Original Article

Method Development and Validation of Ethambutol in Human Plasma by Using LCMS/MS

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Abstract

Objective: To standardize a Ultra Flow liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the determination of Ethambutol in human plasma.

Material and Methods: A simple, specific and sensitive LC-MS/MS method was developed for the determination of Ethambutol in human plasma. Separation in both was achieved by reverse phase chromatography on a Hypurity Advance C18, 50 x 4.6 mm, 5µm column with a mobile phase composition of Methanol: Buffer1 (90:10 % v/v) (Binary pump).

Results: The retention times of Ethambutol and internal standards were 0.97 and 1.04 min respectively. The assay was linear from 99.635 ng/mL to 6100.102 ng/mL. Both intra-day and inter-day accuracy and precision data showed good reproducibility.

Conclusion: The LC-MS/MS method described is sensitive, selective and linear for the wide range of concentrations for Ethambutol in human plasma. Thus, the method developed is well suited for the pharmacokinetic studies.

Key words: Human plasma; Ethambutol; Solid-phase extraction; LCMS/MS

INTRODUCTION

Ethambutol is an oral chemotherapeutic agent which is specifically effective against actively growing microorganisms of the genus Mycobacterium, including M. tuberculosis. It has the chemical formula of: (+)-2, 2’ (Ethylenediimino)-di-1-butanol dihydrochloride.

Detailed survey of literature for Ethambutol revealed several methods based on techniques viz. HPLC [1-5] and liquid chromatography/ tandem mass spectrometry (LC-MS/MS) (6-10) for its determination in pharmaceutical dosage form and in human plasma.

The goal of this study was to develop and validate a sensitive, specific and reproducible LC-MS/MS method of Ethambutol in human plasma by using Diphenhydramine hydrochloride as an internal standard. At the same time, method was efficient in analyzing large number of plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies after therapeutic doses of Ethambutol.
MATERIAL AND METHODS

Chemicals

Methanol of HPLC grade was procured from JT Becker. Water HPLC grade was obtained from a Milli-Q water purification system. Trifluoroacetic of IR and NMR grade acid was procured from spectrochem. Ammonium Acetate was procured from CDH. Formic acid AR grade was procured from Ranbaxy. A reference standard of Ethambutol sodium & Diphenhydramine hydrochloride internal standard was provided by Strides Arcolab Bangalore India.

Chromatographic System

Ultra flow liquid chromatography Tandem Mass Spectrometry was used for method development and validation. Mass Spectrometry Model Waters-Quattro Micro MS/MS, UFLC model is UFLC XR equipped with a model LC-20ADXR a binary pump, SIL-20ACXR auto sampler used to keep temperature at 4°C, CTO-20AC column oven used to keep temperature at 40°C and CBM-20A lite system controller. Detection was made at m/z 205.25/116.10 for Ethambutol and 256.17/167.09 for internal standard using ESI Positive ion spray ionization mode. Dwell time is 200 msec. Masslynx 4.1 software was used for the quantification. The stationary phase was a Hypurity Advance, C18, 4.6 x 50mm, 5µm column.

Preparation of standard solution:

Preparation of standard solution: A stock standard solution of Ethambutol was prepared by dissolving 10 mg of each in 10 ml methanol to obtain approximately 1 mg/ ml. The working standards of Ethambutol in concentrations ranging from 99.635 ng/mL to 6100.102 ng/mL were prepared by appropriate dilution with pooled plasma. The internal standard stock solutions was prepared approximately 1 mg/ml, about 28 µL of the internal standard stock solution was diluted to 25 mL to obtain IS working solution of approximately 1025 ng/ml concentration.

Sample Preparation:

Before extraction, bulk spiked CC and QC samples, were removed from the deep freezer and thawed at room temperature. Calibration standards and QC samples were then made ready for extraction in 4 ml polypropylene tubes. Exactly 100 µl of plasma was pipette out into prelabelled polypropylene tubes, to this 25 µl of internal standard dilution (6 ng/ml) was added, 200 µl Milli-Q water was added and vortexed for 20 seconds. The cartridges {Strata X (30 mg/1cc)} were conditioned with 1.0 mL methanol, equilibrated with the 1.0 ml of milli-Q water and samples were loaded, cartridges were washed with 0.5 ml of milli-Q-water and 0.5 ml of 5mM ammonium acetate buffer (Buffer 2) and were eluted with 0.4 mL of elution solvent and these sample was transferred to the auto sampler vial and 10 µl was injected into the chromatographic system.

System Suitability

The system suitability was performed before starting each day’s activity according to in-house.

Method Validation Parameters:

The optimized LCMS method was validated with respect to the following parameters. The validation was performed as per the US FDA and ANVISA guidelines and in-house operating procedures (11-12). Method was validated for Selectivity, Recovery, Linearity, Accuracy, Precision, Stock Solution Stability, Dilution Integrity and Long Term Stability.

Specificity / Selectivity

Specificity and Selectivity was performed in six different lots of plasma having K₂EDTA as anticoagulant.

Matrix Effect

Blank samples (plasma) from six independent sources of matrix were processed and then spiked with analyte at QCL level and internal standard at the concentration used in the method being validated just before injection into the LC-MS/MS. An aqueous solution of analyte was prepared at QCL level with internal standard in mobile phase.
Carry over

Processed two blank samples, two samples of LLOQ, two samples of ULOQ and Re-injection of first processed two blank samples.

Linearity

A regression equation with a weighting factor of 1/x² of drug to IS concentration was judged to produce the best fit for the concentration-detector response relationship for Ethambutol in human plasma. The representative calibration curves for regression analysis are illustrated in Figure 3.

![Figure 3 - A Representative Calibration Curve for Ethambutol](image)

Precision and Accuracy

The precision of the assay was measured by the percent coefficient of variation over the concentration range of QCLLQ, QCL, QCM and QCH samples respectively during the course of validation. The accuracy of the assay was defined as the absolute value of the ratio of the calculated mean values of the LLOQ, low, middle and high quality control samples to their respective nominal values, expressed in percentage.

Ruggedness

One complete precision and accuracy batch was processed and analyzed by different analyst using different column.

Recovery

Prepared six sets of aqueous recovery comparison samples representing 100 % extraction and injected. The recovery comparison samples of Ethambutol were compared against extracted samples of QCL, QCM and QCH samples.

Dilution Integrity

Six sets of dilution integrity samples (approximately 1.5 times of highest standard concentration) were processed by diluting them twice and another six sets by diluting them four times using pooled plasma. These quality control samples were analyzed along with a freshly spiked and processed calibration curve standards. The quality control sample concentrations were calculated using appropriate dilution factor.

STABILITIES

Bench Top Stability

Bench top stability was determined for 8 hours, using six sets each of QCL and QCH. The quality control samples were quantified against the freshly spiked calibration curve standards.

Freeze-Thaw Stability Three Cycles

The stability in human plasma was determined for three freeze-thaw cycles. Six replicates of QCL and QCH were analyzed after undergoing three freeze-thaw cycles. The freeze-thaw quality control samples were quantified against the freshly spiked calibration curve standards.

Long Term Stability at below -20° C and -50° C

Six replicates of QCL and QCH were stored below -20° C and below -50° C in the freezer and deep freezer respectively for 27 days. These samples were quantified against the freshly spiked calibration curve standards. The stability of the analytes was evaluated by comparing each of the back calculated concentrations of stability QCs with the mean concentrations of the respective QCs analyzed in the first accepted precision and accuracy batch (PA-1).

Auto Sampler Stability

Six replicates of QCL and QCH were analyzed and stored in auto sampler to prove stability. These samples were injected after a period of 47 hours and were quantified against freshly spiked calibration curve standards.

Re-injection Reproducibility

Six replicates of QCL and QCH of the precision and accuracy batch PA-3 were retained in the auto sampler at 5°C for 47 hours to test the re-injection reproducibility of the method. Reinjection reproducibility concentrations were compared against the PA-3 batch concentrations.
Stock Dilution Stability

The stability of stock dilutions of analytes and the internal standard was evaluated at room temperature. Aqueous stock dilutions of the analytes and the internal standard were prepared. One portion of the stock dilution was placed in the refrigerator between 2-8°C, while the other portion was placed at room temperature for 17 hours.

Stock Solution Stability

Stock solution stability was carried out for 27 days by injecting six replicates of stock dilution of stability standards (analyte and internal standard which were prepared and stored in the refrigerator between 2 - 8°C) and freshly prepared stock dilutions of Comparison standard (analyte and internal standard). The response of stability sample was corrected by multiplying with correction factor.

Chromatography

Representative chromatograms of aqueous mixture, blank plasma, QCM & calibration curve of Ethambutol are given in Figure.1 to 3.

Data Processing

The chromatograms were acquired and were processed by peak area ratio method using the Mass lynx Version 4.1 Software. The concentration of the unknown was calculated from the following equation using regression analysis of spiked standard with the reciprocal of the ratio of the (drug concentration) to internal standard concentration as a weighing factor ($1/x^2$):

$$y = mx + c$$

Where, $y$ = peak area ratio of Ethambutol to internal standard

$m$ = slope of calibration curve

$x$ = concentration of Ethambutol

$c$ = y-axis intercept of the calibration curve

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RESULTS AND DISCUSSION

No significant interference was observed at the RT and m/z of Ethambutol and internal standard in all the batches screened. The IS-normalized matrix factor was found to be close to unity for six different matrix lots for Ethambutol and the % CV was 7.01. The % carry over was found to be 0.44 for analyte and 0.01 for internal standard. The within batch precision and accuracy, for a dilution factor of 2 was 7.58% and 106.43%. The within batch precision and accuracy, for a dilution factor of 6 was 4.73 and 115.92%. The percentage
recovery of the drug and the internal standard was calculated and it was 58.98% and 81.38% respectively. The calibration curves for Ethambutol in plasma were plotted. These curves were found to be linear over the concentration range of 99.635 ng/mL to 6100.102 ng/mL with correlation coefficients (r2) greater than 0.998679. Inter and Intra batch precision expressed by relative standard deviation was less than 9%. The accuracy, precision and intraday precision were carried out by preparing six individual samples of QCH, QCM, QCL, QLLQ and QCL. The % CV and % nominal was calculated. Refer Table No: 1 for the results of Within-Batch Precision and Accuracy, Intraday Batch Precision and Accuracy and Between Batch Precision and Accuracy. The Ruggedness mean accuracy ranged from 98.30 % (QCM) to 103.53 % (QLLQ) and the precision ranged from 1.88 % (QCM) to 7.28 % (QLLQ).

Stability studies for the method were carried out by accomplishment of short term and long term stock stability. The percent nominal for bench top stability ranged from 101.59 % (QCL) to 105.13 % (QCH) and the precision ranged from 7.18 % (QCL) to 7.29 % (QCH). The percent nominal for freeze thaw stability ranged from 106.96% (QCL) to 107.94% (QCH) and precision ranged from 2.49 % (QCL) to 5.27 % (QCL) respectively. The percent nominal for short term below -20°C ranged from 96.05% (QCL) to 101.35% (QCL) and precision ranged from 5.06% (QCL) to 96.98% (QCL) respectively. The percent nominal for long term below -50°C ranged from 92.37 % (QCL) to 103.98 % (QCL) and precision ranged from 5.87 % (QCL) to 7.78 % (QCL) respectively. The auto sampler stability at around 50 hours and percent nominal ranged from 92.75 % (QCL) to 108.63 (QCH) and precision ranged from 5.94 % (QCL) to 6.37 % (QCL) respectively. The percent change observed for the internal standard (Diphenhydramine hydrochloride) was 2.31%. The percentage change Re-injection Reproducibility in 4.17 (QCL) and 7.22 (QCH). The stock dilution percent change for Ethambutol was 0.59 % and for Diphenhydramine hydrochloride is 1.17 %, respectively and stock solution percent change for Ethambutol was 6.36 % and for Diphenhydramine hydrochloride is 3.28 %, respectively.

CONCLUSION

The bioanalytical method developed is simple, accurate, precise, sensitive and reproducible. It can be used for the estimation of Ethambutol in biological fluids. The separation method developed produce acceptable values of recovery. The chromatogram developed has well resolved peak of Ethambutol without any interference. Thus, the developed LCMS method can be utilized for bioequivalence and pharmacokinetic studies, for routine analysis during the analysis of Ethambutol.

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REFERENCES


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