hrs	99.1±2.5	101.5 ±2.9
Autosampler stability for 24 hrs	98.3± 1.4	96.6 ± 2.1

3.3. Pharmacokinetic study

In the present study, following a single oral dose of 125 mg/Kg of EMB to rats, drug concentrations were observed for 24h. C_{max} , T_{max} , $t_{1/2}$, MRT and $AUC_{0-\infty}$ are reported in table 3.

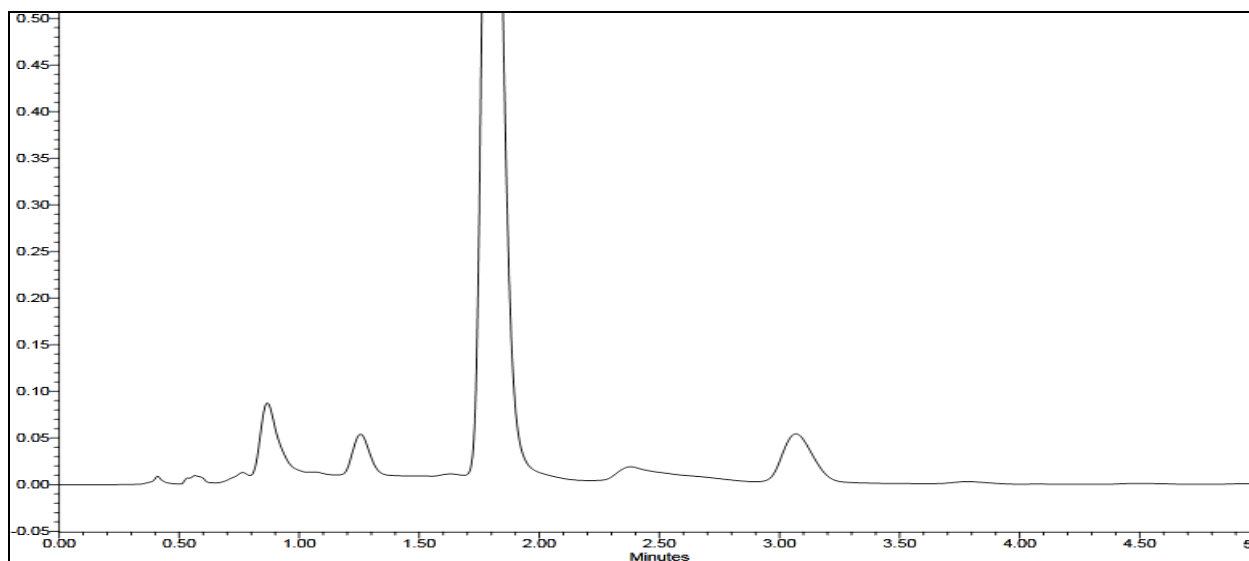


Figure 3: Chromatogram of extracted rat plasma, 1h post oral administration of EMB

Various pharmacokinetic parameters of EMB following oral administration in male wistar rats calculated by pharmacokinetic software PK Solver 2.0 are illustrated in table 3.

Table 3 Pharmacokinetic parameters of free EMB following oral administration

Parameters	FREE EMB
T_{max}	1.96 h
C_{max}	6.64 $\mu\text{g}/\text{mL}$
$T_{1/2}$	2.2 h
Cl	0.26 $\mu\text{g}/\text{mL}/\text{h}$
V_d	4.5 L
K_e	0.057 h^{-1}
AUC_{last}	90.01 $\mu\text{g}\cdot\text{h}/\text{mL}$
AUC_{inf}	96.44 $\mu\text{g}\cdot\text{h}/\text{mL}$
AUMC	1738.88 $\mu\text{g}/\text{ml}\cdot\text{h}^2$
MRT	18.03 h

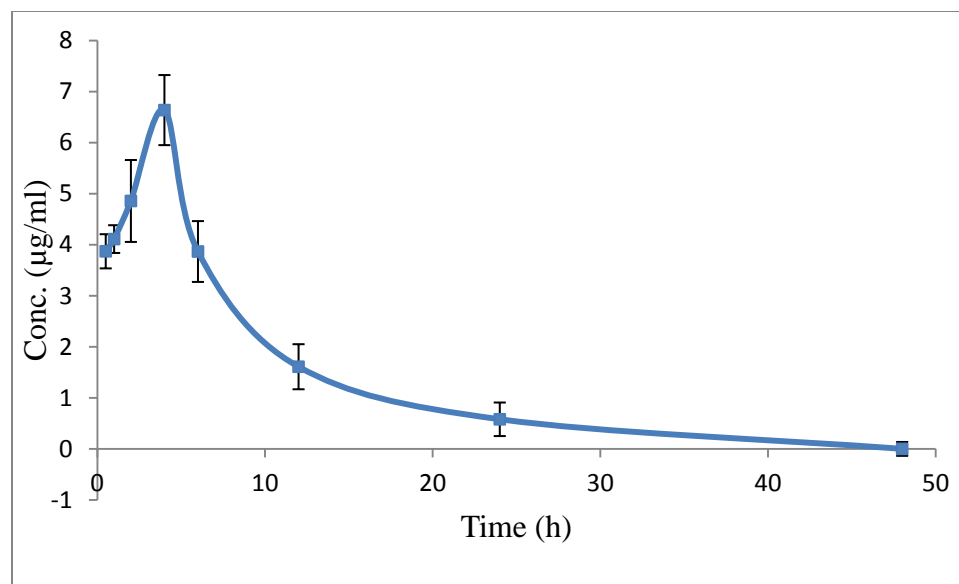


Fig. 4 Observed oral pharmacokinetic profile of EMB in rat, after single oral dose of 125 mg/Kg (n=6)

4. Discussion

Gamberini and Ferioli, in 1988⁽²⁰⁾, assayed EMB employing chemical derivatization with PEIC using HPLC. This assay was used to check the quality of active pharmaceutical ingredient but it could not be used for analysis of EMB in biological samples due to lack of sufficient sensitivity. In 1998, Chenevier et al.⁽¹⁹⁾ developed a HPLC method to determine EMB concentration in plasma and also applied the method for therapeutic monitoring in patients. Although they could attain LOQ of 0.2 µg/ml but the method entails a tedious extraction and sample preparation procedure, involving alkalisation of samples with NaOH, extraction with chloroform followed by derivatization with PEIC, thus requiring large sample volume. A long run time of 15 minutes was another limitation of the method. Pandey et al.⁽²¹⁾ also reported HPLC method with a sensitivity of 0.5 µg/ml using an expensive cyan column (Zorbax CN from Agilent; 150 X 4.6 mm; 5 mm particle size). Further to this, since minimum inhibitory concentration of EMB against *M. tuberculosis* is 0.5 µg/ml, thus it is important to measure the plasma concentrations accurately in a clinical setup at, near or even below this level. By this method it may not be possible to accurately determine concentrations less than 0.5 µg/ml. In a few LC-MS/MS and HPLC-MS/MS methods defining quantification of the EMB, a good sensitivity of 0.05 to 0.9 µg/ml^(16, 17, 25) has been reported, however the included extraction procedures involve a significant number of steps adding to the time of analysis and requiring large volume of solvent

too. Moreover solid phase extraction and use of cartridges makes the analysis tedious and expensive which is in addition to the cost of sophisticated MS equipments which may not be available in all the labs or in a hospital set up. It may also be noted that even though the literature database is replete with validated methods using sophisticated analytical equipments such as LC-MS/MS, HPLC-MS/MS, UFLC-MS/MS methods for determination of EMB in plasma, however use of UPLC method for the quantification of EMB in plasma has not yet been investigated to our knowledge.

In view of above observations, a UPLC method for estimation of EMB in rat plasma is developed and validated presently, according to the principles of good laboratory practices. The developed method is very sensitive to quantify EMB in plasma at significantly low concentrations (LOQ: 0.250 $\mu\text{g/ml}$) utilizing simple C_{18} column. The method was selective for the analyte and there was no interference from endogenous compounds/ plasma components at the retention time of EMB. The method could be extended to determine the total drug content and entrapment efficiency of suitably developed EMB loaded solid lipid nanoparticles carrier system (unpublished work).

In the process of method development a number of mobile phase combinations were tried to resolve the chromatographic peak of PEIC derived EMB. Various ratios of solvent systems which were employed initially, included: acetonitrile: water at a v/v ratio of 70:30; 50:50 and 40:60⁽¹³⁾. These solvent systems were tested at various flow rates ranging from 0.1-0.4 ml/min but invariably resulted in broad chromatograms. In an attempt to attain a selective and a sharp chromatographic peak, acetonitrile was combined with methanol at various concentrations viz acetonitrile: methanol, 30:70, 70:30, and 90:10, v/v, again at flow rates ranging from 0.1-0.4 ml/min. However, the observed chromatograms were again unsatisfactory and non-reproducible. Thereafter mobile phase comprising methanol: water: 30: 70; 50:50 and 80:20, v/v, and finally methanol: water 70:30 v/v at flow rates of 0.10 to 0.40 ml/min was tried and the latter system resulted in a sharp chromatographic peak with significant AUC within a run time of 5 min. The fine tuned method comprised of i) methanol: water 70:30 v/v as the solvent system, ii) 1 μL of injection volume, iii) a flow rate of 0.1ml/min, iv) 3.12 min retention time, and, v) total run time of 5 min. Use of very small injection volume of 1 μL versus a minimum of 20 μL reported for corresponding HPLC method⁽¹⁹⁾ is noteworthy.

Although matrix dilution effect is a suitable and suggested parameter for validation, however the same is not determined and reported presently considering that the method is developed for pharmacokinetic studies and their extension to clinical set up. It is generally observed and reported that the C_{\max} value for EMB is usually low⁽¹⁵⁾ and falls within the presently reported calibration curve range.

A linear calibration curve with low LOD and LOQ, high precision, significant and consistent recovery are the highlights of the developed method. However achievement of high recovery of more than 97% due to the use of large spiking volume (~10%) especially for HQC can not be ruled out.

The results obtained from the single oral dose pharmacokinetic study, substantiate the suitability of the developed method for determining EMB in plasma after oral administration.

The UPLC method being simple, rapid and sensitive is more useful as compared to other reported methods

5. Conclusions

The newly developed and reported UPLC method for EMB gave faster elution, lower retention time of less than 5 min at a very small injection volume of 1 μ l. High resolution than that achieved with conventional HPLC methods, was observed. Developed method exhibited an excellent performance in terms of sensitivity and speed. Further, the method was suitably applied to determine EMB after oral administration, in rat plasma samples.

The developed method allows for the simple, rapid, sensitive and reproducible quantification of EMB, and offers a viable approach for prospective pharmacokinetic studies, and therapeutic drug monitoring.

Conflict of interest

The authors report no conflict of interest.

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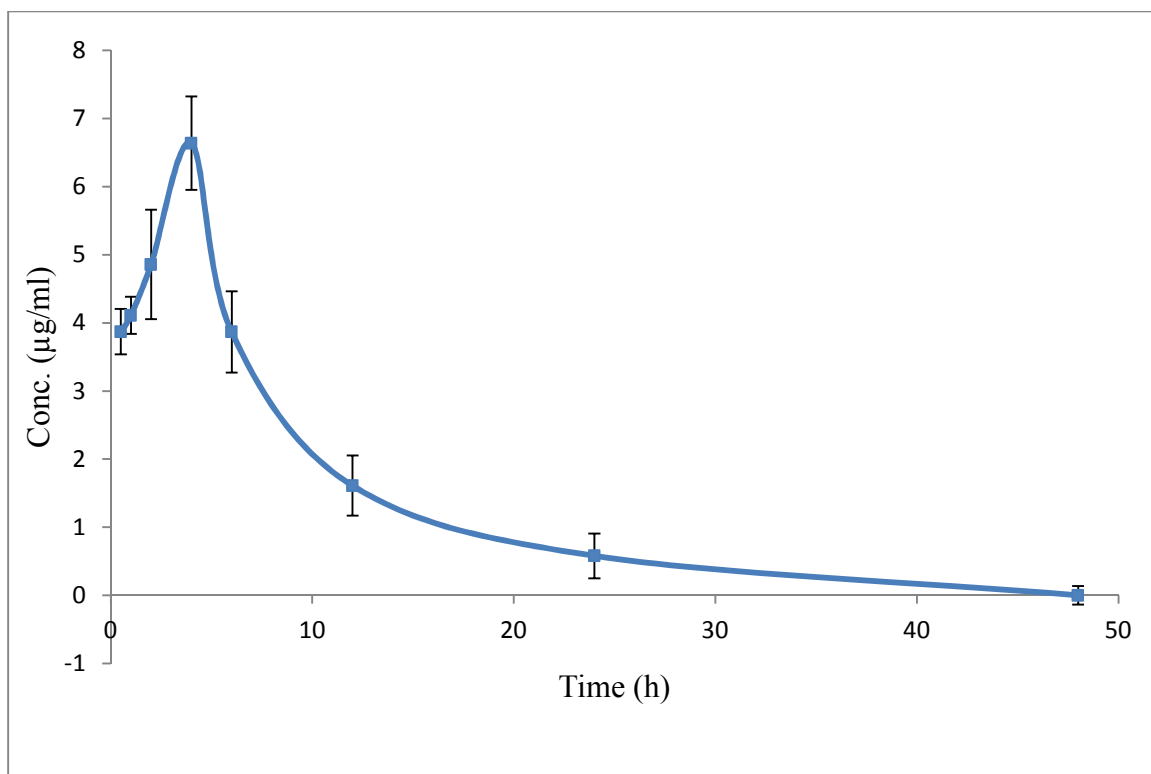
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Method is applicable for Pharmacokinetic studies after oral administration of free EMB, in rats with scope to extend to evaluation of EMB loaded solid lipid nanoparticles.