

Determination of Pyrazinamide in Human Plasma Samples Containing Fixed Dose Combination Molecules by using Liquid Chromatography Tandem Mass Spectrometry

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Abstract

A rapid, simple, sensitive and compatible liquid chromatography tandem mass spectrometric method has been developed and validated for the estimation of Pyrazinamide. Glipizide was used as an internal standard. Detection was performed using TSQ Quantum Discovery max mass spectrometer with ESI source in positive polarity. The detection transition for Pyrazinamide is 124.100 → 79.160 and for Glipizide is 446.200 → 321.200. Chromatographic separation of analyte and internal standard were carried out using a reverse phase column, Hypersil, Gold, 4.6 X 50 mm, 5 μ at a flow rate of 0.400 mL/min. Mobile Phase is composed of Methanol: 0.1 % FA in 10 mM Ammonium Formate (90:10) v/v. Extracted by Solid Phase Extraction with a sample volume of 200 μL plasma. The assay of Pyrazinamide is linear over the range of 0.935 μg/mL to 60.408 μg/mL with a precision of < 9.86%. Mean extraction recovery for Pyrazinamide and Glipizide were more than 61%. Samples are stable at room temperature for 6 hrs, processed samples were stable at least for 28 hrs and also stable at three freeze–thaw cycles.

Keywords: Pyrazinamide; LC-MS/MS; Validation; Human Plasma

Introduction

Pyrazinamide is a pyrazine carboxyl amide; Pyrazinecarboxamide with a molecular formula C₅H₅N₃O and molecular weight 123.11 g/mol [1], pKa of 0.5 Pyrazinamide, the pyrazine analogue of nicotinamide, is an antituberculous agent. It may be used as a bacteriostatic or bacteriocidal against Mycobacterium tuberculosis depending on the concentration of drug attained at the site of infection.

There are several studies that were performed on Fixed Dose Combinations (FDC) medicines and on individual dosage forms. Though, comparative studies between both FDC and individual drugs are available, the response of patients to the treatment depends on the resistance to the drugs. Recommended doses of first line antituberculosis drugs for adults were Isoniazid 8-12mg/kg/dose, Rifampicin 8-12mg/kg/dose, Pyrazinamide 20-30mg/kg/dose and Ethambutol 15-20mg/kg/dose [2].

As per the reported literatures, for the determination of Pyrazinamide in human plasma, no articles has been found as an individual or combined method with more than a recovery of 50%. More over, these methods should also be compatible for the determination of Pyrazinamide in human plasma containing ascorbic acid which is required for the stability of Rifampicin [3,4] especially, in case of samples containing fixed dose combination formulation. Hence, authors propose a method that is developed and validated to overcome the difficulties in achieving the required extraction recoveries that will be useful for carrying out analysis of samples containing multiple drugs.

Experimental

Materials and reagents

Pyrazinamide is purchased from Svizera Labs Pvt. Ltd Mumbai. The Internal Standard Glipizide is purchased from Clearsynth Labs Pvt. Ltd Mumbai. Methanol (HPLC grade), Acetonitrile (HPLC grade), Formic Acid (ULC/MS), Water (Ultra Pure grade), Ascorbic Acid and Ammonium Formate were used.

Instruments

The Liquid chromatography coupled with tandem Mass Spectrometer (LC-MS/MS) system consists of a Finnigan Surveyor Autosampler, Surveyor LC Pump Plus solvent delivery system and a column Oven (Thermo Electron Corporation) is used for ion separations. The Mass spectrometer was Thermo Scientific TSQ Quantum discovery max ultra triple stage quadrupole mass spectrometer used for ion detection. An Electron Spray Ionization (ESI) technique was used. Data was collected and processed using LC Quan Version 4.5.6 Data collection and integration software.

Chromatographic Condition: The Liquid Chromatographic separations were carried out by using Hypersil, Gold, 4.6 X 50 mm, 5 μ Thermo. Column temperature was held at 30°C. The auto sampler tray temperature was 10°C. Mobile phase with a composition of Methanol: 0.1% FA in 10 mM Ammonium Formate (90:10) v/v is pumped by the solvent delivery system at a flow rate of 0.400 mL/min. A typical injection volume was 10.0 μL.

MS/MS detection: Precursor ions for analyte and internal standard were determined from mass spectra obtained by the TSQ mass spectrometer. TSQ mass Spectrometer includes an electronically-controlled, integrated syringe pump. The Mass Spectrometric (MS) conditions for Pyrazinamide and Glipizide were optimized by separate infusion into the MS at a flow rate of 5 μL/min constantly, while adjusting MS parameters to achieve maximal intensity. Electro-spray

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Received February 18, 2012; Accepted April 04, 2012; Published April 08, 2012

Citation: Chaitanya Krishna A, Saravanan RS, Jeevanantham S, Vignesh R, Karthik P (2012) Determination of Pyrazinamide in Human Plasma Samples Containing Fixed Dose Combination Molecules by using Liquid Chromatography Tandem Mass Spectrometry. Adv Pharmacoeconom Drug Safety 1:101. doi:10.4172/2167-1052.1000108

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ionization in positive ion mode (ESI+) was used for ionization and selective reaction monitoring (SRM) mode was chosen for detection. The optimized precursor ions pairs were m/z 124.100 → 79.160 for Pyrazinamide and m/z 446.200 → 321.200 for Glipizide Shown Figure 1 and Figure 2. The optimized MS parameters were as follows: Ion Spray voltage: 5000 volt, Sheath gas pressure: 50 psi, Auxiliary gas pressure: 15 psi, Capillary temperature: 265°C. Collision Pressure: 1.5 psi. Peak areas were automatically integrated using LC Quan Version 4.5.6 (Thermo Corporation).

Preparation of Calibration standards and quality control samples

The calibration standards and the quality control (QC) samples were prepared from separate stock standard solutions. The concentrations of stock solutions were 3974.193 µg/mL and 3973.836 µg/mL for calibration standard and quality control samples. Methanol was used as diluent for preparation of the stock solutions. Spiking solutions for calibration standards and quality control concentrations were prepared in methanol: water (80:20) %v/v. Calibration standards were prepared by spiking corresponding solutions into blank human plasma (containing 0.1% Ascorbic acid) [3,4] to provide concentrations ranging between 0.935 µg/mL (Lower Limit of Quantification-LLOQ) and 60.408 µg/mL (upper limit of quantification-ULOQ). For Quality control plasma samples preparation, the corresponding spiking solutions is spiked to the human blank plasma to attain the four Quality Control (QC)

concentrations: 0.960 µg/mL, 2.865 µg/mL, 23.386 µg/mL and 43.712 µg/mL for achieving LOQQC (limit of Quantification QC), LQC (Low QC), MQC (Middle QC) and HQC (High QC) levels respectively. For preparation of the Calibration curve samples and Quality Control samples, the limits followed were as follows:

LLOQ - atleast equal to or less than 5 half lifes of the drug

ULOQ - two times of the Cmax of drug

LOQ QC - 100 to 105 % of LLOQ

LQC - 2.5 to 3 times of LLOQ

MQC - ± 2 % of Average between LQC and HQC

HQC - 65-85% of ULOQ

For preparation of the plasma samples, volume of 20.000 µL of the spiking solutions was spiked into 0.980 mL of human blank plasma. Internal standard stock solution (1003.915 µg/mL) of Glipizide was prepared in methanol and used as an Internal Standard dilution. Working solutions for internal standard (1.506 µg/mL) was prepared in Methanol: water (80:20) %v/v.

Sample extraction

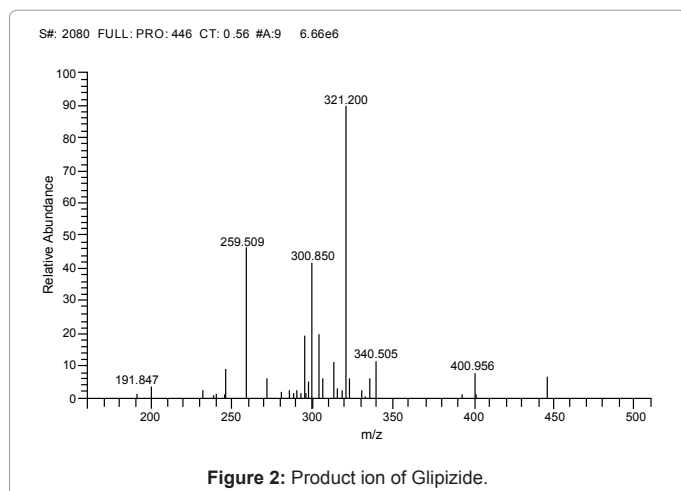
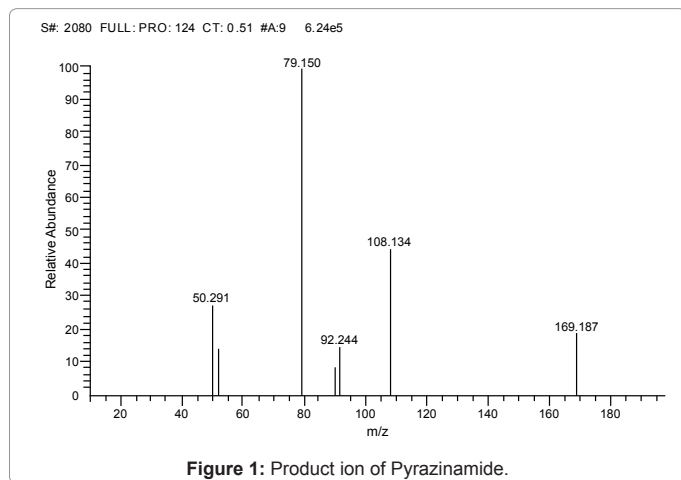
200.0 µL aliquot of plasma samples was mixed with 50.0 µL of internal standard working solution (1.506 µg/mL). Pre-treatment is performed by adding 0.400 mL of 0.1% Formic acid and mixed the samples for approximately 10 secs on a vortex shaker before adapting the SPE procedure. Commercially available cartridge (Analchem PLEXUS 30 mg/1cc) was utilized for extraction. Conditioning and equilibration of the cartridges were performed with 1 mL of methanol and water respectively. Pyrazinamide and Glipizide were extracted into the cartridge by loading the pre-treated plasma samples. Cartridges were washed using 2 x 0.500 mL of Ultrapure Water, in order to remove the unbound substance in the cartridge and to reduce any interfering band in chromatograms. Finally 0.400 mL of mobile phase was used to elute Pyrazinamide. 10.0 µL of the eluent was injected for chromatographic analysis.

Validation [5]

Selectivity and specificity: Blank human plasma from eight different lots (including one Haemolysed and one Lipimic) were processed without Analyte and internal standard, and with the same eight lots LLOQ level is processed to evaluate the presence of any interference at the retention time of Analyte and Internal standard.

Matrix factor: Matrix factor was evaluated at low and high quality control concentrations, to ensure that the precision, selectivity and sensitivity are not compromised due to a change in matrix. Matrix factor can be termed as the quantitative measurement of the matrix effect. Aqueous mixtures of internal standard and analyte, representing 100% extraction of internal standard and analyte, at low and high QC concentrations were prepared. These shall serve as Reference Samples. 8 different lots of blank matrices (from eight individuals, including, one Haemolysed and one Lipimic) were processed in duplicate without addition of IS. Eluted solution volumes were equally diluted with reference sample; it is compared with respective aqueous reference sample equally diluted with mobile phase.

Calibration curve and linearity: The eight-point calibration curve was constructed by plotting, peak area ratio of Pyrazinamide to their corresponding internal standard versus Pyrazinamide concentrations. A linear regression with weighing factor of linear 1/x² was applied.



Intra and inter-day assay accuracy and precision: Intra-day precision and accuracy were determined by analysis of six replicates of each QC sample (n = 6) at LOQQC, LQC, MQC and HQC concentration levels, extracted with a set of standards in one batch. Inter-day precision and accuracy was performed in two different days.

Recovery: Recovery is carried out to evaluate the loss of drugs and/ or internal standards during sample extraction. Peak area counts of drugs and internal standards from extracted QC samples were compared with corresponding QCs reference samples to evaluate any loss of either drugs or internal standards. It is preferable to observe consistent recovery for all three QC levels. For calculating the % recovery, 6 sets of plasma samples at LQC, MQC and HQC levels were prepared, extracted and injected. Simultaneously, aqueous spiked recovery samples at LQC, MQC and HQC concentrations were prepared in singlet and injected 6 times (reference sample).

Stability: Stability of both drugs in different matrices and under different conditions was evaluated. The detailed tests are described below. Stability was assessed by comparing the mean concentration of the stored QC samples with the mean concentration of freshly prepared QC samples. Drug stability in pooled human blank plasma is a function of the storage conditions, the chemical properties of the drug and the matrix effect. The following tests were performed to evaluate the stability, Short-term and Long-term Stock solution stability, Bench top stability, Freeze and thaw stability, Autosampler stability, Wet Extract Stability, Long Term stability In Matrix.

Result and Discussion

Method development

In this method development for determination of Pyrazinamide in human plasma, some of the issues were being addressed. As the molecular weight of Pyrazinamide is low, it would be more difficult to get a good chromatographic condition, where, noise levels were found to be more in several compositions of the mobile phase and affect the sensitivity of the analysis. Finally better results were obtained by optimizing the chromatographic conditions with Methanol: 0.1% FA in 10 mM Ammonium Formate (90:10) v/v as mobile phase. Separation was performed in Hypersil Gold Column, 4.6 X 50 mm, 5 μ at a flow rate of 0.400 mL/min.

Further, a method that can be used for analysis of Pyrazinamide alone is not the only alternative. Because most of the times, Bioequivalence studies are conducted with Fixed Dose Combination drugs. In this context, an analyst should be able to develop a method that can be used for the plasma samples containing all fixed dose combinations. Though the task is multiple, some more criticalities need to be considered by the researchers while developing a method for fixed dose combination molecules. Stability of the molecules is the primary concern, which will change the actual methodology for analysis of Fixed Dose Combination Drugs. For instance, a method for analysis of fixed dose combination formulation of Anti-TB product containing Isoniazid, Rifampicin, Ethambutol and Pyrazinamide will be different from the methodology and different instruments were used for the analysis of samples containing Pyrazinamide alone [5-9]. The main reason for this is being the stability concern of rifampicin [10,3,4]. Due to the Oxidative degradation of Rifampicin, it is better suggested that the plasma sample should contain ascorbic acid to inhibit the oxidative degradation. In this condition, a method that can be used for analysis of Pyrazinamide in Human Plasma containing ascorbic acid will be more beneficial. Previous methods used for the determination

of Pyrazinamide by High Performance Liquid Chromatography (HPLC) [6,11,7,8] has been found to consume more run times because of the lengthy column used for the separation, where they end up in longer time for analysis and also with animal plasma [12]. Some of the methods in LCMS/MS [11] are more tedious to perform because of the chromatographic conditions and sample preparation steps that have been adapted. More over, the new methods have to offer the benefits of cost effectiveness, less run times and simplicity in the process, with the recent advances in the analytical techniques. Recovery of the drug and IS were found to be more (60%) when compared with the previous methods. This method is found to be simple, precise, and compatible, less time consuming for the determination of Pyrazinamide in human plasma by LCMS/MS. Authors propose a simple method that would compensate all the above mentioned problems including the stability issues.

Validation

Selectivity and specificity: No interference from the blank plasma at the retention time of the Analyte and Internal standard.

Matrix Factor: Observed % CV of matrix factor is 8.66% and 5.50% in LQC, 3.38% and 1.80% in HQC for Pyrazinamide and Internal standard respectively. All eight matrix lots showed very similar matrix effect for both analyte and their corresponding internal standard.

Calibration Standard and Linearity: For three consecutive batches, the calibration curves showed an overall accuracy of 90.43%-113.02% with %CV of 0.62%-9.86%. The calibration Standard Linearity has a regression Coefficient of > 0.9929. The detailed results are shown in Table 1. The Calibration Standards meets the acceptance criteria. Chromatograms are shown from Figure 3 to Figure 9.

Accuracy and precision: Table 2 shows inter and intra assay precision and accuracy. The method was found to be highly accurate and precise. For Pyrazinamide, accuracy of 86.27%-105.91% and precision of 1.59%-8.96% for intra-assay, and accuracy of 89.17%-103.41% and precision of 3.04%-8.82% CV for inter-assay were obtained for all QC levels including LOQQC.

Batch ID	Theoretical concentration (μg/mL)							
	0.935	1.869	4.673	11.681	23.363	35.943	51.347	60.408
Batch 01	0.916	1.92	5.633	12.798	23.459	36.463	50.538	54.018
Batch 02	0.848	2.12	5.232	12.594	23.708	34.034	48.702	51.418
Batch 03	0.952	1.74	4.98	12.214	23.72	35.455	49.804	58.451
Mean	0.905	1.927	5.282	12.535	23.629	35.317	49.681	54.629
Precision	5.83	9.87	6.24	2.36	0.62	3.46	1.86	6.51
Accuracy	96.83	103.09	113.03	107.31	101.14	98.26	96.76	90.43

Table 1: Accuracy and Precision for Calibration Standards.

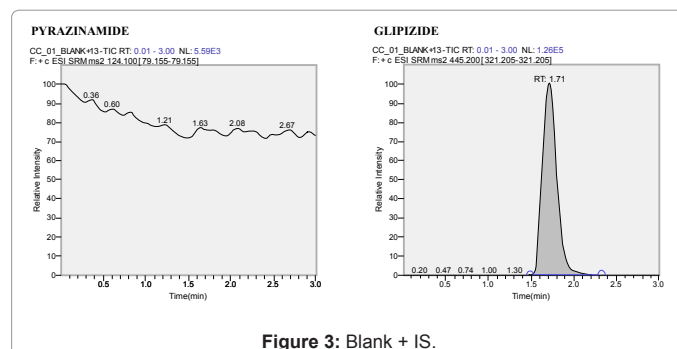
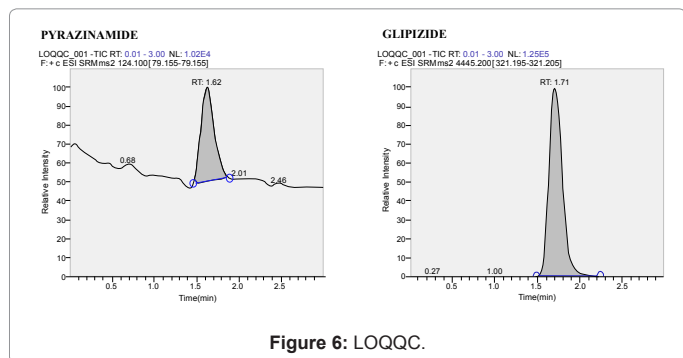
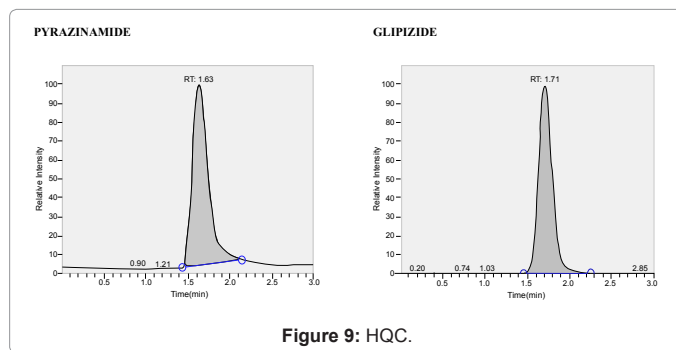
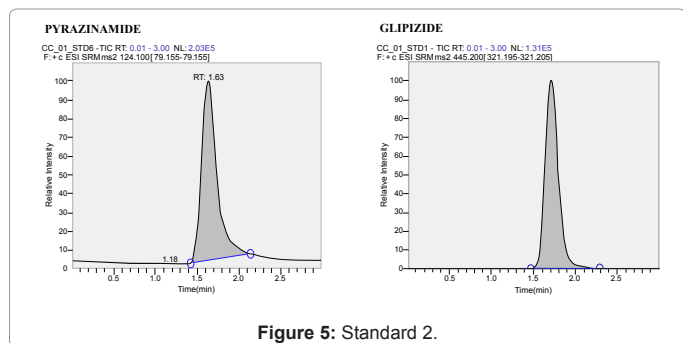
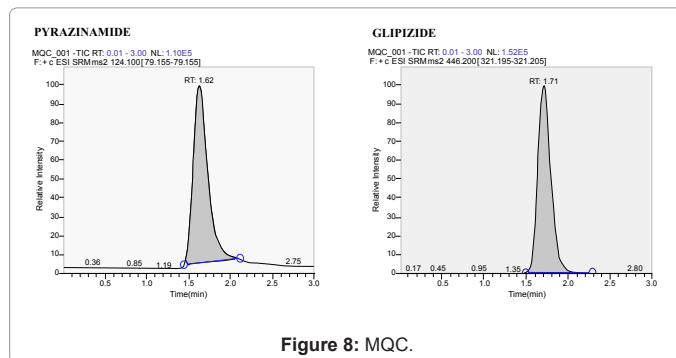
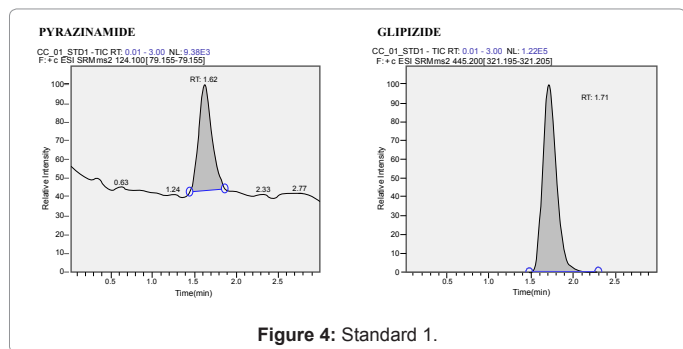
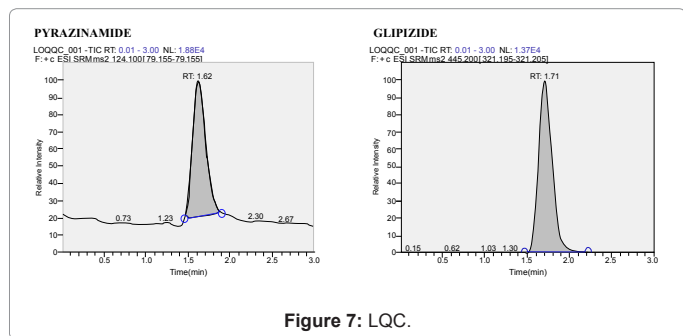


Figure 3: Blank + IS.



Parameter	Intra-Batch	Inter-Batch
Accuracy (%)	86.27 – 105.91	89.17 – 103.41
Precision (%)	1.59 – 8.96	3.04 – 8.82

Table 2: Intra and Inter-day Accuracy and Precision.



QC Level	Recovery (%)	
	Pyrazinamide	Glipizide
LQC	59.80	55.65
MQC	59.64	58.48
HQC	66.07	63.82
Recovery	61.83	59.32
%CV	3.66	4.16

Table 3: Recovery of Analyte and IS.

Parameter	Bench Top Stability		Auto Sampler Stability		Wet extract stability		Freeze and Thaw stability		Long term Matrix stability	
	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC
QC Levels										
Precision	2.37	2.64	2.17	2.61	2	2.64	2.56	2.62	2.8	4.67
% Stability	95.43	93.19	98.17	94.34	99.16	93.26	97.97	93.87	1.0633	0.9695

Table 4: Stability of Pyrazinamide.

Stability	Short term Stock Solution Stability for 7 hrs		Long term Stock solution stability for 15days	
	Analyte	IS	Analyte	IS
%CV	3.01	3.15	3.81	2.24
% Stability	99.84%	100.39%	93.93%	101.98%

Table 5: Stability of Pyrazinamide.

Recovery: Recovery was performed by processing six samples in each level of concentration and compared the area obtained with the area of reference sample (prepared in aqueous), the mean recovery is calculated. The higher mean recovery represents the effectiveness of the extraction techniques. Table 3 show the overall recovery of 61.83% for Pyrazinamide, and 59.32% for Glipizide were obtained. Both compounds show consistent recovery results for all three QC levels.

Stability: Stability of Pyrazinamide in human plasma under differ-

ent conditions was evaluated. The detailed results are shown in Table 4 and Table 5, as seen from the table, three freeze/thaw cycles, 6 h room temperature storage, and 28 h autosampler stability has been estab-

lished. In addition wet extract stability shown for 28 hrs were established. All of these demonstrate the ruggedness of the method.

Conclusion

A simple, rapid, selective method in chromatographic condition and extraction is more precise, sensitive and also compatible for the analysis of Pyrazinamide in human plasma for fixed dose combination drugs with good recovery by using LCMS/MS. The method was developed and validated, which was found to have good in stability. In addition, this method has been used for the determination of Pyrazinamide in human plasma by LCMS/MS for four drug fixed dose combination formulation while conducting the BA and BE studies. Anyway, combination treatment with Rifampicin and Pyrazinamide is accepted as an alternative for Isoniazid therapy. Combination therapy for 2 months offers an equivalent efficacy as 12 months therapy of Isoniazid [13]. Because of these reasons, the FDC formulations are gaining main importance in the therapeutic area of tuberculosis. Hence forth, researchers proposed a method that will be more beneficial for both individual and combinational determination of Pyrazinamide in human plasma by LCMS/MS.

Acknowledgements

We are thankful to Mrs. Gauri Sapte, Director, BBRC, Dr.V.P.Shedbalkar and the management of Maneesh Pharmaceuticals Ltd., for their valuable support and contribution in carrying out this research work with out which it will not be possible to publish the paper.

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