Journal of Chromatography B, 871 (2008) 44–54

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

High throughput and sensitive determination of trazodone and its primary metabolite, *m*-chlorophenylpiperazine, in human plasma by liquid chromatography–tandem mass spectrometry

Bhavin N. Patel[a,b], Naveen Sharma[b], Mallika Sanyal[c], Pranav S. Shrivastav[a,*]

[a] *Chemistry Department, School of Sciences, Gujarat University, Navrangpura, Ahmedabad 380 009, Gujarat, India*
[b] *Analytical Laboratory, BA Research India Ltd., Bodakdev, Ahmedabad 380 054, India*
[c] *Chemistry Department, St. Xaviers' College, Navrangpura, Ahmedabad 380 009, India*

ARTICLE INFO

Article history:
Received 19 March 2008
Accepted 22 June 2008
Available online 3 July 2008

Keywords:
Trazodone
m-Chlorophenylpiperazine
Human plasma
LC–MS/MS
Liquid–liquid extraction

ABSTRACT

A precise, sensitive and high throughput liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for simultaneous determination of trazodone (TRZ) and its primary metabolite, *m*-chlorophenylpiperazine (*m*CPP), in human plasma was developed and validated. The analytes and the internal standard-nefazodone were extracted from 500 μL aliquots of human plasma via liquid–liquid extraction in *n*-hexane. Chromatographic separation was achieved in a run time of 2.5 min on a Betabasic cyano column (100 mm × 2.1 mm, 5 μm) under isocratic conditions. Detection of analytes and IS was done by tandem mass spectrometry, operating in positive ion and multiple reaction monitoring (MRM) acquisition mode. The protonated precursor to product ion transitions monitored for TRZ, *m*CPP and IS were *m*/*z* 372.2 → 176.2, 197.2 → 118.1 and 470.5 → 274.6 respectively. The method was fully validated for its sensitivity, selectivity, accuracy and precision, matrix effect, stability study and dilution integrity. A linear dynamic range of 10.0–3000.0 ng/mL for TRZ and 0.2–60.0 ng/mL for *m*CPP was evaluated with mean correlation coefficient (*r*) of 0.9986 and 0.9990 respectively. The intra-batch and inter-batch precision (%CV) across five validation runs (LLOQ, lower limit of quantitation; LQC, low quality control; MQC, middle quality control; HQC, high quality control and ULOQ, upper limit of quantitation) was ≤8.4% for both the analytes. The method was successfully applied to a bioequivalence study of 100 mg trazodone tablet formulation in 36 healthy Indian male subjects under fasting and fed conditions.

1. Introduction

Trazodone (TRZ) hydrochloride is a triazolopyridine derivative, chemically described as 2-{3-[4-(*m*-chlorophenyl)-1-piperazinyl] propyl}-1,2,4-triazolo-[4,3-*a*] pyridine-3(2*H*)-one hydrochloride. It is a psychoactive compound which belongs to the group of second generation non-tricyclic antidepressants, with a low incidence of serious adverse effects [1–3]. Trazodone is less potent but more selective than conventional tricyclic antidepressants; at low doses, trazodone acts as a serotonin antagonist, while at high doses it acts as a serotonin agonist. TRZ has an overall therapeutic efficacy comparable with imipramine, amitriptyline, desipramine, and placebo in controlled clinical trials. It is nearly completely absorbed after oral administration; although food delays absorption and reduces peak serum concentration, however, the total area under the plasma concentration–time curve is not altered. TRZ has biphasic elimination, with a redistribution half-life of about 1 h and an elimination half-life of 10–12 h [4,5]. Trazodone is nearly completely metabolized hepatically by hydroxylation and oxidation to its active metabolite *m*-chlorophenylpiperazine (*m*CPP), which has antidepressant or anxiogenic effects [6,7]. The usual adult daily dose of trazodone hydrochloride is 150–400 mg given in two divided doses. TRZ is 85–95% protein bound in vitro at concentrations attained with therapeutic doses [2].

Several chromatographic methods have been presented for the estimation of trazodone in biological fluids employing HPLC–UV [8,9], HPLC–fluorescence [10,11], gas chromatography with nitrogen selective [12] and mass spectrometric detection [13,14]. Some of these methods suffer from various drawbacks namely sensitivity, long chromatographic run times, large sample volume for processing or a cumbersome extraction procedure, which prevent their use for routine subject sample analysis. Other reported methodologies present simultaneous determination of trazodone along with several other antidepressant drugs by HPLC–UV [15], GC–MS [16,17] and LC–MS [18,19]. However, some of the side effects

* Corresponding author. Tel.: +91 79 26300969; fax: +91 79 26308545.
E-mail address: pranav_shrivastav@yahoo.com (P.S. Shrivastav).

1570-0232/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2008.06.046

associated with trazodone administration like sedation, dizziness, constipation, headache, blurred vision and psychomotor impairment can be attributed to its active metabolite *m*CPP, which raises particular concern regarding its use in the elderly [20]. Thus, simultaneous determination of trazodone and its active metabolite *m*CPP becomes an integral part of therapeutic drug monitoring for patients treated with trazodone, to improve their quality of life. The simultaneous analysis of trazodone and its active metabolite *m*CPP in human plasma has been the subject of few reports [21–24]. The first two methods report determination of plasma and brain concentration of TRZ and *m*CPP by GC–MS [21] and HPLC–UV [22] respectively. The GC–MS method involved lengthy derivatization of *m*CPP with heptafluorobutyric anhydride prior to detection by electron capture detector. Similarly, the limitation of the HPLC–UV procedure was that of low sensitivity and long chromatographic run time. Ohkubo et al. [23] have determined TRZ and *m*CPP in human plasma by HPLC with ultraviolet and electrochemical detector. The linear dynamic range achieved using SPE 100–2000 ng/mL for TRZ and 5–100 ng/mL for *m*CPP. Another such method proposed by Vatassery et al. [24] involved estimation of TRZ and its metabolite *m*CPP in human plasma and red blood cell samples with a run time of 16 min on a reversed phase HPLC with UV detection. Very recently, a similar method is reported by Mercolini et al. [25] with a sensitivity of 10 ng/mL to determine both the analytes in human plasma.

It is well known that the use of mass spectrometry interfaced with HPLC helps to improve the selectivity and sensitivity compared to traditional HPLC–UV and GC methods. Thus, the aim of the present study was to develop and validate more sensitive, specific and rapid method for the simultaneous estimation of trazodone and its active metabolite in human plasma by LC–MS/MS. Also, the method should be simple, rugged and suitable for routine measurement of subject samples for pharmacokinetic/bioequivalence study.

2. Experimental

2.1. Chemicals and materials

Reference standard material of trazodone hydrochloride and *m*-chlorophenylpiperazine hydrochloride, each having purity of 99.4% were procured from a Pharmaceutical Sponsor based in Secundrabad, India, while nefazodone (IS, 99.9%) a USP reference standard, was procured from Rockville, Maryland, USA. HPLC grade *n*-hexane, methanol and AR grade formic acid (99%), ammonium hydroxide (30%) was procured from S.D. Fine Chemicals Ltd. (Mumbai, India). Ammonium trifluoroacetate used in mobile phase was of Acros Organics (New Jersey, USA). Water used for the LC–MS/MS was prepared using Milli Q water purification system from Millipore (Bangalore, India). Control buffered (K3 EDTA) human plasma was procured from Clinical Department, BA Research India Limited (Ahmedabad, India) and was stored at −20 °C. Platform shaker and Centrifuge were of Innova™ 2100 from New Brunswick Scientific Co. Inc. (New Jersey, USA) and Eppendorf 5810 (Hamburg, Germany) respectively.

2.2. LC–MS/MS Instrumentation and conditions

The liquid chromatography system (Shimadzu, Kyoto, Japan) consisted of a LC-10ADvp pump, an autosampler (SIL-HTc) and an on-line degasser (DGU-14A). Chromatographic column used was Betabasic cyano, 100 mm length × 2.1 mm inner diameter, with 5.0 μm particle size. The mobile phase consisted of 700 mL methanol + 300 mL deionized water + 2.0 mL, 1.0 M ammonium trifluoroacetate + 1.0 mL formic acid. Separation of analytes and IS was performed under isocratic condition at a flow rate of 400 μL/min. The auto sampler temperature was maintained at 4 °C and the injection volume was 5 μL. The total LC run time was 2.5 min. Detection of analytes and IS was performed on a triple quadrupole mass spectrometer, API-4000 equipped with Turbo Ion spray®, manufactured by MDS SCIEX (Toronto, Canada) operating in the positive ion mode. Quantitation was done using multiple reaction monitoring (MRM) mode to monitor protonated precursor → product ion transition of m/z 372.2 → 176.2 for trazodone, 197.2 → 118.1 for *m*-chlorophenylpiperazine and 470.5 → 274.6 for IS (Fig. 1). All the parameters of LC and MS were controlled by Analyst software version 1.4.1.

For trazodone, *m*-chlorophenylpiperazine and nefazodone (IS) the source parameters maintained were Gas 1 (GS1): 55 psi (3.79×10^5 Pa), Gas 2 (GS2): 55 psi (3.79×10^5 Pa), ion spray voltage (ISV): 5500 V, turbo heater temperature (TEM): 550 °C, interface heater (Ihe): ON, entrance potential (EP): 10 V, collision activation dissociation (CAD): 8 psi (5.52×10^4 Pa), curtain gas (CUR): 20 psi (1.38×10^5 Pa). The compound dependent parameters like declustering potential (DP), collision energy (CE) and cell exit potential (CXP) were optimized at 80, 65 and 10 V for trazodone, 75, 45 and 10 V for *m*-chlorophenylpiperazine and 100, 40 and 20 V for nefazodone respectively. Quadrupole 1 and quadrupole 3 were maintained at unit resolution. Dwell time set was 400 ms for both the analytes.

2.3. Preparation of standard stocks and plasma samples

The standard stock solutions of 200 μg/mL for trazodone and 100 μg/mL for *m*-chlorophenylpiperazine and nefazodone were prepared by dissolving requisite amount of trazodone, *m*-chlorophenylpiperazine and nefazodone in methanol. The combined working solution of TRZ (60 μg/mL) and *m*CPP (1.2 μg/mL) was prepared from the stock solutions by diluting 3.0 mL and 0.12 mL respectively in 10 mL volumetric flask using methanol:water (50:50, v/v). This combined working solution was used for spiking plasma calibration and quality control samples using methanol:water (50:50, v/v). IS working solution of 75 ng/mL was prepared using the stock of 100 μg/mL in deionized water. All the standard stock, intermediate stock and working stock solutions were prepared and stored at 4 ± 6 °C until use. Drug free plasma, i.e. control (blank) plasma was withdrawn from the deep freezer and allowed to get completely thawed before use. The calibration standards (CS) and quality control (QC) samples (LLOQ, lower limit of quantitation; LQC, low quality control; MQC, middle quality control; HQC, high quality control; ULOQ, upper limit of quantitation) were prepared by spiking 475 μL of blank plasma with 25 μL of combined working solution (5% of total volume of plasma). Calibration standards were made at 10.0, 20.0, 40.0, 100.0, 200.0, 400.0, 800.0, 1600, 2400 and 3000 ng/mL for TRZ and 0.20, 0.40, 0.80, 2.00, 4.00, 8.00, 16.0, 32.0, 48.0, 60.0 ng/mL for *m*CPP. Quality controls were similarly prepared at 10.0 ng/mL (LLOQ), 25.0 ng/mL (LQC), 230.0 ng/mL (MQC), 2250 ng/mL (HQC) and 3000 ng/mL (ULOQ) for TRZ and 0.20 ng/mL (LLOQ), 0.50 ng/mL (LQC), 4.6 ng/mL (MQC), 45.0 ng/mL (HQC) and 60.0 ng/mL (ULOQ) for *m*CPP. The spiked plasma samples at all the levels were stored at −20 °C for validation and subject sample analysis.

2.4. Protocol for sample preparation

Prior to analysis, spiked plasma samples were withdrawn from −20 °C freezer and thawed for 25–30 min at room temperature. The samples were vortexed adequately using a vortex

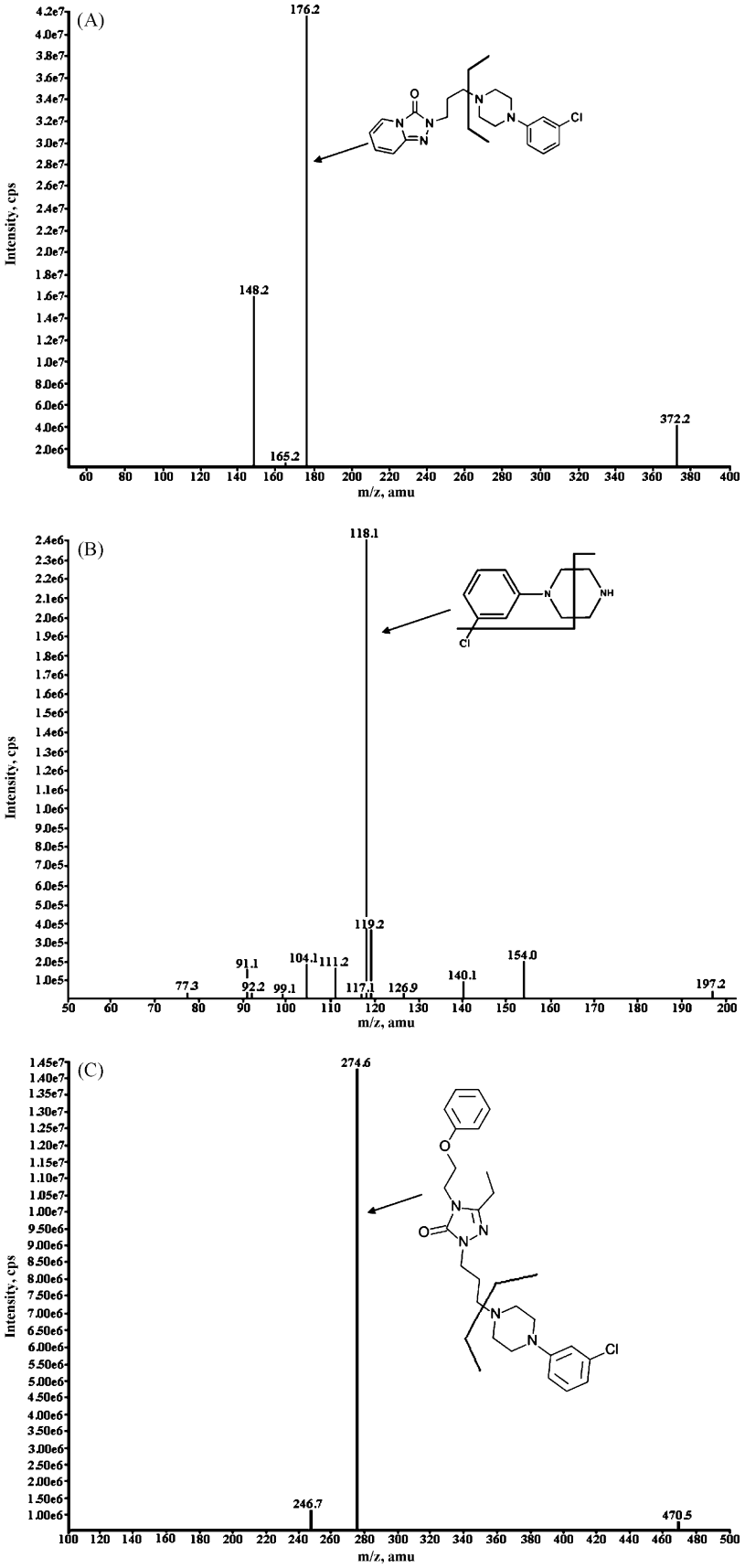

Fig. 1. Product ion spectra of (A) trazodone (372.2 → 176.2, scan range 50–400 amu), (B) *m*-chlorophenylpiperazine (197.2 → 118.1, scan range 50–200 amu) and (C) nefazodone (IS, 470.5 → 274.6, scan range 100–500 amu).

mixer before pipetting. Aliquots of 500 μL plasma were transferred into 15 mm × 125 mm screw cap extraction tubes, 25 μL deionized water along with 100 μL working solution of IS (75 ng/mL) was added and vortexed to mix. To the same tubes, 100 μL of 4.5 M ammonium hydroxide solution was added and vortexed again. Further, 5.0 mL of *n*-hexane was added to all the tubes, capped and shaken for 10 min in a platform shaker. The tubes were then centrifuged for 5 min at 1811 × *g*. The organic layer was transferred to glass tubes by freezing the aqueous part in dry ice bath and evaporated at 40 °C under gentle stream of nitrogen (15 psi, 1.03×10^5 Pa) for 15 min. The residue was taken up in 1.0 mL of mobile phase and 5 μL was used for injection in LC–MS/MS, in partial loop mode.

2.5. Methodology for validation

A thorough and complete method validation of trazodone and *m*-chlorophenylpiperazine in human plasma was done following the USFDA guidelines [26]. The method was validated for selectivity, sensitivity, interference check, linearity, precision and accuracy, recovery, matrix effect, ion suppression/enhancement, cross specificity, stability and dilution integrity.

Test for selectivity was carried out in 10 different lots of blank plasma (with K3 EDTA as anticoagulant), processed by the same extraction protocol and analysed to determine the extent to which endogenous plasma components may contribute to the interference at the retention time of analytes and the internal standard. In this experiment, from each of these 10 different lots, two replicates each of 475 μL were spiked with 25 μL methanol–water solution (50:50, v/v). In the first set, the blank plasma was directly injected after extraction (without analyte and IS), while the other set was spiked with only IS before extraction (total 20 samples). Further, one system suitability sample (SSS) at CS-2 concentration and two replicates of LLOQ concentration (CS-1) were prepared by spiking blank plasma with combined working aqueous standards of trazodone and *m*-chlorophenylpiperazine (5% of total volume of plasma). The blank plasma samples used for spiking of SSS and LLOQ were chosen from one of these 10 lots of plasma. Check for interference due to commonly used medications in human volunteers was done for paracetamol, chlorpheniramine maleate, caffeine, acetylsalicylic acid and ibuprofen. Their stock solutions (100.0 μg/mL) were prepared by dissolving requisite amount in methanol. Further, working solutions (100.0 ng/mL) were prepared in the mobile phase and 5 μL was injected to check any possible interference at the retention time of analytes and IS.

The linearity of the method was determined by analysis of standard plots associated with a ten-point standard calibration curve. Five linearity curves containing ten non-zero concentrations were analysed. Best-fit calibration curves of peak area ratio versus concentration were drawn. The concentration of the analytes were calculated from calibration curve ($y = mx + c$; where y is the peak area ratio) using linear regression analysis with reciprocate of the drug concentration as a weighing factor ($1/x^2$) for TRZ and *m*CPP. The regression equation for the calibration curve was also used to back-calculate the measured concentration at each QC level. The peak area ratio values of calibration standards were proportional to the concentration of the drugs in plasma over the range tested.

Intra-batch and inter-batch (on three consecutive days) accuracy and precision were evaluated at five different concentrations levels (LLOQ, LQC, MQC, HQC and ULOQ) in six replicates for both the analytes. Mean values were obtained for calculated drug concentration over these batches. The accuracy and precision were calculated and expressed in terms of %bias and coefficient of variation (%CV) respectively.

Recovery of the analytes from the extraction procedure was performed at LQC, MQC and HQC levels. It was evaluated by comparing peak area of extracted samples (spiked before extraction) to the peak area of unextracted samples (quality control working solutions spiked in extracted plasma).

Matrix ion suppression effects on the MRM LC–MS/MS sensitivity were evaluated by the post-column analytes infusion experiment. A standard solution containing 100 ng/mL of TRZ and *m*CPP in methanol:water (80:20, v/v) was infused post-column via a 'T' connector into the mobile phase at 5 μL/min employing Harvard infusion pump. Aliquots of 5 μL of extracted control plasma were then injected into the column by the autosampler and MRM LC–MS/MS chromatograms were acquired for both the analytes and IS. To study the effect of matrix on analyte quantitation with respect to consistency in signal, matrix effect was checked in six different lots of K3 EDTA plasma. Four replicates, each at LQC and HQC levels were prepared from these lots of plasma (total 48 QC samples) and checked for the accuracy in terms of %bias in all the QC samples. An ion suppression/enhancement experiment was conducted by continuous infusion of 100 ng/mL combined solution of TRZ and *m*CPP through a three-way splitter. Any dip in the baseline upon injection of double blank plasma would indicate ion suppression, while a peak at the retention times of TRZ, *m*CPP and IS indicates ion enhancement. Cross specificity experiments were conducted for TRZ, *m*CPP and IS at ULOQ level by comparing the peak area at their respective retention times.

Stability experiments were performed to evaluate the analyte stability in stocks solutions and in plasma samples under different conditions, simulating the same conditions, which occurred during study sample analysis. Stock solution stability was performed at room temperature and at 4 ± 6 °C by comparing area response of stability sample of analytes and internal standard with the area response of sample prepared from fresh stock solutions. The results should be within the acceptable limit of ±10% change for stock solution stability experiment. Bench top stability of extracted samples (BTS), room temperature stability (stability in biological matrix, SBM), refrigerated stability of extracted sample (RSS) at 4 ± 6 °C, freeze thaw stability (FTS) and long term stability (LTS) at −20 °C were performed at LQC, MQC and HQC levels using six replicates at each level. To meet the acceptance criteria the %CV should be within ±15%.

The dilution integrity experiment was performed with an aim to validate the dilution test to be carried out on higher analyte concentrations (above ULOQ), which may be encountered during real subject samples analysis. Dilution integrity experiment was carried out at 5 times the ULOQ concentration i.e. 15,000 ng/mL for trazodone and 300 ng/mL for *m*-chlorophenylpiperazine and also at HQC level for both the analytes. Six replicate samples each of 1/10 of 5 × ULOQ (1500/30.0 ng/mL) and 1/10 of HQC (225.0/4.5 ng/mL) concentration were prepared and their concentrations were calculated, by applying the dilution factor of 10 against the freshly prepared calibration curve for trazodone and *m*-chlorophenylpiperazine.

2.6. Bioequivalence study design

The design of study comprised of "An open label, randomized, two period, two treatment, two sequence, balanced, single dose, crossover, comparative evaluation of relative bioavailability of test and reference formulation (100 mg trazodone hydrochloride tablet) in 36 healthy Indian human subjects under fasting and fed conditions". All the subjects were informed of the aim and risk involved in the study and written consents were obtained. The work was approved and subject to review by Institutional Ethics Committee, an independent body comprising of five members which includes a lawyer, medical doctor, social worker, pharmacologist and an academician. The procedures followed while dealing with human

subjects were based on International Conference on Harmonization, E6 Good Clinical Practice (ICH, E6 GCP) guidelines [27]. Health check up for all subjects was done by general physical examination, ECG and laboratory tests like hematology, biochemistry and urine examination. All subjects were negative for HIV, HBSAg and HCV tests. They were orally administered a single dose of test and reference formulation after recommended wash out period of 9 days with 240 mL of water. Drinking water was restricted (at least) from 1 h before dosing and up to 2 h after dosing while supine position was restricted 4 h post dose. Blood samples were collected in vacutainers containing K3 EDTA before (0.0 h) and at 0.25, 0.50, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 8.0, 10.0, 12.0, 16.0, 24.0, 36.0 and 48.0 h of administration of drug. Blood samples were centrifuged at 2061 × *g* for 10 min and plasma was separated, stored at −20 °C until use. An assay reproducibility experiment was conducted by computerized random selection of subject samples (5% of total subject samples analysed). The selection criteria included samples which were near the C_{max} and the elimination phase in the pharmacokinetic profile of the drug and metabolite. The results obtained were compared with the data obtained earlier for the same sample using the same procedure. According to USFDA the percent change should not be more than ±20% [28].

3. Results and discussion

3.1. Method development

As the literature reveals, there is no report yet on the simultaneous determination of TRZ and *m*CPP in human plasma by LC–MS/MS. Thus, in the present study method development was initiated to realize a rugged, sensitive, and specific LC–MS/MS method with a short overall analysis time for the simultaneous quantification of trazodone and *m*-chlorophenylpiperazine in human plasma. To accomplish this aim it was imperative to have a simple, inexpensive and an efficient extraction procedure, with a short chromatographic run time. Also, the sensitivity should be adequate enough to monitor at least three to five half-lives of trazodone concentration with good accuracy and precision for subject samples.

The tuning of MS parameters was carried out in positive as well as negative ionization modes for trazodone, *m*-chlorophenylpiperazine and nefazodone (IS) using 50.0 ng/mL tuning solution. The response observed was much higher in positive ionization mode for all three compounds compared to the negative mode due to their basic nature. Moreover, use of ammonium trifluoroacetate (1.0 M) and formic acid in the mobile phase further enhanced the response for both the analytes and IS with low background noise, resulting in higher sensitivity. The analytes and IS gave predominant singly charged protonated precursor $[M+H]^+$ ions at *m*/*z* of 372.2, 197.2 and 470.5 for TRZ, *m*CPP and IS respectively in Q1 MS full scan spectra. The most abundant ions found in the product ion mass spectra were *m*/*z* 176.2, 118.1 and 274.6 at 65, 45 and 40 V collision energy for TRZ, *m*CPP and IS respectively. To attain an ideal Taylor cone and a better impact on spectral response, nebuliser gas pressure (GS1) was optimized at 55 psi (3.79×10^5 Pa). Fine tuning of nebuliser gas and CAD gas was done to get a consistent and stable response. It was observed that ion spray voltage had a significant effect on the response of both the analytes and IS. At high voltage the response was drastically enhanced and hence an optimum potential of 5500 V was kept which gave consistent and stable signal. The ion source chamber temperature had little effect on the signal and thus was maintained at 550 °C. A dwell time of 400 ms was adequate and no cross talk was observed between the MRMs of analytes and IS.

As trazodone has two pK_a values (basic piperazine unit, pK_a = 11.1 and unsaturated hetero bicyclic structure, pK_a = 6.14), it was difficult to realize an optimized extraction procedure for simultaneous extraction of TRZ and its active metabolite *m*CPP from human plasma. To have a simple and inexpensive extraction procedure, protein precipitation was tried initially with commonly used solvents like acetonitrile, methanol and acetone. In all the three solvents, the recovery was poor with significant matrix interference. Hence, solid phase extraction on Oasis HLB cartridge was utilized to obtain quantitative and precise recovery for TRZ and *m*CPP. The extracts obtained were clearer, with quantitative and precise recovery for trazodone; however, the desired sensitivity was not achieved for *m*CPP. Thus, LLE was tried with different solvent systems viz. hexane, 30% ethyl acetate in hexane, dichloromethane, diethyl ether and methyl *tert*-butyl ether (MTBE). The recovery obtained in MTBE was quantitative but was less selective in blank plasma. However, the results with *n*-hexane were best in terms of selectivity and reproducible response compared to other extraction solvents. Further, addition of 4.5 M ammonium hydroxide assisted in breaking the drug protein binding and at the same time maintaining the basic analytes in a nonionic lipophilic form. Significant efforts were then aimed at improving the method ruggedness during LLE, and transferability by introducing flash freezing step, which helped in retaining the polar matrix in frozen aqueous phase. The mean recoveries obtained were quantitative for *m*CPP (79.7%), but were low for TRZ and IS (53.1% and 53.9%) respectively. Multiple extractions for quantitative recoveries were deliberately avoided as it was time consuming and was less suitable for high throughput analysis. Moreover, according to the USFDA guidelines [26], the recovery need not be 100%, but the extent of recovery of an analyte should be consistent and reproducible at each QC level. The validation results and subject sample analysis support this extraction methodology and hence was accepted in the present study.

The chromatographic conditions were aimed at getting adequate response, sharp peak shape and a short run time per analysis for the analytes and IS. This included mobile phase selection, flow rate, column type and injection volume. Different volume ratios of methanol–water and acetonitrile–water combinations were tried as mobile phase, along with formic acid, ammonium trifluoroacetate and ammonium acetate buffers in varying strengths on Aquasil C18 (100 mm × 2.1 mm i.d., 5 μm), Hypurity cyano (50 mm × 4.6 mm i.d., 5 μm) and Betabasic cyano (100 mm × 2.1 mm i.d., 5 μm). It was observed that 2.0 mL, 1 M ammonium trifluoroacetate and 1.0 mL of formic acid in methanol:water (70:30, v/v) as the mobile phase was most appropriate for faster elution, better efficiency and peak shape. The use of Betabasic cyano chromatography column helped in the separation and elution of all three compounds in a very short time. The elution order/retention mechanism on the reversed phase C-18 column was based on the polarity of analytes, similar to the trend observed in earlier report [24] i.e. metabolite (0.98 min) followed by relatively less polar trazodone (1.02 min). The maximum on-column loading of TRZ and *m*CPP per sample injection was 7.5 ng and 0.15 ng respectively. The total chromatographic run time was 2.5 min for each run using 400 μL/min flow rate.

As per USFDA guideline, an ideal internal standard should mimic the analyte in as many ways as possible. It should have a similar structure, same physicochemical properties or can be a labeled compound. Nefazodone, used as an internal standard has many structural similarities with trazodone and its active metabolite *m*CPP. All three compounds have halogenated phenyl groups and a piperazine unit. Moreover they have similar extractability, chromatographic behaviour and a similar ESI–MS performance. Also, the validation results obtained from this LC–MS/MS methodology encouraged its selection as an IS for the present study.

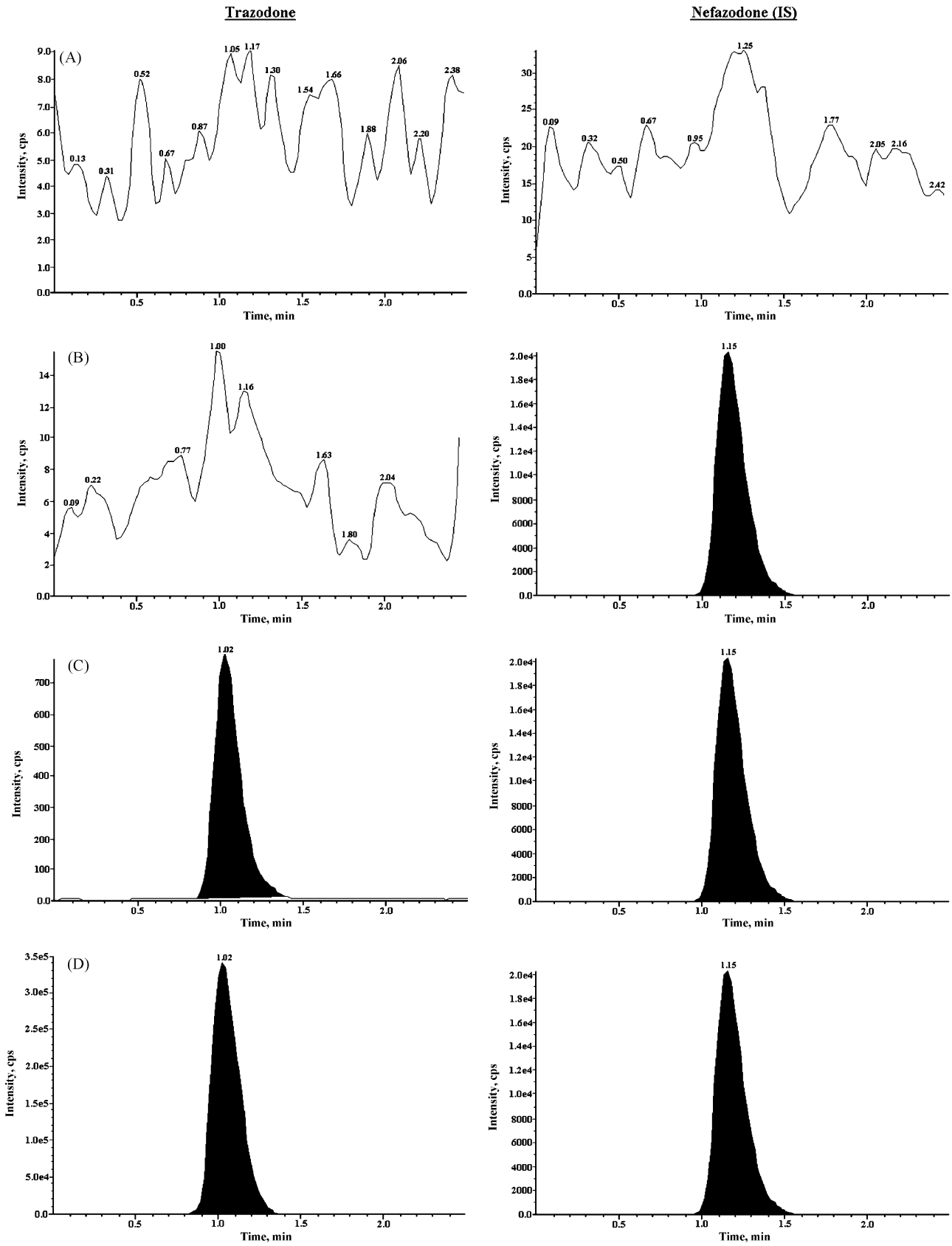

Fig. 2. Chromatograms for trazodone (372.2 → 176.2) and nefazodone (IS, 470.5 → 274.6) in (A) double blank plasma, (B) blank + IS, (C) LLOQ and (D) real subject sample at 0.5 h.

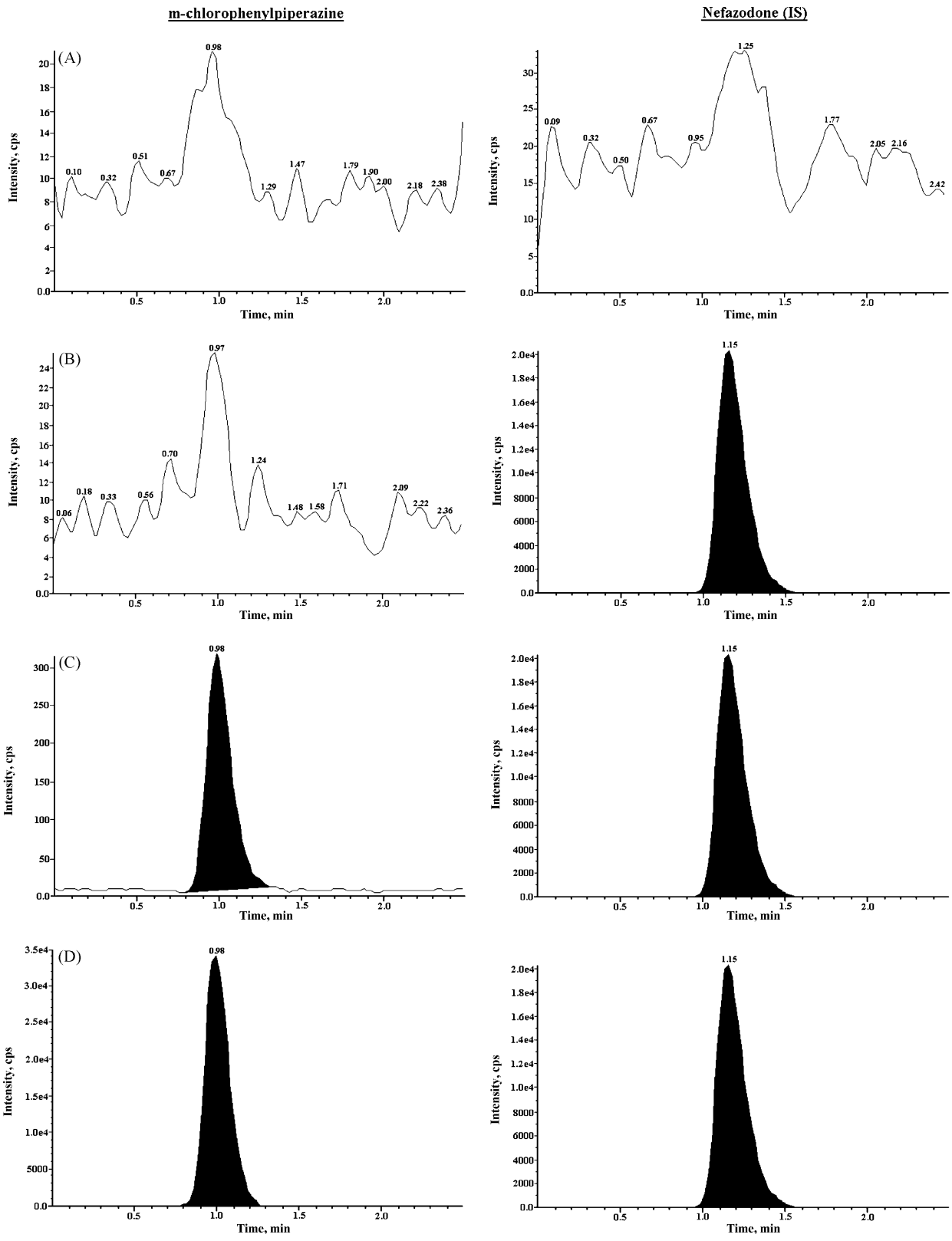

Fig. 3. Chromatograms for *m*-chlorophenylpiperazine (197.2 → 118.1) and nefazodone (IS, 470.5 → 274.6) in (A) double blank plasma, (B) blank + IS, (C) LLOQ and (D) real subject sample at 6.0 h.

3.2. Selectivity and sensitivity (LLOQ)

The aim of performing selectivity check with 10 different types of plasma samples was to ensure the authenticity of the results for study sample analysis. Figs. 2 and 3 demonstrate the selectivity results with the chromatograms of double blank plasma (without IS), blank plasma (with IS), and the peak response of TRZ and *m*CPP at LLOQ (10.0 ng/mL for TRZ and 0.20 ng/mL for *m*CPP) concentra-

Table 1
Evaluation of accuracy and precision of quality control samples for trazodone and *m*-chlorophenylpiperazine ($n = 6$, six replicates per run)

Trazodone	Concentration (ng/mL)				
	LLOQ	LQC	MQC	HQC	ULOQ
	10.0	25.0	230.0	2250	3000
Run 1 (mean ± S.D.)	10.0 ± 0.33	23.7 ± 1.18	244.0 ± 2.06	2167 ± 95.47	2886 ± 59.99
Run 2 (mean ± S.D.)	9.2 ± 0.41	23.6 ± 0.71	234.2 ± 2.36	2037 ± 98.72	2842 ± 8.98
Run 3 (mean ± S.D.)	9.9 ± 0.37	23.1 ± 1.05	227.0 ± 12.05	2213 ± 47.60	2753 ± 41.66
Overall mean	9.7	23.4	235.1	2139	2827
Intra-assay, CV (%)[a]	3.89	4.25	3.29	4.06	1.48
Inter-assay, CV (%)[a]	11.11	3.49	9.05	10.67	5.91
Overall accuracy (%bias)[b]	−3.0	−6.4	2.2	−4.9	−5.8
m-Chlorophenylpiperazine	Concentration (ng/mL)				
	LLOQ	LQC	MQC	HQC	ULOQ
	0.20	0.50	4.6	45.0	60.0
Run 1 (mean ± S.D.)	0.20 ± 0.02	0.46 ± 0.01	4.8 ± 0.07	44.2 ± 1.80	59.8 ± 2.27
Run 2 (mean ± S.D.)	0.22 ± 0.01	0.49 ± 0.03	4.5 ± 0.15	39.8 ± 2.66	60.8 ± 0.33
Run 3 (mean ± S.D.)	0.21 ± 0.01	0.47 ± 0.02	4.5 ± 0.24	42.4 ± 2.85	58.2 ± 3.43
Overall mean	0.21	0.47	4.5	42.4	58.2
Intra-assay, CV (%)[a]	5.90	4.12	3.91	5.51	2.71
Inter-assay, CV (%)[a]	11.66	7.00	11.66	13.79	13.89
Overall accuracy (%bias)[b]	5.0	−6.0	−2.2	−5.8	−3.0

[a] Coefficient of variation; within and between batch assay CVs were calculated by ANOVA.
[b] %Bias = [(overall mean assayed concentration − added concentration)/(added concentration)] × 100.

tion. Also, the real subject sample chromatograms are presented for TRZ and *m*CPP at 0.5 and 6.0 h respectively after oral administration of 100 mg trazodone in these figures. The liquid–liquid extraction method employed gave very good selectivity for the analytes and IS in the blank plasma. The chromatograms show excellent peak shape for both the analytes and IS. No endogenous interferences were found at the retention times of TRZ (1.02 min), *m*CPP (0.98 min) and IS (1.15 min) in the blank plasma. The retention time was short for both the analytes, which makes it suitable for routine analysis. The area observed at the retention time of TRZ and *m*CPP was less than 20% of their LLOQ area whereas, it was less than 5% IS area observed in the LLOQ sample.

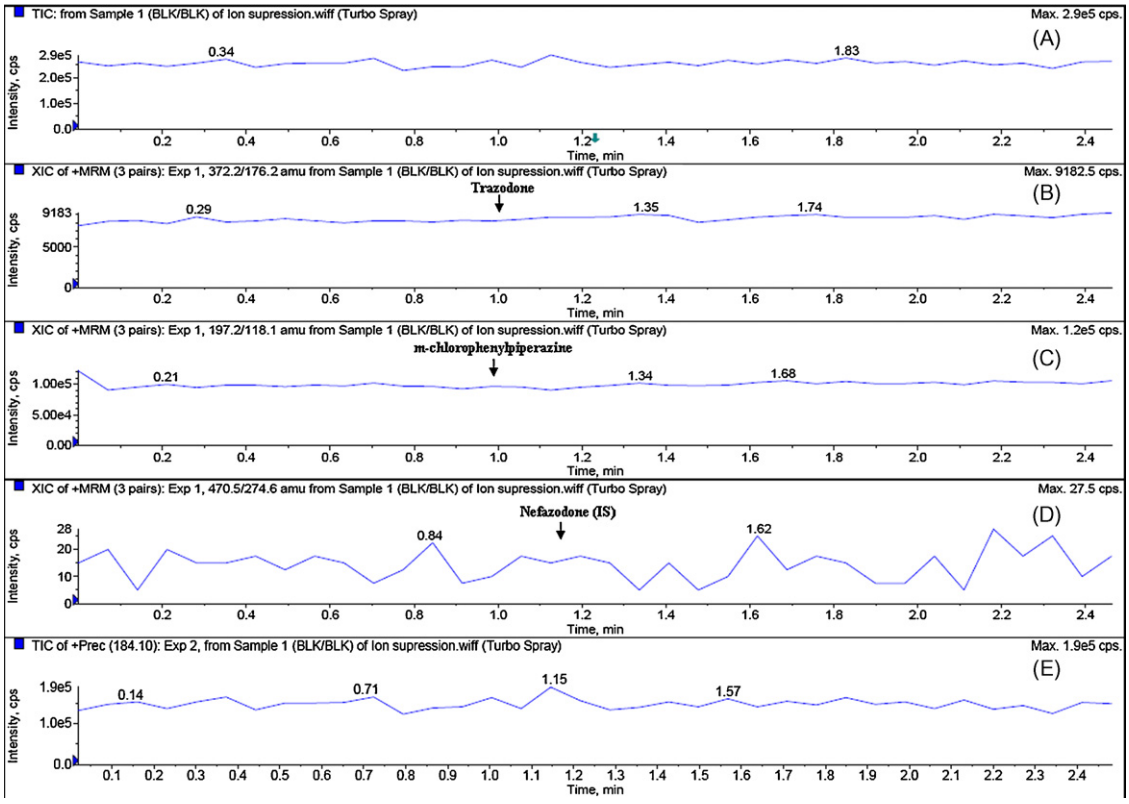

Fig. 4. Ion suppression/enhancement study. Representative post-column analyte infusion MRM LC–MS/MS chromatograms for (A) TIC (total ion current) chromatograms of trazodone, *m*-chlorophenylpiperazine, nefazodone and phospholipid; (B) XIC (exact ion current) chromatogram of trazodone (372.2 → 176.2); (C) XIC of *m*-chlorophenylpiperazine (197.2 → 118.1); (D) XIC of nefazodone (IS, 470.5 → 274.6) and (E) phospholipid precursor ion transition at 184.1, scan range 300–900 amu.

Table 2
Matrix effect in human plasma at LQC and HQC levels

Plasma lot	Trazodone				*m*-Chlorophenylpiperazine			
	LQC (25.0 ng/mL)		HQC (2250 ng/mL)		LQC (0.50 ng/mL)		HQC (45.0 ng/mL)	
	Mean calculated concentration[a]	%Bias	Mean calculated concentration[a]	%Bias	Mean calculated concentration[a]	%Bias	Mean calculated concentration[a]	%Bias
Lot-1	23.4	−6.4	2156	−4.2	0.48	−4.0	42.7	−5.1
Lot-2	23.1	−7.6	2222	−1.2	0.46	−8.0	43.9	−2.4
Lot-3	23.7	−5.2	2198	−2.3	0.48	−4.0	43.4	−3.6
Lot-4	23.4	−6.4	2187	−2.8	0.48	−4.0	43.0	−4.4
Lot-5	23.4	−6.4	2176	−3.3	0.50	0.0	43.9	−2.4
Lot-6	23.5	−6.0	2165	−3.8	0.51	2.0	44.6	−0.9

[a] Mean of four replicate observations at each concentration.

3.3. Linearity, accuracy and precision

The calibration curves for TRZ and *m*CPP were linear from 10.0 to 3000 ng/mL and 0.20 to 60.0 ng/mL with correlation coefficient $r \geq 0.9986$ and $r \geq 0.9990$ respectively across five regression curves. The equations for means ($n = 5$) of five calibration curves for the analyte were: TRZ, $y = 0.0022x + 0.0025$; *m*CPP, $y = 0.0360x + 0.0005$. The standard deviation values obtained for slope and intercept from five linearities were 0.0003 and 0.0019 for TRZ; 0.0039 and 0.0006 for *m*CPP respectively.

The intra-assay precision and accuracy were evaluated in six replicates by one-way analysis of variance using ANOVA. Results presented in Table 1 indicate that intra-assay coefficients of variations, %CV, were between 1.48% and 4.25% for TRZ and between 2.71% and 5.90% for *m*CPP. The inter-assay %CVs were lower than 11.2% for TRZ, while for *m*CPP were lower than 13.9%. The overall accuracy was assessed in terms of %bias that ranged from −6.4% to 2.2% for TRZ, and from −6.0% to 5.0% for *m*CPP.

3.4. Recovery and matrix effect

The overall mean recoveries for TRZ at LQC, MQC and HQC levels were 59.1%, 50.2% and 50.0% and that for *m*CPP were 83.9%, 77.1% and 78.2% respectively with variability (%CV) between them of 9.8% for TRZ and 4.6% for *m*CPP. The recovery of IS was found to be 53.9% with %CV of 4.9. Thus, the consistency in recoveries of TRZ, *m*CPP and IS supports the extraction procedure for its application to routine sample analysis.

Matrix effect is due to co-elution of some components present in biological samples. These components may not give a signal in MRM of target analyte but can certainly decrease or increase the analyte response dramatically to affect the sensitivity, accuracy and precision of the method. Thus assessment of matrix effect constitutes an important and integral part of validation for quantitative LC–MS/MS method for supporting pharmacokinetics studies. Post-column infusion experiment indicates no ion suppression or enhancement at the retention time of TRZ, *m*CPP or nefazodone (IS) as evident from the MRM LC–MS/MS chromatograms in Fig. 4. Fig. 4A gives the total ion current (TIC) chromatogram of trazodone, *m*-chlorophenylpiperazine, nefazodone and phospholipid. The exact ion current (XIC) chromatograms of trazodone ($372.2 \rightarrow 176.2$), *m*-chlorophenylpiperazine ($197.2 \rightarrow 118.1$), nefazodone (IS, $470.5 \rightarrow 274.6$) and TIC of phospholipid precursor ion transition at 184.1 (scan range 300–900 amu) represented in Fig. 4B–E show no region of interference at the retention time of both the analytes and IS.

Assessment of matrix effect was done with the aim to see the effect of different lots of plasma on the back-calculated value of QC's nominal concentration. The results found were well within the acceptable range as shown in Table 2. No ion suppression/enhancement was observed for TRZ and *m*CPP at their respective retention times. Cross specificity experiment indicated no interconversion between TRZ and *m*CPP, as the area observed at the retention time of TRZ in presence of ULOQ area of *m*CPP and vice-versa was negligible (<0.1%). Also, the extraction method was rugged enough and gave accurate and consistent results when applied to real patient samples.

Table 3
Stability results for trazodone and *m*-chlorophenylpiperazine ($n = 6$)

Stability	Storage condition	Level	Trazodone			*m*-Chlorophenylpiperazine		
			A (ng/mL)	%CV	%Bias	*A* (ng/mL)	%CV	%Bias
Stability in biological matrix (SBM)	Room temperature (25 h)	LQC	23.2	3.7	−7.2	0.46	4.3	−8.0
		MQC	231.0	0.6	0.4	4.3	5.0	−6.5
		HQC	2216	1.8	−1.5	42.7	3.1	−5.1
Refrigerator stability of extracted samples (RSS)	Autosampler (4 °C, 96 h)	LQC	25.6	4.6	2.4	0.51	5.0	2.0
		MQC	247.3	1.2	7.5	4.7	1.5	2.4
		HQC	2135	2.5	−5.1	42.1	2.0	−6.4
Bench top stability of extracted samples (BTS)	Room temperature (96 h)	LQC	26.5	3.9	6.0	0.52	4.4	4.0
		MQC	245.4	2.5	6.7	4.6	2.4	0.0
		HQC	2120	2.9	−5.8	42.3	4.5	−6.0
Freeze and thaw stability (FTS)	After 6th cycle at −20 °C	LQC	24.2	3.8	−3.2	0.47	5.1	−6.0
		MQC	253.8	2.5	10.3	4.7	5.5	2.2
		HQC	2345	0.4	4.2	46.1	0.9	2.4
Long term stability (LTS)	70 days at −20 °C	LQC	26.6	5.0	6.4	0.50	6.9	0.0
		MQC	252.3	2.6	9.7	4.7	2.7	2.2
		HQC	2383	1.5	5.9	44.9	1.7	−0.2

A: mean comparison concentration; CV: coefficient of variance.

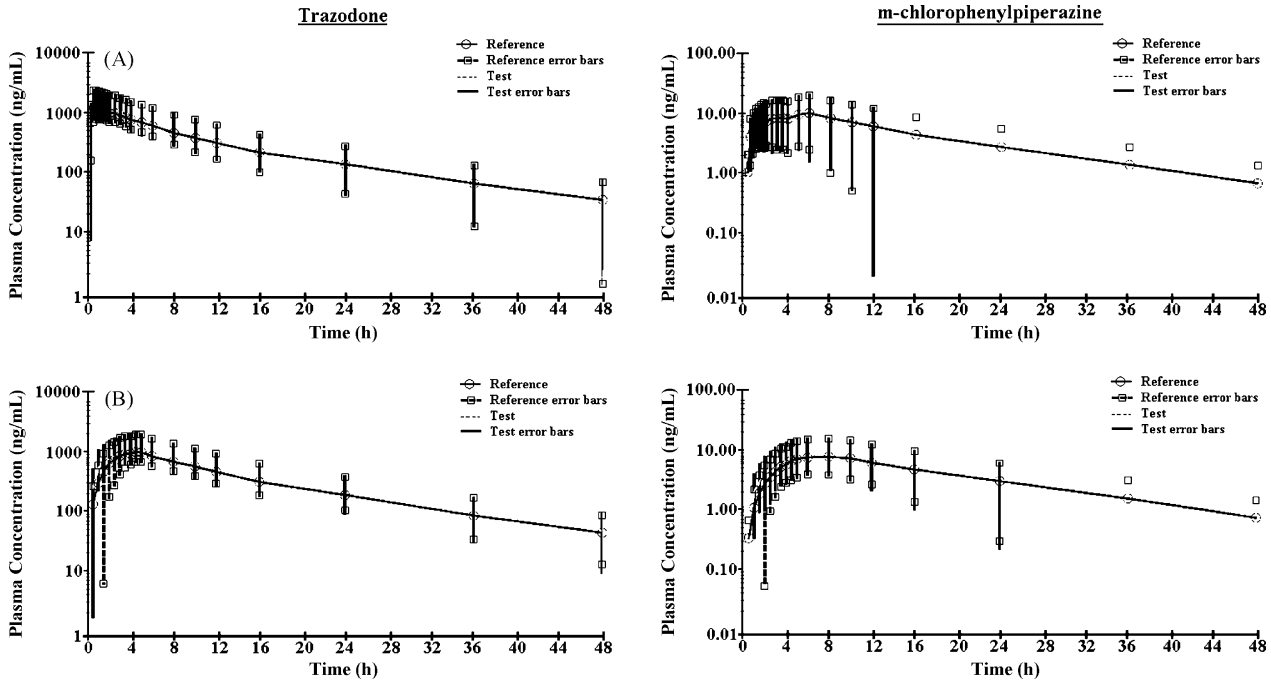

Fig. 5. Mean pharmacokinetic profile of trazodone and *m*-chlorophenylpiperazine after oral administration of 100 mg trazodone tablet to 36 healthy subjects under (A) fasting and (B) fed conditions.

3.5. Stability and dilution integrity

The stability experiments were performed thoroughly to evaluate their stability in stock solutions and in plasma samples under different conditions. The stability of spiked QC samples was compared with freshly prepared quality control samples. Stock solution of TRZ, *m*CPP and IS were stable at room temperature for 6 h and at 4 ± 6 °C for 26 days for TRZ, *m*CPP and IS with mean %change well within 0.8–1.2%. The intermediate solution of TRZ and *m*CPP in methanol–water (50:50, v/v) was stable for 21 days. Both the analytes were found stable in controlled plasma at room temperature up to 25 h and for at least six freeze and thaw cycles. The analytes in extracted plasma samples were stable for 96 h under refrigerated condition of 4 ± 6 °C. Bench top stability of extracted samples was also up to 96 h. The TRZ and *m*CPP spiked plasma samples stored at −20 °C for long term stability were found stable for minimum period of 70 days. The values for the percent change for the above stability experiments are compiled in Table 3.

The mean back-calculated concentrations for 1/10 dilution samples were within 85–115% of their nominal values. The precision (%CV) for 1/10 dilution samples was ≤1.1 for both the analytes.

Table 4
Pharmacokinetic parameters of trazodone and *m*-chlorophenylpiperazine in 36 healthy Indian subjects following oral dose of 100 mg trazodone tablet formulation under fasting and fed conditions

Parameter	Trazodone			
	Fasting		Fed	
	Test Mean ± %RSD	Reference Mean ± %RSD	Test Mean ± %RSD	Reference Mean ± %RSD
C_{max} (ng/mL)	1486.4 ± 25.4	1448.4 ± 25.7	1096.7 ± 25.7	1136.4 ± 26.5
T_{max} (h)	1.1 ± 86.3	1.0 ± 69.6	3.8 ± 52.3	3.6 ± 35.7
$t_{1/2}$ (h)	11.2 ± 28.8	10.8 ± 30.4	10.6 ± 32.1	10.5 ± 30.1
$AUC_{0-48\,h}$ (h ng/mL)	12301.0 ± 39.6	12089.9 ± 39.3	13520.9 ± 30.7	13285.0 ± 31.8
$AUC_{0-\infty}$ (h ng/mL)	13061.7 ± 42.2	12791.2 ± 41.7	14353.1 ± 32.5	14040.6 ± 33.1
K_{el} (1/h)	0.07 ± 33.8	0.07 ± 39.6	0.07 ± 33.6	0.07 ± 32.5
Parameter	*m*-Chlorophenylpiperazine			
	Fasting		Fed	
	Test Mean ± %RSD	Reference Mean ± %RSD	Test Mean ± %RSD	Reference Mean ± %RSD
C_{max} (ng/mL)	11.2 ± 76.6	10.8 ± 68.6	9.1 ± 48.1	9.0 ± 47.1
T_{max} (h)	4.7 ± 45.1	4.6 ± 43.4	5.9 ± 34.3	6.3 ± 34.4
$t_{1/2}$ (h)	11.5 ± 33.2	11.2 ± 38.5	11.2 ± 33.5	10.9 ± 29.3
$AUC_{0-48\,h}$ (h ng/mL)	179.9 ± 95.9	175.6 ± 95.2	169.3 ± 67.8	168.2 ± 66.7
$AUC_{0-\infty}$ (h ng/mL)	195.3 ± 98.7	191.2 ± 98.1	188.3 ± 75.1	183.9 ± 70.8
K_{el} (1/h)	0.07 ± 35.8	0.07 ± 35.4	0.07 ± 32.2	0.07 ± 28.6

3.6. Application of the method on human subjects

The proposed validated method was applied for a pharmacokinetic study of trazodone and *m*-chlorophenylpiperazine in 36 healthy Indian adult male subjects who received 100 mg test and reference formulations of trazodone under fasting and fed conditions. The method was sensitive enough to monitor their plasma concentration up to 48.0 h. In all approximately 2023 samples including the calibration, QC and volunteer samples were run and analysed successfully. The precision and accuracy for calibration and QC samples were well within the acceptable limits. The %change in the randomly selected subject samples for assay reproducibility was less than 12%. This authenticates the reproducibility and ruggedness of the proposed method. The mean pharmacokinetic profile for the treatment, under fasting and fed conditions is presented in Fig. 5. The pharmacokinetic parameters viz. maximum plasma concentration C_{max}, area under the plasma concentration–time curve from zero hour to the last measurable concentration AUC_{0-t}, area under the plasma concentration–time curve from zero hour to infinity $AUC_{0-\infty}$, time point of maximum plasma concentration curve T_{max}, elimination rate constant K_{el} and half-life of drug elimination during the terminal phase $t_{1/2}$ were calculated for trazodone and *m*-chlorophenylpiperazine. The mean pharmacokinetic parameters obtained for the test and reference formulation are presented in Table 4. These observations confirm the bioequivalence of 100 mg test sample with the reference product in terms of rate and extent of absorption.

Further, there was no adverse event during the course of the study. Thus the assay procedure for trazodone in plasma samples demonstrated the linearity, precision and sensitivity needed for the pharmacokinetic studies of this drug.

4. Conclusion

The objective of this work was to develop a simple, cost effective, rugged and a high throughput method for simultaneous estimation of trazodone and its active metabolite, *m*-chlorophenylpiperazine, in human plasma, especially to meet the requirement for subject sample analysis. The simple liquid–liquid extraction employed in the present work gave consistent and reproducible recoveries for both the analytes. The run time per sample analysis of 2.5 min suggests the high throughput of the proposed method. The maximum on-column loading of TRZ and *m*CPP was 7.5/0.15 ng per injection volume of 5 μL. This was considerably less compared to other reported procedures, which helps in maintaining the efficiency and the lifetime of the column. Moreover, the limit of quantification is low enough to monitor at least five half-lives of TRZ and *m*CPP concentration with good intra- and inter-assay reproducibility (%CV) for the quality controls. From the results of all the validation parameters, the method can be useful for therapeutic drug monitoring both for analysis of routine samples of single dose or multiple dose pharmacokinetics and also for the clinical trial samples with desired precision and accuracy.

Acknowledgements

The authors are indebted to Mr. Pankaj Patel, Chairman, BA Research India Ltd., Ahmedabad and Mr. Vijay Patel, Executive Director, BA Research India Ltd., Ahmedabad for providing necessary facilities to carry out this work. We gratefully acknowledge Mrs. Arpana Prasad, Senior Research Scientist, BA Research India Ltd. for her continuous support, motivation and assistance during the course of this project.

References

[1] D.R. Abernethy, D.J. Greenblatt, R.I. Shader, Pharmacology 28 (1984) 42.
[2] R.N. Brogden, R.C. Heel, T.M. Speight, G.S. Avery, Drugs 21 (1981) 401.
[3] S.H. Tariq, S. Pulisetty, Clin. Geriatr. Med. 24 (2008) 93.
[4] S.G. Bryant, L. Ereshefsky, Clin. Pharm. 1 (1982) 406.
[5] S.P. James, W.B. Mendelson, J. Clin. Psychiatry 65 (2004) 752.
[6] R.S. Kahn, S. Wetzler, Biol. Psychiatry 30 (1991) 1139.
[7] R.L. Kraus, Y. Li, A. Jovanovska, J.J. Renger, Neuropharmacology 53 (2007) 308.
[8] B. Gerson, S. Chan, F. Bell, K.M. Pappalardo, J. Psychiat. Res. 20 (1983) 69.
[9] X. Kang, C. Pan, Q. Xu, Y. Yao, Y. Wang, D. Qi, Z. Gu, Anal. Chim. Acta 587 (2007) 75.
[10] R.N. Gupta, M. Lew, J. Chromatogr. 342 (1985) 442.
[11] S. Carda-Broch, M.T. Gil-Agusti, L. Monferrer-Pons, J.S. Esteve-Romero, J. Chromatogr. A 1156 (2007) 254.
[12] O. Andriollo, C. Lartigue-Mattei, J.L. Chabard, H. Bargnoux, J. Petit, J.A. Berger, J. Chromatogr. 575 (1992) 301.
[13] G. Belvedere, A. Frigerio, C. Pantarotto, J. Chromatogr. 112 (1975) 631.
[14] R.E. Gammans, E.H. Kerns, W.W. Bullen, R.R. Covington, J.W. Russell, J. Chromatogr. 339 (1985) 303.
[15] A. Bakkali, E. Corta, J.I. Ciria, L.A. Berrueta, B. Gallo, F. Vicente, Talanta 49 (1999) 738.
[16] S.M.R. Wille, K.E. Maudens, C.H. Van Peteghem, W.E.E. Lambert, J. Chromatogr. A 1098 (2005) 19.
[17] S.M.R. Wille, P. Van Hee, H.M. Neels, C.H. Van Peteghem, W.E.E. Lambert, J. Chromatogr. A 1176 (2007) 236.
[18] T. Shinozuka, M. Terada, E. Tanaka, Forensic Sci. Int. 162 (2006) 108.
[19] H. Kirchherr, W.N. Kuhn-Velten, J. Chromatogr. B 843 (2006) 100.
[20] W.B. Mendelson, J. Clin. Psychiatry 66 (2005) 469.
[21] S. Caccia, M. Ballabio, R. Fanelli, G. Guiso, M.G. Zanini, J. Chromatogr. 210 (1981) 311.
[22] R.L. Miller, C.L. Devane, J. Chromatogr. 374 (1986) 388.
[23] T. Ohkubo, T. Osanai, K. Sugawara, M. Ishida, K. Otani, K. Mihara, N. Yasui, J. Pharm. Pharmacol. 47 (1995) 340.
[24] G.T. Vatassery, L.A. Holden, D.K. Hazel, M.W. Dysken, Clin. Biochem. 30 (1997) 149.
[25] L. Mercolini, C. Colliva, M. Amore, S. Fanali, M.A. Raggi, J. Pharm. Biomed. Anal. 47 (2008) 882–887.
[26] Guidance for Industry: Bioanalytical Method Validation, U.S. Department of Health and Human Services, Food and Drug Administration Centre for Drug Evaluation and Research (CDER), Centre for Veterinary Medicine (CVM), May 2001.
[27] Guidance for Industry: ICH E6 Good Clinical Practice, U.S. Department of Health and Human Services, Food and Drug Administration, Centre for Drug Evaluation and Research (CDER), Centre for Biologics Evaluation and Research (CBER), April 1996.
[28] M.L. Rocci Jr., V. Devanarayan, D.B. Haughey, P. Jardieu, AAPS J. 9 (2007) E336.