Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 858 (2007) 269–275
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Determination of duloxetine in human plasma by liquid chromatography with atmospheric pressure ionization–tandem mass spectrometry and its application to pharmacokinetic study

P. Senthamil Selvan, K. Veeran Gowda, U. Mandal, W.D. Sam Solomon, T.K. Pal *

Bioequivalence Study Centre, Department of Pharmaceutical Technology, Jadavpur University, Kolkata 700032, India

Received 20 February 2007; accepted 2 September 2007
Available online 14 September 2007

Abstract

A rapid, sensitive and accurate liquid chromatographic–tandem mass spectrometry (LC–MS–MS) method is described for the determination of duloxetine in human plasma. Duloxetine was extracted from plasma using methanol and separated on a C18 column. The mobile phase consisting of a mixture of acetonitrile and 5 mM ammonium acetate (45:55, v/v, pH 3.5) was delivered at a flow rate of 0.3 ml/min. Atmospheric pressure ionization (API) source was operated in positive ion mode. Multiple reaction monitoring (MRM) mode using the transitions of *m/z* 298.1 → *m/z* 44.0 and *m/z* 376.2 → *m/z* 123.2 were used to quantify duloxetine and internal standard (I.S.), respectively. The linearity was obtained over the concentration range of 0.1–50.0 ng/ml and the lower limit of quantitation (LLOQ) was 0.1 ng/ml. This method was successfully applied to pharmacokinetic study of a duloxetine formulation product after oral administration to healthy human subjects.

Keywords: Duloxetine; LC-API–MS–MS

1. Introduction

Duloxetine hydrochloride is chemically known as (+)-*N*-methyl-3(1-naphthalenyloxy)-2-thiophenepropanamine hydrochloride (Fig. 1), it is a potent dual inhibitor of serotonin (5-hydroxytryptamine (5-HT)) and norepinephrine (NE) reuptake, possessing comparable affinities in binding to NE and 5-HT transport sites. It has been clinically used for the treatment of major depressive disorder, pain due to diabetic peripheral neuropathy and urinary incontinence [1–5].

For the pharmacokinetic study of a duloxetine formulation product in human subjects, an analytical method with simplicity and high sensitivity was required in our laboratory. A recent survey revealed that few methods were available for the determination of duloxetine in biological samples, which involved HPLC with UV detection, liquid chromatography with single-quadrupole mass spectrometric (LC–MS) method [6], liquid chromatographic–tandem mass spectrometry (LC–MS–MS) with solid phase extraction (SPE) [7,8], gas chromatography-nitrogen–phosphorus detection (GC-NPD) and gas chromatography–mass spectrometry (GC–MS) quantitation [9]. To our knowledge, atmospheric pressure ionization (API) source offers some advantages over atmospheric pressure chemical ionization (APCI), GC-NPD and GC–MS in terms of less background noises and suitability for small molecular compounds [7–9]. Ma et al. described a single-quadrupole mass spectrometry (LC–MS) with selected-ion monitoring (SRM) mode to detect the precursor ion [6]. But in our proposed method, a triple–quadrupole mass spectrometry (LC–MS–MS) with multiple reaction monitoring (MRM) mode was used to detect both the precursor ion and fragment ion. It shows the high specificity of the proposed method. Taking into consideration the low levels of duloxetine in plasma, LC–MS–MS method with simple and economic sample preparation method is the first choice for our purpose. The extraction procedure described by Hoja et al. is cumbersome and involves multi step extraction; basic extraction, acid back-extraction, acid wash and basic re-extraction [10] and the sample preparation procedure reported in Ma et al. also involves a multi step extraction wherein the entire extraction

* Corresponding author. Tel.: +91 33 2414 6967; fax: +91 33 2414 6186.
E-mail addresses: senthamil77@yahoo.com (P. Senthamil Selvan), tkpal_12@yahoo.com (T.K. Pal).

1570-0232/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2007.09.002

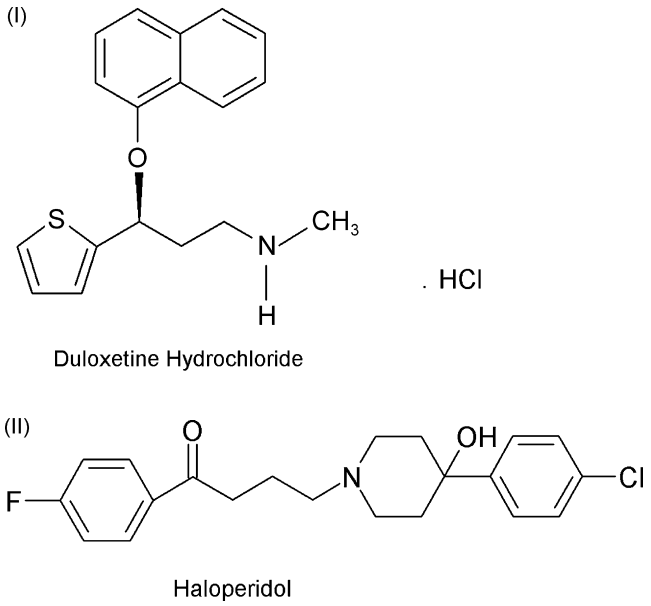

Fig. 1. Chemical structures of duloxetine hydrochloride (I) and haloperidol (II, internal standard).

procedure is repeated twice [6]. Besides, a less sample mass onto the column is often preferred in ordinary laboratories for the benefits of both system maintenance and re-injections of the extracted sample when only a limited sample is available. On the contrary, a simple and single step extraction with low volume of plasma has been reported in the present method. Haloperidol was used as an internal standard (I.S.) to compensate the changes in sample concentration during sample preparation [11].

This paper describes a simple, specific and highly sensitive liquid chromatographic–tandem mass spectrometry with atmospheric pressure ionization (LC-API–MS–MS) method in MRM mode for the determination of duloxetine in human plasma. This method was validated in terms of matrix effect, selectivity, sensitivity, linearity, accuracy, precision, stability and successfully applied to the pharmacokinetic studies of duloxetine hydrochloride tablets in healthy human subjects. In addition, to our knowledge, this is the first, simple and highly validated LC–MS–MS the state-of-the-art technique for the determination of duloxetine in human plasma.

2. Experimental

2.1. Chemicals and reagents

Duloxetine hydrochloride 40 mg as enteric-coated tablet (test product) was supplied by Pentabiotech (Hardwar, India). Symbal 40 mg also as enteric-coated tablet (reference product) and I.S. were procured from Torrent Pharmaceuticals (Mehsana, India). Methanol (LiChrosolv®) and acetonitrile (LiChrosolv®) were purchased from Merck (Mumbai, India). Ammonium acetate and acetic acid were procured from Sigma–Aldrich (Bangalore, India). HPLC grade water was acquired from an in-house water purification system (Milli-Q, Molsheim, France) and used throughout this study. The HPLC mobile phase and sample aliquots were filtered through a 0.45 and 0.2 μm Nylon-66 filters (Agilent Technologies, CA, USA), respectively before use.

2.2. Instrumentation

A liquid chromatographic system consisting of LC-20AD pump; SIL-20AC auto-sampler and CTO-10ASvp column oven (Shimadzu, Kyoto, Japan) were used for the separation. The LC–MS–MS system consisting of turbo spray and atmospheric pressure ionization source (API-2000) with triple–quadrupole tandem mass spectrometer (AB Sciex Instruments, Foster, CA) was used for quantitative determination of duloxetine in plasma. Data integration was performed with Analyst 1.4.1 software version (AB Sciex Instruments).

2.3. Chromatographic conditions

LC separation was performed on a Gemini-C18 column (50 mm × 4.6 mm i.d., 3 μm, Phenomenex, Torrance, CA, USA) with a Security Guard C18 guard column (4 mm × 3.0 mm i.d., Phenomenex). The mobile phase consisting of a mixture of acetonitrile and 5 mM ammonium acetate (45:55, v/v, pH 3.5) was delivered at a flow rate of 0.3 ml/min. The column temperature was maintained at 20 °C. The injection volume was 5 μl.

2.4. Mass spectrometric conditions

A triple–quadrupole mass spectrometer (MS–MS) was used with API source and channel electron multiplier (CEM) detector in positive ion detection mode. In API source, the turbo spray needle was operated at 5500 V current with nebulizer gas at 40 psi (zero air) for better ion spray. And the temperature of the heater in the source was maintained at 400 °C with nebulizer gas at 55 psi (zero air) for the better vaporization of HPLC eluent. For the transition of ions from atmosphere to vacuum region, curtain gas (ultra high purity nitrogen) was served at 10 psi for the effect of collisional induced disassociation (CID) in the curtain plate region. For the precursor ion fragmentation, collisional gas (ultra high purity nitrogen) was served at 5 psi and collision energy of 32 eV for the effect of collisional activated disassociation (CAD) in the collision cell. MRM mode was used for scanning throughout this study. The transitions selected were m/z 298.1 → m/z 44.0 and m/z 376.2 → m/z 123.2 for duloxetine and I.S., respectively, with a dwell time of 200 ms per transition.

2.5. Preparation of calibration standards and quality control samples

Stock solutions of duloxetine hydrochloride and the I.S. were individually prepared at 100 μg/ml in methanol. The stock solution of duloxetine hydrochloride was further diluted with water to give a series of standard solutions with concentration of 0.1, 0.25, 0.8, 2.0, 8.0, 20.0, 40.0 and 50.0 ng/ml. A solution containing 100 ng/ml of I.S. was prepared with methanol. Calibration standards of duloxetine hydrochloride (0.1, 0.25, 0.8, 2.0, 8.0, 20.0, 40.0 and 50.0 ng/ml) were prepared by spiking appropriate amount of the standard solutions in blank plasma obtained from

healthy human volunteers. The quality control (QC) samples were prepared using the pooled plasma at concentrations of 0.25, 2.0 and 40.0 ng/ml. The spiked samples were then treated following the sample preparation procedure as indicated in Section 2.6.

2.6. Sample preparation

In 2.0 ml polyethylene centrifuge tube, 0.2 ml of each plasma sample was transferred and 0.4 ml of HPLC grade methanol which contained 100 ng/ml of I.S. was added. The contents of the centrifuge tubes were briefly mixed by vortexing for 1 min and centrifuged at 14,000 rpm for 10 min to separate the supernatant liquid. The aliquots were filtered through 0.2 μm membrane and injected onto the LC–MS–MS system.

2.7. Method validation

Validation runs were conducted on 3 separate days. Each validation run consisted of a set of the spiked standard samples at eight concentrations over the concentration range (each in triplicate), QC samples at three concentrations ($n = 6$, at each concentration), blank, stability and freeze–thaw samples. Standard samples were analyzed at the beginning of each validation run and other samples were distributed randomly throughout the run. The results from QC samples in three runs were used to evaluate the accuracy and precision of the method developed. Concentrations of the analyte in plasma samples were determined by back-calculation of the observed peak-area ratios of the analyte and I.S. from the best-fit calibration curve using a weighted ($1/x$) linear regression. During routine analysis, each analytical run included a set of standard samples, a set of QC samples in duplicate and plasma samples to be determined.

The matrix effect was investigated by collecting blank plasma from 12 different sources and spiked with low and high QC concentration of duloxetine. After the extraction, the sample aliquots were analyzed and the peak areas of the spiked QC concentrations of 12 different sources were compared.

The extraction recovery of duloxetine was determined at low, medium and high concentrations by comparing the responses from plasma samples spiked before extraction with those from plasma samples spiked after extraction. The same procedure was followed for the calculation of extraction recovery of I.S. also.

Sample stability in terms of freeze–thaw stability, short-term room temperature and long-term stability were tested by analyzing QC samples at concentrations of 0.25, 2.0 and 40.0 ng/ml. The freeze–thaw stability (24 h at −20 °C for four times), short-term stability (24 h, room temperature) and long-term stability (20 days at −20 °C) were calculated. The stock solution standards of duloxetine and I.S. were prepared in methanol, stored at −20 °C for 30 days and the stability was evaluated. The post-preparative stability was tested by comparing after-day analysis with the first-day analysis.

2.8. Application of the LC–MS–MS method

The LC–MS–MS method developed was successfully applied to the pharmacokinetic studies of duloxetine hydrochloride tablets in healthy human subjects. Twelve healthy human volunteers aged 21–27 years were admitted in Bioequivalence Study Center (Jadavpur University, Kolkata, India). After an overnight fast (12 h), each volunteer was given single dose of duloxetine hydrochloride 40 mg tablets either test product or reference product with 240 ml of water. No food was allowed until 3 h after oral administration of the doses. About 5 ml of blood samples were collected from the forehand vein into heparinized tubes before (0 h) and at 0.5, 1–8, 10, 12, 16, 20, 24, 36 and 48 h after dosing. Plasma was separated by centrifugation at 3000 × g for 10 min and kept frozen at −20 °C until analysis.

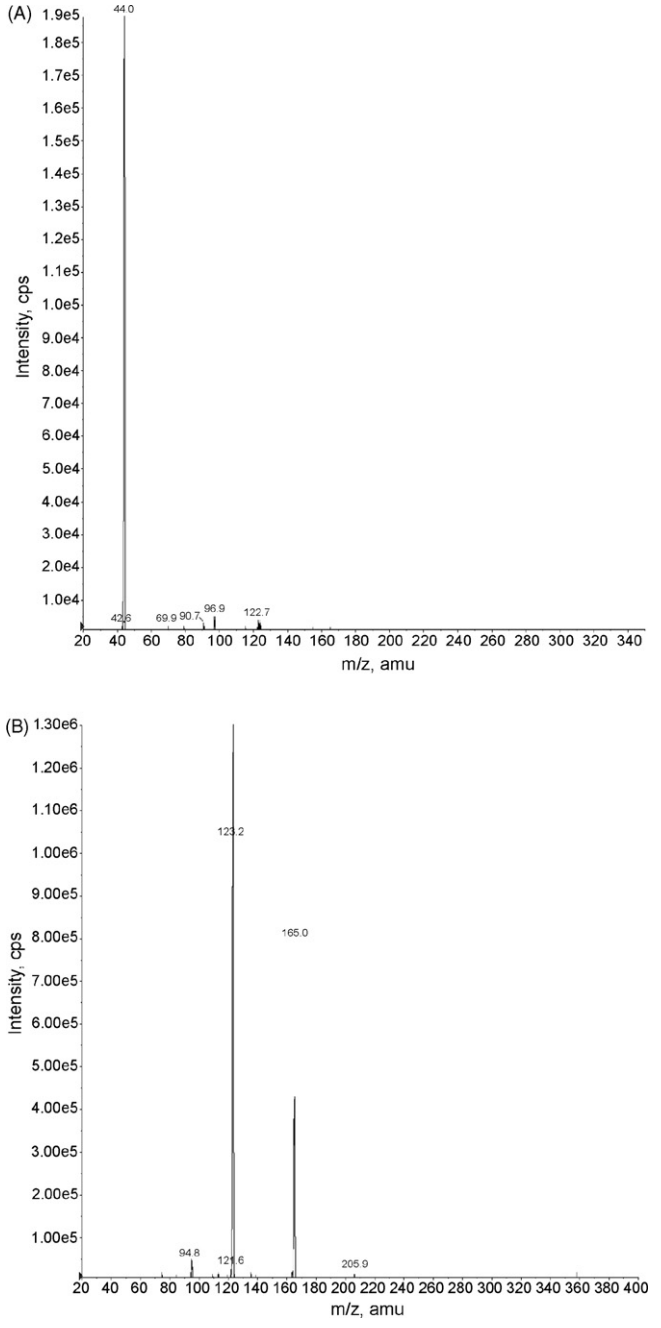

Fig. 2. Full-scan product ion spectra of $[M+H]^+$ of (A) duloxetine; (B) haloperidol.

3. Results and discussion

3.1. Method development

Clean samples are essential for minimizing ion suppression and matrix effect in LC–MS–MS analyses and the other endogenous material in the sample can cause rapid deterioration of columns. In this work the protein precipitation method was used to denature (and precipitate) the proteins present in the plasma samples. Methanol, acetonitrile and their mixtures in different ratios were evaluated as protein denaturants in this work. Methanol produced a flocculent precipitate and not the

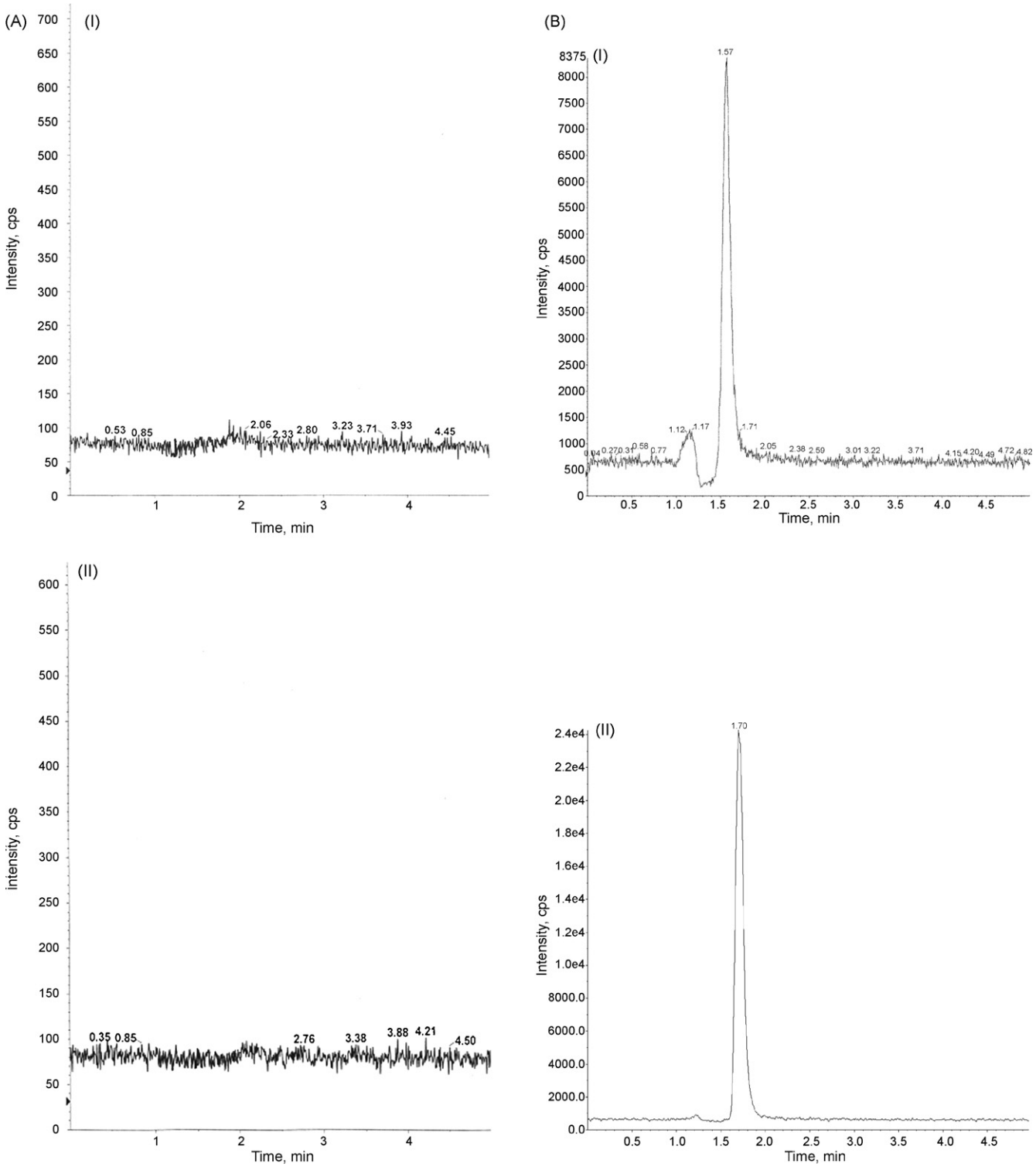

Fig. 3. Representative MRM chromatograms of (A) blank plasma sample, (B) blank plasma sample spiked with analyte (2.0 ng/ml) and I.S. (100.0 ng/ml); (C) plasma sample from forehand vein at 6 h after an oral dose of duloxetine hydrochloride tablets to a human subject. Peak I: Duloxetine, peak II: haloperidol (I.S.).

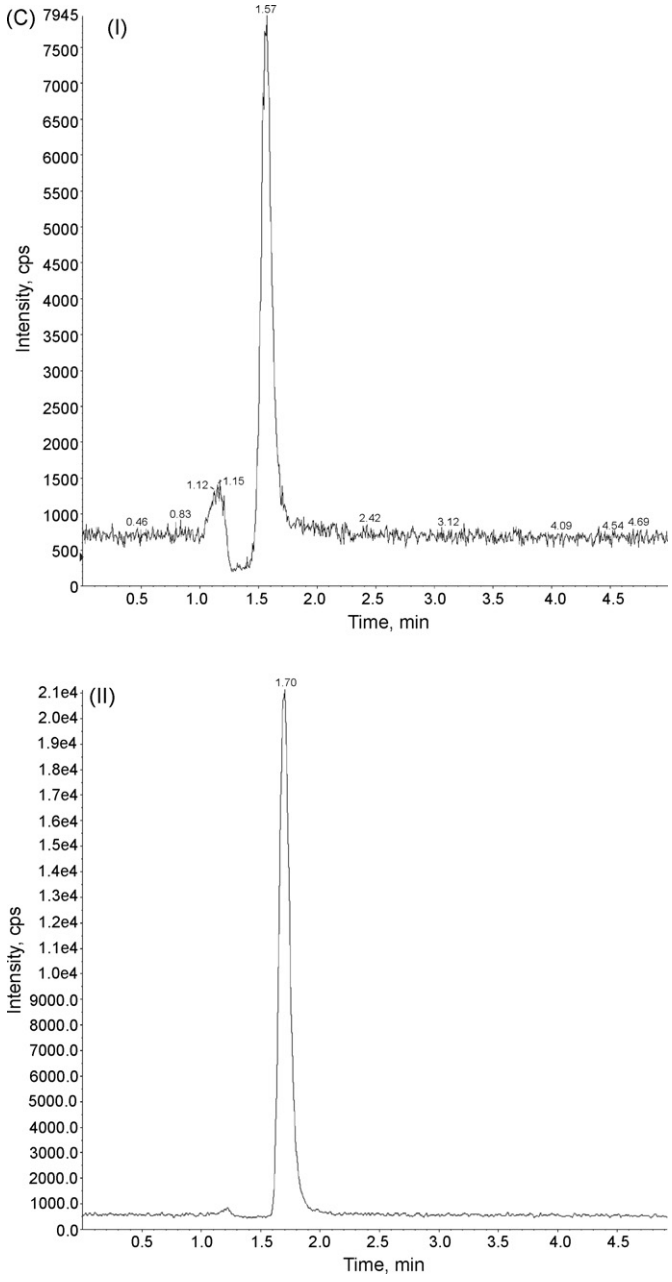

Fig. 3. (*Continued*).

gummy mass obtained with acetonitrile. Methanol also gave a clearer supernatant and prevented the drug entrapment that was observed after acetonitrile precipitation. Finally, methanol was found to be optimal, which produced a clean chromatogram for the blank plasma and the highest recovery for the analyte from the sample plasma.

The chromatographic conditions, especially the composition of mobile phase were optimized through several trials to achieve good resolution and symmetric peak shapes of analytes as well as short run time. It was found that a mixture of acetonitrile and 5 mM ammonium acetate (45:55, v/v) could achieve our purpose and was finally adopted as the mobile phase for the chromatographic separation.

An I.S. is necessary for determination of analyte in biological samples. For an LC–MS–MS analysis, utilization of stable isotope-labeled drugs as I.S. proves to be helpful when significant matrix effect occurs. However, there are also many problems with the use of stable isotope-labeled I.S. The major problems involve inadequate isotopic purity and stability, which often impose unfavorable impact on highly sensitive quantitative analyses. In initial stage of our work, several compounds were tried to find a suitable I.S. and finally haloperidol was found to be optimal for our work. Clean chromatograms were obtained and no significant changes were found in the recovery of duloxetine.

For the quantitation of duloxetine in human plasma, some parameters related with tandem mass spectrometry were investigated. Based on our experiences, API was preferred to APCI to quantify duloxetine in human plasma due to its lower levels of background noises. Parameters involving turbo spray needle temperature, heater temperature, flow rate of nebulizing gas and

curtain gas were optimized to obtain the protonated molecules of duloxetine and I.S. The collision energy was optimized to achieve maximum response of the fragment ion peak. The MRM mode was used for the detection of duloxetine and I.S. with a dwell time of 200 ms per transition.

3.2. Specificity

The specificity of the method was investigated by comparing chromatograms of 12 different sources of human plasma. The positive product ion mass spectra of the molecular ions of duloxetine and I.S. are shown in Fig. 2. The most intensive product ion was observed at *m/z* 44.0 for duloxetine and *m/z* 123.2 for I.S. The representative chromatograms are shown in Fig. 3, indicating no interferences from endogenous substances in plasma with the analyte and I.S. Duloxetine and I.S. exhibited retention times of ca. 1.57 and 1.70 min, respectively. By monitoring the precursor- to product-ion transitions *m/z* 298.1 → *m/z* 44.0 for duloxetine and *m/z* 376.2 → *m/z* 123.2 for I.S. in the MRM mode, a highly sensitive assay for duloxetine was developed.

3.3. Matrix effect

The ion suppression caused by the plasma matrix was evaluated. The matrix effect of the method was considerably reduced and suppressed by utilizing the API source and by eliminating a number of endogenous components from plasma extracts during sample preparation. The matrix effect was determined with blank plasma from 12 different sources which were spiked with low and high QC concentrations of 0.25 and 40.0 ng/ml, respectively. No matrix effect and interferences from endogenous compounds were detected in the 12 different sources of human plasma. It indicates that the extracts were "clean" with no co-eluting "unseen" components interfering with the ionization of the analyte. The relative standard deviation (R.S.D.) value is 4.0% that indicates the reproducibility of peak areas for the 12 extracted aliquots from the blank plasma spiked with low and high QC concentration of duloxetine.

3.4. Linearity and sensitivity

The linearity of each calibration curve was determined by plotting the peak-area ratio (*y*) of duloxetine to I.S. versus the nominal concentration (*x*) of duloxetine. The calibration curves were obtained by weighted (1/*x*) linear regression analysis. To evaluate the linearity of the LC–MS–MS method, plasma calibration curves were determined in triplicate on three separate days. Good linearity was observed over the concentration range of 0.1–50.0 ng/ml for duloxetine on each calibration. In the regression equations, no significant changes were found in the values of slope, intercept and correlation co-efficient on both inter- and intra-day calibrations. Representative regression equation for the calibration curve was $y = 0.5270x + 0.0185$ with a correlation co-efficient of 0.9981. The lower limit of quantitation (LLOQ) for duloxetine was found to be 0.1 ng/ml in human plasma. The percentage relative error (RE) and percentage co-efficient of variation (CV) were found to be less than 15% which is sufficient for pharmacokinetic study of duloxetine in human subjects.

3.5. Accuracy and precision

The accuracy was determined by calculating the percentage deviation observed in the analysis of QC samples and expressed in the relative error. The intra- and inter-run precision was expressed as the R.S.D. As shown in Table 1, for each QC level of duloxetine, the precision was less than 4.0% and the accuracy was less than 5.0% for both intra- and inter-run, indicating the acceptable accuracy and precision of the method developed.

3.6. Extraction recovery

The extraction recovery of duloxetine and I.S. from human plasma was determined by comparing peak areas from plasma samples spiked before extraction with those from plasma samples extracted and spiked after extraction. The results showed that the extraction recoveries of duloxetine from human plasma 86.1 ± 2.4, 85.7 ± 4.1 and $89.0 \pm 3.3\%$ at concentrations of 0.25, 2.0 and 40.0 ng/ml, respectively, and the I.S. was found to be $83.7 \pm 2.9\%$ at concentration of 100.0 ng/ml.

3.7. Stability

The QC plasma samples were stable at 4 °C for 24 h, short- and long-term stability studies; the results showed no significant loss of analyte (Table 2). The stock solutions were stable for at least 30 days and the difference between the assay results were less than 5% for both the analyte and I.S. The post-preparative samples were stable at room temperature for at least 24 h including the residence time in the autosampler. The final stability test

Table 1
Accuracy and precision for the determination of duloxetine in human plasma ($n = 3$ days, six replicates per day)

Added C[a] (ng/ml)	Overall mean C[a] (ng/ml)	Intra-run		Inter-run	
		R.S.D.[b] (%)	RE[c] (%)	R.S.D.[b] (%)	RE[c] (%)
0.25	0.26	2.76	2.73	3.28	2.07
2.00	2.08	3.08	4.17	3.84	3.33
40.0	40.80	1.88	2.43	1.46	1.59

[a] Concentration.
[b] Relative standard deviation.
[c] Relative error.

Table 2
Stability data for duloxetine ($n = 3$ per test and each concentration).

Added C^a (ng/ml)	24 h, 4 °C		24 h, room temperature		20 days, −20 °C	
	Average (%)	CV (%)	Average (%)	CV (%)	Average (%)	CV (%)
0.25	98.87	0.83	100.41	2.76	100.68	5.74
2.0	101.25	4.37	103.62	1.14	101.31	−5.82
40.0	100.90	3.30	97.96	2.32	99.11	−4.99

[a] Concentration.

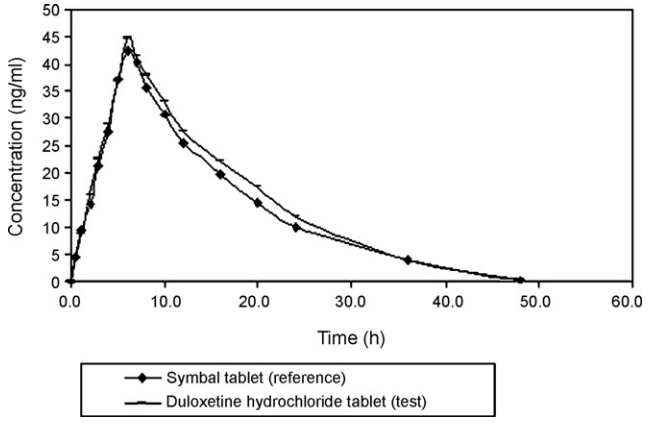

Fig. 4. Mean plasma concentration–time profiles of duloxetine after oral administration of duloxetine hydrochloride tablet (40 mg) and Symbal tablet (40 mg).

was demonstrated after four freeze–thaw cycles. No significant deterioration of analyte was observed under any of these post-preparative and freeze–thaw conditions and the mean recovery was 97.66–101.79% ($n = 6$).

3.8. Application

This method was successfully applied to the pharmacokinetic study of duloxetine hydrochloride tablets. The mean (±S.D.) plasma concentration–time profile for a healthy volunteer after the oral administration of duloxetine hydrochloride tablet at the single dose of 40 mg is shown in Fig. 4. The values of the main pharmacokinetic parameters are shown in Table 3. The duloxetine hydrochloride as enteric-coated tablet was absorbed slowly in accordance with the characteristic of the enteric-coated dosage form. Pharmacokinetic parameters (T_{max}, C_{max}, AUC_{0-t}, $AUC_{0-\alpha}$, $t_{1/2}$ and K_e) were similar between the reference and test products and the AUC agreed with those published pharmacokinetic studies [6].

Table 3
Pharmacokinetic parameters for a single dose of duloxetine hydrochloride (40 mg) tablet after oral administration to 12 healthy human volunteers

Pharmacokinetic parameters	Reference (40 mg)		Test (40 mg)	
	Mean	S.D.	Mean	S.D.
T_{max} (h)	6.23	0.42	6.31	0.51
C_{max} (ng/ml)	42.45	5.84	44.94	7.32
AUC_{0-t} (ng/ml h)	645.21	21.07	662.23	10.23
$AUC_{0-\alpha}$ (ng/ml h)	712.67	60.45	727.51	72.56
$t_{1/2}$ (h)	12.57	1.28	13.24	1.41
K_e (1/h)	0.032	0.004	0.033	0.003

4. Conclusions

A rapid, sensitive and accurate liquid chromatography with atmospheric pressure ionisation–tandem mass spectrometry method (LC-API–MS–MS) was developed for the determination of duloxetine in human plasma. The method offers high sensitivity with a low limit of quantitation of 0.1 ng/ml, wide linearity, and specificity without interferences from endogenous substances and low sample volume. The simplicity of sample preparation facilitates its application in the pharmacokinetic studies of duloxetine products. In addition, this high sensitivity method can be used in bioavailability and bioequivalence studies in human subjects also.

Acknowledgments

The authors wish to thank the DST (Department of Science and Technology, Government of India) for the financial assistance to procure the LC–MS–MS (API-2000) instrument, Pentabiotech for supplying duloxetine hydrochloride standard, Torrent Pharmaceuticals for haloperidol and Bioequivalence Study Centre, Jadavpur University for the technical support.

References

[1] P.V. Tran, F.P. Bymaster, R.K. McNamara, W.Z. Potter, J. Clin. Psychopharmacol. 23 (2003) 78.
[2] S.N. Vaishnavi, C.B. Nemeroff, S.J. Plott, S.G. Rao, J. Kranzler, M.J. Owens, Biol. Psychiatry 55 (2004) 320.
[3] J.E. Turcotte, G. Debonnel, C. de Montigny, C. Hebert, P. Blier, Neuropsychopharmacology 24 (2001) 511.
[4] P. Bech, D.K. Kajdasz, V. Porsdal, Psychopharmacology (Berl.) 188 (2006) 273.
[5] M.H. Skinner, H.Y. Kuan, A. Skerjanec, M.E. Seger, M. Heathman, L. O'Brien, S. Reddy, M.P. Knadler, Br. J. Clin. Pharmacol. 57 (2004) 54.
[6] N. Ma, B.K. Zhang, H.D. Li, B.M. Chen, P. Xu, F. Wang, R.H. Zhu, S. Feng, D.X. Xiang, Y.G. Zhu, Clin. Chim. Acta 380 (2007) 100.
[7] R.J. Lantz, T.A. Gillespie, T.J. Rash, F. Kuo, M. Skinner, H.Y. Kuan, M.P. Knadler, Drug Metab. Dispos. 31 (2003) 1142.
[8] S.A. Chalon, L.A. Granier, F.R. Vandenhende, P.R. Bieck, F.P. Bymaster, M.J. Joliat, C. Hirth, W.Z. Potter, Neuropsychopharmacology 28 (2003) 1685.
[9] D. Anderson, S. Reed, J. Lintemoot, S. Kegler, S. DeQuintana, M. Sandberg, J. Muto, J. Anal. Toxicol. 30 (2006) 576.
[10] H. Hoja, P. Marquet, B. Verneuil, H. Lotfi, J.L. Dupuy, B. Penicaut, G. Lachatre, J. Chromatogr. B. Biomed. Sci. Appl. 688 (1997) 275.
[11] L.R. Snyder, J.J. Kirkland, J.L. Glajch, Practical HPLC Method Development, second ed., John Wiley & Sons, Inc., New York, 1997, p. 657.