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Rapid high Performance liquid Chromatography- Tandem mass Spectrometry Method For Quantitation of Milnacepran in Human Plasma

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ABSTRACT

A highly sensitive, rapid and specific liquid chromatography–mass spectrometric (LC-MS/MS) method for the determination of milnacepran in human plasma using anastrazole as the internal standard (I.S.) was developed and validated. The sample work-up procedure involved a liquid–liquid extraction of the compounds. The chromatographic separation was performed on a Thermo Hypersil–Hypurity C18 column (50 × 4.6 mm, 5 µm). Mass spectrometric data were acquired in MRM monitoring of the $[M + H]^+$ ions at 246.80 > 100.10 and 294.10 > 224.90 for milnacipran and anastrazole, respectively. The method was validated in the concentration range of 2–1,000 ng mL^{-1} ($r^2 > 0.99$). The method was validated and found suitable to high-throughput analysis of human plasma samples for application in pharmacokinetic and bioavailability/bioequivalance studies.

INTRODUCTION

Milnacipran hydrochloride is a selective norepinephrine and serotonin reuptake inhibitor. It is a racemic mixture with the chemical name: (±)-[1R(S), 2S(R)]-2-(aminomethyl)-N, N-diethyl-1-phenylcyclopropanecarboxamide hydrochloride (USP, 2008). Milnacipran is a nontricyclic compound with essentially equal potency for inhibiting the reuptake of both serotonin and noradrenaline, without directly affecting postsynaptic receptor site (Lecrubier, 1997). Milnacepran is in investigation for the treatment of fibromyalgia, preliminary evidence suggests that milnacipran may benefit some patients with a significant improvements in pain and other symptoms of fibromyalgia. The effect sizes were equal to those previously found with Tricyclic antidepressants, and the drug is well tolerated (Gendreau et al., 2005; Owen, 2008). Literature survey reveals that a few methods (Rao et al., 2011; Tondepu et al., 2012) have been reported for the estimation of Milnacipran in solid dosge form. Also few of methods have been employed for the detection of the milnacipran in plasma, including HPLC, LC–MS/MS (Dallet et al., 2002; Castaing et al., 2007; Marcoux et al., 2007; Borkar et al., 2012). However, these reported methods required time-consuming and laborious extraction procedures, relatively large sample volume, long chromatographic analysis time and also showed low sensitivity which may not adequate for pharmacokinetic and bioequivalence studies. Recently, a GC-MS method was reported for the determination milnacepran in human plasma by derivatisation with N-methyl-N-trime thylsilyitrifluroacetamide and (LLOQ) of 30 ng mL^{-1} for milnacepran (Uçaktürk et al., 2010). The method suffered from time-consuming derivatization process, long extraction procedure and large sample volume requirement. Another method was also reported to determine milnacepran in human plasma using liquid chromatography with spectrofluorimetric detection by derivatiza tion with fluorescamine, but the extraction recovery of was approximately 70% and (LLOQ) of 5 ng mL^{-1} (Puozzo et al., 2004). Therefore, a highly sensitive, advanced high throughput LC–MS/MS method was developed and validated for the determination of milnacipran in human plasma. This developed method offered higher sensitivity, simpler sample treatment procedure, smaller sample volume requirement and shorter run time of 2.0 min.

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MATERIALS AND METHODS

Materials and chemicals

A Hypersil Hypurity C18, 5 µm, 50 x 4.6 mm column obtained from Thermo Scientific, Mumbai, India was used for the compound retention. The reference standards of MIlnacepran (99.86) and Anastrozole was obtained from Glenmark pharmaceutical Ltd, India.

High purity water was prepared in-house using a Milli-Q water purification system obtained from Millipore, Bangalore, India. HPLC grade methanol acetonitrile and Methyl tert-Butyl Ether were purchased from J.T.Baker, Mumbai, India. Formic acid was purchased from merck, India. Drug free (blank) human plasma containing K3-EDTA as the anticoagulant was obtained from Yash Laboratory Thane, India. The plasma thus obtained was stored at –20° C prior to use.

Instrumentation

All experiments were performed on an Applied Biosystems API 4000 MS/MS apparatus (AB Sciex, Framingham, USA) fitted with a TurboIonSpray source including a Shimadzu autosampler, Shimadzu Prominence series (Kyoto, Japan) binary pump. Data acquisition and quantitation was performed with Analyst® 1.5 software. For sample evaporation Speedovap caterpillar, Takahe Analytical Instruments, India were used.

LC–MS/MS conditions

The chromatographic separation was achieved on a Hypersil Hypurity C18, 5 µm, 50 x 4.6 mm column obtained from Thermo Scientific Mumbai, India. The mobile phase consisted of 700 mL acetonitrile, 300 mL water and 0.3 mL formic acid, delivered at 0.6 mL min^{-1} by Shimadzu Prominence series (Kyoto, Japan) binary pump. The total run time was 2.0 min and the injection volume was 1.0 µl. Moreover, the column and sample temperature was maintained at 30°C and 5°C, respectively.

Electrospray ionization (ESI) was performed in the positive ion mode with nitrogen as the collision gas. The spray voltage and source temperature were 4500 kV and 400°C respectively. Nitrogen was used as the collision gas. The Declustering Potential (DP), Collision Energy (CE), Entrance potential (EP), Cell Exit Potential (CXP) were optimized during tuning as 75, 19, 10, 11 and 98, 20, 10, 11 eV for Milnacepran and Anastrozole respectively. The collision activated dissociation (CAD) gas and was set at 8 psi, while the curtain gas was set at 30 psi.

The Applied Biosystems API 4000 LC-MS/MS apparatus was operated at unit resolution in the multiple reaction monitoring (MRM) mode, monitoring the transition of the protonated molecular ion m/z 246.80→100.10 for Milnacipran and the transition of the protonated molecular ion m/z 294.10→224.90 for the internal standard, anastrazole. The instrument response was optimized for Milnacepran by infusing a constant flow of a solution of the drug dissolved in mobile phase via a T-piece into the stream of mobile phase eluting from the column. The same methodology was used to optimize the response of the instrument for the internal standard. The summary of optimized instrument parameters is shown in Table 3.

Preparation of standards and quality control samples

Two separate stock solutions of Milnacepran and anastrazole were prepared for bulk spiking of calibration curve and quality control samples for the method validation exercise. Milnacepran and anastrazole (IS) stock solutions were prepared by dissolving them in methanol to obtain a desired concentration. These stock solution of Milnacepran thus prepared were used to spike a pool of blank human plasma which was serially diluted with blank human plasma eight times to obtain calibration standards spanning a range between 2 to 1000 ng/mL for milnacepran . Similarly, quality control standards (QC's) were prepared (using the same methodology) at four different concentrations namely, 2.0(LLOQ), 6.0(LQC), 220.0(MQC), and 890.0 (HQC) ng/mL. The spiked plasma samples (standard and QC samples) were stored at −50 °C until use.

Sample preparation

The plasma samples (100 µl) were transferred to 5.0 mL polyproyline tubes (Tarson, India) and 25µl of the diluted working solution of internal standard (500 ng/mL of Anastrazole) and 100 µl 0.5% of formic acid in water was added. The samples were then vortexed for one minute and 3ml of Methyl tert-Butyl Ether was added and kept on reciprocating shaker at 200 rpm for 10.0 minute. Then centrifuged at 4000 rpm for 5 minutes. 2.5 ml separated supernatant organic layer evaporated at 40 ºC under the stream of nitrogen. Reconstituted the dry residue with the mobile phase and 1.0 µL was injected into the LC-MS/MS system through the autosampler.

Assay validation

The method was validated in terms of selectivity, accuracy, precision, recovery, calibration curve and reproducibility according to the FDA guidelines for validation of bioanalytical method. Chromatogram comparisons of blank and spiked human plasma were used to evaluate the selectivity of the method. Calibration curves were constructed from the peak area ratios of each analyte to internal standard versus plasma concentrations through the linear least squares regression calculation with a weighting factor of 1/X. The LLOQ was determined as the lowest concentration that could be quantified with an acceptable precision and accuracy within ±20% and fulfill the requirement of a signal-to-noise ratio ≥5. Both precision and accuracy of the method were determined by analyzing six replicates of QC samples at low, medium and high concentrations. Intra-day precision was calculated as the relative standard deviation (RSD) resulting from the same day. Inter-assay precision was assessed by the RSD of the mean concentration on three consecutive days. The accuracy was determined by the percent of mean deviation from nominal concentration, bias% = (measured concentration−nominal concentration)/nominal concentration×100%. The recovery of each

analyte from human plasma matrix was evaluated by comparing the mean peak areas obtained from processed QC samples of low, medium and high concentrations with standard solutions of equivalent concentration. Short-term stability of the analytes in human plasma was determined by assessing QC samples after 6 h at room temperature. Freeze–thaw stability was checked after three cycles and long-term stability was determined by assessing QC samples stored at below −50 ◦C for 30 days. QC samples were prepared and injected, and reinjected after the samples of that batch were maintained in the autosampler at 4 ◦C for 48 h. The samples were concluded to be stable if the assay values were within the acceptable limits of ±15% deviation from the nominal concentration.

RESULTS AND DISCUSSION

LC–MS/MS method optimization

During method development quantification was performed using multiple reactions monitoring (MRM) in the positive ion mode and different options were evaluated to optimize sample like extraction, detection parameters and chromatography. Electro spray ionization (ESI) was evaluated to get better response of analytes as compared to APCI (Atmospheric Pressure Chemical Ionization) mode. It was found that the best signal was achieved with the ESI positive ion mode. Fig. 1 and 2 displays the product ion spectra of $[M+H]^+$ ions of Milnacipran and anastrazole (IS). For milnacipran, the product mass spectrum was recorded from the precursor ion m/z 246.8 [M+H] + and the most abundant fragment was monitored at m/z 100.10.

For the Internal standard anastrazole the product mass spectrum was recorded from the precursor ion m/z 294.1 [M+H]+ and the most abundant fragment was monitored at m/z 224.9. A mobile phase with different buffer solution and methanol in varying combinations was tried during the initial development stages. But the best signal for Milnacipran and anastrazole was achieved using a mobile phase containing 700 mL acetonitrile, 300 mL water and 0.3 mL formic acid with a 0.6 mL min^{-1} flow rate and use of a Hypersil Hypurity C18, (50mm x 4.6mm), 5µm column resulted in better peak shape and reduced run time. The retention times for milnacipran and anastrazole were ~ 1.16 minutes and ~ 1.14 minutes, respectively. LC–MS/MS chromatograms showed that the analytes and IS were no interference from each other.

Selectivity

The selectivity of the method was investigated by analyzing human plasma of six different sources, two each of lipemic and heamolysed plasma. Any interference obtained was compared versus six replicates of extracted samples at the LLOQ level prepared in one of the plasma lots with the least interference at the retention time of milnacipran and anastrozole. No endogenous interference peaks were found at the retention times of all analytes and IS. Representative chromatogram obtained from blank plasma and plasma spiked with LLOQ standard for milnacipran and anastrazole is presented in Figs. 3 and 4, respectively.

Linearity and sensitivity

The assay was linear over the concentration range of 2–1000 ng/mL for milnacipran. The correlation coefficients for the calibration regression curve were 0.99 or greater. The calibration standards were back-calculated from the responses. The deviations from the nominal concentrations and coefficient of variation (CV) were less than 15% for all concentrations.

The current assay has an LLOQ of 2 ng/mL for milnacipran. These limits are sufficient for clinical pharmacokinetic studies following oral administration of lowest therapeutic dose.

Accuracy and precision

A summary of inter- and intra-day precisions and accuracies at QC concentrations are shown in Table 1. The intra- and inter day precisions were in the range of 1.96% to 5.69% .The % bias were in range of -7.14% to 4.56%. The results indicated that the assay had remarkable reproducibility with acceptable accuracy and precision.

Recovery

To calculate the absolute recovery, six replicates of spiked plasma QC samples of low, medium and high concentrations were extracted under the conditions noted above. The integrated peak area response of each analyte was compared with those obtained from the standard solutions of equivalent concentration subjected to the same extraction The mean absolute recoveries determined at 6.0, 220, and 890 ng/mL were 88.00, 86.87 and 86.63 %, respectively. The mean absolute recovery of anastrazole was 87.10 %.

Matrix effect

No matrix effect for milnacipran were observed for the six different plasma lots. The RSD of the area ratios of post spiked recovery samples at LQC and HQC levels were less than 3.71% for milnacipran. For the internal standard the RSD of the area ratios over both LQC and HQC levels was less than 6.78%.This indicated that the extracts were “clean” with no co-eluting compounds influencing the ionization of the analyte and the internal standard.

Stability

The milnacipran were found to be stable under following conditions: in plasma at room temperature for at least 6 h, in the autosampler at 4 ◦C for 48 h, three freeze–thaw cycles. The long term stability results also indicated that drug was stable in matrix for up to 30 days at a storage temperature of -50°C. All bias values between the measured concentration and the nominal concentration were in range of −9.90% to 1.59%, which is shown in Table 2. These results suggested that milnacipran was stable under various storage conditions.

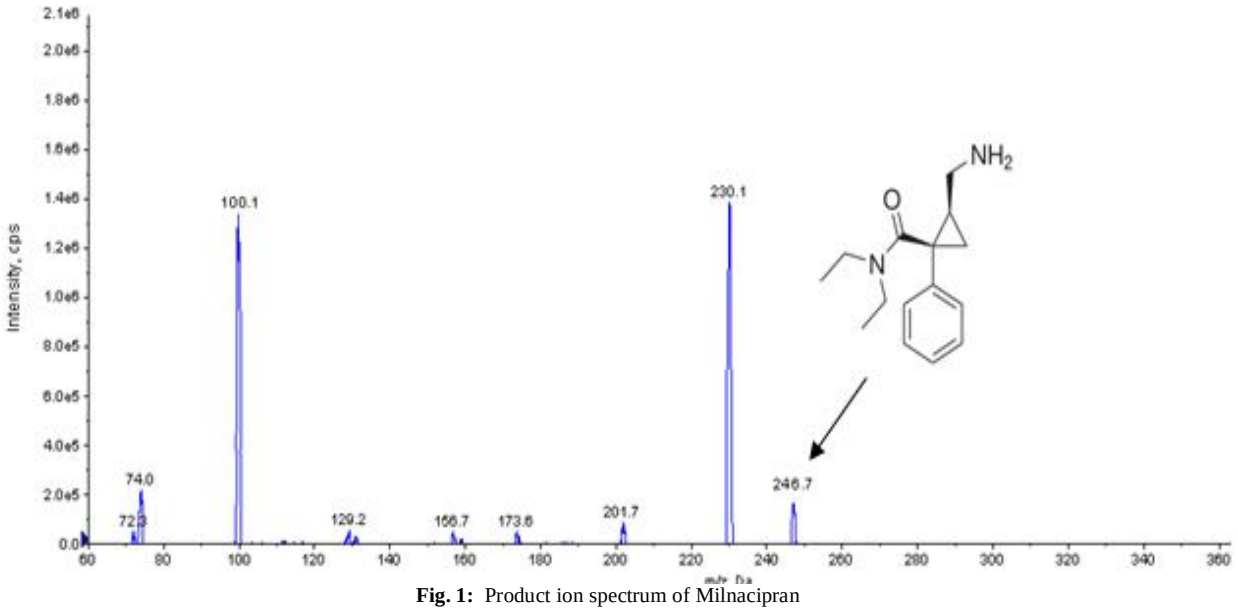

Fig. 1: Product ion spectrum of Milnacipran

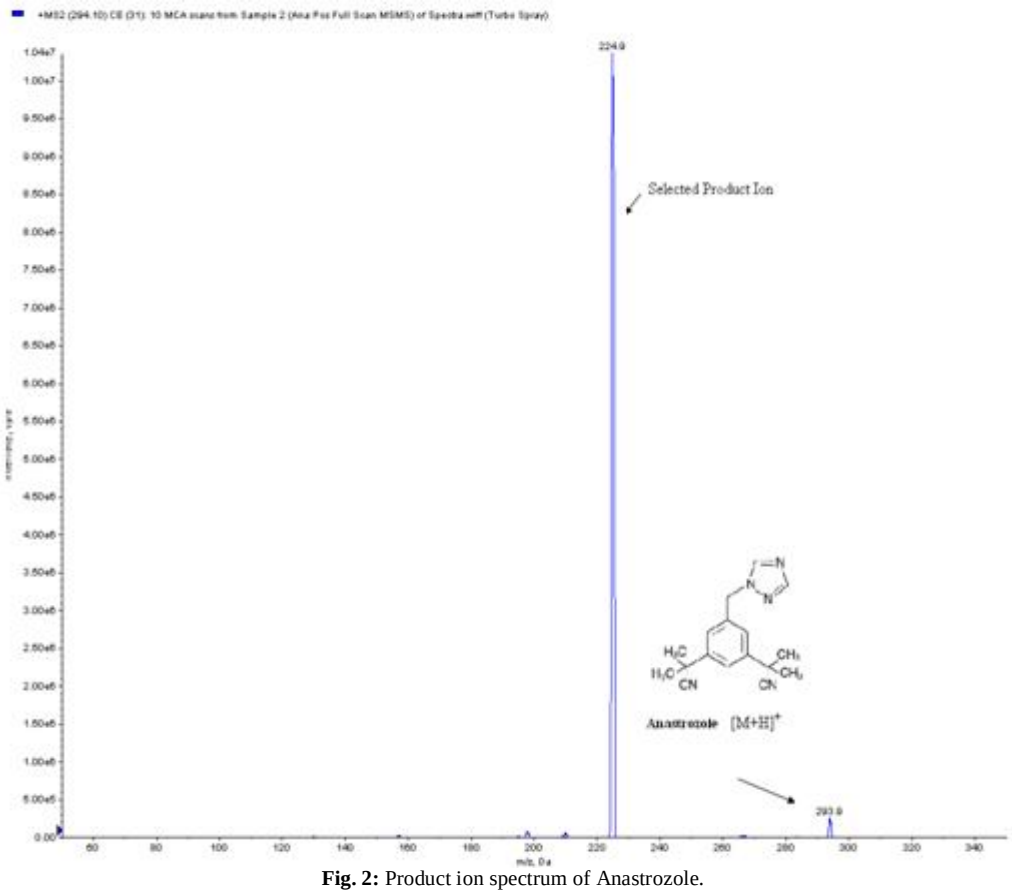

Fig. 2: Product ion spectrum of Anastrozole.

Table1. Precision, accuracy and extraction recovery for milnacipran in plasma QC samples.

Component	Nominal concentration(ng/mL)	Intra-day (n = 6)			Inter-day (n = 18)			Extract recovery (%)
		Measured concentration (ng/mL)	RSD (%)	Bias (%)	Measured concentration (ng/mL)	RSD (%)	Bias (%)	
Milnacepran	2.0	2.2	4.32	-3.69	2.0	5.69	-5.32	-
	6.0	6.1	2.21	4.56	6.0	3.21	2.56	88.00
	223.5	232.4	2.51	3.01	223.5	2.56	3.01	86.87
	894.3	841.774	1.96	-7.14	894.3	4.59	-5.14	86.63

All values are represented as the percent of mean deviation from nominal concentration,
Bias% = (measured concentration−nominal concentration)/nominal concentration×100%

Table. 2: Stability of milnacepran in human plasma under different storage conditions (n = 6).

Components	Nominal concentration (ng/mL)	Room temperature for 24 h	Autosampler at 4◦C for 48 h	Freeze-thaw Stability third cycle	Long Term Storage at −50 ◦C
Milnacipran	**6.0**	**-6.37**	**-5.66**	**-7.72**	**-9.90**
	894.3	-1.87	1.59	-2.84	-1.31

Table. 3: Summary of optimized instrument parameters.

Parameters	Optimized conditions
Sample Cooler Temperature	5°C
Column oven temperature	30°C
Injection volume	1 µL
CAD (collision activated dissociation)	8.00
CUR (curtain gas)	30.00
GS1 (collision gas)	45.00
GS2 (hot air)	50.00
IS (ion spray voltage)	4500.00
TEM (temperature)	400.00
IE1 (Q1 ion energy resolution)	1.200
IE3 (Q3 ion energy resolution)	0.900
DEF (deflector voltage)	0.5
CEM (channel electron multiplier)	2450.0

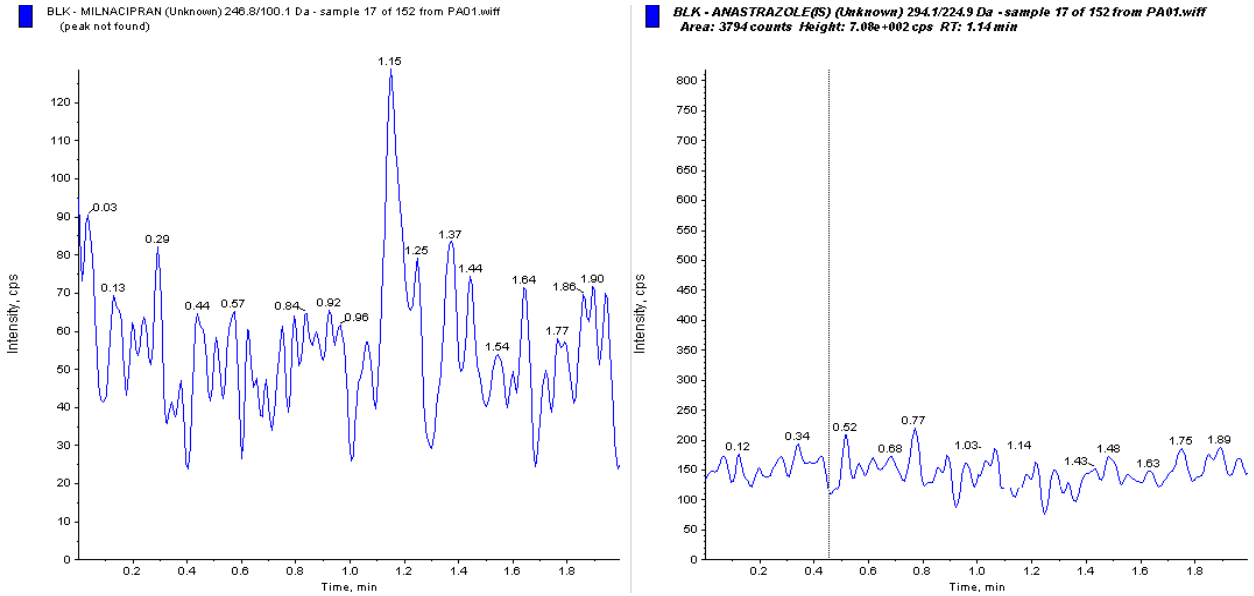

Fig. 3: Representative Chromatogram of blank plasma of milnacipran and its Internal Standard Anastrozole.

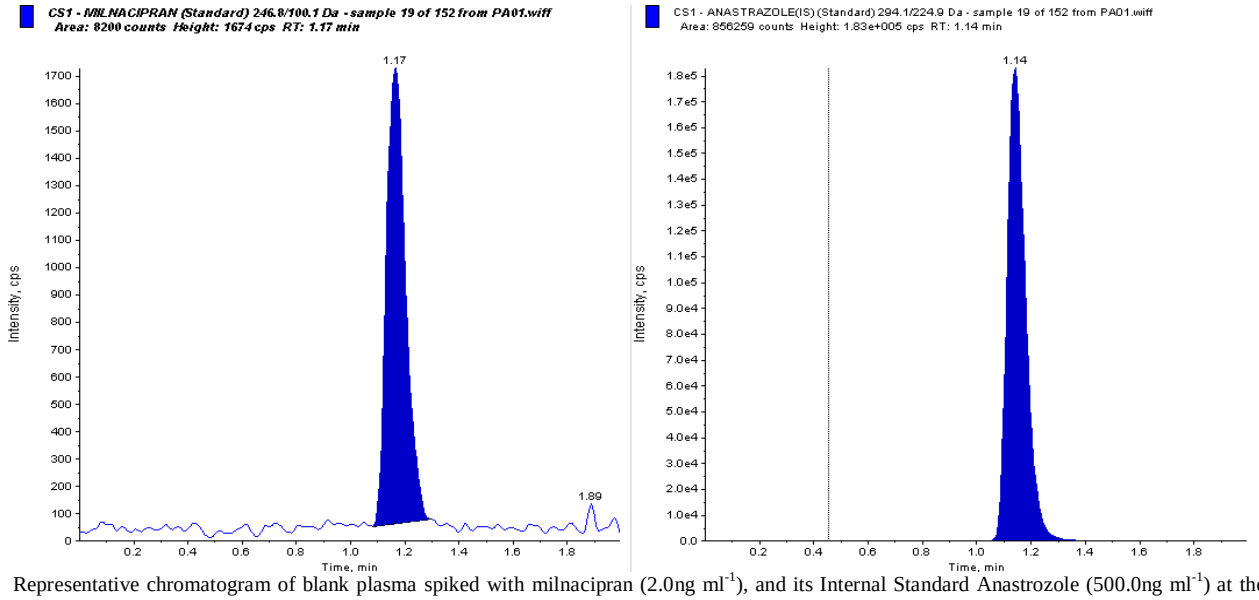

Fig. 4: Representative chromatogram of blank plasma spiked with milnacipran (2.0ng ml^{-1}), and its Internal Standard Anastrozole (500.0ng ml^{-1}) at the lower limit of quantification.

CONCLUSIONS

This is the first fully validated LC–MS/MS method for the quantification of milnacipran in human plasma. The method was proved to be sensitive, accurate, precise and reproducible. Sample preparation showed high recovery for the quantitative determination of analyte in human plasma. The method is very sensitive with a LLOQ of 2.0 ng/mL.

Extraction procedure is simple sample pretreatment procedure,and the high specificity of LC–MS/MS reduces analytical time. This method offers significant advantages in terms of improved sensitivity and selectivity, shorter run time (2.0 min) and smaller sample volume requirements (100µL).

The high sample turnover rate makes this method a suitable and valuable tool in the investigation of the clinical pharmacokinetics and bioequivalence. This method is an excellent analytical option for rapid quantification of Milnacepran in human plasma.

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