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Original Article

DETERMINATION OF TRAZODONE IN HUMAN PLASMA BY REVERSED-PHASE LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY WITH ELECTROSPRAY IONISATION

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ABSTRACT

Objective: The development and validation of LC-MS/MS method for quantification of Trazodone (a serotonin antagonist and reuptake inhibitor (SARI), which is a second generation antidepressant compound belonging to the class of phenyl piperazine) in human plasma is described.

Methods: The method involves protein precipitation (extraction) using Trazodone d6 as an internal standard (IS). Chromatographic separation is achieved on Zorbax eclipse XDB C8 150×4.6 mm, 5 µm column with a mobile phase consisting of 2 mM ammonium formate (pH 3.0) and methanol (30:70 v/v) at a flow rate of 1.0 mL / min and the total run time was 2.5 minute. Detection was carried out by AB Sciex API 3200 tandem mass spectrometer using positive electro-spray ionization mode by multiple reactions monitoring method at m/z 372.00→176.10 and 378.20→182.10 for Trazodone and Trazodone d6 (ISTD) respectively with dwell times of 300 msecs for each of the transitions.

Results: The standard curve was linear from 5.203 ng / mL to 3025.166 ng / mL with goodness of fit (r^2) greater than 0.990 observed during the method validation batches. This assay allows quantification of Trazodone at a concentration as low as 5 ng / mL in human plasma. The observed mean recovery was 88% for the drug.

Conclusions: The method described here is found to be simple, cost effective and suitable for the use in bioequivalence and bioavailability studies.

Keywords: Trazodone, Trazodone d6, LC-MS/MS.

INTRODUCTION

Trazodone is chemically 2-{3-[4-(3-chlorophenyl)piperazin-1-yl] propyl}-2H,3H-[1,2,4]triazolo[4,3-a]pyridin-3-one. It is a serotonin antagonist and reuptake inhibitor (SARI), which is a second generation antidepressant compound belonging to the class of phenylpiperazine. The drug showing antidepressant activity is due to the blockage of serotonin reuptake by inhibiting serotonin reuptake pump at the presynaptic neuronal membrane. Trazodone shows its therapeutic actions through 5-HT2A receptors[1]. Trazodone also induces anti-anxiety and sleep-inducing effects. The mechanism of trazodone hydrochloride's antidepressant action in man is not fully understood. In animals, trazodone selectively inhibits its serotonin uptake by brain synaptosomes and potentiates the behavioral changes induced by the serotonin precursor, 5-hydroxytryptophan. Trazodone is not a monoamine oxidase inhibitor and, unlike amphetamine-type drugs, does not stimulate the central nervous system.

In humans, trazodone hydrochloride is well absorbed after oral administration without selective localization in any tissue. When trazodone hydrochloride is taken shortly after ingestion of food, there may be an increase in the amount of drug absorbed, a decrease in maximum concentration and a lengthening in the time to maximum concentration. Peak plasma levels occur approximately one hour after dosing when trazodone hydrochloride is taken on an empty stomach or two hours after dosing when taken with food. In vitro studies in human liver microsomes show that trazodone is metabolized to an active metabolite, m-chlorophenylpiperazine (mCPP) by cytochrome P450 3A4 (CYP3A4). Other metabolic pathways that may be involved in metabolism of trazodone have not been well characterized. In some patients trazodone may accumulate in the plasma.

Several analytical methods that have been reported for the determination of Trazodone in biological fluids such as spectrophotometry, ion-selective electrode, colorimetry, HPLC, capillary gas chromatography, GC-MS/MS[3] and LC-MS/ MS. Recently, improved analytical methods by LC-MS/MS enabled the detect ability of the drug to low nano gram levels (10 ng / mL) in human plasma. These methods are either liquid-liquid extraction[2] or SPE extraction[1] & also not sensitive. Due to the low quantification requirement & cost effectiveness, it is necessary to develop a simple, specific, selective, sensitive, cost effective, robust and rugged method for the quantification of trazodone in human plasma at low nano gram levels (5 ng / mL).

This article describes a simple, sensitive, cost effective and specific method for the determination of trazodone in human plasma using LC-MS/MS. The assay allows the quantification of trazodone at a concentration of as low as 5 ng / mL in human plasma. The proposed method is a high throughput LC-MS/MS method using protein precipitation extraction, where precision, accuracy and stability [freeze thaw, bench top, dry extract stability, auto sampler stability and long term stability (305 days) in plasma] are demonstrated through the validation data. This method provides excellent specificity and linearity over the range of 5.230 ng / mL to 3025.166 ng / mL of trazodone. This method has been used for the analysis of more than 5000 plasma samples from human volunteers. The method is very sensitive and involves simpler and selective extraction method for the estimation of trazodone in plasma samples compared to the previously reported methods.

MATERIALS AND METHODS

Materials

Trazodone Hydrochloride working standard was provided by Astron research Limited, Ahmedabad, India. Internal Standard Trazodone D6 Hydrochloride was procured from Clearsynth lab limited, Mumbai, India. Methanol (HPLC grade) was purchased from Merck, India. Water (Ultrapure) was generated inhouse through milli Q system, Ammonium formate (GR grade) was purchased from Acros, India and formic acid (Emparta Grade) was purchased from Merck, India. Drug free plasma was obtained from Prathama Blood Center, Ahmedabad, India.

Instrumentation and Equipments

Mass spectrometric analysis was performed on a AB SCIEX API 3200(LC-MS/MS) equipped with atmospheric pressure ionization

interface source, manufactured by MDS Sciex, Canada, attached to a high performance liquid chromatography, manufactured by Simadzu, Japan, equipped with CTO-10 ASvp column oven, LC-10 ADvp pump and SIL-HTc auto sampler. Chromatography was carried out on an Eclipse XDB-C8 5µm 4.6x150 mm column.

The data acquisition was achieved using Analyst 14.2 software.

Samples were centrifuged in a refrigerated centrifuge (Multifuge 3 S-R+) supplied by Thermo scientific, Germany.

Chromatographic condition

The samples were analyzed on an Eclipse XDB-C8 5µm 4.6x150 mm column. A mobile phase consisting of 70:30 mixture of methanol: 2 mM ammonium formate buffer (pH 3.0) was used with a flow rate of 1.0 mL / min at 40°C under isocratic conditions. The auto sampler was set at 4°C and the injection volume was 2 µL. The expected RT for the IS and the drug were 1.61 min and 1.62 min respectively and the total run time for the analysis was 2.5 min.

Mass spectrometric condition

Atmospheric pressure ionization (API) mass spectra were obtained by scanning parent ion scan (MS) and product ion scan (MS/MS) indicated positive molecular ion (M + H)+ for Trazodone and Trazodone d6 (ISTD). Different polarities were employed but the highest product ion intensities were obtained using positive ionization for trazodone and internal standard (IS). The parent ion of trazodone was obtained at 372.00 amu and that of internal standard (IS) was at 378.20 amu. The product ion (MS/MS) spectra indicated that the compounds undergo different collision induced dissociation & collision active dissociation. The MS/MS spectra for trazodone showed a major product ion at 176.10 amu and the internal standard (IS) showed a high intense product ion at 182.10 amu. The molecular ion and product ion were selected for further quantification because of their greater selectivity, specificity and sensitivity.

The trazodone and internal standard were monitored in the positive ion mode using Multiple Reaction Monitoring (MRM) transitions of m/z 372.00 → 176.10 and m/z 378.20 → 182.10 respectively with dwell times of 300 msec for each of the transitions. The mass detection was obtained at unit mass resolution for all channels. Quantification of trazodone in human plasma is based on the peak-area ratios of trazodone versus its internal standard (IS).

The typical mass parameters are summarized in Table 1.

Table 1: It shows mass spectrometry parameters

Parameter ID	Parameters for DRUG	Parameters for ISTD
Nebulizer gas (GS1)	50.00	50.00
Auxiliary gas (GS2)	50.00	50.00
Curtain gas (CUR)	25.00	25.00
Collision activate dissociation (CAD)	3.00	3.00
Ion spray voltage (IS)	5500.0	5500.0
Temperature (TEM)	400	400
De clustering potential (DP)	55.00	55.00
Entrance potential (EP)	10.00	10.00
Cell Entrance Potential (CEP)	20.99	18.06
Collision Energy (CE)	35.00	35.00
Cell exit potential (CXP)	3.00	3.00

Preparation of calibration curve (CC) and quality control (QC) samples

Stock solution of trazodone (1000 µg / mL) was prepared using methanol:water (50:50) solution as diluent. Spiking solutions for CC and QC were prepared from the stock solution by adequate serial dilutions using methanol:water (50:50) solution. Stock solution of trazodone d6 (1000 µg / mL) was prepared using methanol as solvent. The IS spiking solution (1000 ng / mL) was prepared by diluting the stock with methanol.

Calibration standards were prepared by spiking appropriate volume of the respective spiking solutions to control plasma to achieve eight different concentrations from 5.0 ng / mL to 3000.00 ng / mL of trazodone. The standards were then stored in the refrigerator. Quality control (QC) samples were prepared by spiking appropriate volume of the respective spiking solutions to control plasma to achieve four different concentrations at LOQ, low, medium and high levels for trazodone. The QC samples were then stored in the freezer below -65°C.

Sample processing for analysis

The frozen samples were thawed in a water bath at room temperature. The thawed samples were vortexed to ensure complete mixing of the contents. 0.1 mL of each plasma sample was transferred into pre-labeled tubes and 50 µL of internal standard (IS) solution (about 1000 ng / mL) was added to each tube and vortexed to ensure complete mixing. After the addition of 0.5 mL of methanol was added to each tube and vortexed for 3.0 minutes to ensure complete mixing, the samples were centrifuged at rcf 4000 ± 150 for 10 minutes at 10°C. The supernatant was transferred into an appropriate vial for analysis.

RESULTS

Chromatography

The peaks of Trazodone and Trazodone-d6 (ISTD) were free from any overlapping peaks and any closely eluting peaks and appropriately integrated in a manner that adjacent peaks do not influence the analyte and ISTD area.

Representative chromatograms are shown in Figures 1 to 4.

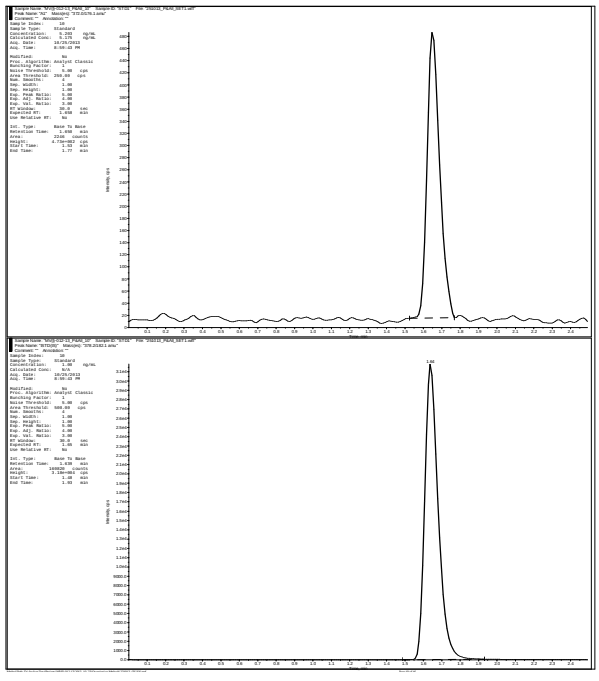

Fig. 1: It shows a representative chromatogram of lower limit of quantification

Selectivity

Eight batches of human blank plasma (six normal, one haemolysed and one lipemic) containing K2EDTA as an anticoagulant were evaluated for selectivity and no interference was observed at the retention times and transitions of Trazodone and Trazodone-d6 (ISTD) in any of the human blank plasma batches

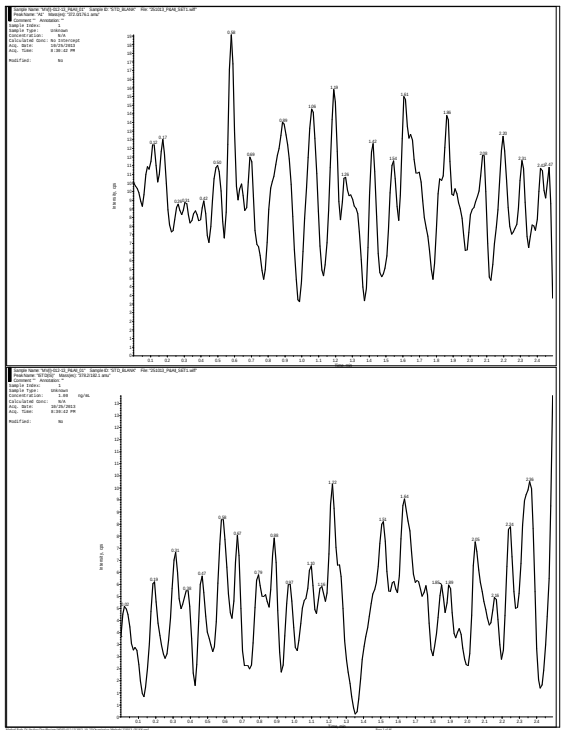

Fig. 2: It shows a representative chromatogram of Blank plasma

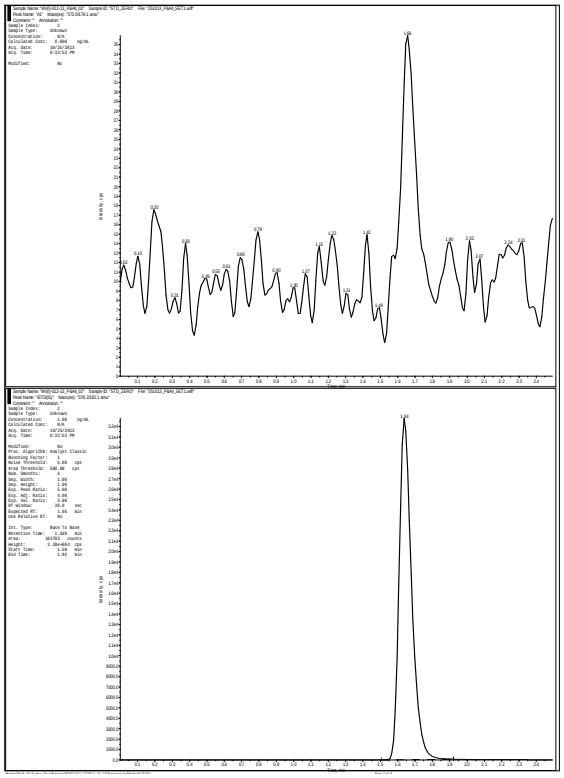

Fig. 3: It shows a representative chromatogram of blank + ISTD

Linearity

A linear equation was judged to produce the best fit for the concentration vs. area response relationship. The regression type was 1/concentration^2 and peak area ratio for an 8-point calibration curve was found linear from 5.203 ng / mL to 3025.166 ng / mL. The coefficient of determination (r^2) was consistently greater than 0.99 during the course of method validation. Calibration curve was processed and run along with each batch that was analyzed on consecutive days using freshly prepared solution each day. The between-batch variability in the calibration curves was reported with the coefficients of variation (CVs) and accuracy obtained during method validation.

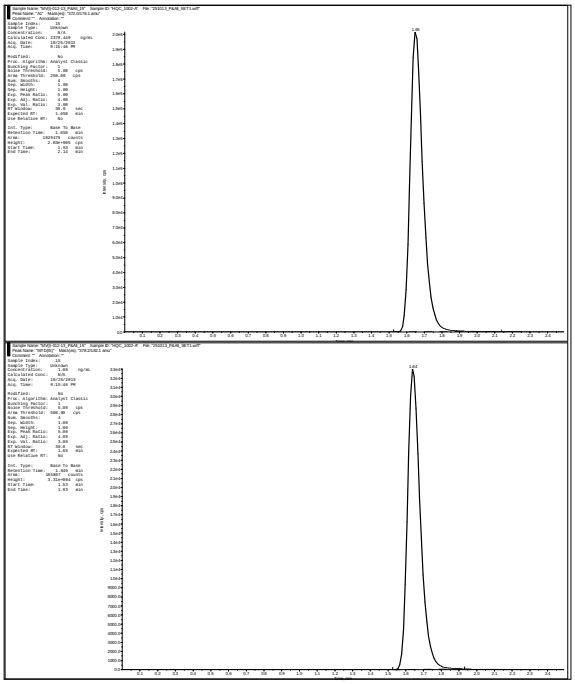

Fig. 4: It shows a representative chromatogram of ULQC

The range of precision and accuracy of the back-calculated concentrations of the standard curve points was from 0.1 % to 2.4 % and from 97.3 % to 102.1 % respectively.

Representative calibration curve is shown in Figures 5.

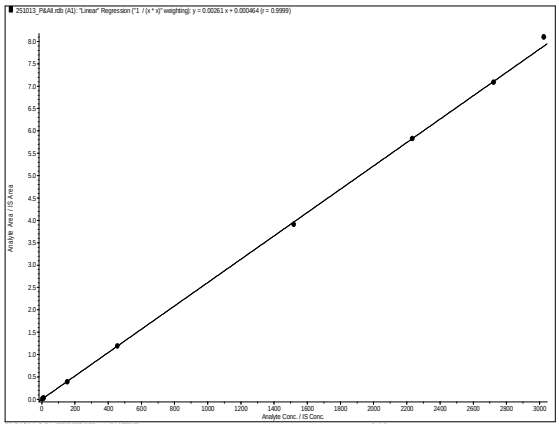

Fig. 5: It shows a representative regression analysis of a calibration curve

Sensitivity & Limit of detection

The lower limit of quantification was 5.203 ng / mL. The limit of detection of the method was 0.656 ng / mL.

Representative chromatograms are shown in Figures 6.

Precisions and Accuracy

Precision of the assay was measured by the percent coefficient of variance over the concentration range of limit of quantification, low, medium, high and dilution quality control samples of Trazodone

during the course of the method validation. Within-batch precision (Intra-day precision) ranged from 0.4 % to 5.7 %. Between-batch precision [Inter-day precision (Global statistics)] ranged from 1.0 % to 6.5 %.

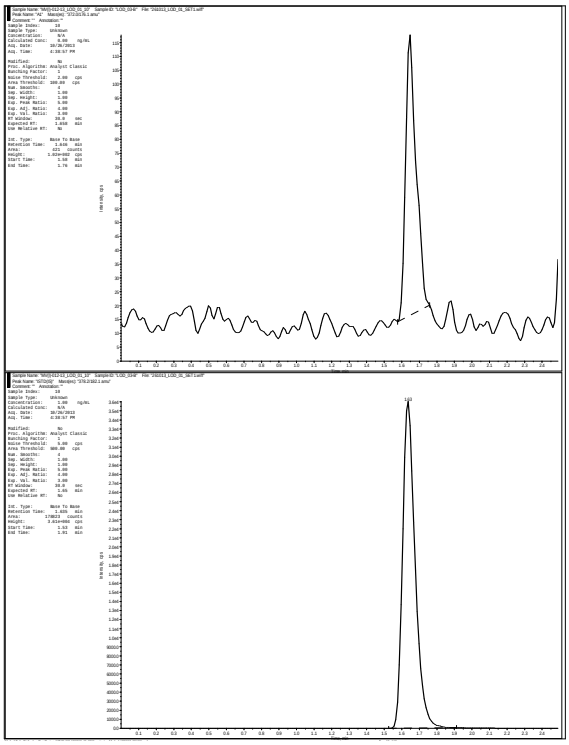

Fig. 6: It shows a representative chromatogram of limit of detection

Accuracy of the assay was defined as the absolute value of the ratio of the mean back-calculated values of the quality control samples to their respective nominal values, expressed in percentage. Within-batch accuracy (Intra-day accuracy) ranged from 102.0 % to 105.8 %. Between-batch accuracy [Inter-day accuracy (Global statistics)] ranged from 101.4 % to 107.2 %.

Stability

Six replicates of low and high quality control samples were used for the determination of the processed sample stability. Results indicated that the difference in the back-calculated concentration from time 0.0 hr to time 73.0 hrs between 2 and 8 °C was -0.3% for LQC and 2.1% for HQC. This showed that processed samples were stable for at least 73.0 hrs when stored between 2 and 8 °C.

The results of long term and short term stock solution stability indicated that the stock solutions of Drug and IS were stable for 11.0 hrs at room temperature and for 06 days while stored between 2 and 8 °C. The long-term stability of the Drug in plasma showed that the spiked Drug samples were stable for at least 61 days after storage at –65.0 °C in a deep freezer. The freeze-thaw stability results indicated that the Drug was stable after going through three freeze-thaw cycles. Furthermore, the results of bench top stability indicated that the processed samples were stable for at least 9.0 hrs at room temperature.

Recovery (extraction yield)

Trazodone

The mean % recovery for the low, medium and high quality control samples was 88.6 %, 86.4 % and 88.6 % respectively.

Trazodone D6

The mean recovery for Trazodone-d6 (ISTD) was 70.3 %.

Robustness and Ruggedness

The robustness and ruggedness of the method was evaluated through long batch of 150 samples.

Matrix effect

The CV (%) for IS normalized matrix factor in ten lots was 0.9 for HQC & 3.1 for LQC. Based on the obtained results it can be concluded that, no significant ion suppression or enhancement was observed during ionization in mass spectrometric detector.

Carryover

No significant carryover was observed when processed blank human plasma was injected immediately after the extracted high standards and when the reconstituted solution was injected immediately after the non-extracted aqueous high standard.

The method validation parameters are summarized in Table 2.

DISCUSSION

Due to the requirement for the quantification of trazodone in human plasma at low nano gram levels this simple, specific, selective, sensitive, cost effective, robust and rugged method for the quantification of trazodone in human plasma at low nano gram levels (5 ng / mL) has been developed. Other reported method of trazodone involves several critical steps of extraction and cleaning of the samples and hence, is very time consuming. Also the sensitivity of the method was not at par. The sensitivity of the drug observed in various HPLC/ UV, LC-MS/MS methods published until now is inadequate for pharmacokinetic studies and monitoring of the therapeutic drugs. Composition of the mobile phase was found to be a critical factor for achieving good shape of the chromatographic peak. In the present method 2 mM ammonium formate was selected as the buffer and methanol as the organic modifier. During the development, it was found the signal of the analyte dropped significantly after the usages of other buffers like formic acid, ammonium acetate, acetic acid & ammonium hydroxide.

Also several analytical columns were tried to obtained good chromatography. Finally good chromatography with lower retention time was obtained in column of Zorbax Eclipse XDB C-8 150X4.6 mm, 5µ using injection volume of 2µL. Various extraction techniques which include protein precipitation, liquid-liquid extraction and solid phase extraction were employed during method development. The liquid-liquid extraction technique using different solvents and back -extraction methods also resulted in low recovery of the drug. Furthermore, the solid phase extraction method involves usages of SPE cartridges, long extraction time and critical extractions steps. As trazodone has a very high protein binding, thus the precipitation methods, using different precipitating agents such as acetonitrile, methanol, acidic-basic acetonitrile / methanol solution were also tried.Hence a method using protein precipitation extraction was adopted utilizing methanol as precipitating agent. Good recovery, better precision & accuracy and very minimal ion suppression or matrix effect was observed during the validation of the method. In this method, sensitivity, linearity and stability improved considerably from the reported method. Tedious SPE & derivatization steps were avoided. This method was validated for calibration range of 5.203 ng / ml to 3025.166 ng / ml, which would be sufficient for the analysis of sample from a single dose (100 mg) administration to humans.

CONCLUSION

The method described here for the assay of trazodone in plasma is found to be simple, specific, selective, sensitive, cost effective, robust and rugged during a method validation. This demonstrates the suitability of this analytical method for the use in bioavailability studies. The method is proved to be suitable for the clinical investigation of trazodone pharmacokinetics and offers higher specificity, speed and greater sensitivity over the previously reported methods.

CONFLICT OF INTERESTS

Declared None

Table 2: It shows a Validation Summary

S. No.	Validation Parameters			Results / Data
01	Linearity (Range)			5.203 to 3025.166 ng / mL
02	Coefficient of determination			Greater than 0.99
03	Limit of quantification			5.203 ng / mL
04	Limit of detection			0.656 ng / mL
05	Selectivity			No interference at the retention times and transitions of drug and internal standard.
06	Precision	Between-batch precision)	(Inter-day	1.0 % to 6.5 %
		Within-batch precision)	(Intra-day	0.4 % to 5.7 %.
07	Accuracy	Between-batch accuracy)	(Inter-day	101.4 % to 107.2 %.
		Within-batch accuracy)	(Intra-day	102.0 % to 105.8 %
08	Robustness and Ruggedness experiment			Method is rugged and robust (up to 150 injections)
09	Recovery (drug) LQC, MQC & HQC			88.6 %, 86.4 % and 88.6 %
10	Recovery (ISTD)			70.3 %
11	Matrix effect			No significant ion suppression or enhancement
12	Stock solution stability (Short term)			11.0 hours (at room temperature)
13	Stock solution stability (long term)			06 days (within 2 to 8°C)
14	Auto sampler / Wet extract stability			73.0 hours (within 2 to 8°C)
15	Freeze and thaw stability			4 cycles at -65 ± 10°C
16	Bench top stability			9.0 hours (at room temperature)
17	Long term stability of drug in plasma			61 Days at -65±10°C

ACKNOWLEDGMENTS

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