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Research Article

Determination of Duloxetine in rat plasma for pharmacokinetic study by developing a highly sensitive method using liquid chromatography-electrospray ionization tandem mass spectrometry

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ABSTRACT

A high sensitive, accurate, rapid assay method has been developed and validated for the estimation of duloxetine in rat plasma with liquid chromatography coupled to tandem mass spectrometry with electro spray ionization in the positive-ion mode. The assay procedure involves extraction of duloxetine and phenacetin (internal standard, IS) from rat plasma by protein precipitation method. Chromatographic separation was achieved using a binary gradient using mobile phase acetonitrile and 0.2% formic acid in water delivered at flow rate of 0.50 mL/min on an Atlantis ODS column with a total run time 4.5 min. The MS/MS ion transitions monitored were 298.4→ 154.1 for duloxetine and 180.2 → 110.1 for IS. As per FDA guidelines, method validation and sample analysis were performed and the results met the acceptance criteria. The linearity was obtained over the concentration range of 0.027 to 1072 ng/mL and the lower limit of quantitation achieved was 27 pg/mL. The intra-day and inter-day precisions were in the range of 1.19-9.51 and 1.98-7.60%, respectively. This method was successfully applied to pharmacokinetic study of duloxetine in rats.

Keywords: Duloxetine; LC-MS/MS; method validation; rat plasma; pharmacokinetics

INTRODUCTION

Duloxetine (Fig. 1), chemically (+)-(*S*)-*N*-Methyl-3-(naphthalen-1-yloxy) 3-(thiophen-2-yl) propan-1-amine, is a potent and balance dual inhibitor of serotonin-norepinephrine (NE) reuptake. It has been demonstrated a relatively evenly balance and potent inhibition of serotonin and NE reuptake at the transport site in both *in vitro* and *in vivo* studies (Wang *et al.,* 1998 and Pitsikas *et al.,* 2000). Duloxetine has no significant affinity for dopaminergic, adrenergic cholinergic, histaminergic, opioid, glutamate and GABA receptor *in vivo* and does not inhibit monoamine oxidage (MAO). Duloxetine is prescribed for the treatment of major depressive disorders (MDD), pain related to diabetic peripheral neuropathy (DPNP) and stress urinary incontinence (Westanmo *et al*., 2005). Duloxetine has no other significant receptor or channel activities other than serotonin and noradrenaline reuptake inhibition mechanisms (Smith *et al*., 2006).

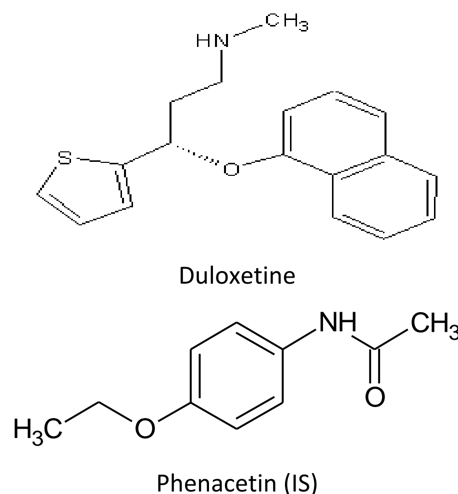
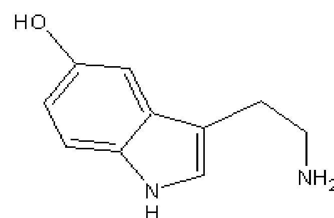


Figure 1: Structural representation of duloxetine and phenacetin (IS)



Serotonin: 5-Hydroxytryptamine or 3-(2-aminoethyl)-1*H*-indol-5-ol

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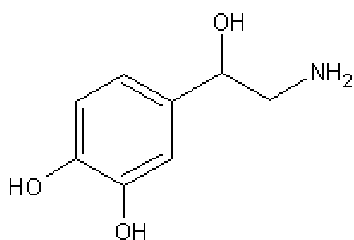

Norepinephrine: 4-[(1R)-2-amino-1-hydroxyethyl] benzene-1, 2-diol

Figure 2: Structural representation of serotonin and norepinephrine

Skinner *et al*., (2004); Hua *et al*., (2004); Suri *et al*., (2005) and Patel D.S *et al.,* (2011) used LC/MS/MS method was applied but there were no analytical details. Few LC-MS/MS method [Zhao *et al*., (2009) and Ma *et al*., (2007)] have been reported for estimation of duloxetine in biological matrices. The LC-MS/MS methods developed and validated by Zhao *et al*., (2009) has longer run time and lower limits of detection (LLOQ) 0.89 ng/mL in 200 µl of human plasma with two step protein procedures as per Ma *et al*.,(2007). Similarly Senthamil Selvan *et al*., (2007) have developed method by using methanol as a precipitating agent and mobile phase consisting of a mixture of acetonitrile and 5 mM ammonium acetate with LLOQ 0.1ng/mL in 200 µl of human plasma and their multiple reaction monitoring (MRM) was m/z 298.1→ 44.0. Patroneva *et al*., (2008) have developed method for desvenlafaxine and duloxetine by using 200 µl of human plasma volume and linearity range was 0.2-50 ng/mL (LLOQ 0.2 ng/mL). This paper describes, a highly sensitive and specific LC-MS/MS method with an LLOQ of 27 pg/mL using a very low volume (50 µL) of rat plasma was developed. The LLOQ achieved is 30-fold lower than the lowest reported LLOQ Zhao *et al*., (2009), Ma *et al*., (2007) and 4-fold lower than Senthamil Selvan *et al.,* (2007) for duloxetine.

EXPERIMENTAL

Chemicals and reagents

Duloxetine (purity: ~99%) and phenacetin were procured from LGC Standard India Private Ltd. HPLC grade acetonitrile (ACN), formic acid, tetra hydro furon (THF) and methanol were purchased from Rankem, Ranbaxy Fine Chemicals Limited, New Delhi, India. Sprague Dawley (S.D) male rats and control S.D rat plasma (Na_2EDTA) were obtained from Animal House, Bioneeds, Bangalore, India. Plasma was stored at -20 ± 5^oC prior to use. All other chemicals/ reagents were of research grade unless otherwise stated.

HPLC operating conditions

A Shimadzu (Shimadzu, Koyoto, Japan) SIL series LC system equipped with degasser (DGU-20A3), isopump (LC-20AD), column oven (CTO-10AS) along with autosampler (SIL-HTc) was used to inject 5 µL aliquots of the processed samples on a Atlantis dC 18 column (4.6 x 50 mm, 3 µm, Waters, Milford, MA, USA) which was maintained at 40 ± 1°C. A binary gradient of mobile phase A (acetonitrile) and B (0.2% formic acid in water) was programmed at 0.50 mL/min. The proportions of mobile phase A: B was initially 10:90 and switched to 100% mobile phase A from 0.01 to 1.8 min and switched back to 90% of mobile phase B from 2.8 min to 2.9 min and continued till 4.5 min.

Mass spectrometry operating conditions

Quantitation was achieved by MS/MS detection in positive ion mode for duloxetine and IS using a MDS Sciex (Foster City, CA, USA) API 4000 mass spectrometer, equipped with a Turboionspray™ interface at 450 °C. The common parameters viz., curtain gas, GS1 gas and GS2 gas were set at 30, 35 and 40 L/min, respectively, whereas the CAD gas (nitrogen) flow was set at 6 L/min. The compounds parameters viz., declustering potential (DP), collision energy (CE) and collision exit potential (CXP) for duloxetine and IS were 46, 9, 8 V and 61, 29, 20 V, respectively. Detection of the ions was performed in the multiple reaction monitoring (MRM) mode, monitoring the transition of the *m/z* 298.4 precursor ion to the *m/z* 154.1 product ion for duloxetine and *m/z* 180.2 precursor ion to the *m/z* 110.1 product ion for IS. Quadrupole Q1 and Q3 were set on unit resolution. The analytical data were processed by Analyst software (version 1. 5).

Preparation of stock and standard solutions

Primary stock solutions of duloxetine for preparation of standard and quality control (QC) samples were prepared from separate weighing. The primary stock solutions were prepared in methanol (357.3 µg/mL). The IS stock solution of 500 µg/mL was prepared in methanol. The stock solutions of duloxetine and IS were stored at 4 °C, which were found to be stable for one month (data not shown) and successively diluted with methanol to prepare working solutions to prepare calibration curve (CC). Another set of working stock solutions of duloxetine was made in methanol (from primary stock) for preparation of QC samples. Working stock solutions were stored approximately at 4 °C for a week (data not shown). Appropriate dilutions of duloxetine stock solution was made in methanol to produce working stock solutions of 0.27, 0.53, 5.36, 53.5, 1071, 2143, 4286,5369,7503 and 10719 ng/mL for CC and 0.27, 0.80, 3751 and 8575 ng/mL for QC. Working stocks were used to prepare plasma calibration standards. A working IS solution (500 ng/mL) was prepared in methanol. Calibration samples were prepared by spiking 45 µL of control rat plasma with the appropriate working solution of duloxetine (5 µL) and IS (10 µL) on the day of analysis. Samples for the determination of precision and accuracy were prepared by spiking control rat plasma in bulk with duloxetine at appropriate concentrations (0.027, 0.08, 375 and 857 ng/mL) and 50 µL

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aliquots were distributed into different tubes. All the samples were stored at -80 ± 10 °C until analysis.

Extraction procedure

The efficiency of duloxetine and IS extraction from rat plasma was determined by comparing the responses of the analytes extracted from replicate QC samples (n=6) with the response of analytes from neat standards at equivalent concentrations (Dams *et al.,* 2003) by protein precipitation method. Recoveries of duloxetine was determined at QC low, QC medium and QC high concentrations viz., 0.08, 375 and 857 ng/mL, whereas the recovery of the IS was determined at a single concentration of 200 ng/mL.

Sample preparation

Protein precipitation method was followed for extraction of duloxetine from rat plasma. To an aliquot of 50 µL rat plasma sample, 10 µL of working IS solution mixed and 150 µL of 10% THF in ACN was added and mixed for 1 min. The sample were centrifuged at 14,000rpm (Sigma-Aldrich 4K15; St. Louis USA) for 5 min at 10ºC. The supernatant were collected and 5 µL was injected to LC-MS/MS system for analysis.

Table 1: Intra- and inter-day precision of determination of duloxetine in rat plasma

Theoretical concentration (ng/mL)	Run	Measured concentration (ng/mL)			
		Mean	SD	RSD	Accuracy (%)
Intra-day variation (Six replicates at each concentration)					
0.02	1	0.21	0.01	5.43	102
	2	0.21	0.01	6.20	100
	3	0.20	0.01	6.30	97.1
	4	0.20	0.01	4.46	97.6
0.08	1	0.66	0.06	8.69	103
	2	0.64	0.04	6.93	99.2
	3	0.66	0.04	5.94	102
	4	0.63	0.06	9.51	98.2
375	1	407	8.22	2.02	108
	2	361	27.3	7.58	96.1
	3	374	16.6	4.42	98.9
	4	389	16.0	4.12	104
858	1	937	25.5	2.73	109
	2	803	18.2	2.27	93.7
	3	835	10.0	1.19	97.4
	4	883	12.5	1.41	103
Inter-day variation (Twenty four replicates at each concentration)					
0.02		0.22	0.00	1.98	103
0.08		0.65	0.05	7.60	98.2
755		374	18.8	5.03	100
858		853	34.2	4.01	99

RSD: Relative standard deviation (SD x 100/Mean)

Table 2: Stability data duloxetine quality controls in rat plasma

Nominal concentration (ng/mL)	Stability	Mean ± SD[a] n = 6 (ng/mL)	Accuracy (%)[b]	Precision (% CV)
0.08	0 h (for all)	0.63 ± 0.06	NA	2.50
	3rd freeze-thaw	0.66 ± 0.06	104	1.53
	8 h (bench-top)	0.65 ± 0.07	103	2.36
	24 h (in-injector)	0.63 ± 0.07	100	1.65
	30 day at -80°C	0.64 ± 0.09	101	2.06
858	0 h (for all)	883 ± 12.5	NA	1.41
	3rd freeze-thaw	872 ± 8.5	98.9	2.94
	8 h (bench-top)	844 ± 10.2	95.6	1.96
	24 h (in-injector)	824 ± 16.2	93.3	2.67
	30 day at -80°C	843 ± 24.6	95.5	2.95

[a]Back-calculated plasma concentrations; [b](Mean assayed concentration / mean assayed concentration at 0 h) x 100

Table 3: Pharmacokinetic parameters of duloxetine

PK parameter	Oral	*Intravenous*
Dose (mg/kg)	30	6
$AUC_{0-\infty}$ (ng*h/mL)	5616 ± 151	1629 ± 252
C_{max}/C_o (ng/mL)	754 ± 64.9	515 ± 87.3
T_{max} (h)	1.00	---
$t_{½ ß}$ (h)	3.2 ± 0.19	3.85 ± 0.81
Cl (mL/min/kg)	---	62.5 ± 10.4
Vd (L/kg)	---	21.3± 8.14
F (%)	68.9	---

$t_{1/2, ß}$: terminal half life. $AUC_{0-\infty}$: area under the plasma concentration-time curve from time zero to infinity. C_{max}: maximum observed plasma concentration. t_{max}: time to the maximum observed plasma concentration. CL: clearance. C_0: xtrapolated concentration at zero time point. V_d: volume of distribution. F: oral bioavailability.

Assay validation procedures

The validation procedures were performed in rat plasma according to FDA guidelines (US DHHS, FDA, CDER, 2001).

Specificity and selectivity

The specificity of the method was evaluated by analyzing rat plasma samples (n=6) to investigate the potential interferences at the LC peak region for analyte and IS.

Matrix effect

The effect of rat plasma constituents over the ionization of duloxetine and IS was determined by comparing the responses of the post extracted plasma standard QC samples (n=6) with the response of analytes from neat standard samples (5 µL of required working stock sample spiked into 45 µL of methanol instead of blank plasma) at equivalent concentrations (Hubert *et al.,* 1999; Dams *et al.,* 2003). Matrix effect was determined at low, medium and high concentrations viz., 0.08, 375 and 857 ng/mL, whereas the matrix effect over the IS was determined at a single concentration of 200 ng/mL.

Applying the Bonfiglio method for Post column infusion to evaluate matrix effect. Briefly, an infusion pump delivers a constant amount of analyte in to LC system outlet entering to mass spectrometer inlet. Mass spectrometer was operated in MRM mode to follow the analyte signal. Rat plasma sample extract was injected on LC column under same chromatographic condition. Since the analyte was infused at constant rate, a steady ion response was obtained as a function of time. Any compound that elutes from the column and causes a variation in ESI response of the infused analyte was seen as a suppression or enhancement in the response of the infused analyte (Fig-7)

Calibration curve and quality control samples

The ten point calibration curve (0.027, 0.05 0.53, 5.36, 53.6, 214, 428, 535, 750 and 1017 ng/mL) and quality control samples (0.08, 375 and 857 ng/mL) were prepared in rat plasma and plotting the peak area ratio of duloxetine: IS against the nominal concentration of calibration standards. Following the evaluation of different weighing factors, the results were fitted to linear regression analysis with the use of $1/X^2$ (*X*-concentration) weighting factor. The calibration curve had to have a correlation coefficient (*r*) of 0.998 or better. The acceptance criteria for each back-calculated standard and quality control concentration were ± 15% deviation from the nominal value except at LLOQ, which was set at ± 20% (US DHHS, FDA, CDER, 2001).

Accuracy and precision

The inter- and intra-assay precision and accuracy were estimated by analyzing six replicates containing duloxetine at four different QC levels i.e., 0.027, 0.08, 375 and 857 ng/mL. The inter-assay precision was determined by analyzing the four levels QC samples on four different runs. The criteria for acceptability of the data included accuracy within ± 15% from the nominal values and a precision of within ±15% relative standard deviation (RSD) except for LLOQ, where it should not exceed ± 20% (US DHHS, FDA, CDER, 2001).

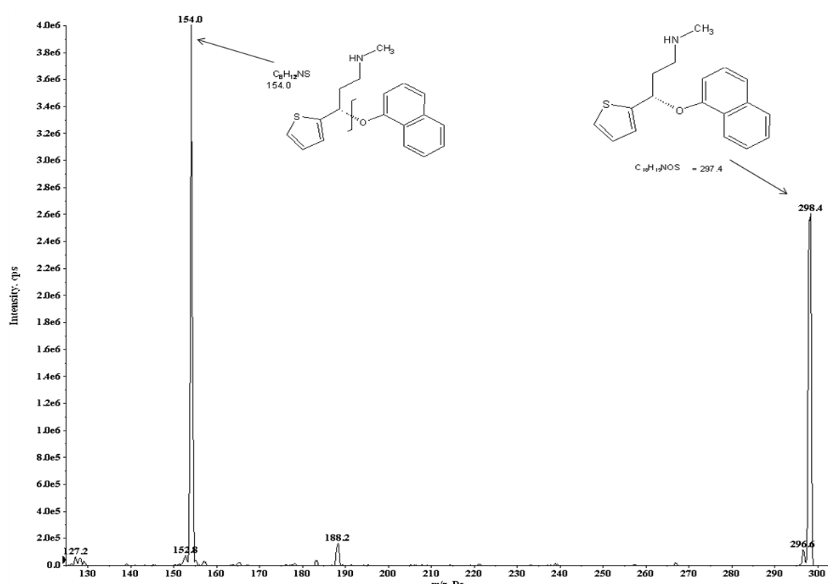

Figure 3: Product ion spectra of I duloxetine

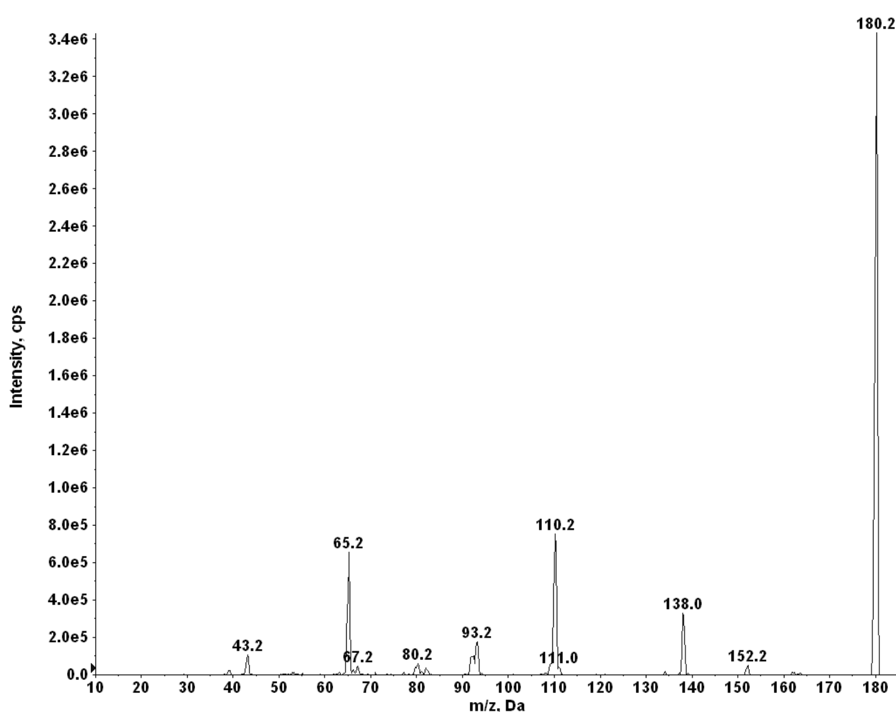

Figure 4: Product ion spectra of IS

Stability experiments

The stability of duloxetine and IS in the injection solvent was determined periodically by injecting replicate preparations of processed samples for up to 24 h (in the auto sampler at 4 °C) after the initial injection. The peak-areas of the analyte and IS obtained at initial cycle were used as the reference to determine the stability at subsequent points. Stability of duloxetine in the biomatrix during 8 h (bench-top) was determined at ambient temperature (25 ± 2 °C) at two concentrations (0.08 and 857 ng/mL) in six replicates. Freezer stability of duloxetine in rat plasma was assessed by analyzing the lower quality control (LQC) and high quality control (HQC) samples stored at -80 ± 10 °C for at least 30 days. The stability of duloxetine in rat plasma following three freeze-thaw cycles was assessed using QC samples spiked with duloxetine. The samples were stored at -80 ± 10 °C between freeze/thaw cycles. The samples were thawed by allowing them to stand (unassisted) at room temperature for approximately 2 h. The samples were then restored to the freezer. The samples were processed using the same procedure as described in the sample preparation section. Samples were considered stable if assay values were within the acceptable limits of accuracy (i.e., ± 15%) and precision (i.e., ± 15% RSD).

Dilution effect

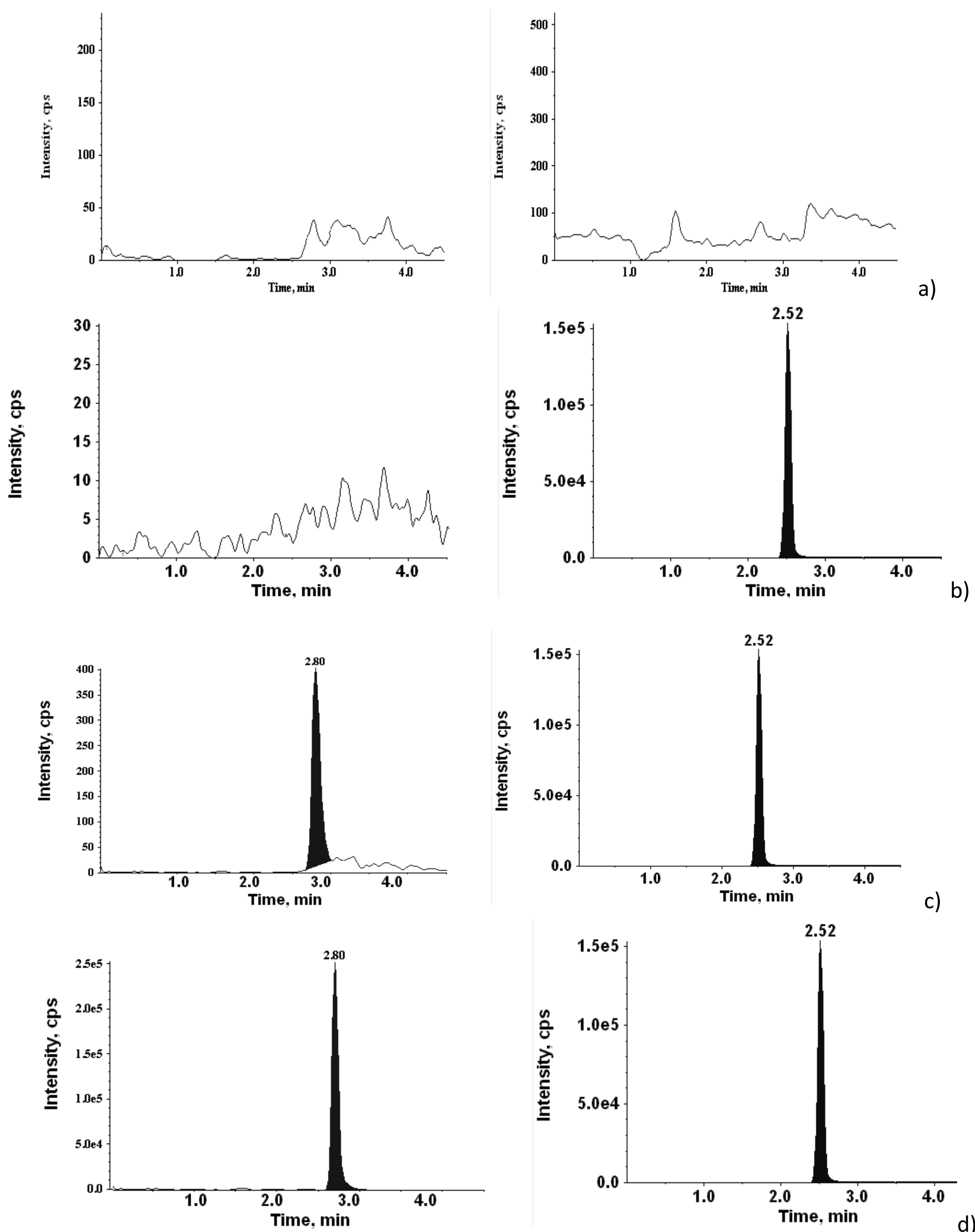

Figure 5: Typical MRM chromatograms of duloxetine (left panel) and IS (right panel) in (a) rat blank plasma (b) rat plasma spiked with IS (c) rat plasma spiked with duloxetine at LLOQ (0.27 pg/mL) and IS and (d) a 1.00 h plasma sample showing duloxetine peak (299 ng/mL) obtained following *intravenous* dose of duloxetine to a rat along with IS peak

Dilution effect was investigated to ensure that samples could be diluted with blank matrix without affecting the final concentration. Duloxetine spiked rat plasma samples prepared at 3.5 time of upper limit of quatification (ULOQ) i.e., 3750 ng/mL and diluted with pooled rat plasma at dilution factors of 5 and 10 in six replicates and analyzed. As part of validation, these twelve replicates had to comply to have both precision of ≤ 15% and accuracy of 100 ± 15% similar to other QC samples.

Pharmacokinetic study in rats

Pharmacokinetic (PK) studies was performed in overnight (~12 h) fasted healthy male Sprague Dawley (SD)

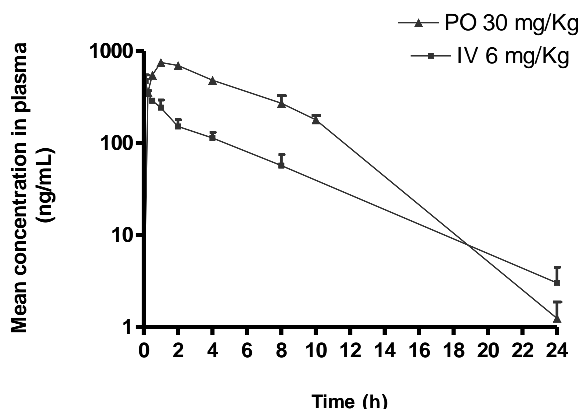

Figure 6: Mean ± S.D plasma concentration-time profile of duloxetine in rat plasma following oral and intravenous administration of duloxetine to rats

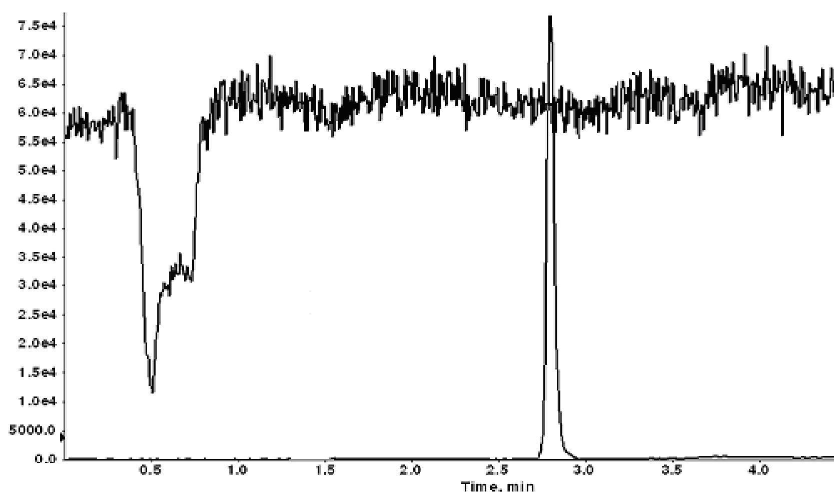

Figure 7: Overlay chromatogram showing the matrix effect for duloxetine

rats (n=6/each study; weight range; 210-220 g) following the approval from ethics committee for animal usage and study design. During fasting time animals had free access to water. Blood samples were obtained following *intravenous* (*i.v*, 6 mg/kg of duloxetine, solution formulation prepared using 3% DMSO, 7% solutol HS-15 : ethanol (1:1), and 90% normal saline) and oral administration (30 mg/kg) of duloxetine, in the form of a suspension, prepared using 30 µL of Tween-80 + 0.5% methyl cellulose) of duloxetine into polypropylene tubes containing Na_2EDTA solution as an anti-coagulant at pre-dose, 0.12 (*i.v* only) 0.25, 0.5, 1, 2, 4, 8, 10 (oral only) and 24 h. Plasma was harvested by centrifuging the blood using (Sigma-Aldrich 4K15; St. Louis, USA) at 1760 *g* for 5 min and stored frozen at -80 ± 10 °C until analysis. In both the experiments animals were allowed to eat feed 2 h post-dose of duloxetine. Plasma (50 µL) samples were spiked with IS and processed as described above. Along with PK samples, QC samples at low, medium and high concentration were assayed in duplicate and were randomly distributed among standard calibrators and unknown samples in the analytical run; not more than 33% of the QC samples were greater than ± 15% of the nominal concentration. Plasma concentration-time data of duloxetine was analyzed by non-compartmental method using WinNonlin Version 5.1 (Pharsight Corporation, Mountain View, CA).

RESULTS

Liquid chromatography

Initial feasibility experiments of various mixture(s) of solvents such as acetonitrile and methanol using different buffers such as ammonium acetate, ammonium formate and formic acid along with altered flow-rates (in the range of 0.2-0.6 mL/min) were performed to optimize for an effective chromatographic resolution of duloxetine and IS (data not shown). The resolution of peaks was best achieved with a binary gradient mobile phase comprising A (acetonitrile) and B (0.2% formic acid in water) with a time program at a flow rate of 0.50 mL/min, on an Atlantis dC 18 (4.6 x 50 mm, 3 µm, Waters; Milford, MA, USA). The chosen conditions were found to be suitable for the determination of electrospray response for duloxetine and IS.

Mass spectroscopy

In order to optimize electro-spray ionization (ESI) conditions for duloxetine and IS, quadrupole full scans were carried out in positive ion detection mode. During a direct infusion experiment, the mass spectra for duloxetine and IS revealed peaks at *m/z* 298.4 and 180.2,

respectively as protonated molecular ions, $[M+H]^+$. Following detailed optimization of mass spectrometry conditions (provided in instrumentation and chromatographic conditions section); MRM reaction pair of *m/z* 298.4 precursor ion to the *m/z* 154.1 was used for quantification for duloxetine. Similarly, for IS MRM reaction pair of *m/z* 180.2 precursor ion to the *m/z* 110.1 was used for quantification purpose. The fragmentation pattern of duloxetine and IS were shown in Fig. 3 & 4 respectively.

Extraction procedure

Simple protein precipitation technique was adequate for striking a balance between adequate recovery samples and cleaner samples. The results of the comparison of neat standards versus plasma-extracted standards were estimated for duloxetine at 0.08, 375 and 857 ng/mL and the mean recovery was found to be 92.7 ± 2.54, 93.6 ± 1.74 and 96.2 ± 3.99%, respectively. The recovery of IS at 200 ng/mL was 87.1 ± 5.8%.

Matrix effect, specificity and selectivity

Average matrix factor values (matrix factor=response of post spiked concentrations/response of neat concentrations) obtained were +0.95 (CV: 2.15%, n= 6), +0.99 (CV: 4.9%, n= 6) and +1.00 (CV: 4.0%, n= 6) for duloxetine in rat plasma at QC low (0.08 ng/mL), QC medium (375 ng/mL) and QC high (857 ng/mL) concentrations, respectively. No significant peak area differences were observed. Matrix effect on IS was found to be +1.11 (CV: 6.1%, n= 6) at tested concentration of 200 ng/mL. While it was found that the plasma extract has a small but negligible impact on the ionization of duloxetine and IS.

Fig. 5 shows a typical chromatogram for the control rat plasma (free of analyte and IS), rat plasma spiked with duloxetine at LLOQ and IS and an *in vivo* plasma sample obtained at 1.0 h after *i.v* administration of duloxetine. No interfering peaks from endogenous compounds were observed at the retention times of analyte and IS in the matrix. The retention time of duloxetine and IS was ~2.80 and 2.52 min, respectively. The total chromatographic run time was 4.5 min.

Calibration curves and quality control

The plasma calibration curve and quality control samples were prepared by using ten calibration standards (viz., 0.027-1071 ng/mL) and quality control (0.08, 375 and 875) respectively . The calibration standard curve had a reliable reproducibility over the standard concentrations across the calibration range. Calibration curve was prepared by determining the best fit of peak-area ratios (peak area analyte/peak area IS) versus concentration, and fitted to the $y = mx + c$ using weighing factor ($1/X^2$). The average regression (n=4) was found to be $\geq$ 0.998. The lowest concentration with the R.S.D < 20% was taken as LLOQ and was found to be 0.027 ng/mL. The % accuracy observed for the mean of back-calculated concentrations for four calibration curves for duloxetine was within 94.1-103; while the precision (% CV) values ranged from 0.56-3.5.

Accuracy and precision

Accuracy and precision data for inter- and intra -day plasma samples are presented in Table 1. The assay values on both the occasions (intra- and inter-day) were found to be within the accepted variable limits.

Stability

The predicted concentrations for duloxetine at 0.08 and 857 ng/mL samples were deviated within ± 15% of the nominal concentrations in a battery of stability tests viz., in-injector (24 h), bench-top (8 h), repeated three freeze/thaw cycles and freezer stability at -80 ± 10 °C for at least for 30 days (Table 2). The results were found to be within the assay variability limits during the entire process.

Dilution effect

The dilution integrity was confirmed for QC samples that exceeded the upper limit of standard calibration curve. The results have shown that the precision and accuracy for two sets of six replicates of diluted samples were within the acceptance range (data not shown).

Pharmacokinetic study in rats

The sensitivity and specificity of the assay were found to be sufficient for accurately characterizing the plasma pharmacokinetics of duloxetine in rats following *oral* and *i.v* administration. Profiles of the mean plasma concentration versus time were shown in Fig. 6 and the pharmacokinetic parameters are summarized in Table 3. Following oral administration of duloxetine maximum concentration (C_{max}) in plasma (754 ± 64.9 ng/mL) was achieved at 1.00 h (T_{max}). Following *i.v* administration the clearance and volume of distribution was found to be 62.4 ± 10.4 mL/min/kg and 21.2 ± 8.14 L/kg, respectively. The terminal half-life was found to be 3.85 ± 0.81h and 3.2 ± 0.19 h by *i.v* and *oral* routes, respectively. The absolute oral bioavailability was found to be 68.9 %.

CONCLUSION

A rapid, sensitive and accurate LC-ESI-MS/MS method was developed for the analyzing the duloxetine in rat plasma by employing simple protein precipitation extraction method. Compared with published methods, the present method has high sensitivity and selectivity . We believe that this high throughput method could provide a useful tool for determination of duloxetine in plasma. The established method was successfully applied to determine the pharmacokinetic disposition of duloxetine in rats.

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