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METHOD DEVELOPMENT AND VALIDATION OF PYRAZINAMIDE IN HUMAN PLASMA BY USING GABAPENTIN AS INTERNAL STANDARD: 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 Pyrazinamide in human plasma.

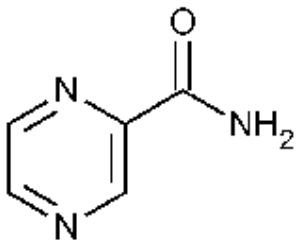
Material and Methods: A simple, specific and sensitive LC-MS/MS method was developed for the determination of Pyrazinamide in human plasma. Separation in both was achieved by reverse phase chromatography on a Hypurity Advance C18, 50 x 4.6 mm, 5µm column with a Methanol: 5 mM Ammonium Acetate Buffer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

Results: The retention times of Pyrazinamide and internal standards were 0.97 and 1.04 min respectively. The method was linear and has been validated over a concentration range of 229.395 ng/mL to 11968.430 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 Pyrazinamide in human plasma. The validation results showed that the method was suitable for analysis of Pyrazinamide in human plasma and well suited for the pharmacokinetic studies.

INTRODUCTION

Pyrazinamide, the pyrazine analogue of nicotinamide, is an antituberculous agent. Pyrazinamide is stable and quite inexpensive. It may be bacteriostatic or bacteriocidal against Mycobacterium tuberculosis depending on the concentration of drug attained at the site of infection. Pyrazinamide has the following structural formula: C5H5N3O. Its molecular weight is 123.11.



Detailed survey of literature for Pyrazinamide 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 aim of this study was to develop and validate a sensitive, specific and reproducible LC-MS/MS method of Pyrazinamide in human plasma by using Gabapentin 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 Pyrazinamide.

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. Ammonium Acetate was procured from CDH. A reference standard of Pyrazinamide sodium & Gabapentin internal standard was provided by Strides Arcolab Bangalore India.

Instrumentation and 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-20Alite system controller. Detection was made at m/z 124.04/78.79 for Pyrazinamide and 172.35/154.32 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. Flow rate was 0.4 mL/min and injection volume was 10 µl.

Preparation of standard solution:

Preparation of standard solution: A stock standard solution of Pyrazinamide was prepared by dissolving 10 mg of each in 10 ml methanol to obtain approximately 1 mg/ ml. The working standards of Pyrazinamide in concentrations ranging from 229.395 ng/mL to 11968.430 ng/mL were prepared by appropriate dilution with pooled plasma. The internal standard stock solutions was prepared approximately 1 mg/ ml, about 30 µL of the internal standard stock solution was diluted to 25 mL to obtain IS working solution of approximately 3000 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 (3 µg/ml) was added, 30 µl Milli-Q water was added and vortexed for 20 seconds. The cartridges (Analchem plexus 30mg and 1 cc) 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 1 ml of milli-Q-water and samples were eluted with 0.2 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 K_2EDTA 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 $1/x^2$ of drug to IS concentration was judged to produce the best fit for the concentration-detector response relationship for Pyrazinamide in human plasma. The representative calibration curves for regression analysis are illustrated in Figure 3.

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 Pyrazinamide were compared against extracted samples of QCL, QCM and QCH samples.

Dilution Integrity

Six sets of dilution integrity samples (approximately 1.6 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 9 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 31 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 56 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 56 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 24 hours.

Stock Solution Stability

Stock solution stability was carried out for 39 days by injecting six replicates of stock dilution of stability standards (analyte and internal standard which 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 Pyrazinamide 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)^2 to internal standard concentration as a weighing factor ($1/x^2$):

$$y = mx + c$$

Where, y = peak area ratio of Pyrazinamide to internal standard
m = slope of calibration curve
x = concentration of Pyrazinamide
c = y-axis intercept of the calibration curve

RESULTS AND DISCUSSION

All the six plasma lots met the above acceptance criteria. The method was specific and selective. The pooled plasma also met the acceptance criteria for specificity and selectivity. The IS-normalized matrix factor was close to unity for six different matrix lots. The variability (%CV) was 2.21 % (<15%). The % carry over was found to be 0.51 for analyte and 0.03 for internal standard. The percentage recovery of the drug and the internal standard was calculated and it was 41.36% and 37.80% respectively. The within batch precision and accuracy, for a dilution factor of 2 was 1.62% and 104.23%. The within batch precision and accuracy, for a dilution factor of 6 was 3.29% and 103.21%. The calibration curves for Pyrazinamide in plasma were plotted. These curves were found to be linear over the concentration range of 229.395 ng/mL to 11968.430 ng/mL ng/mL with correlation coefficients (r2) greater than 0.998566. 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 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 93.50 % (QCM) to 100.30 % (QCH) and the precision ranged from 1.78 % (QCM) to 6.93 % (QCLLQ).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 97.09% (QCL) to 99.63% (QCH) and the precision ranged from 4.08% (QCH) to 6.74% (QCL). The percent nominal for freeze thaw stability ranged from 95.20% (QCL) to 99.23% (QCH) and precision ranged from 3.63% (QCH) to 5.26% (QCL) respectively. The percent nominal for long term below -20°C ranged from 95.84% (QCL) to 100.32% (QCH) and precision ranged from 2.65% (QCH) to 3.76% (QCL) respectively. The percent nominal for long term below -50°C ranged from

97.81% (QCL) to 102.44% (QCH) and precision ranged from 2.65% (QCH) to 3.76% (QCL) respectively. The percent for auto sampler stability nominal ranged from 97.81% (QCL) to 102.44% (QCH) and precision ranged from 2.18% (QCH) to 3.53% (QCL) respectively. The percentage change Re-injection Reproducibility in 1.10 (QCH) and 3.07 (QCL). The stock dilution percent change for Pyrazinamide was 1.89% and for Gabapentin is 0.25 %, respectively and stock solution percent change for Pyrazinamide was 0.84 % and for Gabapentin is 1.85 %, respectively.

CONCLUSION

The method was specific / selective with respect to the analyte and internal standard. The method was precise, accurate and rugged. The analyte was stable in the biological matrix for when stored below -50°C & -20°C. The results showed that the method was valid even if the samples were diluted upto 2 and 6 dilutions. The recovery was consistent at all levels. The analyte was stable in human plasma on bench top. The analyte was stable till four freeze-thaw cycles. The analyte and internal standard in stock dilution and stock solution were stable. Thus, the developed LCMS method can be utilized for bioequivalence and pharmacokinetic studies.

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REFERENCES

1. Chenevier P, Massias L, Gueylard D, Farinotti R. Determination of Pyrazinamide in plasma by high performance liquid chromatography after pre-column derivatization. J Chromatogr B Biomed Sci Appl 1998; 708:310-5.

2. Philippe Cheneviera, Laurent Massiasa, Delphine Gueylarda and Robert Farinottia, Determination of Pyrazinamide in plasma by high-performance liquid chromatography after pre-column derivatization. Journal of Chromatography B: Biomedical Sciences and Applications Volume 708, Issues 1-2, 24 April 1998, Pages 310-315.

3. M. Breda, P. Marrari, E. Pianezzola and M. Strolin Benedetti. Determination of Pyrazinamide in human plasma and urine by high-performance liquid chromatography with fluorescence detection. Journal of Chromatography A Volume 729, Issues 1-2, 5 April 1996, Pages 301-307.

4. Yan M, Guo T, Song H, Zhao Q, Sui Y. Determination of Pyrazinamide hydrochloride in the combination tablets by precolumn derivatization. J Chromatogr Sci. 2007 May-Jun;45(5):269-72.

5. Breda M, Marrari P, Pianezzola E, Strolin Benedetti M. Determination of Pyrazinamide in human plasma and urine by high-performance liquid chromatography with fluorescence detection. J Chromatogr A. 1996 Apr 5;729(1-2):301-7.

6. Zhilong Gong, Yousef Basir, David Chu, Melanie McCort-Tipton. A rapid and robust liquid chromatography/tandem mass spectrometry method for simultaneous analysis of anti-tuberculosis drugs Pyrazinamide and Pyrazinamide in human plasma. Journal of chromatography. B, Analy 06/2009; 877(16-17):1698-704.

7. Notari S, Mancone C, Sergi M, Gullotta F, Bevilacqua N, Tempestilli M, Urso R, Lauria FN, Pucillo LP, Tripodi M, Ascenzi P. Determination of antituberculosis drug concentration in human plasma by MALDI-TOF/TOF. IUBMB Life. 2010 May; 62(5):387-93.

8. Conte JE Jr, Lin E, Zhao Y, Zurlinden E. A high-pressure liquid chromatographic-tandem mass spectrometric method for the determination of Pyrazinamide in human plasma, bronchoalveolar lavage fluid, and alveolar cells. J Chromatogr Sci. 2002 Feb;40(2):113-8.

9. Sekai Chideya, Carla A. Winston, Charles A. Peloquin, William Z. Bradford, Philip C. Hopewell, Charles D. Wells, Arthur L. Reingold, Thomas A. Kenyon, Themba L. Moeti, and Jordan W. Tappero. Isoniazid, Rifampin, Pyrazinamide, and Pyrazinamide Pharmacokinetics and Treatment Outcomes among a Predominantly HIV-Infected Cohort of Adults with Tuberculosis from Botswana. Tuberculosis Drugs and Treatment Outcomes • CID 2009:48; June; 1685-94.

10. Chandra Immanuel, Prema Gurumurthy, Geetha Ramachandran, P. Venkatesan, V. Chandrasekaran & R. Prabhakar. Bioavailability of rifampicin following concomitant administration of Pyrazinamide or isoniazid or pyrazinamide or a combination of the three drugs. Indian J Med Res 118, September 2003, pp 109-114.

11. ICH, Stability Testing of New Drug Substances and Products. International Conference on Harmonization, IFPMA, Geneva, 2003.

12. Guidance for Industry US-FDA Bioanalytical Method Validation. May (2001).

Figure 1 - A Representative Chromatogram of Pyrazinamide for Blank

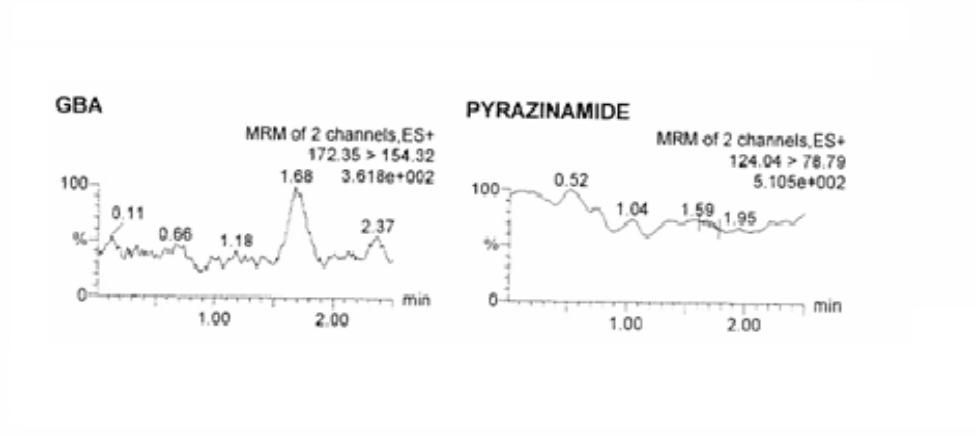

Figure 2 - A Representative Chromatogram of Pyrazinamide for QCM

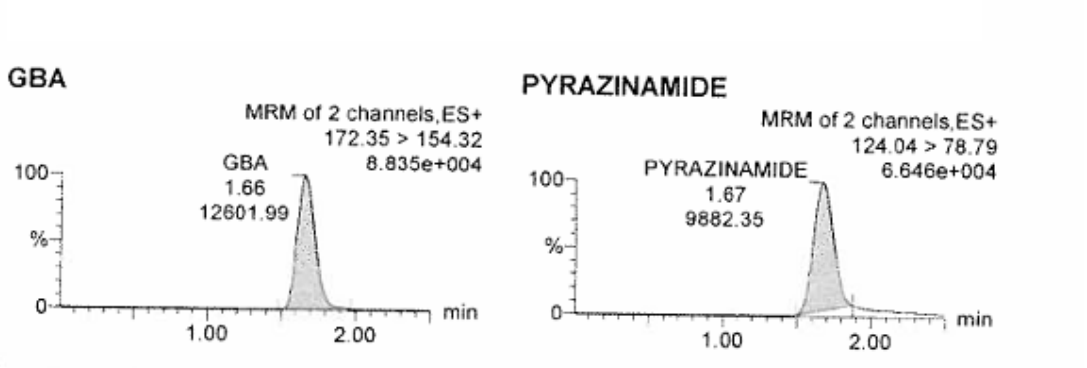

Figure 3 - A Representative Calibration Curve for Pyrazinamide

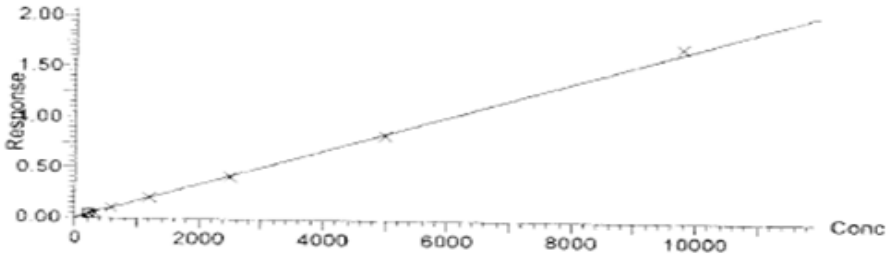

Table No. 1: Precision and Accuracy

Within Batch Precision	PA1	1.83 % - 6.33 % (QCM- QCLLQ)
	PA2	4.17 % - 6.18 % (QCM-QCLLQ)
	PA3	2.93 % - 6.44 % (QCL-QCH)
Within Batch Accuracy	PA1	98.99 % - 104.73 % (QCM- QCLLQ)
	PA2	106.39 % - 107.89 % (QCLLQ -QCM)
	PA3	99.23 % - 101.46 % (QCM-QCH)
Intraday Batch Precision	Day-1	6.53 % - 1.81 % (QCLLQ - QCM)
	Day-2	5.24 % - 6.89 % (QCL - QCLLQ)
Intraday Batch Accuracy	Day-1	98.64 % - 100.46% (QCM -QCLLQ)
	Day-2	102.81 % - 104.27 % (QCLLQ-QCH)
Between Batch Precision		6.53 % - 1.81 % (QCLLQ - QCM)
Between Batch Accuracy		100.89 % - 103.47 % (QCM -QCLLQ)