Validation of an UPLC-MS/MS method for simultaneous Quantification of two Selective Serotonin and nor Epinephrine Reuptake Inhibitors and one Selective Serotonin Reuptake Inhibitor in human plasma

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ABSTRACT

A simple and sensitive Ultra Performance Liquid chromatography-mass spectrometry (UPLC-MS/MS) method was developed and validated for simultaneous estimation of two Selective Serotonin and Nor Epinephrine Reuptake Inhibitors (Duloxetine &Venlafaxine) and one Selective Serotonin Reuptake Inhibitor (Paroxetine) in human plasma using Sertraline as an internal standard. The above mentioned compounds and the internal standard were extracted from 0.5 ml plasma by solid phase extraction method. The analytical separation was carried out in a reverse liquid chromatography at acidic pH 3.9 at isocratic mode. All analytes were monitored in multiple reaction monitoring mode using the respective $[M+H]^+$ ions, m/z 298.06/153.90 for Duloxetine, 279.20/57.75 for Venlafaxine,330.61/162.83 for Paroxetine and m/z 306.14/158.71 for the internal standard. The proposed method was validated with linear range of 0.5 -100ng/ml for Duloxetine, Venlafaxine and Paroxetine. The %R.S.D of intra-day and inter-day assay was lower than 15%. For its sensitivity and reliability, the proposed method is particularly suitable for pharmacokinetic studies.

Key words: SSNRI, SSRI, Duloxetine, Venlafaxine, Paroxetine, UPLC-MS/MS

INTRODUCTION

Antidepressant drugs are widely used in different psychiatric disorders and these drugs are frequently encountered in emergency toxicology screening, drug-abuse testing and forensic medical examinations [1]. Various methods for determination of antidepressant drugs have been reported, including high-performance liquid chromatography (HPLC) [2], Capillary gas chromatography [3], gas chromatography/mass spectrometry (GC–MS) [4] and high-performance liquid chromatography–mass spectrometry (LC–MS) [5].Most of the LC-MS method shows determination of one compound and its main metabolites [6–9], or some compounds belonging to the same antidepressant group [10–12].

The present study describes development and validation of an UPLC-MS/MS method for the estimation of three main marketed anti depressant drugs Duloxetine, Venlafaxine and Paroxetine belonging to the category Selective Serotonin and Nor Epinephrine Reuptake Inhibitors and Selective Serotonin Reuptake Inhibitor, in human plasma. Duloxetine hydrochloride [(+)-(S)-N-methyl-3-(1-naphthalenyloxy)-2-thiophenepropanamine hydrochloride] is a balanced selective serotonin and nor epinephrine-reuptake inhibitor and it has been approved by the US Food and Drug Administration for the treatment of major depressive disorder (MDD) [13], and diabetic peripheral neuropathic pain (DPNP) [14]. The empirical formula is $C_{18}H_{19}NOS \cdot HCl$, *Fig.1 (a)* which corresponds to a molecular weight of 333.88. [15].
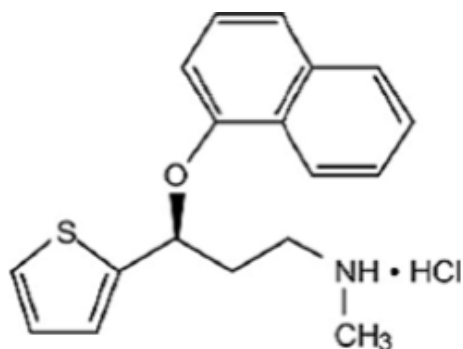
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Fig.1 (a) - Chemical structure of Duloxetine. - [(+)-(S)-N-methyl-3-(1-naphthalenyloxy)-2-thiophenepropanamine hydrochloride]

Venlafaxine (1-[2-(dimethylamino)-1-(4-methoxyphenyl) ethyl] cyclohexanol, and it is a second generation antidepressant drug [16]. It is one of the most potent selective serotonin and nor epinephrine reuptake inhibitors (SNRI), and its therapeutic effects are attributed to this activity. It has also been used for patients who are non-responders to SSRI or whose response to these drugs decreases over time [17].Venlafaxine has the empirical formula of $C_{17}H_{27}NO_2$ HCl *Fig.1(b)* and its molecular weight is 313.87[18].
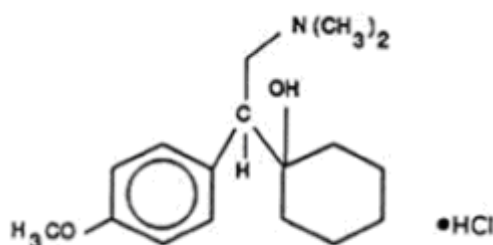

Fig.1 (b) - Chemical structure of Venlafaxine - (1-[2-(dimethylamino)-1-(4-methoxyphenyl) ethyl] cyclohexanol.

Paroxetine ((3S, 4R)-3-[(1, 3-benzodioxol-5-yloxy) methyl]-4-(4-fluorophenyl) piperidine, is a second generation antidepressant drug whose therapeutic activity is due to the selective inhibition of the reuptake of serotonin (SSRI). It is also prescribed in the treatment of related disorders, such as obsessive-compulsive disorder, panic fits, social phobia, post-traumatic stress and pavor nocturnus [19] and ADHD[20] It has the empirical formula of $C_{19}H_{20}FNO_3•HCl•1/2H_2O$ Fig.1(c).
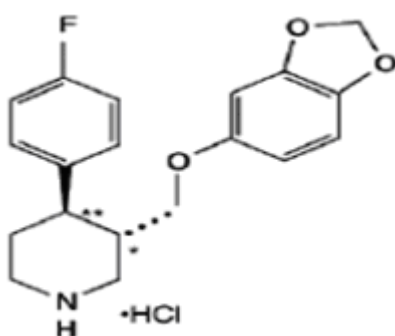

Fig.1 (c) - Chemical structure of Paroxetine - ((3S, 4R)-3-[(1, 3-benzodioxol-5-yloxy) methyl]-4-(4-fluorophenyl) piperidine.

The molecular weight is 374.8 (329.4 as free base) [21].The internal standard used in this method was Sertraline HCL. Sertraline hydrochloride is a Selective Serotonin Reuptake Inhibitor (SSRI) and it has a molecular weight of 342.7[22]. Sertraline hydrochloride has the following chemical name: (1S-cis)-4-(3, 4-dichlorophenyl)-l, 2, 3, 4-tetrahydro-N-methyl-l-naphthalenamine hydrochloride. The empirical formula C17H17NCl2•HCL. *Fig.1 (d)*
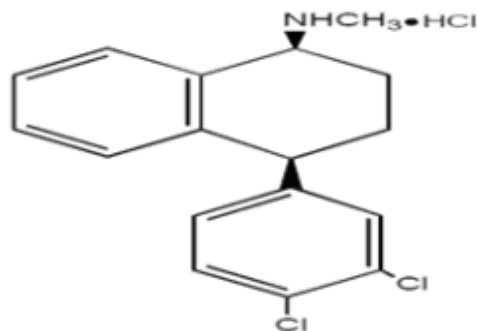
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Fig.1 (d) - Chemical structure of Sertraline- (1S-cis)-4-(3, 4-dichlorophenyl)-l, 2, 3, 4-tetrahydro-N-methyl-l-naphthalenamine hydrochloride.

MATERIALS AND METHODS

Chemicals & Reagents

Duloxetine HCl (purity 99.5%), Venlafaxine HCL (purity 99.3%), Paroxetine HCL (purity 100.1%) & Sertraline HCl (purity 99.6%) were obtained from Orchid Chemicals and pharmaceuticals ltd. Methanol (HPLC grade, manufactured by J.T. Baker), Acetonitrile (HPLC grade, manufactured by J.T. Baker), Water (Milli Q water), Ammonium acetate (AR grade, manufactured by Merck India. ltd), Formic acid (AR Grade, Manufactured by S.D fine chemicals Ltd). Blank human plasma received from VHS blood bank, Chennai.

Preparation of Standard and Quality control samples

The stock solution of Duloxetine, Venlafaxine, Paroxetine and Sertraline internal standard was prepared by dissolving the accurately weighed reference compounds in Methanol to give a final concentration of 1 mg/mL, stored at 2-8°C in the refrigerator and is used for a maximum of 5 days. The solutions were then serially diluted with Methanol–water (50:50, v/v) to obtain standard working solutions at concentrations of 2.0µg/ml,1.6 µg/ml,1.2 µg/ml,0.8 µg/ml,0.4 µg/ml,0.2 µg/ml,0.1 µg/ml,0.020 µg/ml,0.010 µg/ml separately. All the solutions were stored at 2-8°C and were brought to room temperature before use. Calibration solutions were prepared by spiking blank human plasma with standard solutions of each drug standard to give concentrations of 0.5, 1.0, 5.0, 10.0, 20.0, 40, 60, 80, and 100.0 ng/ml. Quality control (QC) samples, which were used both in pre study validation and during each experimental run of the validation study, were prepared by spiking control human plasma with standard solutions of each drug standard solutions to give concentrations of 1.5, 30.0 and 70 ng /ml.

Preparation of Plasma Samples for estimation

To 500 µL of spiked plasma sample in a clean vial, 25µl of Internal solution was admixed and vortexed for 60sec. The analytes were seperated in OASIS HLB solid phase extraction cartridges using 1ml of 0.1%Ammonia in Acetonitrile solution as eluent. Separated mixture was transferred and 10 µL of the supernatant was directly injected onto the UPLC/MS/MS system.

Instrumentation
UPLC-and Mass spectrometric conditions

Acquity binary solvent manager and an Acquity sample Manager were used for solvent and sample delivery.

Chromatographic separation was achieved by using Acquity 100 x 2.mm, 1.7µm, and 10 µL at column temperature 45°C. The mobile phase consisted of Acetonitrile–Ammonium Acetate buffer pH 3.9 (80:20) pumped at a flow rate of 0.2mL/min. Total run time was 3.0 min for each injection.

A Waters Micro mass Quattro premier mass spectrometer equipped with an ESI source was used for mass analysis and detection. Mass spectrometric analysis was performed in the positive ion mode (ESI+) and set up in the multiple reaction monitoring (MRM) mode. Nitrogen was used as desolvation gas (800L/Hr) and cone gas (100 L/Hr). The capillary temperature was 3.30kV. Cone voltage was 15 V.

Argon was used as the collision gas and the collision energy used for Duloxetine was 10 V, 15V for Venlafaxine, 24V for Paroxetine &25V for internal standard. Based on the full-scan mass spectra of the analytes, the most abundant ions were selected and the mass spectrometer was set to monitor the transitions of the precursors to the product ions as *m/z* 298.06/153.90 for Duloxetine,279.20/57.75 for Venlafaxine,330.61/162.83 for Paroxetine and

m/z 306.14/158.71 for the internal standard. The scan time for each analyte was set to 0.1 s. Data acquisition, Peak integration and calibration were performed with MassLynx 4.0 software.
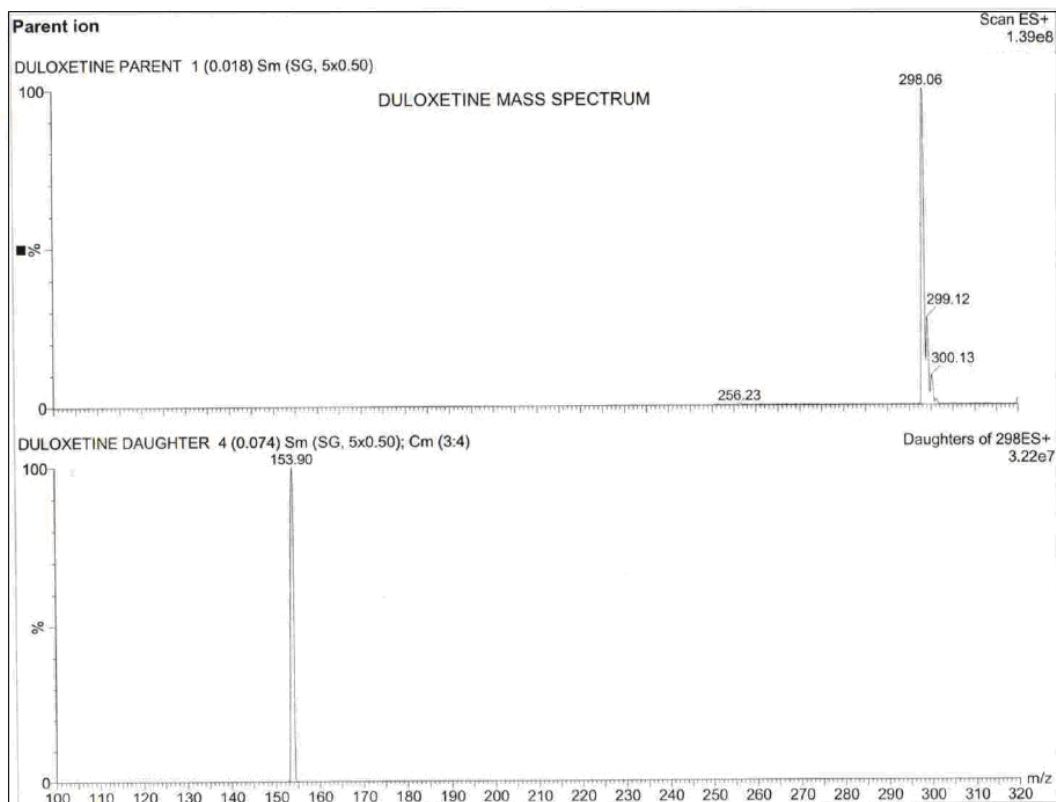

Fig.2 (a).Mass spectrum of Duloxetine

Fig.2 (b).Mass spectrum of Venlafaxine
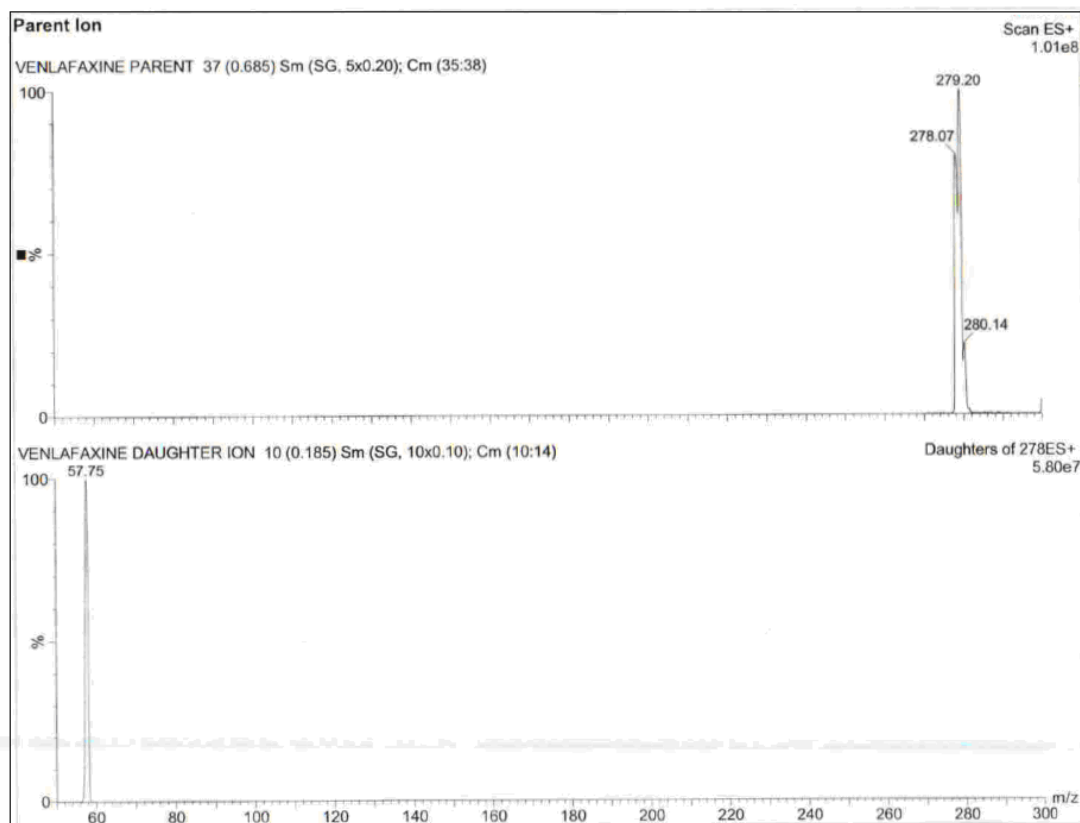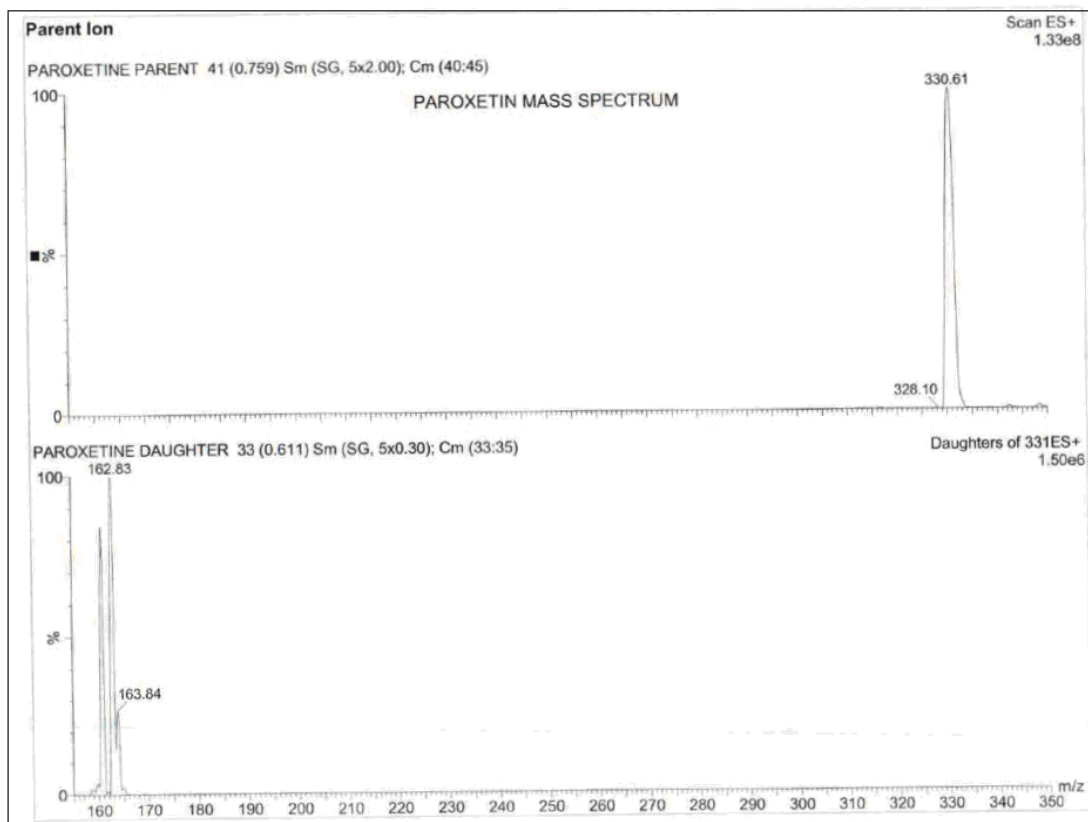
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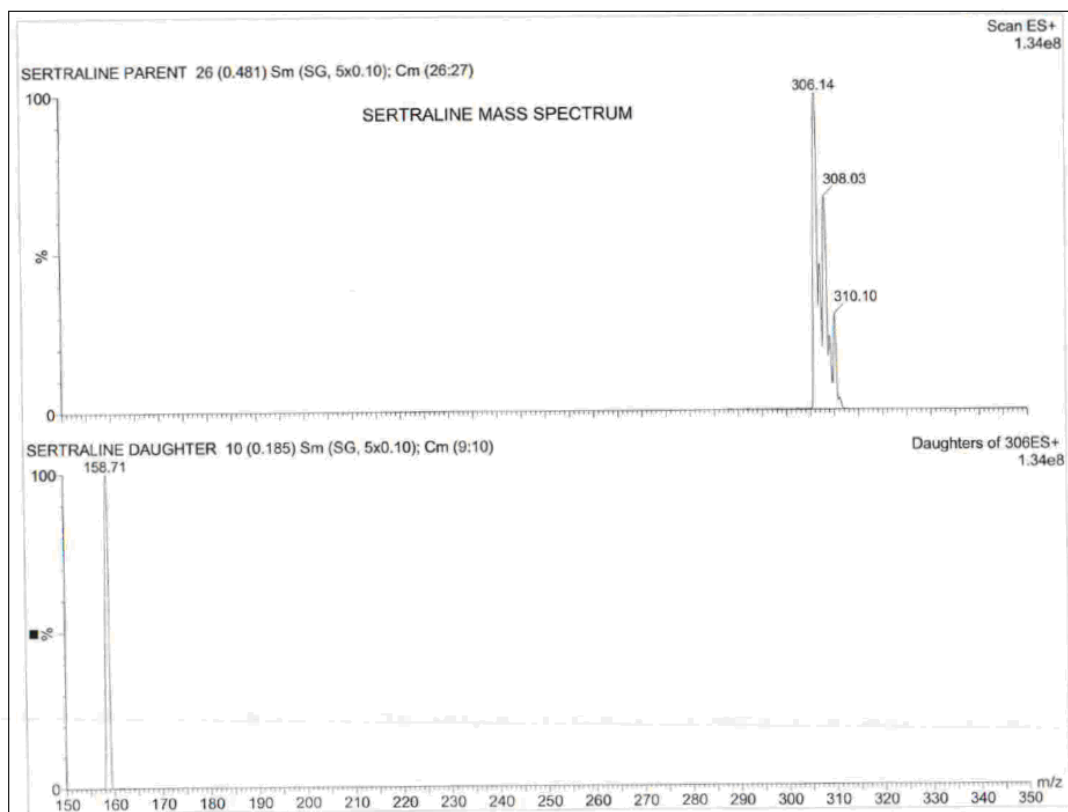**Fig.2 (c).Mass spectrum of Paroxetine**

Fig.2 (d).Mass spectrum of Internal standard (Sertraline)

Method Validation

The method was validated for Specificity, Accuracy, Precision, Matrix effect, sensitivity, bench Top stability, Auto sampler stability, Freeze thaw stability, Recovery, & Linearity according to the FDA guideline for Validation of bio

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analytical methods (FDA, 2001)[23]. The Specificity was investigated by preparing and analyzing six individual human blank plasma samples at LLOQ level. The LLOQ was defined as the lowest concentration of the analyte measured with acceptable precision and accuracy [relative standard deviation (RSD) and relative error ≤20%], and the analytes response at this concentration level was NMT 5 times the baseline noise. Linearity was assessed by analyzing Analyte standards (0.5–100 ng /ml) in human plasma. Calibration curves were analyzed by weighted linear regression ($1/x$) of assayed–nominal drug peak area ratios. Accuracy and precision were assessed by determining QC samples at three concentration levels (six samples each concentration) on three different validation days. The precision as determined as %RSD and the accuracy was expressed as a percentage of the nominal concentration. The criteria used to assess the suitability of precision and accuracy was as follows: the RSD should not exceed 15% and the accuracy should be within 85 - 115%. Furthermore, the recovery (extraction efficiency) of analyte from human plasma was determined by comparing the areas of spiked plasma samples before and after sample processing.

The stability of analyte was assessed by determining QC samples at three concentrations (six samples each). The stability studies included: (a) stability at room temperature (22–25°C) for 4 h; (b) stability after two freeze–thaw cycles; (c) stability of the extracted samples at room temperature (22–25°C) for 12 h; and (d) the long-term stock solution stability at 4°C for 5 days. During routine analysis, each analytical run included blank plasma, blank plasma with internal standard, a set of calibration samples, a set of QC samples and unknowns.

RESULTS AND DISCUSSION

Method development
In this study, ESI was chosen as the ionization source. It was found that the signal intensity of analytes in human plasma was high using ESI source and the ESI source provided satisfactory data on method validation and subsequent quantitation for plasma samples from healthy volunteers. By ESI, the analytes formed predominantly protonated molecules [M+H] $^+$ ions in full-scan spectra. To determine antidepressant analytes using MRM mode, full-scan and product ion spectra of the analyte were investigated. The most abundant ion in the product ion mass spectrum was at 153.90 for Duloxetine, 57.75 for Venlafaxine, 162.83 for Paroxetine & 158.71 for the internal standard which is presented in Fig. 2(a), 2(b), 2(c) &2(d).

It was found that the capillary temperature and the spray voltage did not significantly influence the MS behaviour of the analyte and remained unchanged at the recommended value of 350°C and 3.3 kV. Therefore, the SRM transition of *m/z* [298.06/153.90, 279.20/57.75, 330.61/162.83 and 306.14/158.71] was selected to obtain maximum sensitivity. In the present study, a simple Solid Phase Extraction technique was used. All selected analytes were not detectable with protein precipitation and inconsistent with liquid-liquid extraction during our method development. On the other hand, it was found that the extraction efficiency was increased when Solid phase Extraction (SPE) using 0.1%Ammonia in Acetonitrile solution as extraction solvent. The % recovery was increased when the ratio changed from 0.5%, 1.5% & 2.0%. A mobile phase consisting of Acetonitrile-ammonium acetate buffer pH3.9 (80:20) was finally used. Each chromatographic run was completed within 3.0 min.

Method validation
Specificity
 The UPLC/MS/MS method demonstrated high specificity because only ions derived from the analytes of interest were monitored. The selectivity towards endogenous plasma matrix was tested in six different batches of human plasma samples by analyzing blanks and samples at LLOQ levels. Observing the chromatographs indicated no significant visible interference at the expected retention times of the selected analytes was modified to elute in a region where visible interference is not observed. The method had the shortest total running time (3.0 min) for determination of various anti depressant drugs in human plasma compared with those reported in the literature [24].

Matrix effects
 To evaluate the absolute matrix effect, i.e. the potential ion suppression or enhancement due to the matrix components, six different batches of blank plasma were eluted by 0.1%Ammonia in Acetonitrile solution and then spiked with the analyte at QC concentrations. The corresponding peak areas of the analyte in spiked plasma post-extraction (*B*) were then compared with those of the aqueous standards in mobile phase (*A*) at equivalent concentrations. The ratio (*B/A* x□100) is defined as the ME (Matrix Effect). A ME value of 100% indicates that the response in the mobile phase and in the plasma extracts was the same and no absolute matrix effect was observed. A value of >100% indicates ionization enhancement, and a value of <100% indicates ionization suppression. The result of ME at QC concentrations of selected analytes in five different lots of human plasma shows that there was ME, as indicated by values of >100% in the area of the analyte in spiked plasma samples post-extraction. This indicated ionization enhancement for selected analytes under the present chromatographic and extraction conditions

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when ESI interface was employed. Fortunately, the ionization enhancement observed was similar and kept consistent over the QC concentration ranges of the analyte (1.5 – 70 ng/ml) without showing any analyte concentration-dependence as well as for different lots of human plasma. Moreover, such ionization did not affect the slopes and linearity of the established calibration curves over the whole analytical period. The assessment of the relative ME was made by a direct comparison of the analyte peak area values between different lots (sources) of plasma. The variability in the values, expressed as RSD (%), is a measure of the relative ME for the target analyte. The variability was acceptable with an RSD value of 1.7% at different concentrations of analyte in five different lots of human plasma, indicating that the relative ME for the analyte was minimal in this study. In the present study, an ionization enhancement effect due to the undetected matrix components in human plasma was observed. However, such ionization enhancement remained consistent over the QC concentration ranges of the analyte without showing any analyte concentration-dependence and did not significantly affect the behaviours of calibrations curves, precision and accuracy data. Thus, despite the presence of the ME, the present analytical method was reliable.

Linearity and lower limit of quantification

The slope, the intercept and the correlation coefficient (*r*) for each standard curve from each analytical run were determined automatically by Mass Lynx software programme. The concentration range was estimated on the basis of the regression curve (y= a × x + b) and correlation coefficient. For each point on the calibration curves for the analyte, the concentrations back-calculated from the equation of the regression analysis were within acceptable limits for accuracy and precision of ±15%. Overall, selected anti depressant drugs gave linear response as a function of the concentration ranges studied and showed excellent linearity over 0.5 - 100 ng/ml *[Fig.3]*
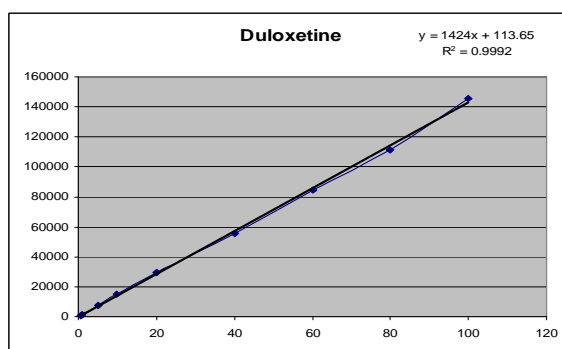

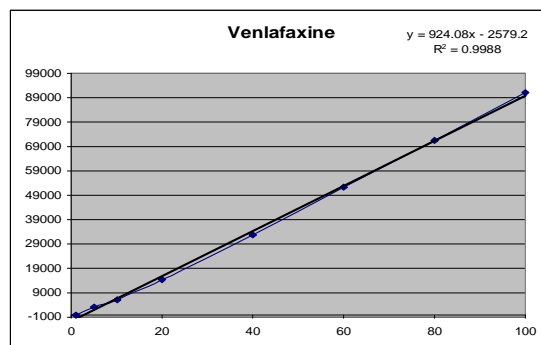**Fig.3 (a). Representative Linearity curve of Duloxetine**

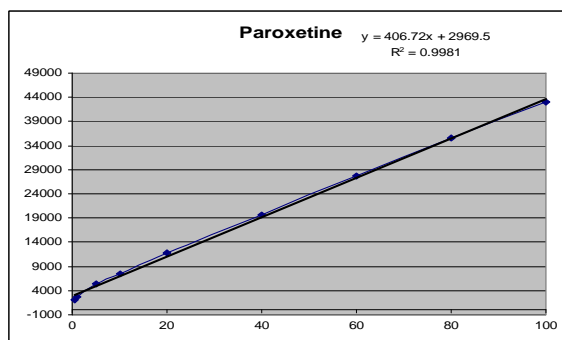**Fig.3 (b). Representative Linearity curve of Venlafaxine**

Fig.3 (b). Representative Linearity curve of Paroxetine

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The lowest concentration on the calibration curve was 0.5ng/ml. The analyte response at these concentration levels was >20 times the baseline noise. The precision and accuracy at these concentration levels were acceptable and within the acceptance criteria. Thus, the lowest concentration on the calibration curve was accepted as the LLOQ. However, the LLOQ could be lowered by injecting a more concentrated solution into the UPLC/MS/MS system. However, the current LLOQ (Achieved for 0.5 mL samples) was already sufficient for the estimation in human plasma.

Precision and accuracy
The intra-batch and inter batch precision and accuracy data for selected analytes are summarized in Table 1.

All values of accuracy and precision were within recommended limits (FDA, 2001) [22].

The intra-batch precision for Duloxetine was 0.618 to 5.013% and accuracy was 93.067 to 100.278%. Intra batch precision for Venlafaxine was 0.126 to 3.818% and accuracy was 97.333 to 100.800%. For Paroxetine intra batch precision was 0.499 to 8.311 & accuracy was 96.533 to 100.0.

The inter-batch precision& Accuracy for Duloxetine was 0.314 to 0.796 and 94.318 to 99.684% respectively, for Venlafaxine 0.333 to 0.986% and 98.700 to 100.148% respectively and for Paroxetine 0.376 to 2.246% & 97.533 to 99.818% respectively.

Table-1 Summary of Precision and Accuracy Batch

	Analyte Name	Duloxetine				Venlafaxine				Paroxetine			
	Analyte conc. level	LLOQ QC	Low QC	Mid QC	High QC	LLOQ QC	Low QC	Mid QC	High QC	LLOQ QC	Low QC	Mid QC	High QC
Intra Batch P&A 1	Mean Conc. (ng/ml) n.=6*	0.465	1.477	29.766	69.916	0.487	1.504	30.070	70.026	0.483	1.498	29.965	70.000
	±SD	0.022	0.030	0.444	0.432	0.013	0.016	0.124	0.088	0.020	0.024	0.584	0.349
	%CV	4.752	2.000	1.492	0.618	2.697	1.065	0.411	0.126	4.133	1.581	1.950	0.499
	%Nominal	93.067	98.478	99.220	99.880	97.333	100.289	100.234	100.036	96.533	99.833	99.884	100.000
Intra Batch P&A 2	Mean Conc. (ng/ml) n.=6*	0.476	1.502	30.022	69.537	0.495	1.512	29.982	69.909	0.494	1.480	29.730	69.909
	±SD	0.024	0.023	0.259	0.881	0.009	0.017	0.086	0.518	0.041	0.034	0.353	0.518
	%CV	5.043	1.521	0.863	1.267	1.905	1.151	0.286	0.741	8.311	2.303	1.187	0.741
	%Nominal	95.120	100.144	100.073	99.338	98.900	100.800	99.941	99.870	98.767	98.644	99.101	99.870
Intra Batch P&A 3	Mean Conc. (ng/ml) n.=6*	0.474	1.504	29.928	69.667	0.499	1.490	29.902	70.331	0.487	1.477	29.799	69.708
	±SD	0.020	0.046	0.381	0.627	0.019	0.026	0.358	0.145	0.036	0.032	0.352	0.894
	%CV	4.192	3.055	1.273	0.900	3.818	1.730	1.197	0.207	7.318	2.193	1.181	1.283
	%Nominal	94.767	100.278	99.760	99.524	99.867	99.356	99.674	100.473	97.300	98.433	99.329	99.583
Inter Batch P&A	Mean Conc. (ng/ml) n.=6*	0.472	1.495	29.905	69.707	0.494	1.502	29.985	70.089	0.488	1.485	29.831	69.872
	±SD	0.002	0.012	0.094	0.225	0.005	0.005	0.148	0.234	0.011	0.006	0.134	0.279
	%CV	0.438	0.796	0.314	0.323	0.986	0.352	0.492	0.333	2.246	0.376	0.449	0.399
	%Nominal	94.318	99.633	99.684	99.581	98.700	100.148	99.950	100.126	97.533	98.970	99.438	99.818

P&A: Precision and Accuracy,   SD: Standard Deviation, CV: Coefficient of Variance
*LLOQ: Lower limit of Quantitation,   QC: Quality control, *n = number of samples*

Recovery
Table- 2 shows the recovery (extraction efficiency) of Selected Anti depressant drugs from human plasma. The mean recovery as 80.022% to 87.434% for Duloxetine, 79.03% to 88.626% for Venlafaxine and 81.561% to 83.996% at all different concentrations, which indicated that the extraction efficiency using 0.1% Ammonia in Acetonitrile solution was satisfactory without concentration dependence.

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Table-2   Summary of Recovery Studies

Analyte name	Duloxetine			Venlafaxine			Paroxetine		
Analyte Conc. Level	Low QC	Mid MQC	High QC	Low QC	Mid MQC	High QC	Low QC	Mid MQC	High QC
%Mean recovery	80.022	81.341	87.434	79.030	80.544	88.626	81.561	80.327	83.996

Stability
The stability of Selected Anti depressant drugs in human plasma under different storage conditions are presented in Table 3. No degradation products were detected under the selected MS conditions. Hence Selected Anti depressant drugs in human plasma can therefore be stored at room temperature (25°C) for 4hrs and at -20°C and after two freeze–thaw cycles.

These results indicate that selected analytes are stable under routine laboratory conditions and no specific procedure (e.g. acidification or addition of organic solvents) is needed to stabilize the compounds for daily clinical drug monitoring.

Table-3   Summary of Stability Studies

Analyte name	Duloxetine		Venlafaxine		Paroxetine	
Analyte concentration level	Low QC	High QC	Low QC	High QC	Low QC	High QC
Bench Top stability (%Mean stability, n=6*)	99.149	99.703	99.634	99.929	99.259	99.370
Auto sampler Stability (%Mean stability, n=6*)	98.030	99.040	98.959	99.770	99.502	98.293
Freeze Thaw stability (%Mean stability, n=6*)	98.243	98.667	98.094	99.153	97.633	99.354

n = number of samples

CONCLUSION

In this study, we reported on a newly developed and validated UPLC/ MS/MS method for the determination of SSNRI and SSRI drugs in human plasma. The sample pre-treatment was easy and extraction efficiency was more with 0.1% Ammonia in Acetonitrile. The selected analytes were subjected to UPLC/ MS/MS analysis using ESI technique with satisfactory mass spectral response generated. Detailed validation following FDA guideline indicated that the developed method had high sensitivity, reliability, specificity and excellent efficiency with a total running time of 3.0 min per sample. The method was successfully applied to pharmacokinetic studies and Therapeutic Drug Monitoring (TDM) of selected drugs in human plasma.

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