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DEVELOPMENT AND VALIDATION OF SENSITIVE UPLC-MS/MS BASED METHOD FOR THE ESTIMATION OF CRIZOTINIB IN HUMAN PLASMA

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With the objective of decreasing analysis time and retaining good efficiency (UPLC-MS/MS) is an outstanding analytical approach for speedy biomedical analysis. The aim of this study was to develop and validate a simple, rapid, sensitive and specific UPLC-MS/MS method for quantification of CZT in human plasma. After a simple protein precipitation using acetonitrile and methanol, CZT and paroxetine (IS) were separated on Acquity UPLC BEH^{TM} C_{18} column (50 × 2.1 mm i.d., 1.7 µm, Waters, USA) using a mobile phase composed of methanol:0.1%*(v/v)* ammonium hydroxide (80:20) pumped at a flow rate of 0.4 mL min^{-1}. CZT and IS were eluted at 0.46 and 0.66 min, respectively. The mass spectrometric determination was carried out using an electrospray interface operated in the positive mode with multiple reaction monitoring mode. The mass transitions *m*/*z* 450.0 → 260.0 and *m*/*z* 330.11 → 192.11 were used to measure CZT and internal standard, respectively. Mass transition *m*/*z* 450.0 → 176.99 was used as qualifying ion for CZT. The method was linear in the concentration range of 5–500 ng mL^{-1} with correlation coefficient of 0.997 and lower limit of quantitation of 5 ng mL^{-1}. This study represents the first report describing the determination of CZT in human plasma by UPLC-MS/MS method. The proposed method is superior to the previously reported LC-MS methods in terms of the sensitivity and simplicity as the method described herein is based on simple one step protein precipitation for sample preparation and isocratic flow of mobile phase. The run time was only 2 min which is suitable for high-throughput analysis.

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Keywords: Crizotinib, Ultra-performance liquid chromatography; Tandem mass spectrometry; Human serum

1. Introduction

Crizotinib {CZT, 3-[(1R)-1-(2,6-dichloro-3-fluorophenyl)ethoxy]-5-(1-piperidin-4-ylpyrazol-4-yl)pyridine-2-amine, Fig. 1} is a novel small-molecule inhibitor of tyrosine kinases. It functions by competitive binding with the ATP-binding pocket of target kinases. Some patients with non-small cell lung carcinoma have a chromosomal rearrangement that generates a fusion gene between (echinoderm microtubule-associated protein-like 4' (EML4) and anaplastic lymphoma kinase (ALK), which results in constitutive kinase activity that contributes to carcinogenesis and seems to drive the malignant phenotype. The kinase activity of the fusion protein is inhibited by crizotinib (1-4) and caused tumor shrinking in 90% of lung cancer patients with non-small cell lung cancer (NSCLC) carrying anaplastic lymphoma kinase (ALK) fusion gene (5). Based on two successful clinical multi-center studies, CZT was granted an accelerated approval by the Food and Drug Administration (FDA) on August 26, 2011 (under the trade name of Xalkori® capsules made by Pfizer, Inc.) for the treatment of patients with advanced local or metastatic NSCLC that is ALK-positive as detected by FDA-approved test (Abbot Molecular, Inc.)

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(6) There has been reported no effect of food on the absorption of CZT (7). The peak plasma concentration reached 4 hours after a single dose of crizotinib (1). Pharmacokinetics were linear from a 100 mg once daily dose to a 300 mg twice daily dose (7, 8).

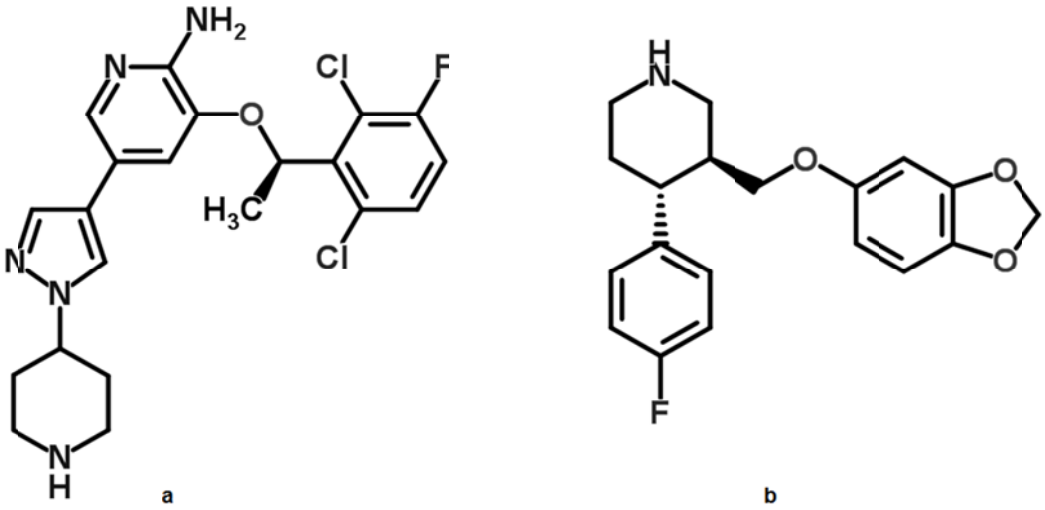

Fig. 1. Chemical structures of: a) crizotinib and b) paroxetine (IS).

UPLC is a new category in separation science which builds upon well-established principles of liquid chromatography, using sub 2-µm porous particles. These particles operate at elevated mobile phase linear velocities to produce significant reductions in separation time and solvent consumption. Literature indicates that UPLC system allows approximately nine fold decreases in analysis time as compared to the conventional HPLC system using 5-µm particle size analytical columns, and approximately threefold decrease in analysis time in comparison with 3-µm particle size analytical columns without compromise on overall separation (9-13).

Literature review revealed that there was only one liquid chromatography-tandem mass spectrometric assay for CZT in mouse plasma (9) and no published literature was found for ultra-performance liquid chromatography-tandem mass spectrometric assay for CZT in human plasma. The present study describes the development and validation of an UPLC method coupled with tandem mass spectrometry (UPLC-MS/MS) for the determination of CZT in human plasma.

2. Experimental

2.1 Reagents

CZT was obtained from Tocris Bioscience China. The CZT purity determined by the manufacturer using HPLC analysis was 99.7 and 99.8% for batch 1 and 2 respectively. Internal standard (IS), paroxetine hydrochloride, was obtained from SmithKline Beecham Pharmaceuticals, UK. Plasma of normal healthy human volunteers was obtained from King Khalid University Hospital (Riyadh, Saudi Arabia), and kept frozen at -80 ºC until analysis. Acetonitrile and methanol (HPLC-grade), and ammonium acetate were acquired from Winlab Laboratory, UK. Formic acid was obtained from BDH Laboratory, UK,and ammonia from Merck, Germany. Other chemicals were of analytical grade. All aqueous solutions was prepared using water that was purified using Milli-QR Gradient A10R (Millipore S.A.S - Molsheim, France) having pore size 0.22 µm.

2.2 Instrumental parameters and samples

Liquid chromatography. - The instrument consisted of a binary pump, autosampler, quaternary solvent manager, degasser and column heater-cooler. The mobile phase consisted of methanol: 0.1% *(v/v)* ammonium hydroxide (80:20), and used at a flow rate of 0.4 $mLmin^{-1}$. The separating coloumn used was C_{18} Acquity UPLC BEH^{TM} with following dimensions (50 × 2.1 mm, i.d., 1.7 µm, Waters, USA) maintained at 25 °C. In the partial loop mode the injection volume was 5 µL with autosampler temperature of 8 °C.

Mass spectrometric conditions. - Waters TQD triple quadrupole mass spectrometer with Waters Acquity liquid chromatography system (Waters, USA) was used for the study. Positive mass ionization mode with multiple reaction monitoring (MRM) using an electrospray interface (ESI) to carry out spectrometric detection for both CZT and IS was performed. Desolvating gas (nitrogen) was used as a gas at a flow rate of 500 L h^{-1} at a desolvation temperature set at 300 °C with source temperature of 150 °C. Capillary voltage of 3.10 kV was found to be optimum for the study. Flow rate for the collision gas (argon) used was 0.1 mL min^{-1}. The parameters used for the MS analyzer were as: HM1 and LM1 resolution 8.0 and 5.0; HM2 and LM2 resolution 10.0 and 10.0 respectively; dwell time 0.146 s; ion energy 1 0.2 V; ion energy 2 0.5 V. The collision energy and cone voltage were optimized for each analyte to maximize the signal corresponding to the major transition observed in the MS/MS spectra, following the fragmentation of the $[M+H]^+$ ions corresponding to the selected compounds. The UPLC-MS/MS system was controlled by Mass Lynx software (SCN 805; Version 4.1, Waters, USA.

2.3 Calibration standards and quality control samples.

CTZ and paroxetine (IS) standard stock solutions were prepared in methanol (final concentration 1 mg mL^{-1}). Standard stock solution of CTZ was serially diluted to prepare working solutions in the required concentration range with diluent methanol–water (60:40, v/v). The calibration standards were prepared by spiking with working solutions yielding concentration range from 5 to 500 ng mL^{-1} for CTZ. CTZ quality control (QC) stock solutions have been prepared separately in methanol–water (60:40, v/v). QC samples at four different levels: 5 ng mL^{-1} lower limit of quantitation (LLOQ), 15 ng mL^{-1} low quality control (LQC) (LQC, within three times of the LLOQ), 100 ng mL^{-1} middle quality control (MQC) and 400 ng mL^{-1} high quality control (HQC) were prepared similarly as calibration standards. Upper limit of quantitation (ULOQ) is the highest standard of the calibration curve. Internal standard working solution was prepared by diluting the paroxetine stock solution in methanol to get 50 µg mL^{-1}.

2.4 Sample preparation.

Plasma samples stored at around −80 °C were thawed, left for 1 hour and vortexed for 30 s at room temperature before extraction to ensure homogeneity. To 50 µL of plasma sample, 20 µL of IS (50 µg mL^{-1}) (except for blank sample) was added. The samples were vortex mixed for about 30 s and then 120 µL of methanol was added to it and vortex mixed again for another 30 seconds. After vortex mixing further 800 µL of acetonitrile was added to the sample. The samples were again vortex mixed gently for 1.5 min and then cold centrifuged for 10 min at 10000 rpm. After centrifugation, 800 µL of supernatant was transferred into UPLC vial, evaporated to dryness and reconstituted with 100 µL of combination of methanol and acetonitrile in the ratio of 50:50 and 5-µL volumes (in partial loop with needle over fill mode) of the sample were subjected to the analysis by UPLC–MS/MS.

2.5 Method validation

A full method validation was performed according to guidelines set by the United States Food and Drug Administration US-FDA (15) and European Medicines Agency (EMEA) guidelines (16). The validation of this procedure was performed in human plasma in order to evaluate the method in terms of selectivity, linearity of response, accuracy, precision, recovery,

dilution integrity and stability of analytes during both short-term sample processing and long-term storage. Selectivity, linearity, accuracy and precision exercise was also performed in human plasma. For toxicological studies use of at least one qualifying ion in addition to the internal standard is required for the confirmatory purposes when selected ion monitoring is used for identification of an analyte. Therefore qualifying ion *m*/*z* 450.0 → 176.99 was measured along with the target ion *m*/*z* 450.0 → 260.0.

2.6 System suitability and carryover effect.

System performance experiment was performed by injecting six consecutive injections using aqueous standard mixture of CZT and internal standard at the start of each batch during the method validation using LQC and MQC solutions. Carryover effect was evaluated to ensure that the rinsing solution used to clean the injection needle and port was able to avoid any carry-forward of injected sample in subsequent runs. The design of the experiment comprised blank plasma, *LLOQ* and upper limit of quantitation (*ULOQ*) followed by blank plasma to check for any possible interference due to carryover.

2.7 Linearity and standard curve.

The linearity of the method was determined by a weighted least square regression analysis of standard plot associated with an seven-point standard curve (5-500 ng mL-1).. The calibration curves were generated by plotting area ratio (CZT/IS) as a function of CZT concentration. Calibration curves from accepted three precision and accuracy batches were used to establish linearity. Curves were best fitted using a least square linear regression model Back-calculations were made from these curves to determine the concentration of CZT in each calibration standard and the resulting calculated parameters were used to determine concentrations of analyte in quality control samples. The determination coefficient R2 > 0.98 was desirable for all the calibration curves.

2.8 Limit of detection and limit of quantitation.

The limit of detection (*LOD*) was determined by the signal to noise ratio. and lower limit of quantitation (*LLOQ*) of the method was the lowest standard on the calibration curve. The LOD was determined as the lowest concentration level resulting in the peak area of three times of the base line noise.

2.9 Precision and accuracy.

Intra- and inter-day accuracies expressed as a percentage of deviation from the respective nominal value. The precision of the assay was measured by relative standard deviation (%) at four concentrations in human plasma. Intra-day precision and accuracy were assessed by analyzing six replicates of the quality control samples at four levels during a single analytical run. The inter-day precision and accuracy were assessed by analyzing 18 replicates of the quality control samples at each level through three precision and accuracy batches runs on 3 consecutive validation days. The deviation at each concentration level from the nominal concentration was expected to be within ±15.0% except LLOQ, for which it should not be more than 20.0%. Similarly, the mean accuracy should not deviate by ± 15.0% except for the LLOQ where it can be ± 20.0% of the nominal concentration.

2.10 Extraction recovery.

To investigate extraction recovery, a set of samples (*n*=6) at each LQC, MQC and HQC was prepared by spiking CTZ into unique lots plasma at 15, 100, and 400 ng mL^{-1}, respectively. Each of the samples were processed as per the procedure sample preparation protocol. A second set of plasma samples was processed and spiked post-extraction with the same

concentrations of CTZ and IS that actually existed in the pre-extraction spiked samples. Extraction recovery for each analyte was determined by calculating the ratios of the raw peak areas of the pre-extraction spiked samples to those of the samples spiked after extraction.

2.11 Selectivity and matrix effect.

Six different batches of blank human plasma were tested to identify the peaks due to the possible biogenic plasma components. Among the analyzed batch, plasma batch showing no or minimal interference at the retention time of analytes and internal standards was selected. Samples were spiked with standard CZT at lower limit of quantification level (5 ng mL-1) and paroxetine at 2 µg mL-1 and processed using the proposed extraction protocol and analyzed after spiking. The assessment of matrix effect (co-eluting, undetected endogenous matrix compounds that may influence the analyte ionization) was performed and was evaluated by analyzing LLOQ sample. The matrix effect was evaluated by analyzing LLOQ sample. The matrix factor and matrix effect was calculated via the formula:

Matrix factor = X2/X1

Matrix effect (%) = [(X1 – X2) / X1] × 100 (%)

Where X1= response of neat concentrations and X2 is response of post-spiked concentrations

2.12 Stability and dilution integrity.

Under a variety of storage and processing conditions six replicates of QC samples at low and high concentrations were assessed to establish stability of CTZ in plasma. Freeze-thaw stability, bench top stability (short term stability), auto sampler storage stability and long term stability was determined at low and high concentration quality control samples using six aliquots at each. To establish the bench-top stability plasma samples were exposed to room temperature for ~6 h, which exceeded the actual sample processing procedure time. For accessing freeze/thaw stability three cycles of freeze (at around −80 ○C)–thaw (room temperature) was carried out. To establish stability of the samples in the autosampler, reconstituted QC samples were stored for ~48 h under autosampler condition (maintained at 8 ○C) before being analyzed. Determination of long-term stability was carried out by storing test samples at around −80○C for 60 days. For the stability of stock solutions and working solutions of crizotinib and paroxetine were kept at room temperature for 24 h and in the refrigerator temperature (below 10 ○C) for 30 days prior to analysis. Freshly spiked calibration standards were used to perform all the stability tests. If the deviation was within ±15% from the mean calculated concentration of stability quality control, the samples were considered stable in plasma. In order to validate the dilution test, dilution integrity experiment was carried out on higher analyte concentrations (above ULOQ i.e. 500 ng mL-1), which may be encountered during real subject samples analysis. Blank matrix was spiked with a concentration of 1.8 times of that of the ULOQ to give a concentration of 900 ng mL-1. This sample was further diluted with blank matrix by applying the dilution factor of 2 and 4, respectively, and their concentrations were calculated against the freshly prepared calibration curve. Six replicate samples were run for each dilution factor. If deviation was within ± 15 % of nominal value and RSD ≤ 15 % at both diluted levels, the integrity of the samples was considered to be maintained.

2.13 Ruggedness/robustness.

Robustness was examined by evaluating the influence of small variation in the assay variables on its analytical performance. In these experiments, one parameter was changed whereas the others were kept unchanged. Ruggedness was also tested by applying the proposed method to the assay of CZT using the same operational conditions by two different analysts (analyst 1 and 2) with two different columns of Acquity UPLC BEH^{TM} C_{18} column (50 × 2.1 mm i.d., 1.7 µm, Waters, USA). In each time, the recovery percentage was calculated for the LQC and HQC samples.

3. Results and discussion

3.1 Optimization of chromatographic conditions

Initial feasibility experiments of various mixture(s) of organic solvents such as acetonitrile and methanol along with Millipore water, both having 0.1 % formic acid, were tested. Also these organic solvents along with 0.1% ammonium hydroxide were tried by altering flow-rates (range 0.2–0.4 mL min^{-1}) to optimize effective chromatographic conditions for CZT and IS. The best conditions were achieved with an isocratic elution of mobile phase comprising methanol: 0.1% *(v/v)* ammonium hydroxide (80:20) at a flow-rate of 0.4 mL min^{-1}, on Acquity UPLC BEH® C_{18} column (50 × 2.1 mm i.d., 1.7 µm). The selected conditions were found to be suitable for the determination of electrospray response for CZT and IS (Table I).

Table I. Chromatographic and linearity parameters for the analysis of CZT by the proposed method

Parameter	Value
Range (ng ml^{-1})	5-500
Intercept[a]	0.05145 ± 0.001
Slope[a]	0.03265 ± 0.0023
Correlation coefficient	0.9986
LLOQ (ng ml^{-1})	5.00
LOD (ng ml^{-1})	1.80

[a] n=3

UPLC-MS/MS operation parameters were carefully optimized for the determination of CZT *(Table I).* Analytes were detected by tandem mass spectrometry using MRM of precursor–product ion transitions with 0.146 s dwell time. Precursor-to-product ion transitions of *m/z* 450.0 → 260.0, and *m/z* 330.11 → 192.11 were used to measure CZT and internal standard. Mass transition *m/z* 450.0 → 176.99 was used as qualifying ion for CZT. A standard solution (100 ng mL^{-1}) of CZT and the paroxetine were directly infused along with the mobile phase into the mass spectrometer with ESI as the ionization source. The mass spectrometer was tuned initially in both positive and negative ionization modes for CZT. It was observed that the signal intensity of positive ion was much higher than that of negative ion. Parameters, such as capillary and cone voltage, desolvation temperature, ESI source temperature and flow rate of desolvation gas and cone gas, were optimized to obtain the optimum intensity of protonated molecules of CZT and IS for quantification. Among the parameters, capillary and cone voltage, especially cone voltage, were important parameters. The precursor ion intensity increased significantly when cone voltage was raised gradually. Lastly, analytes produced the strongest ion signals when cone voltage was set up at 48 V. The cone voltage was optimized using cone ramp (0-100) V and it was noticed that when the cone voltage was lesser than 48 V, the ion signals decreased rapidly. The collision energy was investigated from 2 to 80 eV to optimize the response of product ion, and the best values were found to be 28 eV and 42 eV for the chosen product ions *m/z* 260 and *m/z* 176.99 respectively. For IS, *m/z* spectra at 192.11 was produced at optimum collision energy of 26 eV. The product ion spectra of CZT and IS are shown in Fig. 2.

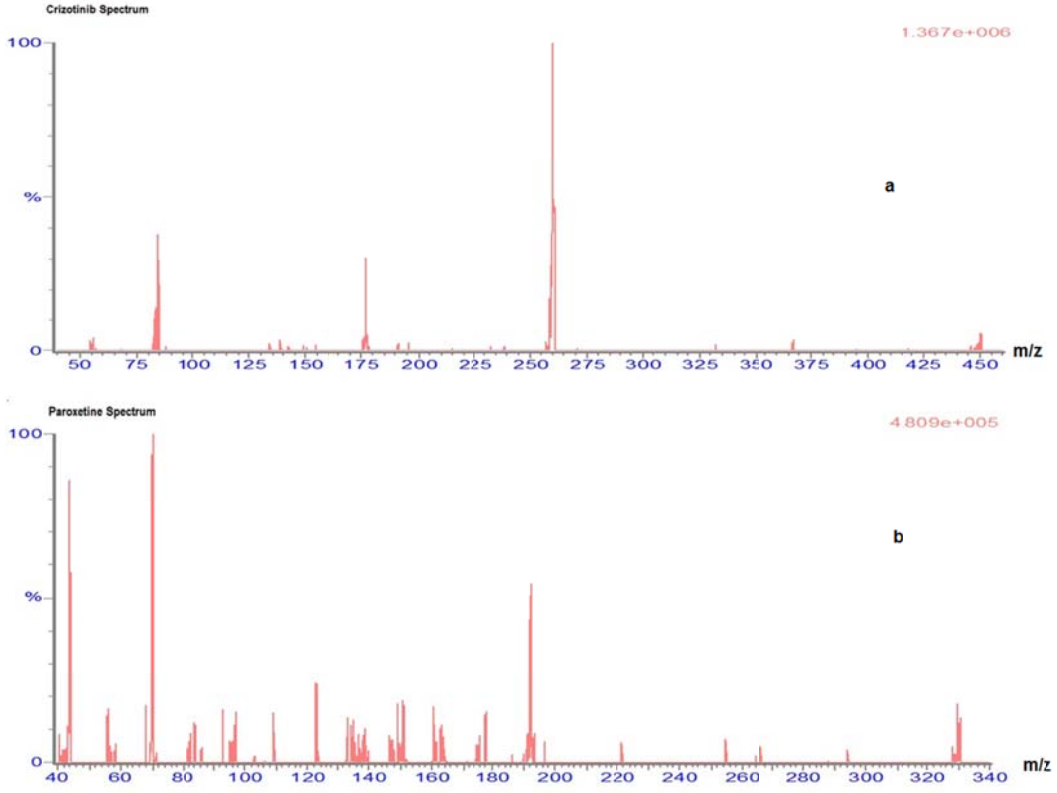

Fig. 2. The product ion spectra of: a) crizotinib and b) paroxetine (IS).

3.2 Optimization of sample processing

Clean samples are essential for minimizing ion suppression and matrix effect in UPLC–MS/MS analysis. Protein precipitation can be helpful in producing a clean sample and avoiding endogenous substances in plasma with the analytes and IS onto the column and MS system. Two organic solvents were tried, methanol and acetonitrile. Finally a combination of acetonitrile and methanol (87:13) was found to be optimal, which can produce a clean chromatogram for a blank plasma sample and yield the highest recovery for the analytes from the plasma.

3.3 Linearity and sensitivity

It was found to be linear from 5 to 500 ng mL^{-1} for CZT in human plasma *(Table II)*. The determination coefficient (R^2) was found consistently greater than 0.997 during the course of validation. *LOD* was 1.8 ng mL^{-1} and *LLOQ* was 5 ng mL^{-1} in human plasma. This *LLOQ* was low enough to investigate the pharmacokinetic behavior of CZT in human plasma (Table I).

Table II. Intra- and inter-day precision and accuracy of CZT in human plasma.

Spiked CZT (ng mL^{-1})	Run	Human plasma	
		Recovery (%)	Precision (RSD, %)
		Intra-day variation[a]	
5.00	1	100.83	7.2
	2	100.23	11.0
	3	105.50	6.8
15.00	1	103.30	7.1
	2	103.81	8.9
	3	104.96	7.9
100.00	1	101.08	3.0
	2	100.41	2.4
	3	99.73	4.3
400.00	1	101.07	2.4
	2	103.52	1.9
	3	100.95	2.3
Mean Recovery		102.11 ± 1.98	
		Inter-day variation[b]	
5.00		103.30	2.3
15.00		105.13	1.8
100.00		100.41	0.7
400.00		101.85	1.4
Mean Recovery		102.67 ± 2.02	

At each concentration: [a] n=6, [b] n=18.

3.4 System suitability and carryover effect

Overall RSD value for peak area of CZT and that of IS did not exceed 4.6 % for CZT and 1.8 % for IS and the overall RSD for retention time of CZT and IS didn't exceed 0.32 and 0.26 % respectively. These values were within the prescribed limits of 5% for peak area and 2% for retention time. The ion ratio of target ion i.e. m/z 450.0 → 260.0 to that of qualifying ion m/z 450.0 → 176.99 did not exceed the acceptance criteria of 20% and the RSD of the ion ratios did not exceed 3.54%. The carry over effect was evaluated as described in the experimental section and no carryover effect was observed during method validation.

3.5 Selectivity and matrix effect. -

Selectivity of the method was assessed by comparing the chromatogram of blank plasma with the corresponding spiked *LLOQ* sample. Six different batches of blank human plasma were tested to identify the peaks due to the possible biogenic plasma components. Thus the method looks to be selective enough for determination of CZT and paroxetine in plasma. Representative chromatograms obtained from blank plasma showing no interference at the retention time of analyte and IS are shown in Figs. 3a and e, respectively. Representative chromatogram of *LLOQ* and IS are shown in Figs. 3b and f, respectively, whereas representative chromatogram of LQC and HQC are shown in Figs. 3c and d, respectively.

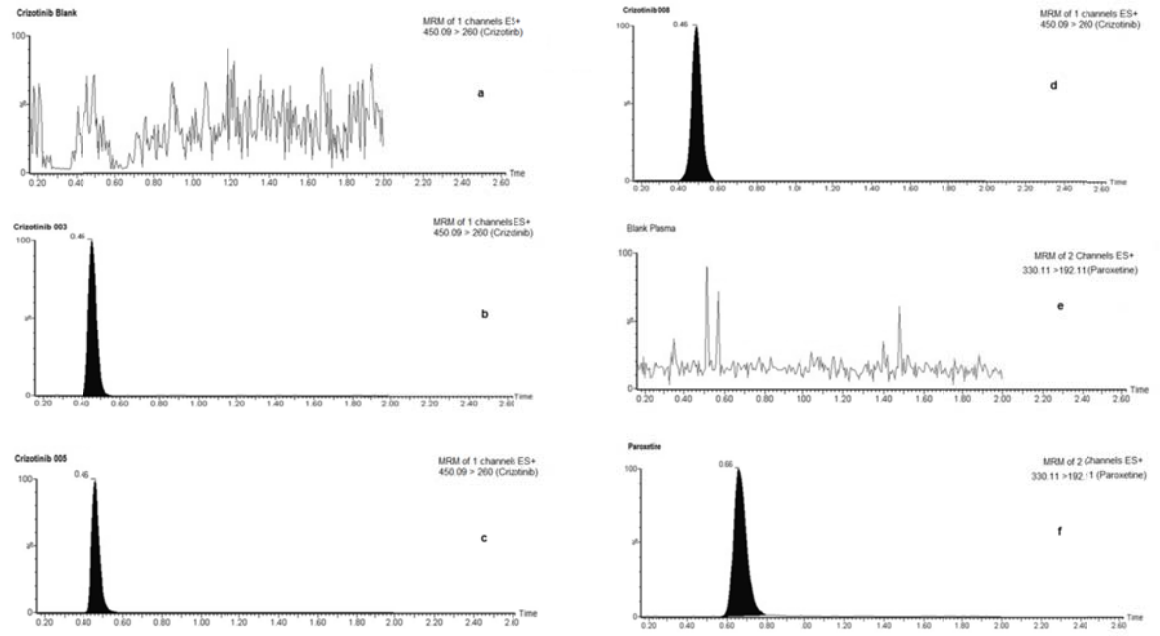

Fig. 3. Representative chromatograms of: a) blank human plasma; b) CZT in human plasma at: LLOQ, c) CZT in human plasma at: LQC, d) CZT in human plasma at: HQC, e) CZT in blank plasma with paroxetine (IS) and f) at HQC of CTZ in human plasma depicting paroxetine (IS).

The matrix effect is a noticeable problem while determining compounds in human plasma. To evaluate the possibility of ion suppression or enhancement in the present experiments, chromatographic peak area of CZT from the spike-after-precipitation samples at LLOQ concentration level was compared to that obtained from the standard solution at the same concentrations in neat solvent. The matrix effect obtained for CZT were in the range of 6.56-8.57% (n= 6) at LLOQ level. It was also observed that CZT showed the matrix factor of 0.91 calculated as ratio of response of post-spiked concentrations to that of response of neat concentrations at *LLOQ*.

3.6 Precision and accuracy

Table II summarizes % recovery and RSD values for both intra- and inter-day results of QC samples in plasma. They RSD values were 2.3 – 11.0 and 0.7 – 2.3 %, respectively for intra and inter day precision and the recovery values were 99.73-105.20 and 100.41-105.13. These results indicate that the method has acceptable precision and accuracy.

3.7 Stability and dilution integrity

The stability data as summarized in Table V suggest that CTZ spiked plasma was stable for a minimum of 6 h at room temperature. It was also found that under autosampler storage condition CTZ was quite stable at 8 °C for a minimum of 48 h. CTZ was also stable during the three freeze–thaw cycles and at −80 °C for 60 days. The stock solutions and working standards of CTZ and IS were found to be stable at refrigerator temperature (below 10 °C) for 30 days and for a minimum of 24 h at room temperature.

For the dilution integrity test %RSD did not exceed 4.14 which is within the acceptable criteria. The dilution integrity results are indicated in (Table III) and conclude that the dilution of the concentrated plasma sample upto four times doesn't affect the analysis of CTZ estimation.

Table III. Stability and dilution integrity data of CZT in human plasma.

Stability[a]	Spiked conc. (ng mL^{-1})	Recovery (%)	Precision (RSD, %)[a]
Bench top (6 h)	15.00	99.72	6.1
	400.00	100.42	2.0
Freeze thaw (3 cycle)	15.00	99.53	6.3
	400.00	99.51	2.8
Auto sampler (48 h)	15.00	98.98	6.6
	400.00	99.49	2.4
60 days at -80 °C	15.00	97.90	6.2
	400.00	98.10	2.8
30 days at 8 °C	15.00	100.67	8.7
	400.00	101.33	3.0
Dilution integrity	225.00	99.40	4.1
	450.00	99.93	2.1
Mean Recovery		99.58 ± 0.97	

[a] *n*=6.

3.8 Extraction recovery

At three QC concentration levels (15, 100 and 400 ng mL^{-1}), the percent recoveries of CZT obtained from plasma are given in Table IV. The mean recovery for CZT was 90.6± 1.4 %. The mean recovery for the IS paroxetine at the concentration employed was 80.0 ± 4.7%. These results indicate that the extraction efficiency for CZT using protein precipitation method was satisfactory, consistent and concentration independent.

Table IV. Recovery data of CZT and paroxetine in human plasma

Compound	Spiked conc. (ng mL^{-1})	Recovery (%)[a]
CZT (analyte)	15.00	89.10 ± 7.1
	100.00	91.15 ± 3.1
	400.00	91.66 ± 2.6
	Mean ± SD	90.64 ± 1.3
Paroxetine (IS)	2000.00	80.39 ± 4.7

[a] Mean±SD, *n*=6

3.9 Robustness and ruggedness

There was no significant change recovery of CZT when mobile phase composition, flow rate, or temperature were changed slightly. The mean recovery % values ranged from 96.56 ± 1.98 - 104.48 ± 1.89 (Table V) which indicate the method is robust. Ruggedness of the proposed method was evaluated and it was found the results obtained from analyst to analyst and column to column variations were reproducible, as the mean recovery % values ranged from 96.57 ± 7.47 – 101.57 ± 7.47 (Table V).

Table V: Robustness and ruggedness of analytical method

Robustness		
Condition	**Modification**	**Recovery (% ± SD)[a]**
Mobile phase composition (ratio)	79:21	98.30 ± 2.19
	80:20	104.48 ± 1.89
	81:19	101.70 ± 1.70
Mobile phase flow rate (mL $min^{-1)}$	0.44	101.67 ± 0.93
	0.40	97.34 ± 1.55
	0.36	98.68 ± 1.75
Temperature (°C)	20.00	96.56 ± 1.98
	25.00	101.18 ± 1.02
	30.00	99.35 ± 0.80
Mean Recovery		99.91 ± 2.53
Ruggedness		
Instrument	**Spiked Concentarion**	**Recovery (% ± SD)[a]**
Column I	15.00	97.23 ± 7.23
	400.00	99.21 ± 5.75
Column II	15.00	100.65 ± 4.19
	400.00	98.63 ± 4.78
Analyst I	15.00	101.85 ± 3.72
	400.00	99.81 ± 2.68
Analyst II	15.00	98.81 ± 7.95
	400.00	96.57 ± 7.47
Mean Recovery		99.09 ± 1.71

[a] n=5.

4. Conclusions

A novel simple, economical high-throughput and highly sensitive UPLC-MS/MS method was successfully developed and validated for the determination of CZT in human plasma. The method involved simple one step protein precipitation method for plasma sample preparation for analysis and short runtime (2 min). The proposed method might be of use for pharmacokinetic and toxicokinetic study for CZT in humans.

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