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An accurate, rapid and sensitive determination of tramadol and its active metabolite *O*-desmethyltramadol in human plasma by LC–MS/MS

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ABSTRACT

A rapid, sensitive and accurate liquid chromatography–tandem mass spectrometry (LC–MS/MS) assay for the simultaneous determination of tramadol and its active metabolite, *O*-desmethyltramadol in human plasma is developed using propranolol as internal standard (IS). The analytes and IS were extracted from 200 μL aliquots of human plasma via protein precipitation using acetonitrile. Chromatographic separation was achieved in a run time of 2.0 min on an Aquasil C18 (100 mm × 2.1 mm, 5 μm) column 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 method was fully validated for its selectivity, sensitivity, linearity, precision and accuracy, recovery, matrix effect, ion suppression/enhancement, stability and dilution integrity. A linear dynamic range was established from 1.0 to 600.0 ng/mL for tramadol and 0.5–300.0 ng/mL for *O*-desmethyltramadol. The method was successfully applied to a bioequivalence study of 200 mg tramadol tablet formulation in 27 healthy Indian male subjects under fasting condition.

1. Introduction

Tramadol hydrochloride is a centrally acting analgesic, used in the treatment of moderate to severe acute and chronic pain [1]. It acts as an opiate agonist by selective activity at the μ-opioid receptors [2]. It inhibits reuptake of norepinephrine and serotonin, which appears to contribute to the drug's analgesic effect [3]. Tramadol (T) has been found to produce numerous positive responses in vertebrates; antitussive, antidepressant, anti-inflammatory and immunostimulatory effects [4,5]. The therapeutic plasma concentration level of tramadol is in the range of 100–300 ng/mL and has plasma protein binding of about 20% [6]. Tramadol is rapidly absorbed after oral administration and has a bioavailability of 65–70% due to first-pass metabolism [7]. It is extensively metabolized in the liver by cytochrome P4502 isoenzymes D6 to *O*-desmethyltramadol (ODT) and *N*-desmethyltramadol (NDT). The metabolite ODT is pharmacologically active and is mainly responsible for the analgesic efficacy of tramadol [8]. About 10–30% of the parent drug is excreted unchanged in the urine [9].

Several bioanalytical methods are reported to determine tramadol alone or in combination with its metabolites in a variety of biological matrices. Methods for estimating only tramadol have been described employing HPLC with UV [10–13], fluorescence [13,14], diode array detector [15], GC with flame ionization detection [16], and mass spectrometry detection [17–20]. An interesting study for the simultaneous determination of 11 opioids used in palliative care, with five of their metabolites by an automated LC–MS/MS procedure has been reported by Musshoff et al. [21]. The analytes were extracted from blood plasma using solid phase extraction and separated under gradient conditions.

Simultaneous quantification of tramadol and its metabolites in brain tissue of mice and rats [22], saliva [23], urine [23,24], amniotic fluid [25] and plasma [22,26–39] are reported using different analytical techniques. Tao et al. [22] have presented a gas chromatographic method using nitrogen–phosphorous detector to measure tramadol and its active metabolite *O*-desmethyltramadol. A stereospecific high performance liquid chromatographic analysis of tramadol and its *O*-demethylated and *N, O*-demethylated metabolites has been described using fluorescence detection [37]. An improved HPLC method for T and ODT in human plasma is given by Gu and Fawcett [35] with a sensitivity of 3 and 1.5 ng/mL respectively. Other HPLC methods [23,27,31,32,36] reported for simultaneous determination of T and ODT have lower limits of quantification in the range of 2.5–50 ng/mL, with high chromatographic run time of 5–20 min per sample analysis. Three simultaneous stereoselective methods for tramadol and its main

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Table 1
Comparison of selected analytical methods developed for simultaneous determination of tramadol (T) and *O*-desmethyltramadol (ODT) in human plasma.

Sr. no.	Extraction procedure (human plasma volume); mean recovery (T/ODT)	Elution; mobile phase; injection volume	Maximum on-column loading[a] (ng)	Analytical run time (min)	Detection technique	LLOQ (ng/mL)	Reference no.
1[b]	LLE[c] with ethyl acetate (0.12 mL);(89.55/82.1%)	Isocratic; methanol:H_2O 19:81), pH 2.5 with PA[d]; 100 μL	30 for T and ODT	7	HPLC-fluorescence	2.5 for T and ODT	[23]
2	SPE[e] (1.0 mL); (94.36/93.52%)	Isocratic; ACN[f]:phosphate buffer:TEA[g] (30:70:0.1 v/v); 20 μL	1400 for T and 200 for ODT	2	HPLC-UV	50 for T and ODT	[31]
3	LLE[c] with TBME[h] (1.0 mL); (–/–)	Gradient; ACN[f]-0.01 M phosphate buffer pH 2.8; 100 μL	16.6 for T and ODT	18	HPLC-fluorescence	2.5 for T and ODT	[32]
4	LLE[c] with DEE[i]-DCM[j]-hexane (1.0 mL); (87.2/89.8%)	Isocratic; ACN[f]:buffer, pH 3.9 (35:65, v/v); 100 μL	384 for T and 192 for ODT	15	HPLC-fluorescence	3.0 for T and 1.5 for ODT	[35]
5[k]	LLE[c] with EA[l] (0.25 mL); (77.75/82.1%)	Isocratic; methanol:H_2O (13:87, v/v), pH 2.5; 100 μL	50 for T and ODT	5	HPLC-fluorescence	2.5 for T and 1.25 for ODT	[36]
6	LLE[c] with EA[l] (0.7 mL); (78.18%/80.04%)	Gradient; PA[d]-TEA[g]-ACN[f]-methanol; 90 μL	103.23 for T and 51.27 for ODT	27	HPLC-fluorescence	4.078 for T and 3.271 for ODT	[38]
7	PP[m] with PA (0.2 mL); (102.7/92.0%)	Isocratic; 0.2% TFA[n]:ACN[f] (90:10, v/v); 2 μL	0.3056 for T and 0.3143 for ODT	4	LC–MS/MS	2.0 for T and ODT	[39]
8	PP[m] with ACN[f] (0.2 mL); (97.2/95.8%)	Isocratic; methanol:H_2O:ATF[o]:FA[p] (700 + 300 + 1 + 0.1 mL); 5 μL	0.250 for T and 0.125 for ODT	2	LC–MS/MS	1.0 for T and 0.5 for ODT	Present method

[a] At ULOQ level.
[b] Including *N*-desmethyltramadol and *O*,*N*-di desmethyltramadol.
[c] Liquid–liquid extraction.
[d] Phosphoric acid.
[e] Solid phase extraction.
[f] Acetonitrile.
[g] Triethylamine.
[h] tert-butyl methyl ether.
[i] Diethyl ether.
[j] Dichloromethane.
[k] Including *N*-desmethyltramadol.
[l] Ethyl acetate.
[m] Protein precipitation.
[n] Trifluoroacetic acid.
[o] Ammonium trifluoroacetate.
[p] Formic acid.

phase I metabolites in human plasma by on-line capillary zone electrophoresis-electrospray ionization mass spectrometry [29] and HPLC-fluorescence [33,34] have been proposed. Very few methods are available so far for the determination of tramadol and its active metabolite using liquid chromatography tandem mass spectrometry in human plasma. An enantiomeric determination of tramadol and its main metabolite *O*-desmethyltramadol in human plasma is presented by Ceccato et al. using LC–MS/MS [28]. The method involves an automated SPE procedure for extraction, followed by separation on normal phase chiral column in the presence of other metabolites such as N-desmethyltramadol (NDT) and N,*O*-desmethyltramadol (NODT). The limits of quantitation for the enantiomers were 0.5 ng/mL using 1.0 mL human plasma. Zhao et al. [25] have reported an LC–MS/MS method employing atmospheric pressure chemical ionization as interface detection for T and ODT. The calibration curves were linear in the range of 8.0–800 μg/mL in human plasma and 1.0–400 μg/mL in amniotic fluids. Very recently, Vlase et al. [39] have presented a sensitive LC–MS/MS method for T and ODT using protein precipitation. The linear dynamic range established was 2–300 ng/mL for both the analytes in human plasma. The salient features of some selected methods for simultaneous determination of tramadol and *O*-desmethyltramadol are complied in Table 1.

Thus, there is a need to develop rapid, rugged and adequately sensitive methods for simultaneous determination of T and ODT. The method presented in this study using LC-ESI-MS/MS is rapid and sensitive for routine subject sample analysis. The chromatographic separation of the analytes and IS was achieved in a run time of 2.0 min, giving a high turnaround for the analysis. The method was successfully applied to study the pharmacokinetics/bioequivalency of 200 mg tramadol tablet formulation in 27 healthy Indian male subjects under fasting condition.

2. Experimental

2.1. Chemicals and materials

Reference standard material of tramadol (99.0%) and *O*-desmethyltramadol (95.5%) were procured from Cadila Healthcare Ltd. (Ahmedabad, GUJ, India) and SynFine Research Inc. (Toronto, Ont., Canada), while propranolol (IS, 99.0%) was procured from IPCA Laboratories Ltd. (Ratlam, MP, India). HPLC grade acetonitrile and methanol were procured from S.D. Fine Chemicals Ltd. (Mumbai, MAH, India). Ammonium trifluoroacetate used in mobile phase was of Acros Organics (Springfield, NJ, USA) while AR grade formic acid (99%) was obtained from S.D. Fine Chemicals Ltd. (Mumbai, MAH, India). Water used for the LC–MS/MS was prepared using Milli Q water purification system from Millipore (Bangalore, KAR, India). Control buffered (K3 EDTA) human plasma was procured from Clinical Department, BA Research India Limited (Ahmedabad, GUJ, India) and was stored at −20 °C. Centrifuge was of Eppendorf 5810 (Hamburg, Germany).

2.2. LC–MS/MS instrumentation and conditions

The liquid chromatography system from Shimadzu (Kyoto, Japan) consisted of a LC-10ADvp pump, an autosampler (SIL-HTc) and an on-line degasser (DGU-14A). Chromatographic column used was Aquasil C18, 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 + 1.0 mL, 1.0 M ammonium trifluoroacetate + 0.1 mL formic acid. Separation of analytes and IS was performed under isocratic condition at a flow rate of 400 μL/min. The autosampler temperature was maintained at 4 °C and the injection volume was 5 μL. The total LC run time was 2.0 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, Ont., 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* 264.2 → 58.1 for tramadol, 250.2 → 58.1 for *O*-desmethyltramadol and 260.2 → 116.1 for IS (Fig. 1). All the parameters of LC and MS were controlled by Analyst software version 1.4.1.

For tramadol, *O*-desmethyltramadol and propranolol (IS) the source parameters maintained were Gas 1 (GS1): 40 psi, Gas 2 (GS2): 60 psi, ion spray voltage (ISV): 1500 V, turbo heater temperature (TEM): 500 °C, interface heater (Ihe): ON, entrance potential (EP): 10 V, collision activation dissociation (CAD): 8 psi, curtain gas (CUR): 20 psi. The compound dependent parameters like declustering potential (DP), collision energy (CE) and cell exit potential (CXP) were optimized at 40, 45 and 5 V for tramadol, 45, 37 and 5 V for *O*-desmethyltramadol and 65, 26 and 10 V for propranolol respectively. Quadrupole 1 and quadrupole 3 were maintained at unit resolution. Dwell time set was 400 ms for both the analytes and IS.

2.3. Preparation of standard stocks and plasma samples

The standard stock solutions of 100 μg/mL were prepared by dissolving requisite amount of tramadol, *O*-desmethyltramadol and propranolol in methanol. These stock solutions were further diluted in methanol:water (50:50, v/v) to get an intermediate concentration of 12.0 and 6.0 μg/mL for T and ODT respectively. Combined working solutions of tramadol and *O*-desmethyltramadol, required for spiking plasma calibration and quality control samples were subsequently prepared using the standard and intermediate stock solutions in methanol:water (50:50, v/v). IS working solution of 75.0 ng/mL was prepared using the stock of 100 μg/mL in acetonitrile. 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 blank plasma with respective working solutions (5% of total volume of plasma). Calibration standards were made at 1.00, 2.00, 5.00, 20.0, 40.0, 90.0, 150.0, 300.0, 500.0 and 600.0 ng/mL for T and 0.50, 1.00, 2.50, 10.0, 20.0, 45.0, 75.0, 150.0, 250.0, 300.0 for ODT. Quality controls were prepared at 1.00 ng/mL (LLOQ), 3.00 ng/mL (LQC), 37.5 ng/mL (MQC), 450.0 ng/mL (HQC) and 600.0 ng/mL (ULOQ) for T and 0.50 ng/mL (LLOQ), 1.50 ng/mL (LQC), 18.75 ng/mL (MQC), 225.0 ng/mL (HQC) and 300.0 ng/mL (ULOQ) for ODT. 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 30–45 min at room temperature. The samples were vortexed adequately using a vortex mixer before pipetting. Aliquots of 200 μL plasma were transferred into polypropylene micro centrifuge tubes, 10 μL deionized water along with 400 μL working solution of IS in acetonitrile (75.0 ng/mL) was added. The tubes were capped and vortexed for 60 s vigorously. Further, the samples were centrifuged for 3 min at 17,949 × *g*. The supernatant (100 μL) was taken in labeled riavials and 300 μL of

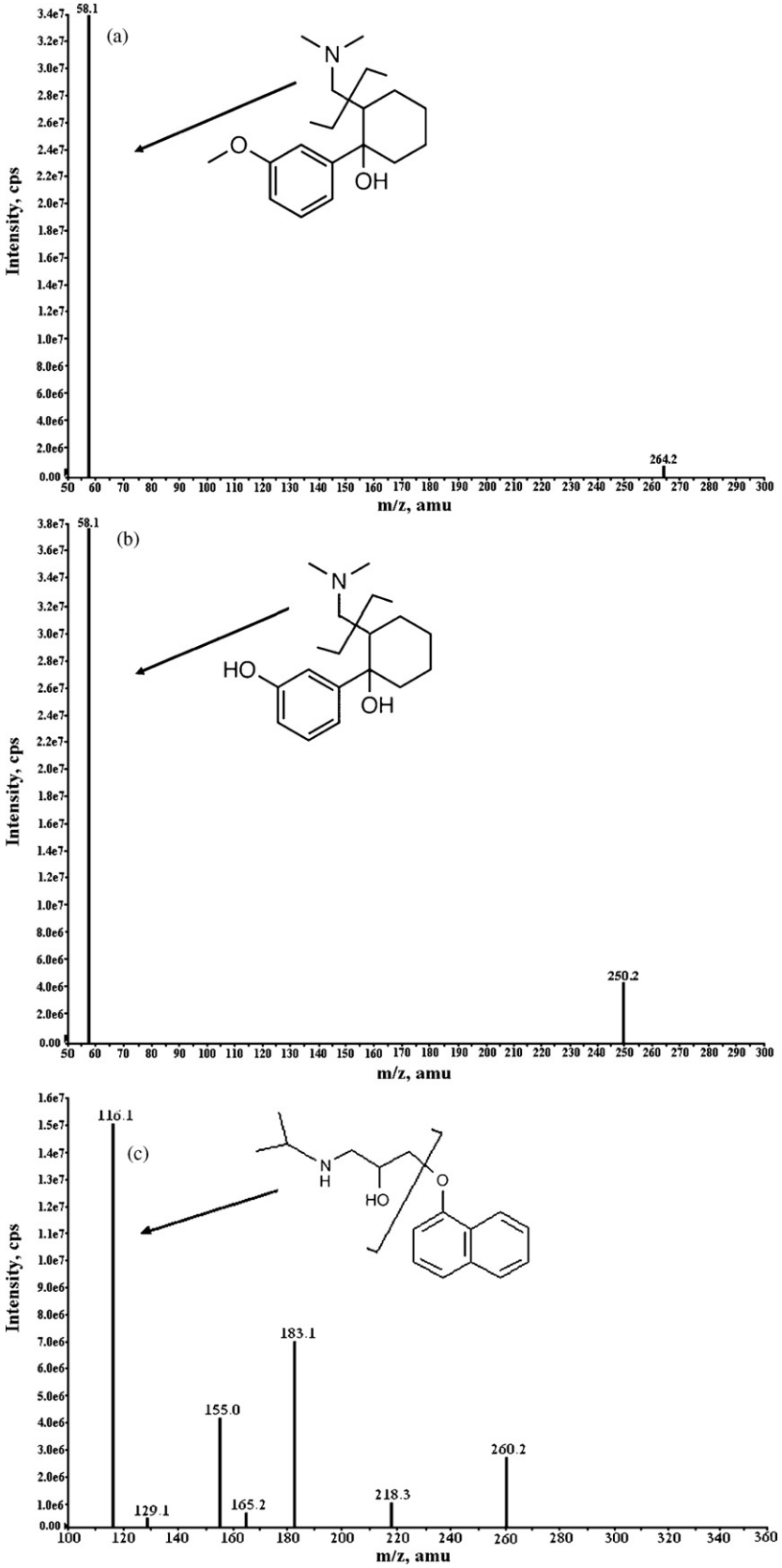

Fig. 1. Product ion spectra of (a) tramadol (264.2 → 58.1, scan range 50–300 amu), (b) *O*-desmethyltramadol (250.2 → 58.1, scan range 50–300 amu) and (c) propranolol (IS, 260.2 → 116.1, scan range 100–360 amu).

mobile phase was added. After through mixing, the samples were transferred to labeled autoinjector vials and 5 μL was used for injection in LC–MS/MS, in partial loop mode.

2.5. Validation methodology

A thorough and complete method validation of tramadol and *O*-desmethyltramadol in human plasma was done following the USFDA guidelines [40]. The method was validated for selectivity, sensitivity, interference check, carryover 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 collected with K3 EDTA as anticoagulant. The samples were processed through the protein precipitation extraction protocol and analyzed to determine the extent to which endogenous plasma components might 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 190 μL were spiked with 10 μL methanol–water (50:50, v/v) solution. In the first set, the double 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 tramadol and *O*-desmethyltramadol (5% of total volume of plasma). The blank plasma sample 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.

Carry over experiment was performed to verify any carry over of analytes, which may reflect in subsequent runs. The design of the study comprised of the following sequence of injections, i.e. double blank plasma sample → two samples of LLOQ → double blank plasma → ULOQ sample → double blank plasma → ULOQ sample → double blank plasma to check for any interference due to carry over.

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 analyzed. 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 T and ODT respectively. 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 was 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).

To evaluate the magnitude of matrix ion suppression/enhancement effects on the MRM LC–MS/MS sensitivity, post column analytes infusion experiment was conducted. A standard solution containing 100 ng/mL of T and ODT 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. Any dip in the baseline upon injection of double blank plasma would indicate ion suppression, while a peak at the retention time of T, ODT and IS indicates ion enhancement. 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. Cross specificity experiment were conducted for T, ODT 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 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 (BTS), room temperature stability (SBM), refrigerated stability of extracted sample (RSS), freeze thaw stability (FTS) and long term stability (LTS) 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. 3000 ng/mL for tramadol and 1500 ng/mL for *O*-desmethyltramadol and also at HQC level for both the analytes. Six replicate samples each of 1/10 of 5 × ULOQ (300.0/150.0 ng/mL) and 1/10 of HQC (45.0/22.5 ng/mL) concentration were prepared and their concentrations were calculated, by applying the dilution factor of 10 against the freshly prepared calibration curve for tramadol and *O*-desmethyltramadol.

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 (200 mg tramadol hydrochloride extended release tablet) and reference formulations (TOPALGIC® L.P., 200 mg tramadol hydrochloride extended release tablet) in 27 healthy Indian human subjects under fasting conditions". All the subjects were informed of the aim and risk involved in the study and written consent 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 [41]. Health check up for all subjects was done by general physical examination, ECG and laboratory tests like hematology, biochem-

istry 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 1.0, 2.0, 3.0, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 9.0, 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 also conducted by computerized random selection of subject samples (5% of total samples analyzed). 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% [42].

3. Results and discussion

3.1. Method development

As the literature reveals (Table 1), there is a need to develop LC–MS/MS methods which can encompass all the merits of an optimum bioanalytical method applicable for routine sample analysis. Thus, in the present study method development was initiated to realize a rugged, sensitive, and specific LC-ESI-MS/MS method with a short overall analysis time for the simultaneous quantification of tramadol and *O*-desmethyltramadol 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 five half lives of tramadol 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 tramadol, *O*-desmethyltramadol and propranolol (IS) using 100.0 ng/mL tuning solution. The response observed was much higher with low background noise in positive ionization mode for all the three compounds compared to the negative mode. The analytes and IS gave predominant singly charged protonated precursor $[M+H]^+$ ions at *m*/*z* of 264.2, 250.2 and 260.2 for T, ODT and IS respectively in Q1 MS full scan spectra. Further, fragmentation was initiated using sufficient nitrogen for collision activated dissociation and by applying 15 V collision energy to break the precursor ions. The most abundant ions found in the product ion mass spectra were *m*/*z* 58.1, 58.1 and 116.1 at 45, 37 and 26 V collision energy for T, ODT and IS respectively. To attain an ideal Taylor cone and a better impact on spectral response, nebuliser gas pressure (GS1) was optimized at 40 psi. 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 (4500 V), the response was drastically reduced and hence an optimum potential of 1500 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 500 °C. A dwell time of 400 ms was adequate and no cross talk was observed between the MRMs of analytes and IS.

Most of the methods reported have employed either liquid–liquid extraction [23,27,32,35,36] or solid phase extraction [31] to extract T and ODT from human plasma. So far only one procedure [39] has applied protein precipitation with perchloric acid for quantitative extraction of T and ODT from human plasma. Thus, the extraction was initiated using a simple, quick and inexpensive protein precipitation technique using common solvents like methanol, acetonitrile and acetone. Quantitative and precise recoveries were obtained using acetonitrile as the precipitating agent compared to other solvents with negligible ion suppression. The mean recoveries for T, ODT and IS obtained were 97.2%, 95.8% and 104.0% respectively. Moreover, 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 strength 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 1.0 mL, 1 M ammonium trifluoroacetate and 0.100 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 Aquasil C18 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 reports [23,31,32,36], i.e. metabolite (0.84 min) followed by relatively less polar tramadol (0.96 min). The maximum on-column loading of T and ODT per sample injection was 250 and 125 pg respectively. The total chromatographic run time was 2.0 min for each run using 400 μL/min flow rate.

A general internal standard was used to minimize analytical variation due to solvent evaporation, integrity of the column and ionization efficiency. Thus, propranolol was selected as an internal standard which had similar chromatographic behaviour and was easily precipitated with acetonitrile along with the analytes. There was no significant effect of IS on analyte recovery, sensitivity or ion suppression. Also, the validation results obtained from this LC–MS/MS methodology encouraged its selection as an IS for the present study.

3.2. Selectivity, sensitivity, interference and carryover check

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 T and ODT at LLOQ (1.00 ng/mL for T and 0.50 ng/mL for ODT) concentration. Also, the real subject sample chromatograms are presented for T and ODT at 5.5 and 7.5 h respectively after oral administration of 200 mg tramadol in these figures. The protein precipitation method in combination with mass spectrometry 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 T (0.96 min), ODT (0.84 min) and IS (1.14 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 T and ODT was less than 20% of their LLOQ area whereas, it was less than 5% IS area observed in the LLOQ sample. Though the present method was intended for T and its pharmacological active metabolite ODT, the potential interference of another major metabolite of tramadol, *N*-desmethyltramadol (NDT) was also considered. Like ODT, NDT too has the same precursor ion at *m*/*z* 250 but a different product ion at *m*/*z* 45. Thus, the possible interference

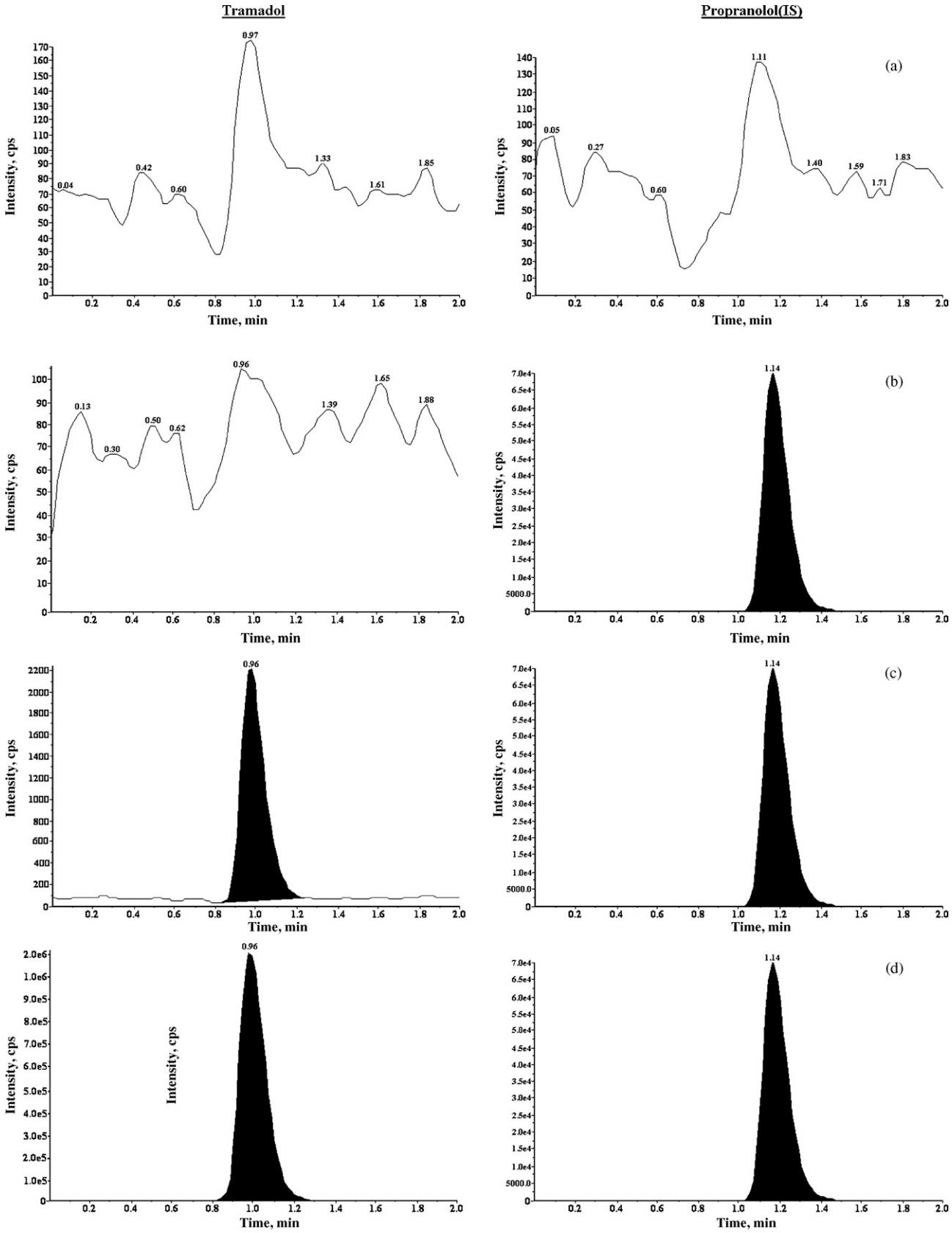

Fig. 2. Chromatograms for tramadol (264.2 → 58.1) and propranolol (IS, 260.2 → 116.1) in (a) double blank plasma, (b) blank+ IS, (c) LLOQ and (d) real subject sample at 5.5 h.

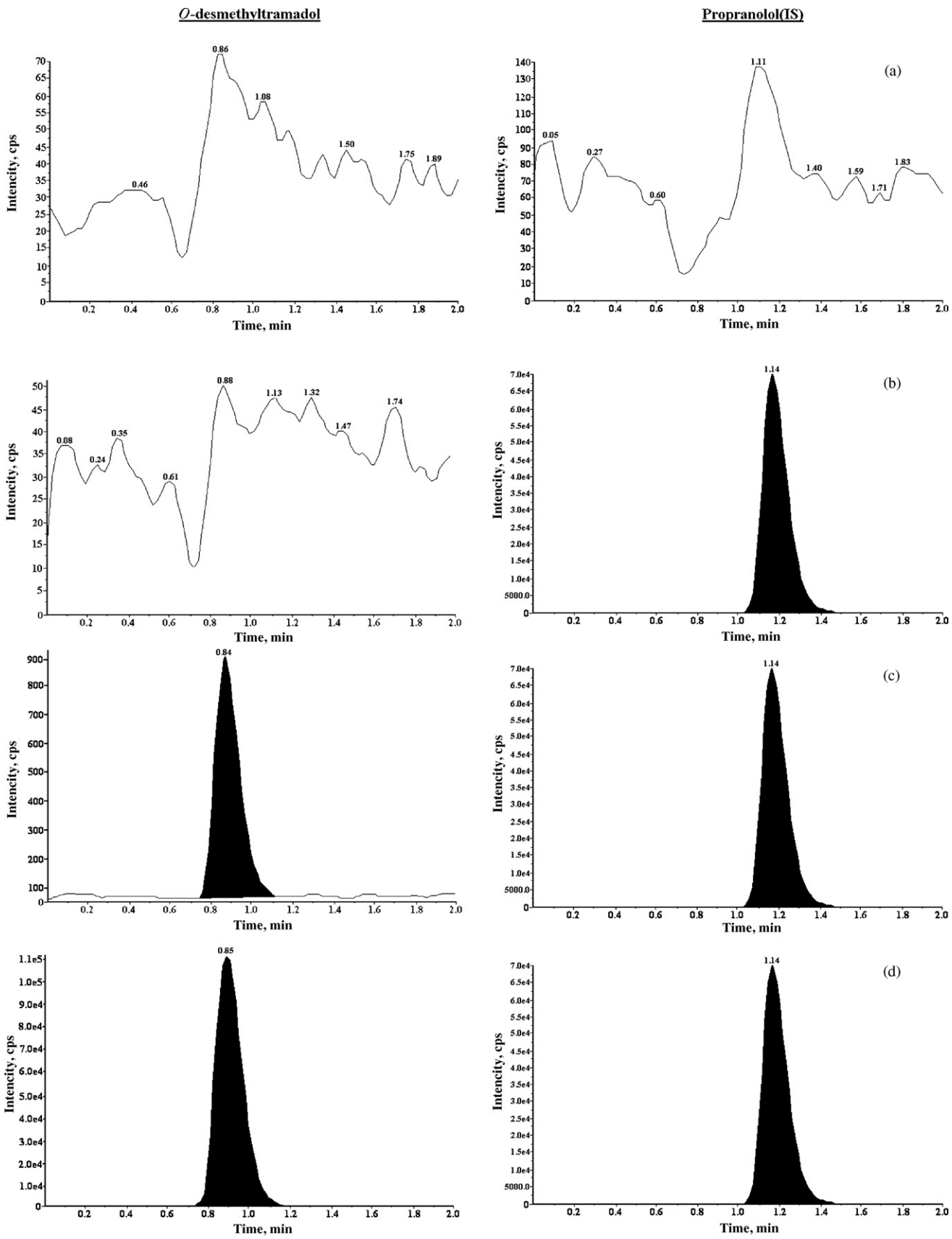

Fig. 3. Chromatograms for *O*-desmethyltramadol (250.2 → 58.1) and propranolol (IS, 260.2 → 116.1) in (a) double blank plasma, (b) blank + IS, (c) LLOQ and (d) real subject sample at 7.5 h.

Table 2
Summary of calibration curves for tramadol and *O*-desmethyltramadol with back calculated concentrations.

ID no.	Tramadol, concentration in ng/mL												
	CS-1	CS-2	CS-3	CS-4	CS-5	CS-6	CS-7	CS-8	CS-9	CS-10	Regression Parameters		
	1.00	2.00	5.00	20.0	40.0	90.0	150.0	300.0	500.0	600.0	Slope	Intercept	r^a
1	0.98	2.03	5.13	20.7	41.0	95.1	141.7	292.2	485.7	581.7	0.02673	0.00028	0.9992
2	1.02	1.92	5.01	20.3	40.0	89.2	150.1	308.6	503.2	587.6	0.02617	0.00073	0.9997
3	0.96	2.19	4.96	19.8	40.1	85.6	148.9	302.8	512.8	591.5	0.02800	−0.00120	0.9991
4	1.00	2.03	4.82	19.8	41.0	90.3	152.5	295.7	513.9	582.9	0.02749	0.00052	0.9997
5	1.05	1.79	4.94	20.6	39.6	91.7	152.8	303.6	499.5	598.1	0.02733	0.00292	0.9990
Mean	1.00	2.00	5.00	20.2	40.3	90.4	149.2	300.6	503.0	588.4	0.02714	0.00065	0.9993
S.D.	0.04	0.15	0.11	0.40	0.63	3.46	4.50	6.57	11.47	6.70	0.0007	0.0015	0.0004
%CV[b]	3.7	7.5	2.2	2.0	1.6	3.8	3.0	2.2	2.3	1.1			
%bias[c]	0.0	0.0	0.0	1.0	0.7	0.4	−0.5	0.2	0.6	−1.9			
ID no.	*O*-desmethyltramadol, concentration in ng/mL												
	CS-1	CS-2	CS-3	CS-4	CS-5	CS-6	CS-7	CS-8	CS-9	CS-10	Regression parameters		
	0.50	1.00	2.50	10.0	20.0	45.0	75.0	150.0	250.0	300.0	Slope	Intercept	r^a
1	0.50	0.99	2.55	10.1	20.5	47.0	72.2	146.3	248.3	294.8	0.02299	−0.00052	0.9996
2	0.51	0.96	2.46	10.0	19.9	44.3	74.6	155.6	259.0	296.3	0.02260	−0.00037	0.9996
3	0.48	1.10	2.45	9.8	19.9	42.4	74.2	154.6	257.1	300.3	0.02494	−0.00082	0.9989
4	0.49	1.04	2.46	9.8	20.1	44.8	75.7	148.5	259.2	294.5	0.02440	−0.00050	0.9997
5	0.51	0.96	2.47	10.1	19.6	45.4	75.3	152.3	250.9	303.1	0.02531	−0.00144	0.9998
Mean	0.50	1.01	2.48	9.94	20.0	44.8	74.4	151.5	254.9	297.8	0.02405	−0.00073	0.9995
S.D.	0.01	0.06	0.04	0.15	0.34	1.67	1.37	3.97	4.99	3.76	0.0012	0.0004	0.0004
%CV[b]	2.8	5.8	1.7	1.5	1.7	3.7	1.8	2.6	2.0	1.3			
%bias[c]	0.0	1.0	−0.8	−0.6	0.0	−0.4	−0.8	1.0	2.0	−0.7			

[a] Correlation coefficient.
[b] Coefficient of variance.
[c] Standard deviation.

due to NDT was eliminated as the MRM selected for ODT was *m*/*z* 250.2 → 58.1.

No interference was observed for commonly used medications by subjects like paracetamol, chlorpheniramine maleate, caffeine, acetylsalicylic acid and ibuprofen; this is evident from the ion chromatograms at LLOQ level and real subject sample chromatograms of T and ODT at C_{max} respectively. Almost negligible area (less than 1% of LLOQ area) was observed in double blank plasma run after highest calibration standard (ULOQ), which suggests no carry over of the analyte in subsequent runs.

3.3. Linearity, accuracy and precision

The calibration curves for T and ODT were linear from 1.00 to 600.0 ng/mL and 0.50–300.0 ng/mL with correlation coefficient $r \geq 0.9990$ and $r \geq 0.9989$ respectively within five regression curves. The standard deviation values obtained for slope and intercept from five linearties was 0.0007 and 0.0015 for T; 0.0012 and 0.0004 for ODT respectively. Their observed mean back calculated concentration with accuracy (%) and precision (% CV) of five linearties are given in Table 2.

Table 3
Intra-batch and inter-batch accuracy and precision for tramadol and *O*- desmethyltramadol.

Level	Conc. added (ng/mL)	Tramadol							
		Intra-batch				Inter-batch			
		n^a	Mean conc. found[b] (ng/mL)	% bias	% CV[c]	n^a	Mean conc. found[d] (ng/mL)	% bias	% CV[c]
LLOQ	1.00	6	1.06	5.7	1.7	18	1.04	4.0	5.5
LQC	3.00	6	2.96	−1.3	1.4	18	2.93	−2.2	4.5
MQC	37.5	6	35.9	−4.2	0.8	18	35.8	−4.5	5.2
HQC	450.0	6	421.1	−6.4	0.5	18	434.1	−3.5	6.8
ULOQ	600.0	6	565.8	−5.7	0.6	18	581.5	−3.1	4.4
Level	Conc. added (ng/mL)	*O*-desmethyltramadol							
		Intra-batch				Inter-batch			
		n^a	Mean conc. found[b] (ng/mL)	% bias	% CV[c]	n[a]	Mean conc. found[d] (ng/mL)	% bias	% CV[c]
LLOQ	0.50	6	0.51	1.0	3.2	18	0.51	1.9	4.2
LQC	1.50	6	1.47	−2.2	1.1	18	1.47	−1.7	4.4
MQC	18.75	6	17.93	−4.4	0.7	18	17.79	−5.1	4.9
HQC	225.0	6	214.7	−4.6	0.6	18	222.2	−1.2	6.6
ULOQ	300.0	6	285.7	−4.8	0.4	18	295.5	−1.5	4.4

[a] Total number of observation.
[b] Mean of six replicate observations at each concentration.
[c] Coefficient of variation.
[d] Mean of eighteen replicate observations over three different analytical runs.

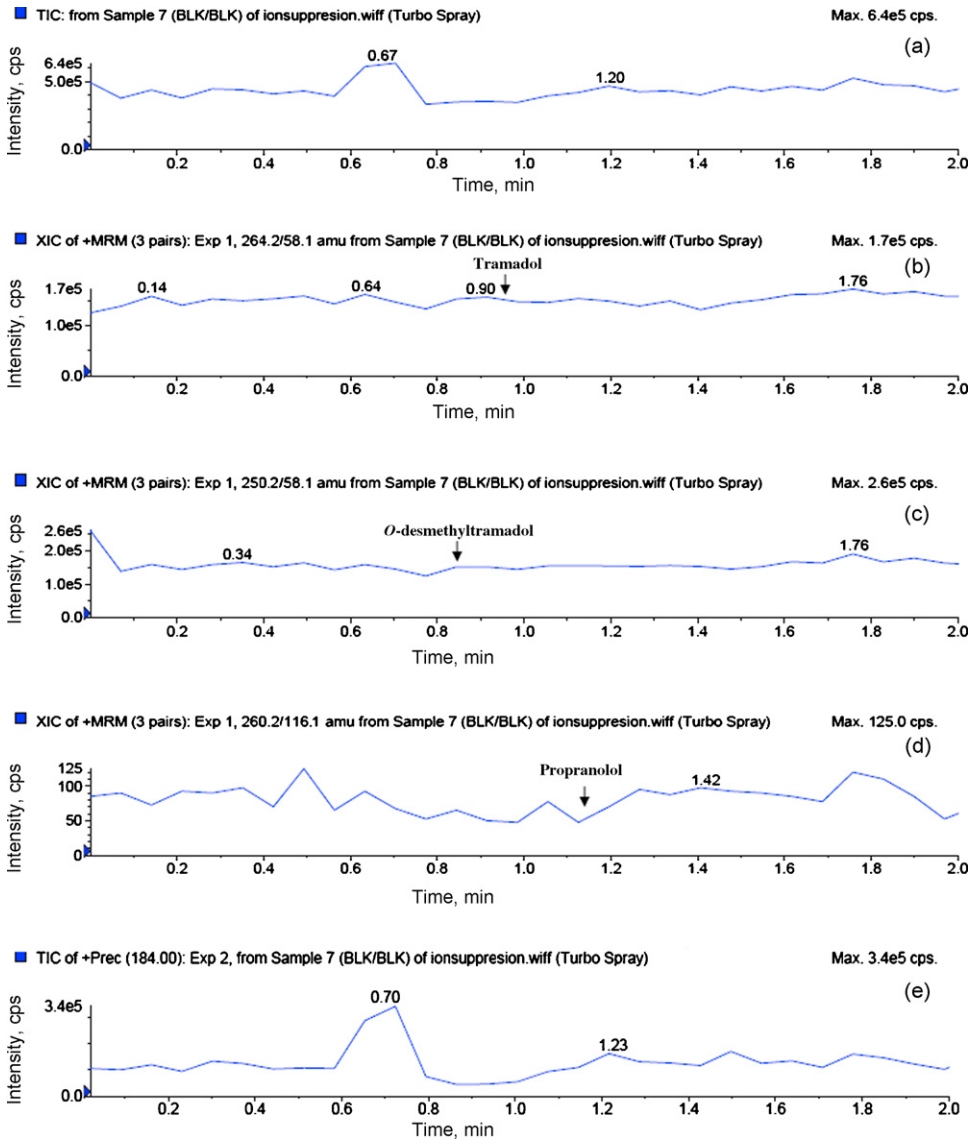

Fig. 4. Representative post column analyte infusion MRM LC–MS/MS chromatograms for (a) combined total ion current (TIC) chromatograms of tramadol, *O*-desmethyltramadol, propranolol and phospholipids. (b) Exact ion current (XIC) chromatograms of tramadol (264.2 → 58.1). (c) XIC of *O*-desmethyltramadol (250.2 → 58.1). (d) XIC of propranolol (IS, 260.2 → 116.1) and (e) phospholipid precursor ion transition at 184.1, scan range 300–900 amu.

The intra-assay precision and accuracy were evaluated in six replicate analyses for T and ODT at five concentration levels viz. LLOQ, LQC, MQC, HQC and ULOQ each on the same analytical run. Inter-assay precision and accuracy was calculated after repeated analysis in three different analytical runs. The QC concentrations were calculated from calibration curve and the intra-batch and inter-batch precision was less than 6.9% for both the analytes. Accuracy expressed in terms of % bias was within −6.4 to +5.7%. The detailed results for intra-assay and inter-assay accuracy and precision are given in Table 3.

Table 4
Evaluation of matrix effect in human plasma at LQC and HQC levels.

Plasma lot	Tramadol				*O*-desmethyltramadol			
	LQC (3.00 ng/mL)		HQC (450.0 ng/mL)		LQC (1.50 ng/mL)		HQC (225.0 ng/mL)	
	Mean calculated conc.[a]	% bias	Mean calculated conc.[a]	% bias	Mean calculated conc.[a]	% bias	Mean calculated conc.[a]	% bias
Lot-1	3.43	14.2	470.8	4.6	1.60	6.5	237.7	5.6
Lot-2	3.29	9.7	496.2	10.3	1.61	7.5	247.2	9.9
Lot-3	3.33	10.9	485.4	7.9	1.59	5.7	250.2	11.2
Lot-4	3.38	12.8	503.8	12.0	1.64	9.3	256.7	14.1
Lot-5	3.25	8.2	495.7	10.2	1.56	4.3	251.6	11.8
Lot-6	3.43	14.2	451.5	0.3	1.53	2.3	229.9	2.2

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

3.4. Recovery, ion suppression and matrix effect

The overall mean recoveries for T at LQC, MQC and HQC levels were 98.0%, 97.9% and 95.8% and that for ODT was 98.0%, 95.8% and 93.6% respectively with variability (%CV) between them of 1.3% for T and 2.3% for ODT. The recovery of IS was found to be 104.0% with %CV of 4.3. Thus, the consistency in recoveries of T, ODT and IS supports the extraction procedure for its application to routine sample analysis.

Matrix effect may be defined as a composite of some undesirable effects that originate from a biological matrix. These components may result in ion suppression/enhancement, decrease/increase in sensitivity of analytes over a period of time, increased baseline, imprecision of data, drift in retention time and distortion or tailing of a chromatographic output [43]. The extent of ion suppression/enhancement depends on the sample extraction procedure and it is also compound dependent. Protein precipitation has the most severe effect on ion suppression/enhancement compared to solid phase extraction and liquid–liquid extraction. In the present work as the extraction procedure employed is protein precipitation, it becomes extremely essential to assess matrix effect for the proposed LC–MS/MS method. Post column infusion experiment indicates no ion suppression or enhancement at the retention time of T, ODT or propranolol (IS) as evident from the MRM LC–MS/MS chromatograms in Fig. 4. Fig. 4a gives the total ion current (TIC) chromatograms of tramadol, *O*-desmethyltramadol, propranolol and phospholipid. The exact ion current (XIC) chromatograms of tramadol (264.2 → 58.1), *O*-desmethyltramadol (250.2 → 58.1), propranolol (IS, 260.2 → 116.1) and TIC of phospholipid precursor ion transition at 184.1 (scan range 300–900 amu) are represented in Figs. 4b–e. As shown in Fig. 4e, the phospholipid is eluted well before the analytes and IS at 0.7 min. The retention of T at 0.96 min and ODT at 0.84 min was adequate to avoid the interference due to phospholipid. 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 4. No ion suppression/enhancement was observed for T and ODT at their respective retention times. Cross specificity experiment indicated no interconversion between T and ODT, as the area observed at the retention time of T in presence of ULOQ area of ODT and vice-versa was negligible (<0.1%). Thus, the extraction procedure was sufficiently rugged and gave accurate and consistent results when applied to real patient samples.

3.5. Stability and dilution integrity

The stability experiments were performed thoroughly to evaluate the stability of T and ODT in stock solutions and in plasma samples under different conditions. The stability of spiked QC samples was compared with freshly prepared quality control samples. The results obtained were well within the acceptable limits. Stock solution of T, ODT and IS were stable at room temperature for 6 h and at 4 ± 6 °C for 49 days for T, ODT and 16 days for IS with mean % change well within 0.5 to −5.8%. The intermediate solution of T and ODT in methanol–water (50:50, v/v) was stable for 12 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 95 h under refrigerated condition of 4 ± 6 °C. Bench-top stability of extracted samples was also up to 95 h. The values for the percent change for the above stability experiments are compiled in Table 5.

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 ≤2.0 for both the analytes.

3.6. Application of the method on human subjects

The proposed validated method was applied for a pharmacokinetic study of tramadol and *O*-desmethyltramadol in 27 healthy Indian adult male subjects who received 200 mg test and reference formulations of tramadol under fasting condition. The method was sensitive enough to monitor their plasma concentration up to 48.0 h. In all approximately 1506 samples including the calibration, QC and volunteer samples were run and analyzed 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 condition is presented in Fig. 5. The pharmacokinetic parameters, viz. maximum plasma concentration

Table 5
Stability results for tramadol and *O*-desmethyltramadol ($n = 6$).

Stability	Storage condition	Level	Tramadol			*O*-desmethyltramadol		
			A[a] (ng/mL)	% CV[b]	% bias	*A*[a] (ng/mL)	% CV[b]	% bias
Stability in biological matrix (SBM)	Room temperature (25 h)	LQC	2.96	2.2	−1.3	1.45	2.7	−3.1
		MQC	35.7	1.2	−4.7	17.74	1.4	−5.4
		HQC	410.7	1.1	−8.7	209.4	1.0	−6.9
Refrigerator stability of extracted samples (RSS)	Autosampler (4 °C, 95 h)	LQC	2.75	2.3	−8.2	1.36	2.0	−9.4
		MQC	35.0	1.2	−6.6	17.28	1.6	−7.8
		HQC	400.4	2.1	−11.0	201.9	1.8	−10.3
Bench top stability of extracted samples (BTS)	Room temperature (95 h)	LQC	2.86	1.7	−4.5	1.40	1.8	−6.9
		MQC	36.0	3.5	−4.2	16.99	2.2	−9.4
		HQC	410.5	1.6	−8.8	203.7	1.2	−9.5
Freeze and thaw stability (FTS)	After 6th cycle at −20 °C	LQC	2.91	3.4	−2.9	1.48	1.7	−1.1
		MQC	36.9	1.3	−1.7	18.00	1.9	−4.0
		HQC	425.7	1.1	−5.4	211.1	1.1	−6.2
Long term stability (LTS)	72 days at −20 °C	LQC	2.97	4.1	−1.0	1.54	3.5	2.7
		MQC	38.9	0.7	3.7	20.01	2.8	6.7
		HQC	457.7	2.2	1.7	235.6	1.8	4.7

n = Number of replicates at each level.
[a] Mean comparison concentration.
[b] Coefficient of variance.

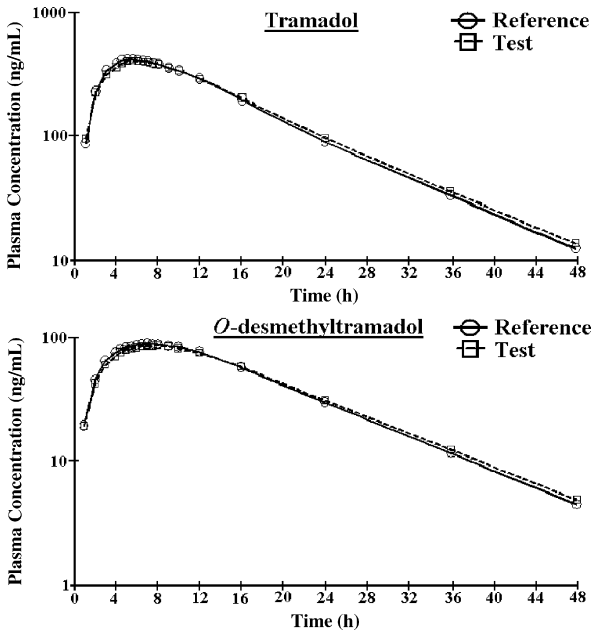

Fig. 5. Mean pharmacokinetic profile of tramadol and *O*-desmethyltramadol after oral administration of 200 mg tramadol tablet to 27 healthy subjects under fasting condition.

C_{max}, area under the plasma concentration–time curve from 0 h to the last measurable concentration AUC_{0-t}, area under the plasma concentration–time curve from 0 h 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 tramadol and *O*-desmethyltramadol. The mean pharmacokinetic parameters obtained for the test and reference formulation are presented in Table 6. These observations confirm the bioequivalence of 200 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 tramadol in plasma samples demonstrated the linearity, precision and sensitivity needed for the pharmacokinetic studies of this drug.

Table 6
Pharmacokinetic parameters of tramadol and *O*-desmethyltramadol in 27 healthy Indian subjects following oral dose of 200 mg tramadol tablet formulation under fasting condition.

Parameter	Tramadol	
	Test Mean ± S.D.	Reference Mean ± S.D.
C_{max} (ng/mL)	429.93 ± 117.76	435.80 ± 89.95
T_{max} (h)	5.67 ± 1.24	5.57 ± 1.34
$t_{1/2}$ (h)	7.86 ± 1.45	7.71 ± 1.48
$AUC_{0-48\,h}$ (h ng/mL)	6964.74 ± 2267.39	6913.30 ± 1941.32
AUC_{0-inf} (h ng/mL)	7141.85 ± 2447.10	7071.59 ± 2094.42
Kel (1/h)	0.091 ± 0.015	0.093 ± 0.016
Parameter	*O*-desmethyltramadol	
	Test Mean ± S.D.	Reference Mean ± S.D.
C_{max} (ng/mL)	95.75 ± 36.05	98.58 ± 37.94
T_{max} (h)	7.63 ± 1.83	7.48 ± 1.73
$t_{1/2}$ (h)	8.49 ± 1.77	8.27 ± 1.43
$AUC_{0-48\,h}$ (h ng/mL)	1867.37 ± 697.51	1872.84 ± 700.02
AUC_{0-inf} (h ng/mL)	1931.57 ± 730.73	1928.58 ± 723.16
Kel (1/h)	0.085 ± 0.015	0.086 ± 0.014

4. Conclusion

The objective of this work was to develop a simple, cost effective, rugged and a high throughput method for simultaneous estimation of tramadol and its active metabolite *O*-desmethyltramadol in human plasma, especially to meet the requirement for subject sample analysis. The simple protein precipitation employed in the present work gave consistent and reproducible recoveries for both the analytes. The run time per sample analysis of 2.0 min suggests high throughput of the proposed method. The maximum on-column loading of T and ODT was 250/125 pg for 5 μL injection volume. 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 T and ODT 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.

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