World Journal of Pharmaceutical Sciences
ISSN (Print): 2321-3310; ISSN (Online): 2321-3086
Published by Atom and Cell Publishers © All Rights Reserved
Available online at: http://www.wjpsonline.org/
Original Article

Bioanalytical method development and validation of trigonelline by tandem mass spectra and its application to pharmacokinetic study

Kanaga Sabapathi S[1, 2], Vrushabendra Swamy B. M[3]

[1]Department of Pharmacokinetics and Drug metabolism, Syngene International Limited, Biocon Park, Plot 2&3, Bommasandra Industrial Estate - Phase-IV, Bommasandra-Jigani Link Road, Bangalore 560 099, India
[2]Shri JagdishprasadJhabarmalTibrewala University, Jhunjhunu-Churu Road, Vidyanagari, P.O. Chudela, Bhasawtakhurad, KajiwadaMohalla, Jhunjhunu, Rajasthan 333001
[3]Department of Pharmacology, East Point College of Pharmacy, Biderahalli, Bangalore 560049, India

Received: 25-09-2015 / Revised: 20-10-2015 / Accepted: 25-10-2015

ABSTRACT

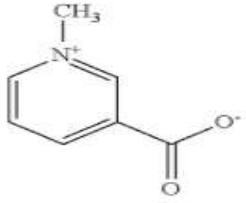
A rapid, simple and sensitive LC–MS/MS analytical method was developed and validated for the determination of trigonelline in Sprague Dawley Rat Plasma, using Atenolol as an internal standard. AB SCIEX QTRAP® 4000 LC-MS/MS was used. Chromatographic separation was achieved using a Gemini 5µ C6 Phenyl 100x4.6mm (Phenomenex), maintained at 40°C. The samples were eluted using 0.1% Formic acid in Water (solvent A) and 0.1% formic acid in Acetonitrile (solvent B), at a flow rate of 0.8 ml/min with a total run time of 4.0 min. The atmospheric pressure ionization source (API-4000) triple quadruple mass spectrometer equipped with an electro spray ionization source, operating in positive mode. Analysis was performed in multiple reaction-monitoring (MRM) mode by monitoring the ion transitions from m/z 138.0→92.1 (Trigonelline) and m/z 267.3→145.2 (IS). Calibration curves in spiked plasma were linear over the concentration range of 1–2000 ng/mL with determination coefficient >0.9989. The developed method was validated in terms of selectivity, accuracy, precision, linearity, Matrix effect, dilution integrity and stability study. The proposed method uses less biological material and the method is compatible for different biological matrix also. Method can be applicable for pharmacokinetic studies and in-vitro related studies using LC-MS/MS or HPLC.

Key words: Bio-analytical, Trigonelline, Atenolol, LC-MS/MS, SD Rat plasma

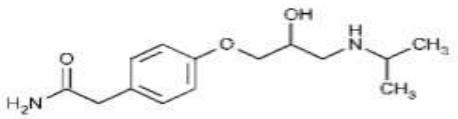
INTRODUCTION

Methods for measuring drugs in biological media are becoming increasingly important for the study of bioavailability & bioequivalence studies, quantitative evaluation of drugs and their metabolites, new drug development, clinical pharmacokinetics, research in basic biomedical and pharmaceutical sciences and therapeutic drug monitoring. There is evidence that Trigonella foenum-graecum L. (fenugreek), a traditional Chinese herb, and its components are beneficial in the prevention and treatment of diabetes and central nervous system disease [1]. The pharmacological activities of trigonelline, a major alkaloid component of fenugreek, have been more thoroughly evaluated than fenugreek's other components, especially with regard to diabetes and central nervous systemdisease [2, 3, 4].

CHEMICAL STRUCTURE



(A) TRIGONELLINE



(B) ATENOLOL

***Corresponding Author Address:** Mr.Kanagasabapathi S, Research Scientist, Syngene International Limited, Biocon Park, Plot 2&3, Bommasandra Industrial Estate - Phase-IV, Bommasandra-Jigani Link Road, Bangalore 560 099, India; E-mail: kspathi123@gmail.com*

MATERIALS AND METHODS

Chemicals: Trigonelline and atenolol (IS) was purchased from Sigma Aldrich, India. HPLC Grade solvents (Acetonitrile, Methanol) were obtained from Merk, India. Dimethyl sulfoxide (DMSO) was purchased from sigma Aldrich, India. Formic acid was obtained from Sigma Aldrich, Germany. And milli-Q water was from SG Series Compact Pretreatment Module. Blank Rat plasma was purchased from bio chemed USA.

Stock Solution, Calibration Standards and Quality Control Samples: Standard stock solutions of Trigonelline and Atenolol (IS) were prepared in DMSO with a final concentration of 1mg/ml. These solutions were stored at 2–8ºC until use. The IS stock solution was diluted to achieve a final concentration of 1µg/ml in acetonitrile. Analytical standards for Trigonelline were prepared in acetonitrile:water (70:30, v/v) over a concentration range of 1-2000 ng/ml by serial dilution, and same concentration range for calibration curve were prepared in blank SD rat plasma. Quality control (QC) samples at three different concentration levels (4, 1200, 1600 ng/ml as, low, medium and high, respectively)

Chromatographic Condition: A PerkinElmer Series 200 pump (Norwalk, CT, USA) consisting of flow control valve, vacuum degasser (Series 200 vacuum degasser) operated in gradient mode to deliver the mobile phase at flow rate of 0.8ml/min. The chromatographic system consisted of reverse phase column (Phenomenex, Gemini 5µ C6 Phenyl 100x4.6mm) and mobile phase consists solvent A: 0.1% Formic acid in Water and solvent B: 0.1% formic acid in Acetonitrile. The samples (5 µl) were injected on to the LC–MS/MS system through an auto injector. The auto sampler temperature was kept at 10°C and the column oven was maintained at 40°C.

Gradient Method

Total Time (min)	Flow Rate (µL/min)	Mobile Phase-A	Mobile Phase-B
0.01	800	100	0
0.40	800	100	0
1.00	800	5	95
2.20	800	5	95
2.40	800	100	0
4.00	800	100	0

Mass Spectrometric Condition: Mass spectrometric detection was performed on API 4000–QTRAP mass spectrometer (Applied Biosystems/MDS SCIEX, Concord, Ontario, Canada) equipped with a Turbo V Ion Spray ionization source operating in ESI positive ion mode, In mass spectrometer, zero air was used as nebulizing gas (GS1) And turbo gas (GS 2) while nitrogen as curtain gas (CUR) and collision activated dissociation (CAD) gas and these gases were constantly supplied from a gas generator (Peak Scientific, USA). MS and MS/MS condition for pure standards of Trigonelline, and IS were optimized by continuous infusion at 5µL/min using syringe pump (Model '11', Harvard apparatus, Inc., Holliston, MA, USA). MS/MS analyses of all analysts were performed using nitrogen as CAD gas. The most abundant product ion of each component was selected to build multiple reaction monitoring (MRM) method. The transitions monitored were m/z 138>92, and 267.3>145.2 for component trigonelline and IS, respectively.

Ion source gas (GS 1), ion source gas (GS 2), curtain gas (CUR), collision gas (CAD), ion spray voltage and temperature were set to 35, 45, 10, medium, 5500 kV and 550 °C, respectively. The mass spectrometer was operated in unit resolution for both Q1 and Q3 in the MRM mode, with a dwell time of 100 msec MRM channel. All data were acquired within 4.0 min using Analyst 1.6.1 software (Applied Biosystems/MDS SCIEX).

Sample Preparation: Trigonelline from the plasma was extracted using Acetonitrile precipitation extraction technique. Aliquot of 50µl plasma was taken into eppindorf tubes and added 150µl of Acetonitrile contain internal standard, vortex (Type 37600 mixer, Thermolyne, USA) for 5 min and centrifugation (Megafuge 3SR, Heraeus, Germany) at 10,000 rpm for 5 min, the supernatant collected and 5 µL was injected onto the LC–MS/MS system.

METHOD VALIDATION

The method was validated to meet the acceptance criteria of industrial guidance for the bioanalytical method validation (US Food and Drug Administration, 2001) [5]. The method was validated for selectivity, linearity, precision and accuracy, recovery, Matrix effect, dilution integrity and stability.

Selectivity: The selectivity was assessed by comparing the chromatograms of six different batches of blank plasma obtained from six different batches with those of the corresponding standard plasma spiked with trigonelline and IS. The area response of analytes in blank extract should not be

greater than 20% compared with the area response of LLOQ concentration.

Linearity: The linearity was tested by analyzing calibration standards at 10 concentration levels over the range of 1–2000ng/mL of the analyte. The samples were run in the order from low to high concentrations. A blank sample (without trigonelline and IS) and a zero sample (with IS) were also analyzed to confirm the absence of any interferences, these data were not included to construct calibration plots. The calibration curve was prepared by determining the best fit of peak area ratios (peak area analyte/peak area IS) vs concentration and fitted to y = mx + c using weighing factor (1/x2).

Recovery: Recovery of trigonelline was determined by comparing the peak area of extracted analyte standard with the peak area of non-extracted standard. The recovery of IS was determined in the same way. Recovery of the analyte need not be 100%, but the extent of recovery of an analyte and that of IS should be consistent, precise and reproducible at all QC levels.

Precision and Accuracy: For precision and accuracy studies, samples of three concentration levels were prepared as low (LQC), medium (MQC) and high (HQC) quality controls, corresponding to 4, 1200 and 1600µg/ml respectively with six replicates each. Precision was evaluated with inter and intra batches.

Stability studies: The autosampler stability of trigonelline in reconstitution was examined at 8°C for 24 h using LQC and HQC samples insix replicate injections and comparing with the fresh quality control samples. The bench top stability was evaluated at ambient temperature of 24±2°C for 6 h using LQC and HQC samples in six replicate injections and comparing with the fresh quality control samples. Long-term stability of analyte was studied for 10 days by analyzing the quality control samples at two different levels. Freeze–thaw stability quality control samples containing analyte were tested after four freeze (-80°C) and thaw cycles (room temperature) and compared with freshly spiked plasma samples. All stability evaluations were based on back calculated concentrations. Sample were considered to be stable if the assay values were within the acceptance limits of percentage mean ratio of the fresh quality control samples and the stability samples.

Matrix effect: The matrix effect was evaluated by comparing the peak area obtained from analytes in post-extracted blank plasma samples, with those of the respective compound dissolved at the same concentrations in reconstitution solution (acetonitrile–water, 50:50). The matrix effect was determined at two levels (LQC and HQC) for HMB, whereas the matrix effect over the IS was determined at a single concentration of 2 mg/mL.

Dilution integrity: Dilution integrity was performed to extend the upper concentration limit with acceptable precision and accuracy.

RESULTS AND DISCUSSION

Liquid chromatography: The chromatographic conditions including mobile phase composition and column type were optimized for better resolution and sensitivity. Several trials were carried out to achieve good resolution and increase the signal of the analyte. The feasibility of several mixtures such as acetonitrile and methanol using different buffers such as ammonium acetate and ammonium formate along with altered flows (in the range of 0.4–1.0 mL/min)was tested for complete chromatographic resolution of Trigonelline and IS from interfering biological matrix. The presence of a small amount of formic acid in mobile phase and sample preparation improved the detection and recovery of the analyte. The versatility, suitability and robustness of the method were checked with several C18 and C8 columns. The Phenomenex, Gemini 5µ C6 Phenyl column provided very good selectivity, sensitivity and peak shape for Trigonelline and atenolol (IS). shows typical chromatograms for blank plasma without Trigonelline, blank plasma without IS , blank plasma spiked with LLOQ and ULOQ concentration (Fig. 6). The mobile phase comprising 0.1% formic acid with acetonitrile and water (Gradient) with a flow rate of 0.8 mL/min was found to be suitable during LC optimization.

Selectivity: The described method used reversed-phase HPLC for separation of trigonelline and atenolol (IS) was shown to be selective for the analyte and IS (retention times for trigonelline and atenolol were 1.65 and 1.98 minutes respectively). No interfering peaks were observed with the same retention time of the analyte when different plasma samples were analyzed.

Linearity: Linearity was demonstrated from 1.0-2000.0ng/ml. The calibration curve includes 10 calibration standards which are distributed throughout the calibration range. Correlation coefficient was demonstrated for the evaluation of goodness fit. The average correlation coefficient was found to be 0.9989 with goodness of fit.

Accuracy and Precision: Accuracy and precision was evaluated by analyzing 3 batches. Each batch consists of six replicates of LQC, MQC and HQC. Precision was evaluated both interday and intraday batches. The interday and intraday precision and accuracy of the method for each trigonelline concentration levels (4.0, 1200.0 and 1600.0ng/ml) are represented in Table 2. The mean accuracy for each concentration level ranged from 99.92% to 104.63%.

Stability Studies: Trigonelline was stable in rat plasma after short term storage on bench top for 6 h at room temperature (24±2°C), storage in the autosampler for 24 h at 8°C, long-term storage for 10 days at -80°C and four freeze–thaw cycles. In stability studies six replicates of LQC and HQC control levels were analyzed. The results are presented in Table 3 and were found to be within the assay variability limits during the entire process.

Recovery: The recovery in terms of extraction efficiency was determined at three concentration (LQC, MQC and HQC) levels by comparing peak areas obtained from processed QC samples and the peak area obtained from samples with equal concentration with those of QC samples spiked in reconstitution solution (acetonitrile–water,50:50). The recovery values of Trigonelline were 83.07, 78.06 and 80.41% respectively. The mean recovery of IS found to be 95.49% represented in table 4&5.

Matrix effect: The average matrix factor values (matrix factor = peak area of post-spiked concentrations/peak area of neat concentrations) obtained were +0.02 (CV 4.1%) and +0.01 (CV 1.8%) at LQC and HQC concentrations, respectively. The matrix effect of IS was found to be +0.07 (CV 5.6%). Overall it was found that the plasma extract has no impact on the ionization of analyte and IS.

Dilution integrity: The upper concentration limits can be extended to 100000 ng/mL by a 10- or 50-fold dilution with rat plasma with %CV of1.8% and an accuracy of 98.5%.

Application to pharmacokinetic study: The method described above was successfully applied to a PK with and without P-glycoprotein inhibitor (Elacridar 1mg/kg) study in which plasma concentration of pure markers was determined for up to 24 h after the intravenous at 1mg/kg and oral administration at 10 mg/kg doses of trigonelline with and without P-glycoprotein inhibitor (Elacridar 1mg/ml) in male SD rats., bioavailability was found greater with P-glycoprotein inhibitor. Pharmacokinetic parameters were calculated from the plasma concentration time data by non-compartmental analysis using Win Nonlin (Phoenix version 6.2, Pharsight Inc., USA). The pharmacokinetic parameters of trigonelline were presented in Table 6.The plasma concentration time profiles of trigonelline are shown in Fig. 3, 4 and 5.

CONCLUSION

The current validated LC-MS/MS method for trigonelline offers good accuracy and significant advantages in terms of sensitivity, selectivity and sample preparation. It can be used for the estimation of trigonelline in biological fluids. The separation method developed produce acceptable values of recovery. The chromatogram developed has well resolved peak of trigonelline without any interference. And we conclude that the developed method can be applied in bioequivalence, pharmacokinetic, in-vitro related studies and toxicokinetic studies with desired precision and accuracy along with high-throughput.

Table 2. Intra-day and inter-day precision and accuracy for the determination of Trigonelline

Intra-day (n = 6)				**Inter-day (n = 18)**		
Concentration added(ng/mL)	**Concentration found (ng/mL)**	**Accuracy (%)**	**CV (%)**	**Concentration found (ng/mL)**	**Accuracy (%)**	**CV (%)**
4	4.05	101.25	1.5	4.12	103.00	2.4
1200	1298.59	108.22	2.4	1255.58	104.63	1.8
1600	1624.54	101.53	2.4	1598.78	99.92	3.2

Table 3. Stability of Trigonelline under different storage conditions(n=6)

Storage conditions	Concentration (ng/mL)		Accuracy (%)
	Actual concentration	observed concentrations	
Autosampler stability for 24 h (8 °C)	4.00	4.08	102.00
	1600.00	1612.89	100.81
Bench-top stability for 6 h (24±2° C)	4.00	4.15	103.75
	1600.00	1605.56	100.35
Long term freeze–thaw stability for10 days (-80° C)	4.00	4.06	101.50
	1600.00	1613.58	100.85
Four freeze–thaw cycles stability	4.00	4.12	103.00
	1600.00	1602.59	100.16

Table 4: Recovery of Atenolol in Plasma

Sample Name	Analyte Peak Area (counts)	Average Analyte Peak Area (counts)	% of recovery	%CV
Plasma Extracted LQC-01	1530294	1503291	95.49	3.59
Plasma Extracted LQC-02	1454280			
Plasma Extracted LQC-03	1569941			
Plasma Extracted MQC-01	1583187			
Plasma Extracted MQC-02	1512230			
Plasma Extracted MQC-03	1495830			
Plasma Extracted HQC-01	1465981			
Plasma Extracted HQC-02	1458952			
Plasma Extracted HQC-03	1458924			
Aqueous LQC-01	1534086	1574336		
Aqueous LQC-02	1563885			
Aqueous LQC-03	1571672			
Aqueous MQC-01	1611238			
Aqueous MQC-02	1549874			
Aqueous MQC-03	1574669			
Aqueous HQC-01	1600458			
Aqueous HQC-02	1598248			
Aqueous HQC-03	1564897			

Table 5: Recovery of Trigonelline in Plasma

Sample Name	Analyte Peak Area (counts)	Average Analyte Peak Area (counts)	% of recovery	%CV
Plasma Extracted LQC-01	2724	2678	83.07	1.76
Plasma Extracted LQC-02	2743			
Plasma Extracted LQC-03	2568			
Aqueous LQC-01	3245	3224		
Aqueous LQC-02	3369			
Aqueous LQC-03	3059			
Plasma Extracted MQC-01	50078	51245	78.16	2.57
Plasma Extracted MQC-02	51199			
Plasma Extracted MQC-03	52459			
Aqueous MQC-01	65489	65565		
Aqueous MQC-02	65947			
Aqueous MQC-03	65258			
Plasma Extracted HQC-01	534665	548129	80.41	1.64
Plasma Extracted HQC-02	550174			
Plasma Extracted HQC-03	559548			
Aqueous HQC-01	675894	681667		
Aqueous HQC-02	684589			
Aqueous HQC-03	684519			

Table 6: Pharmacokinetics data with and without the P-glycoprotein inhibitor (Elacridar 1mg/ml)

Intravenous PK data of (1mg/kg dose)					
PK Parameters	**Plasma Conc. (ng/ml) IV**			**Mean IV (1mpk)**	**SD**
	Animal 1	**Animal 2**	**Animal 3**	**(ng/ml)**	
C_0 (ng/ml)	967.80	856.97	725.46	850.08	121.3
$AUC_{(inf)}$ (hr*ng/ml)	547.03	564.69	551.07	554.26	9.3
$AUC_{(0-t)}$ (hr*ng/ml)	543.23	560.27	544.97	549.49	9.4
Vd (L/kg)	3.85	3.92	3.26	3.68	0.4
CLp (L/hr/kg)	1.83	1.78	1.82	1.81	0.0
Vdss (L/kg)	1.92	1.95	2.27	2.05	0.2
$t_{1/2}$ (h)	1.46	1.53	1.24	1.41	0.2

Oral PK data of (10mg/kg dose) without P-glycoprotein inhibitor (Elacridar 1mg/ml)					
PK Parameters	**Plasma Conc. (ng/mL) PO**			**Mean PO (10mpk)**	**SD**
	Animal 4	**Animal 5**	**Animal 6**	**(ng/ml)**	
Cmax(ng/ml)	70.40	68.70	58.80	65.97	6.3
Tmax(h)	2.00	2.00	2.00	2.00	0.0
AUC(inf) (hr*ng/ml)	n.d.	n.d	n.d	n.d.	n.d
AUC(0-t) (hr*ng/ml)	185.49	184.37	183.39	184.42	1.1
Bioavailability* (%)	3.41	3.29	3.37	3.36	0.1

Oral PK data of (10mg/kg dose) with P-glycoprotein inhibitor (Elacridar 1mg/ml)					
PK Parameters	**Plasma Conc. (ng/mL) PO**			**Mean PO (10mpk)**	**SD**
	Animal 7	**Animal 8**	**Animal 9**	**(ng/ml)**	
Cmax(ng/ml)	602.50	750.70	750.50	701.23	85.5
Tmax(h)	0.50	0.50	0.50	0.50	0.0
AUC(inf) (hr*ng/ml)	2240.74	2845.29	2501.22	2529.08	303.2
AUC(0-t) (hr*ng/ml)	2189.18	2746.27	2430.16	2455.20	279.4
Bioavailability* (%)	40.30	49.02	44.59	44.64	4.4
t1/2 (h)	4.02	6.73	4.25	5.00	1.5

Fig.1. Q1 Scan of the triconelline

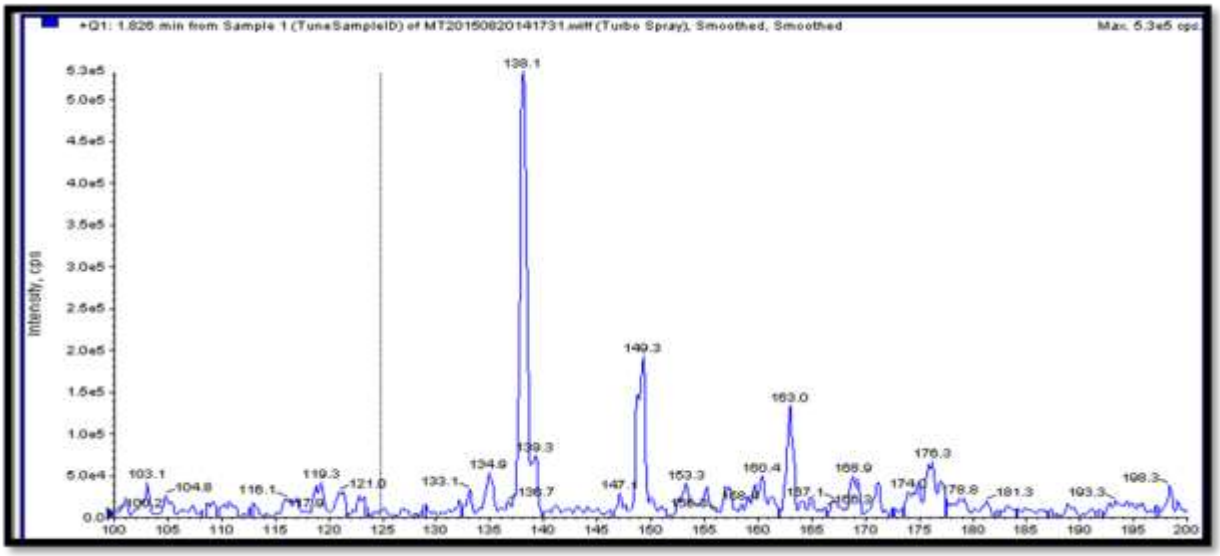


Fig.2. Q3 Scan of the triconelline

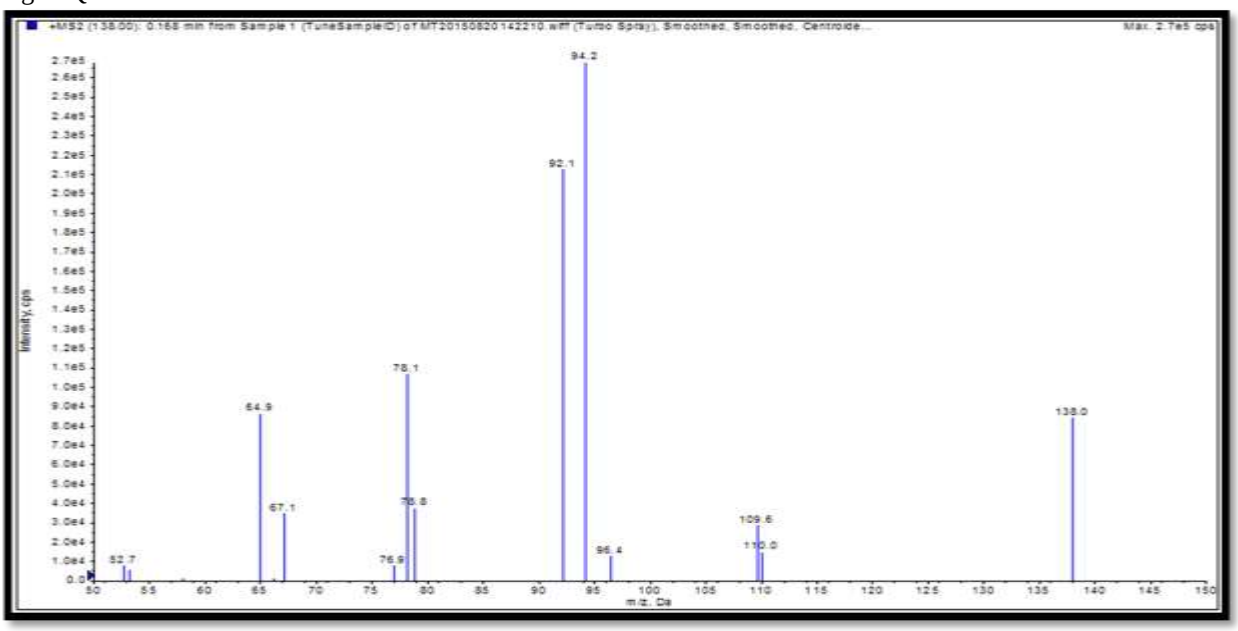


Fig.3.Intravenous PK data of (1mg/kg dose)

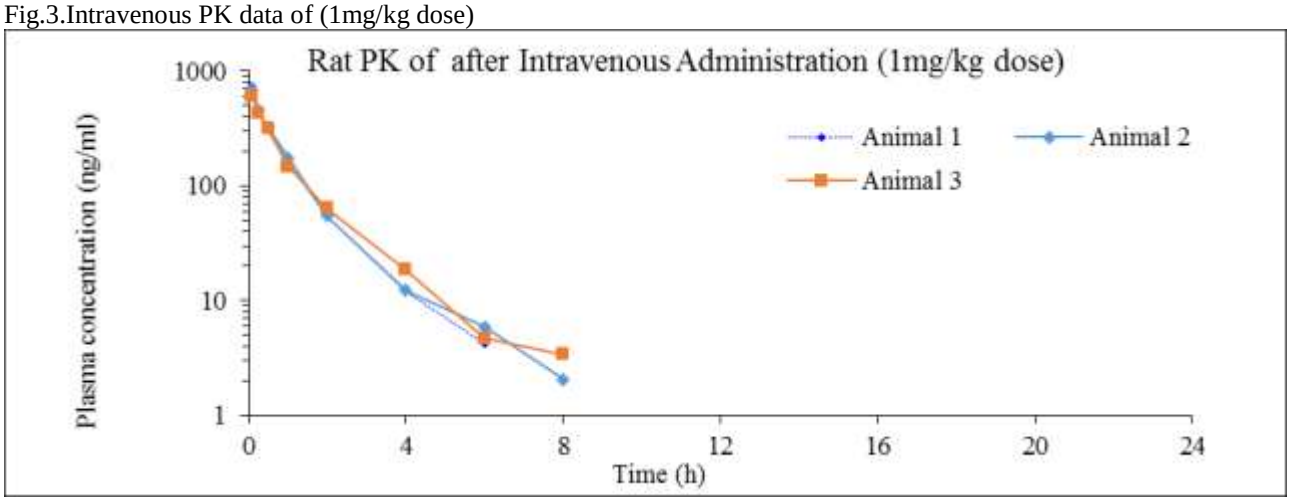


Fig.4.Oral PK data of (10mg/kg dose) without P-glycoprotein inhibitor

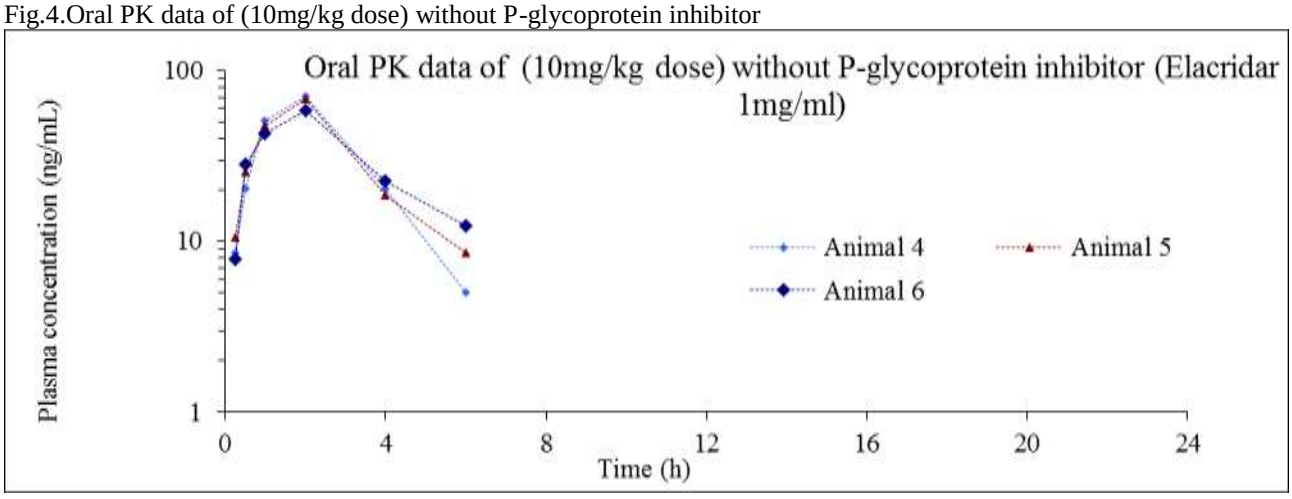


Fig.5.Oral PK data of (10mg/kg dose) with P-glycoprotein inhibitor (Elacridar 1mg/ml)

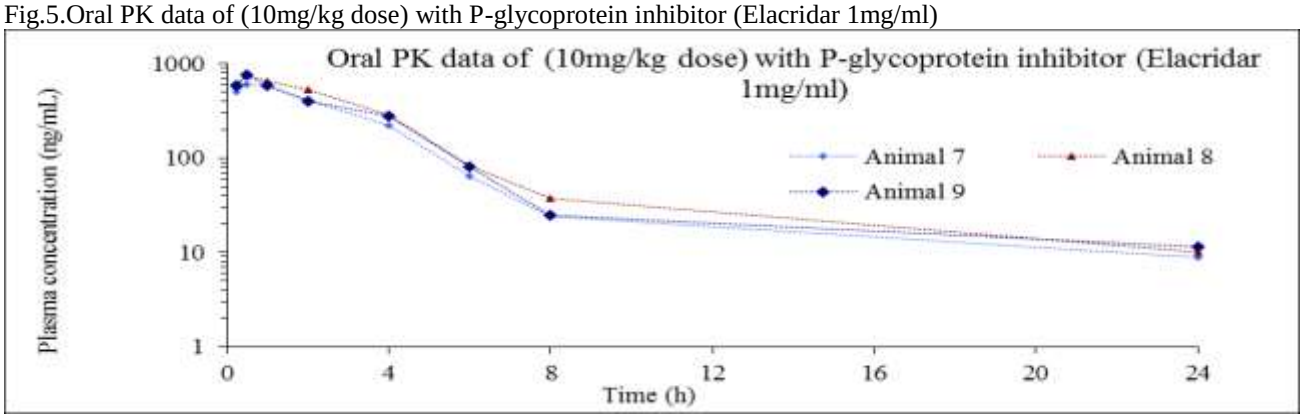
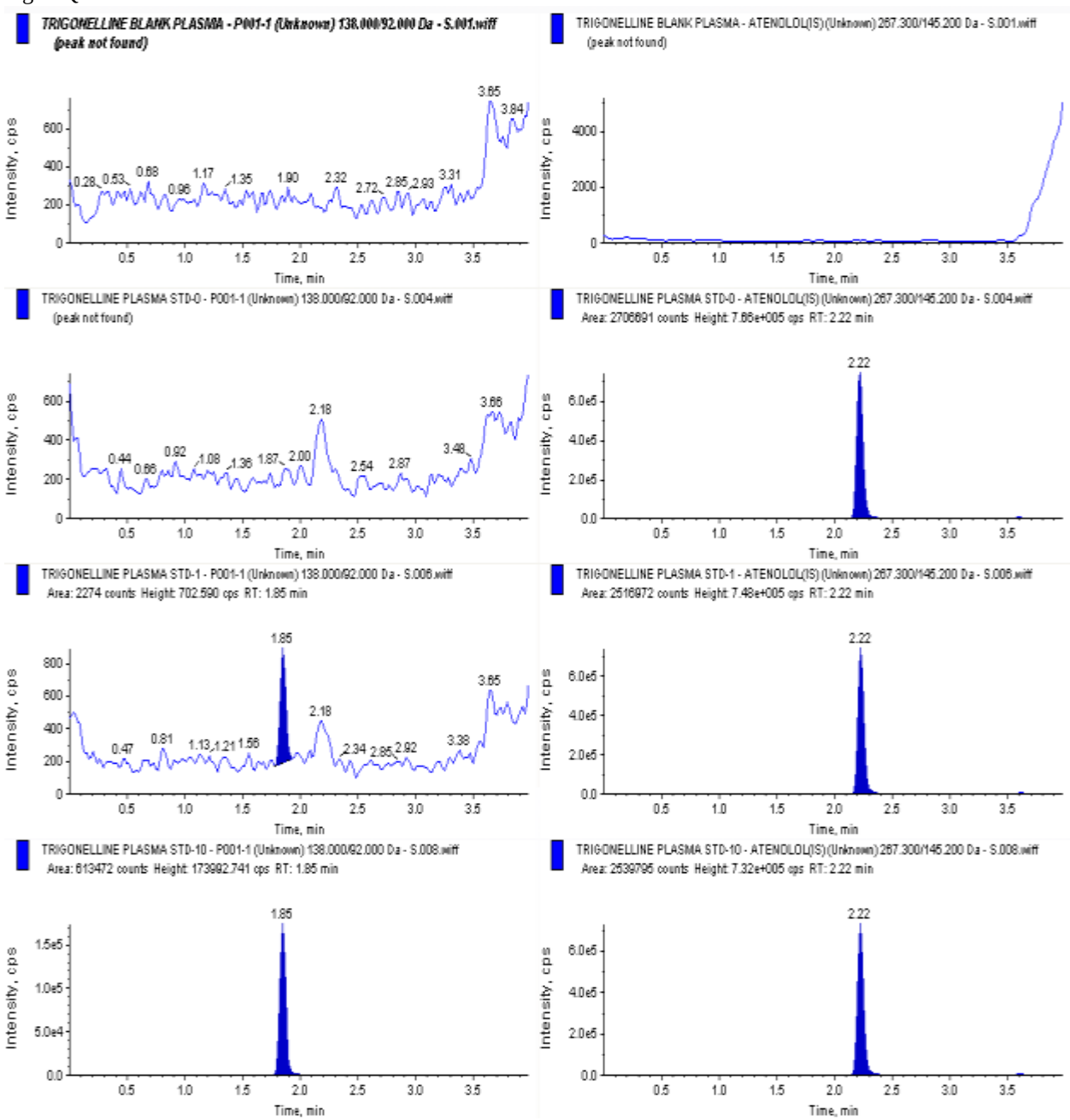


Fig.6. Q1 Scan of the triconelline



REFERENCE

[1]. Zhou J1, Chan L, Zhou S. Trigonelline: a plant alkaloid with therapeutic potential for diabetes and central nervous system disease.Curr Med Chem. 2012; 19(21):3523-31.
[2]. Lang R1, Yagar EF, Eggers R, Hofmann T. Quantitative investigation of trigonelline, nicotinic acid, and nicotinamide in foods, urine, and plasma by means of LC-MS/MS and stable isotope dilution analysis. J Agric Food Chem. 2008 Dec 10; 56(23):11114-21
[3]. Daniel Perrone, Carmen Marino Donangelo, Adriana Farah Fast simultaneous analysis of caffeine, trigonelline, nicotinic acid and sucrose in coffee by liquid chromatography–mass spectrometry Food Chemistry 110 (2008) 1030–1035
[4]. Roman Lang, Anika Wahl, Timo Stark and Thomas Hofmann Urinary N-methylpyridinium and trigonelline ascandidate dietary biomarkers of coffee consumption. Mol. Nutr. Food Res. 2011, 55, 1–11
[5]. Guidance for Industry: Bioanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Rockville, MD, 2001, Available from: http://www.fda.gov/CVM.