ISSN: 2279 - 0594

Journal of Biomedical and Pharmaceutical Research 1 (3) 2012, 51-59

RESEARCH ARTICLE

Pharmacokinetic Evaluation of Duloxetine Enteric Coated Pellets and Development of LC-MS/MS Method for Quantification of Duloxetine in Rat Plasma.

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ABSTRACT

The aim of this study was to develop and evaluate enteric coated pellets (ECP) to improve stability, solubility, dissolution and enhance oral systemic exposure of novel serotonin (5-HT) and nor-epinephrine (NE) reuptake inhibitor (SNRI), duloxetine. Along with this our study also aimed to develop a sensitive LC-MS/MS method for estimation of duloxetine in rat plasma. An attempt has been made to improve the stability and systemic exposure of duloxetine by formulating it in the form an extended release pellets and simultaneously the PK of enteric coated pellets (ECP) of duloxetine was performed in rats in parallel with suspension formulation. The optimized formulation showed approximately 2 hr lag time in drug release in both *in-vitro* and *in-vivo* system. The systemic exposure (AUC) and maximum concentration in plasma (Cmax) of enteric coated pellets (ECP) of duloxetine was significantly higher than conventional suspension formulation. A highly sensitive and rapid LC-MS/MS method has been developed and validated for the estimation of Duloxetine in rat plasma. The chromatographic separation was performed with 0.2% formic acid: acetonitrile at flow rate of 0.4 mL/min on Symmetry Shield RP-18 column with a total run time of 4.0 min. The MS/MS ion transitions monitored were 297.9 →154.1 for duloxetine hydrochloride and 515.1 → 276.2 for IS (telmisartan). Method validation and pre-clinical sample analysis were performed as per FDA guide lines and the results met the acceptance crieteria. The lower limit of quantification achieved was 0.1 ng/mL and the linearity was observed from 0.1 to 1500ng/mL. This novel method has been applied to pharmacokinetic study of duloxetine hydrochloride in rats. Finally it can be concluded that delayed release pellets in capsule approach can be used to improve the stability, dissolution and systemic exposure of pH senestive and poorly water-soluble drugs such as duloxetine.

KEY WORDS: solid oral dosage form, LC-MS/MS, duloxetine, enteric coated pellets

INTRODUCTION:

Mostly new chemical entities (NCEs) are unstable in gastric pH, poorly water-soluble and pose a challenge in developing an optimum solid oral dosage form. Oral route has been the major route of drug delivery for the treatment of various diseases. Delivery of pH sensitive and poorly water soluble molecule to oral route is difficult because, approximately 40% of the drug compounds are limited to low aqueous solubility and instability in gastric pH, which leads to limited oral bioavailability, high intra-subject and inter-subject variability and lack of dose proportionality [1]. To increase the oral bioavailability of pH sensitve, poorly water soluble compounds and discussed drawbacks, various other formulation strategies have been adopted including the use of cyclodextrins, nanoparticles, solid dispersions and permeation enhancers [2-3]. In recent years, much attention has focused on delayed release formulations to improve the oral bioavailability of pH sensitve and poorly water-soluble compounds. In fact, the most popular approach is the incorporation of the drug compound into entric coated pellets, which delivers the drug to the small intestine their by aiding for its better absorption. Duloxetine hydrochloride [(+)-(S)-N-methyl-3-(1-naphthalenyloxy)-2-thiophenepropanamine hydrochloride] (Figure.1), is an novel serotonin (5-HT) and nor-epinephrine (NE) reuptake inhibitor (SNRI) that has been approved by USFDA for the treatment of major depressive disorder (MDD) and vasomotor symptoms associated with menopause. Duloxetine is also used for the treatment of stress urinary incontinence (SUI) and diabetic peripheral neuropathic pain [4-6].

Literature survey revealed that few LC-MS methods have been reported for estimation of duloxetine in biological matrices. Although the methods are sensitive and has an efficient extraction procedure but the total chromatographic run time is too long, whichmay not be favourable for routine subject analysis. Also, all these reported procedures have a very high on-column loading

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of the analytes at the ULOQ level, which may reduce the efficiency of the column and may affect the column life [7-9]. Normal-phase HPLC with fluorescence detection [10] was described by Ishigooka et al., a complicated mobile phase component was needed, and a long run time (20.1 min). Radioactivity assays has also been reported by Lantz et al. [11], but the method needs special instrument. LC/MS/MS techniques frequently provide specific, selective and sensitive quantitative results, often with reduced sample preparation and time of analysis compared with other commonly employed techniques, but it is too expensive to be available in most laboratories.

Thus, the aim of the present work was to develop duloxetine enteric-coated pellets, to investigate its pharmacokinetic behaviors in rats and establish a simple, accurate, rapid and sensitive LC–MS/MS method with a lower limit of quantification (LLOQ) of 0.1 ng/mL using 50µL of rat plasma. Our method involves simple liquid-liquid extraction sample processing using ter-butyl methyl ether (amicable for plasma) and has a run time of 4.0 min for the separation of both the analyte and IS; hence our method gives higher throughput. The newly developed LC-MS/MS method was successfully used in a rat pharmacokinetic study and to assess the plasma concentration of duloxetine following administration of a 50 mg/kg oral dose.

MATERIALS AND METHODS:

CHEMICALS AND REAGENTS:

Duloxetine hydrochloride and telmisartan (IS) were procured from Dr Reddy laboratory Pvt Ltd (Hyderabad, India). HPLC-grade acetonitrile and methanol were purchased from Rankem (Ranbaxy Fine Chemicals Limited, New Delhi, India). Analytical grade formic acid was purchased from S.D. Fine Chemicals (Mumbai, India). Albino male rat were purchased from Reliance Life Sciences (Mumbai, India).

HPLC OPERATING CONDITIONS:

A Shimadzu VP (Shimadzu, Japan) LC system equipped with degasser (G1379A), quaternary pump (10ADvp), column oven (CTO-10ASvp) and auto-sampler (SIL-HTC) along with a system controller (SCL-10Avp) was used to inject 2 µL aliquots of the processed samples on a Symmetry Shield RP18 column (50 x 4.6 mm, 3.5 µm, Waters Corporation, Ireland, UK), which was kept at ambient temperature (24 ± 2°C). The isocratic mobile phase, a mixture of 0.2% formic acid and acetonitrile mixture (20:80, v/v) was filtered through a 0.45 µm membrane filter (Millipore) and then degassed ultrasonically for 5 min was delivered at a flow rate of 0.40 mL/min into the mass spectrometer electro spray ionization chamber.

MASS SPECTROMETRY OPERATING CONDITIONS:

Quantification was achieved by MS/MS detection in positive ion mode for analyte and IS using a MDS Sciex (Foster City, CA, USA) API 4000 mass spectrometer, equipped with a Turboionspray™ interface at 500°C. The common parameters, i.e. curtain gas, nebulizer gas, auxillary gas and collision gas, were set at 12, 45, 55 and 8 psi, respectively. The compounds parameters, i.e. declustering potential (DP), collision energy (CE), collision exit potential (CXP) and entrance potential (EP) for duloxetine and IS were 80, 45, 12, 10 V and 60, 30, 10, 10 V, respectively. Detection of the ions was performed in the multiple reaction monitoring (MRM) mode, monitoring the transition of the m/z 297.90 precursor ion to the m/z 154.10 product ion for duloxetine and m/z 515.10 precursor ion to the m/z 276.20 product ion for IS. Quadrupole Q1 was set on low resolution where as Q3 was set on unit resolution. The analytical data were processed by Analyst software (version 1.4.2).

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 (1000 µg/mL). The IS stock solution of 1000 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 the calibration curve (CC). Another set of working stock solutions of duloxetine were made in methanol (from primary stock) for preparation of QC samples. Working stock solutions were stored at approximately 4°C for a week (data not shown). Appropriate dilutions of duloxetine stock solution were made in methanol to produce working stock solutions of 0.1, 0.5, 10, 50, 100, 500, 750 and 1500 ng/mL. Working stocks were used to prepare plasma calibration standards. A working IS solution (200 ng/mL) was prepared in 50% methanol. Calibration samples were prepared by spiking 45 µL of control rat plasma with the appropriate working solution of the analyte (5 µL) and IS (5 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 (0.1, 1.50, 500 and 800 ng/mL) and 50 µL plasma aliquots were

distributed into different tubes. All the samples were stored at −80 ± 10°C.

RECOVERY:

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 post extracted plasma standard sample at equivalent concentrations by liquid–liquid extraction [12]. Recoveries of duloxetine were determined at QC low and QC high concentrations, i.e. 1.50 and 800 ng/mL, whereas the recovery of the IS was determined at a single concentration of 200 ng/mL.

SAMPLE PREPARATION:

A simple liquid–liquid extraction method was followed for extraction of duloxetine from rat plasma. To an aliquot of 50 µL plasma, IS solution (5 µL of 200ng/mL) was added and mixed for 15 s on a cyclomixer (Remi Instruments, Mumbai, India). After the addition of 2 mL of ter-butyl methyl ether (TBME), the mixture was vortexed for 2 min, followed by centrifugation for 10 min at 3200 rpm on Multifuge 3SR (Heraus, Germany). The organic layer (1.8 mL) was separated and evaporated to dryness at 40°C using a gentle stream of nitrogen (Turbovap®, Zymark® Kopkinton, MA, USA). The residue was reconstituted in 200 µL of the mobile phase and 2 µL was injected onto LC-MS/MS system.

VALIDATION PROCEDURES:

A full validation according to the FDA guidelines (US DHHS, FDA, CDER, 2001) was performed for the assay in rat plasma [13].

SPECIFICITY AND SELECTIVITY:

The specificity of the method was evaluated by analyzing rat plasma samples from at least six different lots 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 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 [14]. Matrix effect was determined at low and high concentrations, i.e. 1.50 and 800 ng/mL, whereas the matrix effect over the IS was determined at a single concentration of 200 ng/mL.

CALIBRATION CURVE:

The eight point calibration curve (0.10, 0.50, 10, 50, 100, 500, 750 and 1500 ng/mL) was constructed by plotting the peak area ratio of duloxetine: IS against the nominal concentration of calibration standards in rat plasma. Following the evaluation of different weighing factors, the results were fitted to linear regression analysis with the use of 1/X2 (X = concentration) weighting factor. The calibration curve had to have a correlation coefficient (r) of 0.99 or better. The acceptance criteria for each back-calculated standard concentration were ± 15% deviation from the nominal value except at LLOQ, which was set at ± 20% (US DHHS, FDA, CDER, 2001).

PRECISION AND ACCURACY:

The intra-assay precision and accuracy were estimated by analyzing six replicates containing duloxetine at four different QC levels: 0.50, 1.50, 500 and 800ng/mL in plasma. 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% deviation (SD) 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).

STABILITY EXPERIMENTS:

The stability of duloxetine and IS in the injection solvent was determined periodically by injecting replicate preparations of processed plasma samples for up to 12 h (in the autosampler at 4°C) after the initial injection. The peak-areas of the analyte and IS obtained in the initial cycle were used as the reference to determine the stability at subsequent points. Stability of duloxetine in plasma during 6 h (bench-top) was determined at ambient temperature (24 ± 2°C) at two concentrations (1.50 and 800 ng/mL) in six replicates. Freezer stability of duloxetine in rat plasma was assessed by analyzing the LQC and 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 returned 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% SD) and precision (i.e. ±15% RSD).

***IN VIVO* STUDIES IN RATS:**

A pharmacokinetic (PK) study was performed in over night (~12 h) fasted healthy male albino rats (n = 3, weight range 230–250 g) following approval from the ethical committee. During fasting time animals had free access to water. Blood samples (0.2mL via jugular vein) were obtained following oral administration of 5 mg/kg duloxetine, [in the form of a suspension and enteric coated pellets with 5mg equivalent weight of duloxetine, via modified oral gavage needle to their respective groups **(Figure.2)] into** polypropylene tubes containing K_2EDTA solution as an anti-coagulant at pre-dose, 0.25, 0.5, 1, 2, 4, 6, 8 and 24 h [15-16]. Plasma was harvested by centrifuging the blood using Biofuge (Hereaus, Germany) at 1760g for 5 min and stored frozen at −80 ± 10°C until analysis. 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 distributed among 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, USA).

RESULTS AND DISCUSSION:

LIQUID CHROMATOGRAPHY:

The feasibility 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 0.1–0.5 mL/min) was tested for complete chromatographic resolution of duloxetine and IS (data not shown). The resolution of peaks was achieved with 0.2% formic acid: acetonitrile (20:80, v/v) with a flow rate of 0.40 mL/min, on a Symmetry Shield RP18 column (50 × 4.6 mm, 3.5 µm, Waters, UK) and was found to be suitable for the determination of electrospray response for duloxetine and IS (Figure.3).

MASS SPECTROSCOPY:

In order to optimize 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 297.90 and 515.10, respectively, as protonated molecular ions, [M + H]. Following detailed optimization of mass spectrometry conditions (provided in the instrumentation and chromatographic conditions section) the m/z 297.90 precursor ion to the m/z 154.10 was used for quantification of duloxetine. Similarly, for IS the m/z 515.10 precursor ion to the m/z 276.20 was used for quantification purpose.

RECOVERY:

A simple liquid–liquid extraction with TBME proved to be robust and provided the cleanest samples. The results of the comparison of neat standards vs plasma-extracted standards were estimated for duloxetine at 1.50 and 800 ng/mL and the mean recovery was found to be 75.31 ± 5.58 and 72.37 ± 2.44%, respectively. The recovery of IS at 200 ng/mL was 85.32 ± 6.92%.

MATRIX EFFECT, SPECIFICITY AND SELECTIVITY:

Average matrix factor values (matrix factor = response of post spiked concentrations/response of neat concentrations) obtained were +0.60 (CV: 3.21%, n = 6) and +0.71 (CV: 12.70%, n = 6) for duloxetine in rat plasma at QC low (1.50 ng/mL) and QC high (800 ng/ mL) concentrations, respectively. No significant peak area differences were observed. The matrix effect on IS was found to be +1.16 (CV: 14.95%, n = 6) at the tested concentration of 200 ng/ mL. Overall it was found that the plasma extract has a small impact on the ionization of analyte and IS. No interfering peaks from endogenous compounds are observed at the retention times of analyte and IS in the matrix (Data not shown). The retention times of duloxetine and IS were 1.72 and 1.75 min, respectively. The total chromatographic run time was 4.0 min.

CALIBRATION CURVE:

The plasma calibration curve was constructed using eight calibration standards (0.1–1500 ng/mL). The calibration standard curve had a reliable reproducibility over the standard concentrations across the calibration range. The calibration curve was prepared by determining the best fit of peak-area ratios (peak area analyte/peak area IS) vs concentration, and fitted to the y = mx + c using a weighing factor (1/X2). The average regression (n = 4) was found to be ≥0.996. The lowest concentration with the RSD < 20% was taken as LLOQ and was found to be 0.5ng/mL. The percentage accuracy observed for the mean of back-calculated concentrations for four calibration curves for duloxetine was within 90.1–113, while the precision (%CV) values ranged from 0.71 to 7.12.

ACCURACY AND PRECISION:

Accuracy and precision data for intra- and inter-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 1.50 and 800 ng/mL samples deviated within ±15% of the nominal concentrations in a battery of stability tests: in-injector (12 h), bench-top (6 h), repeated three freeze–thaw cycles and freezer stability at −80 ± 10°C for at lea st for 30 days. The results were found to be within the assay variability limits during the entire process.

***IN-VITRO* EVALUATION:**

Duloxetine resistance to acidic media is 99.80% for 2 hr in 0.1N Hcl dissolution media. These indicated that release of duloxetine was delayed greatly (Figure.4), suggesting that duloxetine delayed release pellets are stable at gastric pH and which will help to enhance the bioavailability.

***IN VIVO* STUDIES:**

The sensitivity and specificity of the assay were found to be sufficient for accurately characterizing the plasma pharmacokinetics of duloxetine in rats. Profiles of the mean plasma concentration vs time were shown in (Figure.5). Pharmacokinetic parameters were tabulated in (Table-2). Maximum concentration in plasma (Cmax 74.3 ± 6.31ng/mL, 116 ± 5.15ng/mL) was achieved at (0.5 h, 3.0h) Tmax respectively for duloxetine suspension and enteric coated pellets. The AUC $_{(0-t)}$ was (256 ± 8.1 h*ng /mL, 1030 ± 11.2 h*ng /mL) respectively for duloxetine suspension and enteric coated pellets. The higher sensitivity of this method compared with the current existing methods in literature facilitates the quantification of duloxetine at lower concentrations with high turnover.

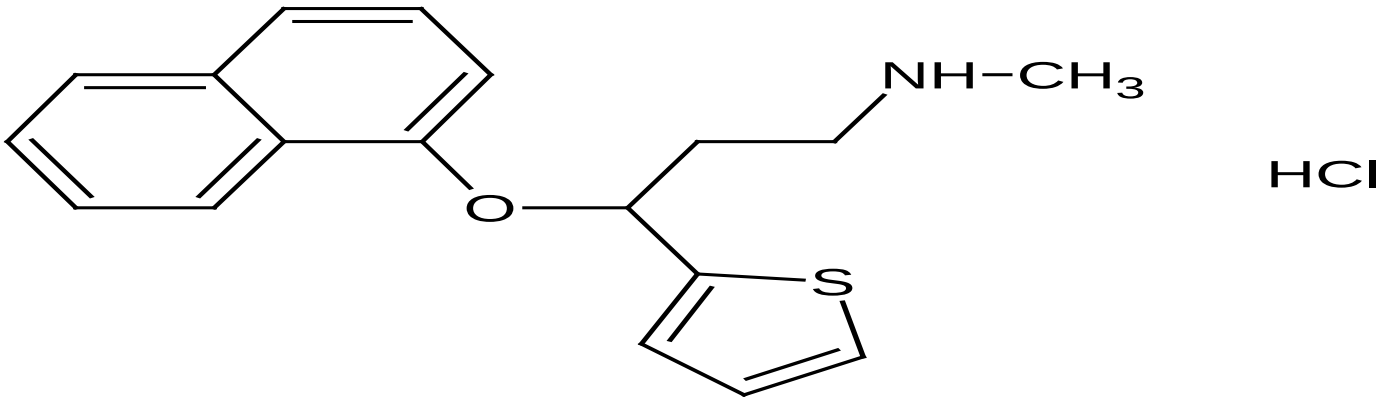

Figure No.1: Structural representation of Duloxitine hydrochloride

Figure No.2: A special devices designed for oral adminstration of enteric coated pellets to rats.

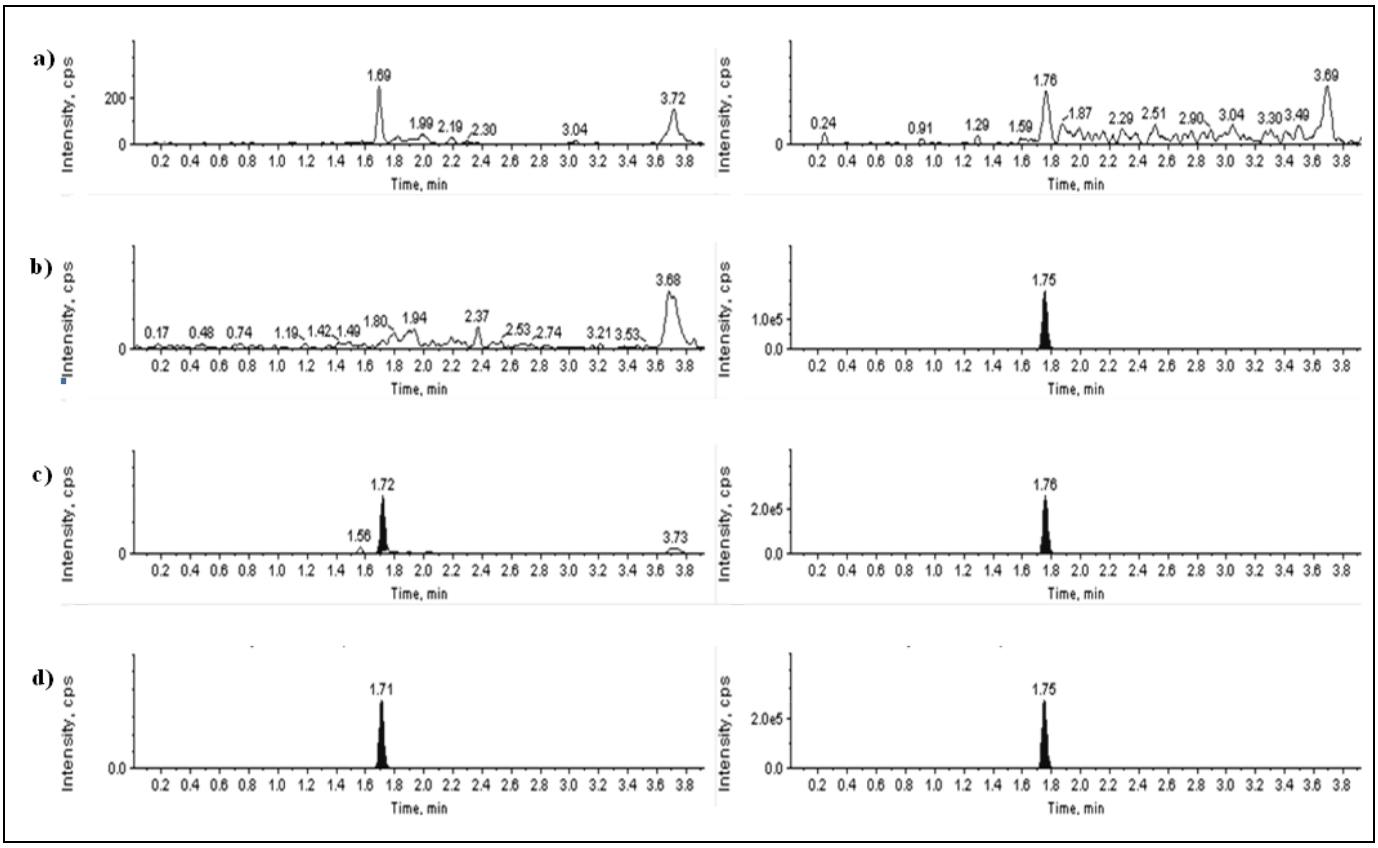

Figure No.3: Typical multiple reaction monitoring (MRM) chromatograms of duloxetine (left panel) and internal standard (right panel). a) Blank plasma, b) Blank palsma spiked with internal standard, c) Blank palsma spiked with duloxetine at LLOQ (0.1 ng/mL) and internal standard, d) Blank palsma spiked with duloxetine at ULOQ (1500ng/mL) and internal standard

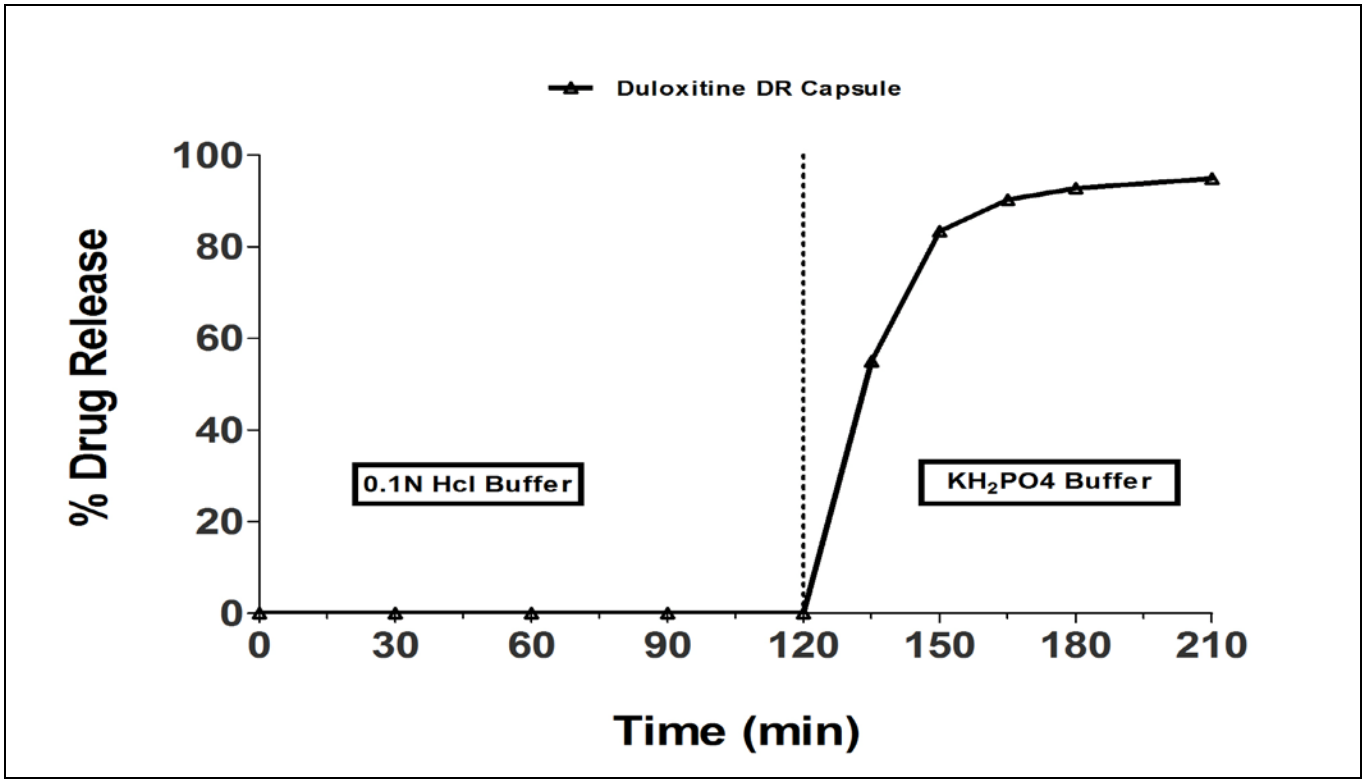

Figure No. 4: *In-vitro* stability of duloxitine enteric coated pellets at gastric pH

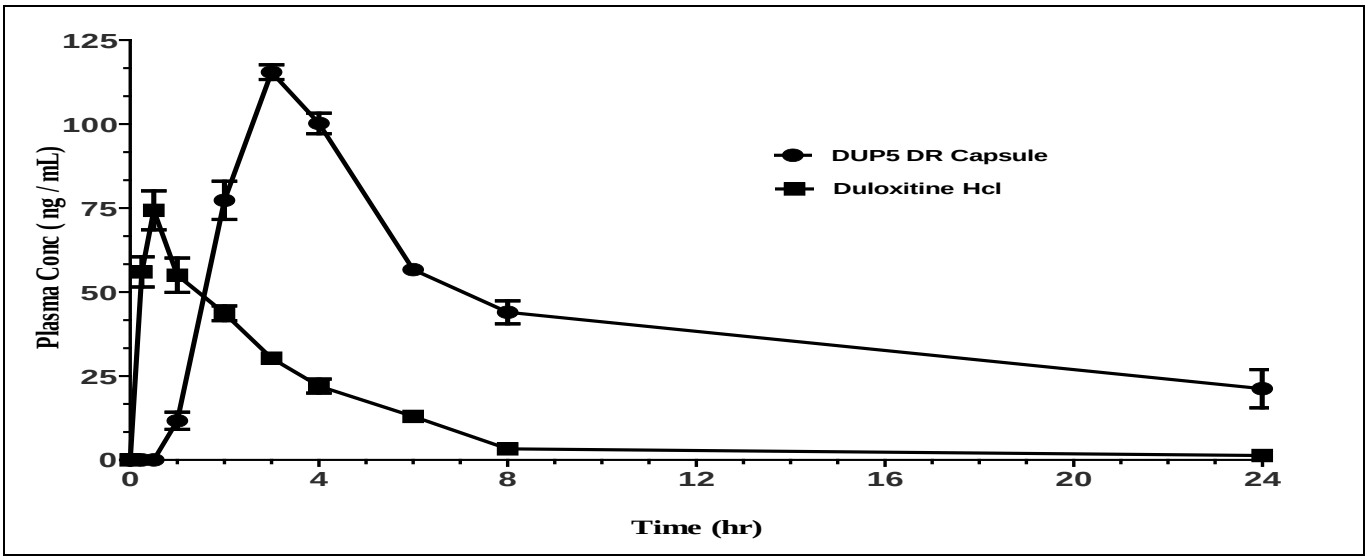

Figure No. 5: Mean ± SD plasma concentration–time profile of Duloxetine suspension (5mg/kg) and ECP (enteric coated pellets containing Duloxitine Hcl equivelent to 5mg/kg) in rat plasma following oral dosing of duloxitine to rats.

Intra-day variation (six replicates at each concentration)					
Theoretical concentration (ng/mL)	Run	Measured concentration (ng/mL)			
		Mean	SD	RSD	Accuracy %
0.10	1	0.11	0.08	1.19	110
	2	0.10	0.06	12.5	100
	3	0.12	0.09	10.1	120
	4	0.11	0.04	8.07	110
1.50	1	1.49	0.07	12.1	99.3
	2	1.55	0.07	8.47	103
	3	1.58	0.11	4.21	105
	4	1.53	0.08	2.74	102
500	1	495	2.21	1.90	99.0
	2	501	1.14	3.97	100
	3	514	5.08	5.78	103
	4	502	1.97	6.74	100
800	1	811	4.91	4.77	101
	2	809	4.28	8.41	101
	3	816	5.28	7.07	102
	4	819	6.37	4.71	102
Inter-day variation (twenty four replicates at each concentration)					
Theoretical concentration (ng/mL)		Mean	SD	RSD	Accuracy %
0.10		0.11	0.14	13.0	110
1.50		1.55	0.28	12.1	103
500		516	3.27	7.78	103
800		817	7.13	5.20	102

Table No. 1: Intra- and inter-day precision of determination of Duloxitine in rat plasma

RSD, Relative standard deviation (SD × 100/mean).

Group	Dose (mg/kg)	Cmax (ng/mL)	Tmax (hr)	AUClast (hr*ng/mL)
ECP	5	116	3.00	1030
Duloxetine Suspension	5	74.3	0.50	256

Table No. 2: Mean ± SD pharmacokinetic parameters of duloxitine (5mg/kg) and enteric coated pellets containing duloxitine equivalent to 5mg following oral dosing to rats.

CONCLUSION:

An optimized enteric coated pellet system for duloxetine was successfully developed with an increased acidic stability, dissolution rate and solubility, which ultimately increased the systemic exposure of duloxetine in rats. Simultaneously a method using LC-MS/MS for the determination of duloxetine in rat plasma employing simple liquid–liquid extraction was developed. The method is rapid, simple, specific and sensitive, and additionally demonstrates good accuracy and precision. Compared with the published methods, the present method features high selectivity and sensitivity with an LLOQ of 0.1ng/mL. We believe that this high-throughput method could provide a useful tool for the determination of in plasma. The established method was successfully applied to a rat pharmacokinetic study and to assess the plasma concentration.

ACKNOWLEDGEMENTS:

The authors are thankful to Dr. Vinay babu, (MD), Bioneed preclinical services Pvt Ltd. for providing facilities to carry out the work and for his valuable suggestions during the work.

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