IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)
e-ISSN:2278-3008, p-ISSN:2319-7676. Volume 11, Issue 5 Ver. II (Sep. - Oct.2016), PP 98-104
www.iosrjournals.org

Validated Liquid Chromatography-Electrospray Ionization Tandem Mass Spectrometric Method for Quantification of Milnacipranin Human Plasma for Pharmacokinetic Study: ARandomized, Open-Label, Two-Period, Comparative Crossover Study in Healthy Korean Male Volunteers

YongSun Park[a,*], Dong Pil Kim[b], Ki Tak Kim[b], Hong Chul Park[b],Yong Hwan Kim[b], Ye Na Kim[a], JinYoung Kim[a], Bo Ra Jin[a],Soo Ji Sim[a], Heon Soo Lee[a]
[a]*Drug Development Supporting Service Division, Bio-Medieng Co. Ltd., Seongnam-si, Gyeonggi-do, Korea*
[b]*Nexpharm Korea. Co. Ltd., Seoul, Korea*

Abstract: *A sensitive and specific liquid chromatography combined with electrospray ionization (ESI) tandem mass spectrometry (LC-MS/MS) method, operating in the positiveionization mode, for quantifying of milnacipran in human plasma using milnacipran-d_{10} as internal standard (IS) was developed and validated. The analyte and IS were extracted by simple one step protein precipitation (PP). The chromatographic separation was performed on a Gemini C_{18} column (150 X 2.0 mm, 5 um) under isocratic conditions using a mixture of acetonitrile/0.1% formic acid (50:50, v/v) as mobile phase at a flow rate of 0.25 ml/min. Quantitation was performed using multiple reaction monitoring (MRM) mode to study parent → product ion transitions of m/z 247.2 →100.1 for milnacipran and m/z257.2→240.3 for IS, respectively. Linearity in plasma was obtained over the concentration range 2 ~ 500 ng/ml, with a coefficient of determination (r^2) of 0.9999. The method was has been successfully applied for routine assay to support pharmacokinetic study of milnacipran in human plasma after an oral administration of 50 mg milnacipran.*

Keywords: *Milnacipran, Human plasma, LC–MS/MS, Pharmacokinetics*

I. Introduction

Milnacipran (1-phenyl-1-diethylamino-carbonyl-2-amino-methyl-cyclopropane hydrochloride) is a new antidepressant which is characterized by serotonin and norepinephrine reuptake inhibitor (SNRI), without directly affecting postsynaptic receptor site and is used in the clinical treatment of fibromyalgia [1]. Fibromyalgia syndrome (FMS) is a complex syndrome characterized by chronic widespread muscular pain associated with other symptoms such as fatigue, cognitive dysfunction, sleep disturbance, depression, anxiety, and stiffness. Due to its antidepressant activity, milnacipran can be beneficial to FMS patients with coexisting depression. Inhibition of both neurotransmitters works synergistically to treat both FMS as well as depression [2-8].

Several studies on humans and animals are necessary for the development of a new chemical entity in order to gather extensive knowledge on its pharmacokinetics and metabolism. Also few methods have been employed for the detection of the milnacipran in plasma, including HPLC, LC-MS/MS [9-12]. 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 milnacipran in human plasma by derivatization with N-methyl-N-trimethylsilyitrifluroacetamide and low limited of quantitative (LLOQ) of 30 ng/ml for milnacipran [13]. The method suffered from time-consuming derivatization process, long extraction procedure and large sample volume requirement. Another method was also reported to determine milnacipran in human plasma using liquid chromatography with spectrofluorimetricdetection by derivatization with fluorescamine, but the extraction recovery of was approximately 70% and LLOQ of 5 ng/ml [14].

Therefore, a highly sensitive, advanced high throughput LC-MS/MS method was developed and validatedfor 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 2.5 min.

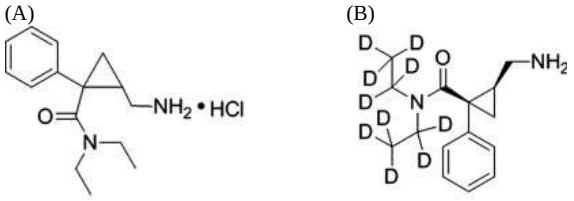

Fig.1. Structural representation of milnacipran (A) and deuterated milnacipran-d_{10}(B).

II. Experimental

2.1. Materials and Instrument

Milnacipranhydrochloride(99.9%) and milnacipran-d_{10}hydrochloride (IS, 98.0%) were purchased from Tokyo chemical industry Co., Ltd (Japan) and Toronto Research Chemicals(Canada), respectively.Acetonitrile (J.T. Baker, USA) was HPLC grade, and other chemicals were of analytical grade. All aqueous solutions including the bufferfor the mobile phase were prepared with Milli Q (Millipore, Milford, MA, USA) grade water. Drug-free plasma for the preparation of calibration standard was obtained from metrolhospitalblood donor service (Anyang, Korea). Before analysis, the blank samples were analyzed by the present LC-MS/MS method. No significant peaks were observed at the retention times of the analyte and IS.

An Agilent 1200 system consisting of G1312A quaternary pump, G1379B degasser, 1367B autosampelr, G1316A thermostat,G1316A column oven (TTC) compartment (Agilent, Waldbronn, Germany) was used for solvent and sample delivery. An API 4000 triple-quadruple mass spectrometer equipped with a TurboIonSpray (ESI) source was used for mass analysis and detection (Applied Biosystems, Foster City, CA, USA). Data processing was performed on Analyst 1.6 software package.

2.2. Chromatographic and Mass Spectrometric Conditions

Isocratic chromatographic separation was achieved on a Gemini C_{18} column, 150mm X 2.0mm, i.d., 5 um (Phenomenex, USA). The mobile phase consisted of acetonitile / 0.1% formic acid (50:50, v/v) at a flow rate of 0.25ml/min. The column and auto-sampler temperature were maintained at 35 ℃ and 8℃, respectively.

The mass spectrometer was operated in positive ionization mode. The tuning parameters were optimized for milnacipran and the I.S. by infusing a solution containing 500 ng/ml of both analytes at a flow rate of 10 ul/min into the mobile phase (0.20 ml/min) using a analyte column 'T' connection. Optimized instrument settings specific milnacipran and IS were as follows: curtain gas was 20 psi, ion source gas 1 was 50 psi, ion source gas 2 was 50 psi, ionspray voltage was 5500 V, turbo heater temperature was 600 ℃. Quantitation was performed using multiple reaction monitoring (MRM) of the transitions m/z 247.2→100.1 for milnacipran and m/z 257.2→240.3 for the IS, respectively, with a dwell time of 300 ms per transition. The precursor ions of milnacipran and IS were formed using declustering potentials of 31 and 41 V, respectively, and their precursor ions were fragmented at collision energies of 27 and 17 eV by collision-activated dissociation with nitrogen at a pressure setting of 5 (arbitrary units). Both quadrupoles were maintained at unit resolution.

2.3. Preparation of Calibration Standard and QC Samples

A stock solution of milnacipran with a concentration of 1.0 mg/ml (calculated from purity) was prepared by dissolving 11.5mg of milnacipranhydrochloridein 10 ml of acetonitrile. Seven standard working solutions of 20, 50, 100, 500, 1000, 2500 and 5000 ng/mlof milnacipran were made by further dilution of the stock solution with water. The quality control (QC) samples were similarly prepared at concentrations of 60, 2000 and 4000ng/ml, by a separate weighing of the pure standard. A 16 ng/mlworking solution of the IS was also prepared by diluting the 0.1ug/ml stocksolution of milnacipran-d_{10} with 90% acetonitreile.

Matrix-matched calibration standards and QC samples of milnacipran were prepared by spiking 10 ul of the working solutions into 90 ul of drug-free plasma. The calibration standards were prepared at concentrations of 2, 5, 10, 50, 100, 250 and 500 ng/mlof milnacipran in plasma, while the corresponding QC samples were prepared at 6, 200 and 400ng/ml.

These standard-spiked plasma calibration solutions and QC samples were stored at −20 ℃. For each batch of unknown samples to be analyzed, the appropriate standard and QC solutions were brought to room temperature, and processed through the plasma sample preparation procedure in parallel with the unknown samples.

2.4. Sample preparation

To 2.0mlpolypropylene centrifuge tube, 500 ul of the IS solution (milnacipran-d_{10}, 16 ng/ml) was added to 100 ul of plasma samplesandvortexed for 30 sec followed by centrifugation at 12000 rpm for 5 min.

The supernatant layer 200 ul was added to 500 ul of deionized water and vortexed for 30 sec. The content was finally transferred into appropriate auto-sampler vials and an aliquot (5 ul) was injected onto the LC-MS/MS for analysis.

2.5. Method validation

Plasma samples were quantified using the ratio of the peak area of analyte to IS as the assay response.The specificity of the method was determined by analyzing six different batches of human plasma as is, to demonstrate the lack of chromatographic interference from endogenous plasma components. Sets of spiked calibration curve (CC) standards and QC samples (n=4 at eachconcentration) were prepared and analyzed on three different occasionsto evaluate linearity, precision and accuracy. To evaluate linearity, plasma calibration curves were prepared and assayed on three consecutive days over the range of 2 ~ 400 ng/ml. Least-squares linear regression was used for curve fitting with $1/x^2$ as the weighting factor. For determining the intra-day precision and accuracy, a replicate analysis of plasma samples of milnacipran in human plasma was performed on the same day. The run consisted of a CC and five replicates of each the lower limit of quantification (LLOQ), low, mid and high concentration QC samples. The inter-day precision and accuracy were assessed by analysis of three batches on different days. The precision was expressed as the coefficient of variation(CV %) and the accuracy as the relative error (R.E.%).The extraction recovery of the analytes from the plasma was evaluated by comparing the mean detector responses of three replicates of processed QC samples at low and high concentration to the detector responses of standardsolutions of same concentration. Endogenous matrix components may change the efficiency of droplet formation or droplet evaporation, which in turn affects the amount of charged ion in the gas phase that ultimately reaches the detector. Three set of samples were prepared by directly spiking the analytes into reconstitution solution and without the presence of residue extracted from blank plasma. Post-preparative stability, three aliquots each of low and high QC samples were stored at 8℃ in an auto-sampler for 27 hr, analyzed and the concentrations were compared with the actual values. Three aliquots of each low and high QC samples were kept in deep freezer at -70 ℃for 35 day. The samples were processed and analyzed and the concentrations obtained were compared with the actual value of QC samples to determine the long term stability of analyte in human plasma. Three aliquots each of low and high unprocessed QC samples were kept at ambient temperature (25 ℃) for 19 hr in order to establish the short term stability of the analytes. The stability of the analytes after three freeze and thaw cycles was determined at low and high QC samples. The samples were stored at -70 ℃ for 24 hr and thawed unassisted at room temperature. After completely thawing, the samples were refrozen for 12 ~ 24 hr. After three freeze-thaw cycles, the concentration of the samples were analyzed. Separate standard working solutions containing 60ng/ml, 4000ng/ml of milnacipran and 16 ng/ml of IS were prepared and stored at 2 ~ 8 ℃ for 29 day. The response obtained from the two drugs was calculated and compared with that of the freshly prepared solutions of the same concentration.

2.6. Pharmacokinetic study

The validated method was used to determine the plasma concentrations of milnacipran from a clinical trial in which 28 healthy male volunteers received a single oral dosage of 50 mg milnacipran. Eligible volunteers were Korea men aged 25 to 46 years (29.3 ± 4.9) and the average body weight was 70.1 ± 8.2 kg. The study protocol was approved by the Human Investigation Ethics Committee of Metro hospital, Anyang-ci, Korea. Blood samples were collected into heparinized glass tubes before and 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 12, 24 and 36 hr post-dosing, and centrifuged at 4000 rpm (4 ℃) for 10 min to separate the plasma fractions. The collected plasma samples were stored at −70 ℃until analysis.

Determination of the pharmacokinetic parameters was performed by non-compartmental assessment of data using the computer program WinNonlin (WinNonlin V5.0.1, Pharsight Corporation, California, USA). Mean and individual concentration–time profiles were generated and used to determine the maximum plasma concentration (C_{max}) and the time to attain these maximum concentrations (T_{max}). The area under the plasma concentration–time curve from time zero to the time of the last measurable concentration (AUC_{0-t}) was calculated by the linear trapezoidal rule. The terminal elimination rate constant (ke) was estimated by log-linear regression of concentrations observed during the terminal phase of elimination.

III. Results and Discussion

3.1. Optimization of the mass spectrometric condition

Mass parameters were tuned in both positive and negative ionization modes for theanalytes. Good response was achieved in positive ionization mode. By positive electrospray ionization (ESI) mode, The detector was operated at unit resolution in the multiple-reaction monitoring (MRM) mode using the transitions

of the protonated molecular ions of milnacipran at *m*/*z* 247.2→ 100.1 and IS at *m*/*z* 257.2→240.3 Optimized parameters were as follows: curtain gas, gas 1 and gas 2 (nitrogen)20, 50 and 50 units, respectively; dwell time 300 ms; source temperature 600 ℃; ion spray voltage 5500 V. Declustering potential and collision energy were 31V and 27 eV for milnacipran and 41V and 17 eV for IS, respectively.

3.2 Optimization of the chromatographic condition

In pursuit of symmetric peak shape and retention time of ~1.54 min, feasibility of various mixture(s) of solvents such as acetonitrile and methanol using different buffers such as ammonium acetate, ammonium formate and formic acid with variable pH range of 3.0 ~ 7.0, along with altered flow rates (in the range of 0.2 ~ 0.5 ml/min) were tested for complete chromatographic resolution of milnacipran and IS (data not shown). The resolution of peaks was achieved with 0.1% formic acid and acetonitrile mixture (50:50, v/v) with a flow rate of 0.25 ml/min, on a Gemini C_{18} column and was found to be suitable for the determination of electrospray response for milnacipran and IS.

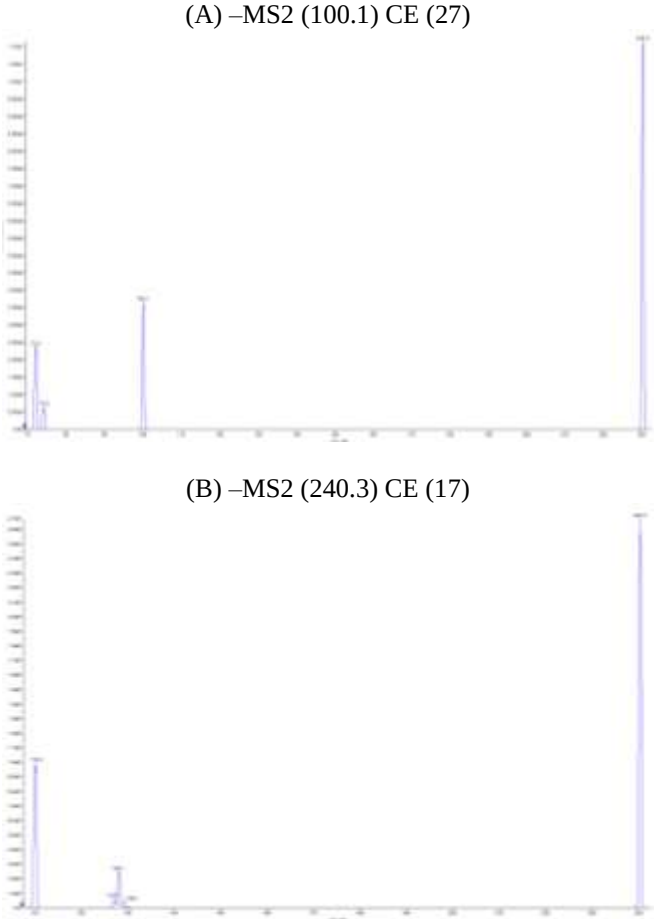

Fig. 2. Product ion spectra of $[M\text{-}M]^+$ of milnacipran (A) and milnacipran-d_{10} (B).

3.3. Sample pre-treatment

In order to achieve cleanliness in extract, protein precipitation (PP)was optimized for extraction of analytes from plasma. The protein precipitation reagent (milnacipran-d_{10} in 90% acetonitreile) gave consistent results in terms of recovery of milnacipran and IS and also gave cleaner plasma blank samples.

3.4. Assay specificity

A typical chromatogram for the control human plasma (free of analyte and IS) and human plasma spiked with milnacipran at LLOQ are shown in Fig. 3, respectively. No interfering peaks from endogenous compounds are observed at the retention times of analytes and IS. The retention time of milnacipran and IS was 1.04 and 1.04 min. The total chromatographic run time was 2.5 min.

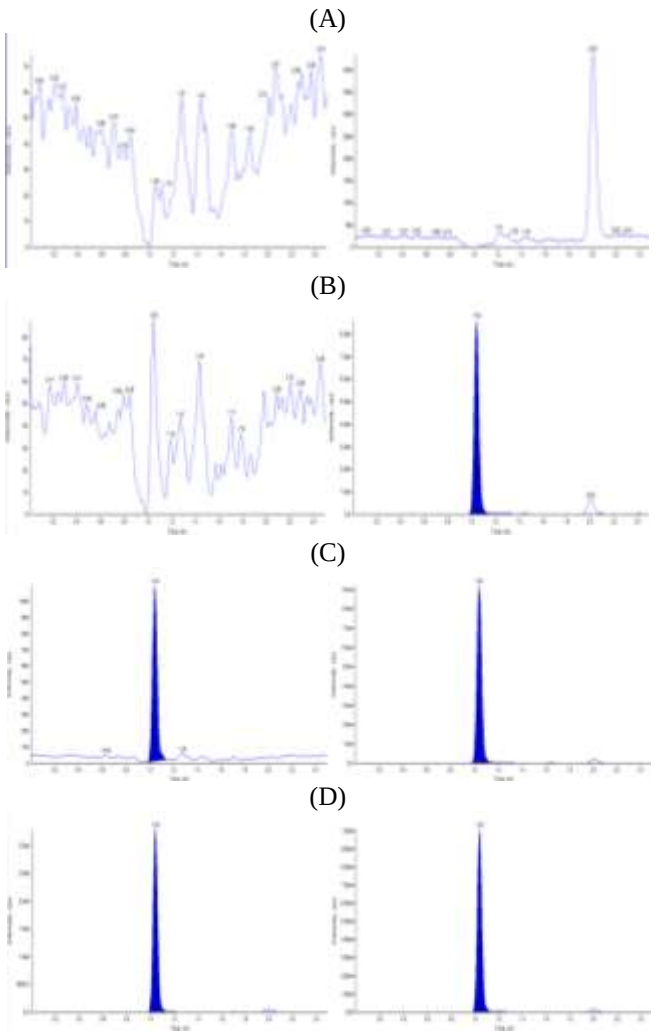

Fig.3. Typical MRM chromatograms of milnacipran (left panel) and IS (right panel) in (A) human blank plasma, (B) human blank plasma spiked with IS, (C) human plasma spiked with milnacipranat LLOQ (2 ng/ml) and IS, (D) a representative chromatogram(2 hr)of extracted a male volunteer.

3.5. Linearity and Lower limit of Quantification

The linear regression of the peak-area ratios versus concentrations was fitted over the concentration range of 2 ~ 500ng/ml in human plasma. A typical equation of the calibration curves was as follows: y = 0.0053x + 0.0075

(r^2=0.9999), where y represents the peak-area ratio of analyte to IS and x represents the plasma concentration of milnacipran. Good linearity was seen in this concentration range.

The lower limit of quantification was 2ng/ml for determination of milnacipran in plasma. The precision and accuracy at the concentration of LLOQ are shown in Table 1.

3.6 Precision and Accuracy

The method showed good precision and accuracy. Table 1 summarizes the intra- and inter-day precision and accuracy for milnacipran from QC samples. The intra-day precision (CV %) for QC samples (2, 6, 200, 400ng/ml) were 1.20%, 1.10%, 2.11% and 1.37%, respectively and that of inter-day analysis were 7.97%, 2.48%, 1.43%, 0.90% with a relative errors (R.E. %) within 0.23% to 8.70%.

3.7. Recovery and Matrix Effect

The extraction recoveries of milnacipran from human plasma were 95.07% (CV=1.50%),98.52% (CV=0.66%) and 98.28 (CV=1.39%) at concentration levels of 6, 200 and 400 ng/ml, respectively, and the mean extraction recovery of IS was 99.41% (CV=0.97%).

The endogenous components are mainly the cause of ion suppression effects during electrospray ionization. The extent of this effect is mainly dependent on sample extraction procedure and is also compound dependent. The result indicated that the matrix components did not alter or deteriorate the performance of the

proposed method as the % coefficient of variation (CV%) of two QC samples was less than 98.26% and 99.61% for milnacipran and IS respectively indicates the reproducibility of peak area as well as the extracts were 'clean' and no unseen component interfere with the ionization of the analytes. The matrix effect on the estimation of the analytes was shown in Table 2.

3.8. Stability

The result of stability experiments showed that no significant degradation occurred during the chromatography, extraction and sample storage of milnacipran plasma samples. Stability data are shown in Table 3.

Table 1. Precision and accuracy data for the analysis of milnacipran in human plasma (n=5)

Added	Intra-day			Inter-day		
(ng/ml)	Found (ng/ml)	CV (%)	R.E (%)	Found (ng/ml)	CV (%)	R.E (%)
2	1.83	1.20	8.70	2.01	7.97	0.63
6	5.83	1.10	2.90	5.99	2.48	0.23
200	198.57	2.11	0.71	196.87	1.43	1.57
400	389.45	1.37	2.64	388.08	0.90	2.98

Table 2. Extraction recovery and matrix effect of milnacipran and milnacipran-d_6(IS).

Analyte	Concentration (ng/ml)	Recovery (%)	Matrix effect (%)
Milnacipran	6	95.07 (CV=1.50%)	98.26 (CV=1.49%)
	200	98.52 (CV=0.66%)	-
	400	98.28 (CV=1.39%)	99.16 (CV=0.71%)
Milnacipran-d_{10}	16	99.41 (CV=0.97%)	99.61 (CV=1.65%)

Table 3. Summary of stability of milnacipran in human plasma under various storage conditions (n=3).

Storage conditions	Concentration (ng/ml)			CV (%)	Variation
	Added	Initial	After		(%)
Post preparative (27 hr at 8℃)	6	5.84	6.16	2.30	5.42
	400	388.14	400.42	0.44	3.16
Short-term (19 hr at 25 ℃)	6	5.84	6.07	2.57	3.82
	400	388.14	397.41	0.55	2.39
Long-term (35 day at -70 ℃)	6	5.84	6.19	6.56	5.93
	400	388.14	401.24	0.28	3.38
Three freeze/thaw (3 cycles)	6	5.84	6.08	1.08	4.05
	400	388.14	389.88	1.61	0.45
Stock solution (29 day at 2 ~ 8℃)*	60	8352	8197	0.86	-1.86
	4000	507929	511112	0.51	0.63

* Stability of stock solution was evaluated peak area.

Table 4. Pharmacokinetic parameters of milnacipran after a single oral dose 50 mg milnaciprancapsules of the test and reference.

Parameters	Test*	Reference**
C_{max}, ng/ml	119.13±28.62	116.99±30.50
T_{max}, hr	2.04±1.09	2.36±1.30
AUC_{0-t}, ng·hr/ml	1141.28±166.61	1153.71±184.12
$AUC_{0-\infty}$, ng·hr/ml	1194.74±181.44	1204.97±196.76
$t_{1/2}$, hr	7.94±0.96	7.75±0.90

* Milpran Cap., 50 mg (Nexpharm Korea Co. Ltd. Korea)
** Ixel Cap., 50mg (Bukwang Pharmaceutical Co. Ltd. Korea)

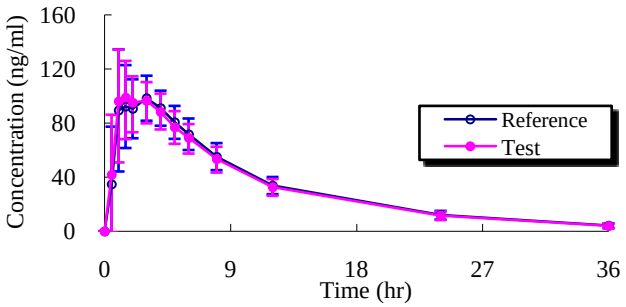

Fig. 4. Mean plasma concentration–time curve of milnacipran after a single oral dose of 50 mg milnacipran to 28 healthy male volunteers. Each point represents the mean±SD.

3.9 Application in Pharmacokinetic Study

This validated analytical method was applied to investigate the pharmacokinetic profiles of milnacipran in human plasma after an oral administration of 50 mg milnacipran. Profile of the mean plasma concentration of milnacipran versus time is shown in Fig. 4. The main pharmacokinetic parameters of milnacipran in 28

volunteers were calculated.

For the pharmacokinetic analysis of plasma, the mean (SD) values obtained for the test and reference products were as follows: C_{max}, 119.13 (28.62) and 116.99 (30.50) ng/ml; T_{max}, 2.04 (1.09) and 2.36 (1.30) hr; $T_{1/2}$, 7.94 (0.96) and 7.75 (0.90) hr; AUC_{0-t}, 1141.28 (166.61) and 1153.71 (184.12) ng·hr/ml; and $AUC_{0-\infty}$, 1194.74 (181.44) and 1204.97 (196.61) ng·hr/ml, respectively (Table 4).The parametric 90% confidence intervals for AUC_{0-t}, and C_{max}values were 96.74 ~ 101.40% and 94.16 ~ 110.79%, respectively, and were entirely within the bioequivalence acceptance limits.

IV. Discussion

To the best of our knowledge, we have developed for the firsttime fully validated LC–MS/MS method for the determination ofmilnacipran, which provides the highest sensitivity (2ng/ml)using a simple PP extraction procedure. A good internalstandard should track the analyte during extraction and any inconsistentresponse due to matrix effect. This is also established withalmost the same recovery of IS compared to the analyte. The mostappropriate IS for typical anions are none other than deuteratedcompounds and hence milnacipran-d_{10} was used as IS. Resultsobtained by usage of d_{10} internal standard were consistent andreproducible which was evident by incurred sample analysis conductedon this study.

V. Conclusions

An LC-MS/MS assay for milnacipran in human plasmawas developed and validated with respect to linearity, precisionand accuracy, and analysis of real samples was demonstrated.It was proved to be superior in sensitivity, sample pretreatmentand speed of analysis in comparison to the previously reportedanalytical methods. This method was successfullyapplied to pharmacokinetic studies for milnacipran andwas found to be sensitive and reliable.

Reference

[1] Y. Lecrubier. Milnacipran: The clinical properties of a selective serotonin and noradrenaline reuptake inhibitor (SNRI), *Humanpsychopharmacology*, 12, 1997, S127-S34.

[2] Owen R. T., Milnacipran Hydrochloride: Its Efficacy, Safety and Tolerability Profile in Fibromyalgia Syndrome, *Drugs Today*, 44(9), 2008, 653-660.

[3] Gendreau R. M., Thorn M. D., Gendreau J. F. *et. al*., Efficacy of Milnacipran in Patients with Fibromyalgia, *J Rheumatol*, 32(10), 2005, 1975-1985.

[4] Pae C. U., Marks D. M, Shah M. *et. al*., Masand, Milnacipran: Beyond a Role of Antidepressant, *Clini Neuropharmacol*, 32(6), 2009, 355-363.

[5] Nakagawa A., Watanabe N., Omori I. M. *et. al*., Milnacipran versus Other Antidepressive Agents for depression, *Cochrane Database Syst Rev*, 8(3), 2009.

[6] Leo R. J, Brooks V. L., Clinical Potential of Milnacipran, a Serotonin and Norepinephrine Reuptake Inhibitor, in Pain, *Curr Opin Investig Drugs*, 7(7), 2006, 637-642.

[7] Gracely R. H., Jensen K., Petzke F., *et al.*, The Effect of Milnacipran on Pain Modulatory Systems in Fibromyalgia: An fMRI Analysis, A*nnals of the Rheumatic Diseases*, 67, 2008, 255.

[8] H. Naseem, W. Thomason, A. Silman *et al.*, The PTPN22* C1858T Functional Polymorphism Is Associated with Susceptibility to Inflammatory Polyarthritis but Neither This Nor Other Variants Spanning the Gene Is Associated with Disease Outcome, *Ann Rheum Dis*, 31;67(2), 2007, 251-255.

[9] Dallet P., Labat L, Richard M., *et. al*., A reversed phase HPLC method development for the separation of new antidepressents, *Journal of liquid chromatography & related technologies*, 25, 2002, 101-111.

[10] Castaing N., Titier K., Receveur-Daurel M., *et. al*., Quantification of eight new antidepressants and five of their active metabolites in whole blood by High-performance liquid chromatography-Tandem mass spectrometry, *J Anal Toxicol*, 31(6), 2007, 334-341.

[11] Saint-Marcoux F., Sauvage F., Marquet P., Current role of LCMS in therapeutic drug Monitoring, *analytical and Bioanalytical chemistry*, 388(7), 2007, 1327-1349.

[12] Roshan M. B., B. Raju, Prashant S. D., *et. al*., Liquid chromatography/electrospray ionization tandem mass spectrometric study of milnacipran and its stress degradation products, *Rapid commun mass spectrom*, 27(2), 2013, 369-374.

[13] Uçaktürk E., Şafak C., Determination of Milnacipran in Human Plasma Using GC–MS, *Chromatographia*, 72(1), 2010, 111-119.

[14] Puozzo C., Filaquier C., Zorza G., Determination of milnacipran, a serotonin and noradrenaline reuptake inhibitor, in human plasma using liquid chromatography with spectrofluorimetric detection, *J Chromatogr B Analyt Technol Biomed Life Sci*, 806(2), 2004, 221-228.