Quantification of Fluoxetine in Human Plasma by the Development of Liquid Chromatography-tandem Mass Spectrometry Method

Geeta Negi[1], Jaseela Majeed[2]*, Saurabh Arora[3], Sachin Rana[3], Manoj Singh[3], Jaideep Tomar[3]

[1]Department of Pharmaceutical Chemistry, School of Pharmaceutical Sciences, Delhi Pharmaceutical Sciences and Research University, New Delhi, INDIA.
[2]School of Allied Health Sciences, Delhi Pharmaceutical Sciences and Research University, New Delhi, INDIA.
[3]Arbro Analytical Division, Arbro Pharmaceuticals Private Limited, Kirti Nagar Industrial Area, Kirti Nagar, New Delhi, INDIA.

ABSTRACT

Aim: The aim of this research paper is the development and validation of an easy, selective and sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the quantification of fluoxetine in human K3EDTA plasma and application of this method on bioequivalence studies of fluoxetine. **Methods:** As Amitriptyline belongs to same category drug so, it was selected as an internal standard for the quantification of fluoxetine. The protein precipitation (PPT) method was used to extract analyte from 250 μl aliquot of human plasma. Chromatographic separation was achieved on BDS Hypersil C$_{18}$ (50 x 4.6 mm, 5 μm) column at in 4.0 min run time using isocratic mobile phase consisting of acetonitrile and mixture of ammonium acetate containing 0.15% formic acid (55:45 % v/v) at a flow rate of 0.5 ml/min. The ionization was carried out through Electron spray ionization (ESI) operating in positive ion mode and detection was via multiple reaction monitoring (MRM) acquisition mode using the respective *m/z* 310.1→44.2 for fluoxetine and 278.1→233.1 for IS. The method was validated to be linear over the concentration range 0.25 to 40.00 ng/ml. **Results:** This LC-MS/MS method was found to be accurate and precise with intra-batch and inter-batch accuracy (% Nominal) of ±15 % and precision of <15 % and the method was successfully applied in analyzing human plasma samples of fluoxetine.

Key words: Fluoxetine, LC-MS/MS, Amitriptyline, Human plasma, Protein Precipitation, Pharmacokinetic.

INTRODUCTION

Depression is the most common mental illness in United States and other countries as well as it is the second leading cause of death among people ages between 15 to 24. According to World Health Organisation (WHO),[1] 100 million people in the worldwide suffers from depression of which 16.2 million adults in United States have experienced a major depressive disorder. Depression has become the leading cause of disability worldwide. Since, Fluoxetine has fewer side effects, it is recommended as a Drug of Choice over Tricyclic antidepressants.[2] Fluoxetine shown in Figure 1 (N-methyl-3-phenyl-3-[4-(trifluoromethyl)phenoxy] propan-1-amine, sold under the brand name Prozac®), is the most stimulating selective serotonin-reuptake inhibitors (SSRIs) which belongs to the class of antidepressants. It is the first agent anti-depressant drug, used to treat major depressive disorder (MDD), premenstrual dysphoric disorder (PMDD), moderate to severe bulimia nervosa and obsessive-compulsive disorder (OCD). It also treats bipolar I depression when given in combination with olanzapin.[3] Fluoxetine undergoes first pass metabolism in which it gets metabolized into its active metabolite. Norfluoxetine.[4] Norfluoxetine tends to have same pharmacologic potency as
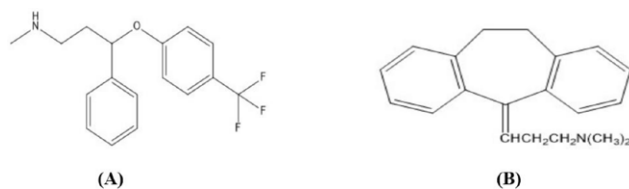
Figure 1: Structure of (A) Fluoxetine and (B) Amitriptyline.

of fluoxetine. The generation of norfluoxetine from fluoxetine generally occurs in 7-8 days due to which the elimination life of fluoxetine gets longer, so, it is very difficult to collect blood samples from human volunteers for longer duration. Therefore, bioanalytical methods are developed to focus only on biological samples for the detection of fluoxetine.[5]

Literature review shows that various methods have been used for the determination of the fluoxetine and its metabolite in human plasma. It includes high performance liquid chromatography (HPLC)[6] with UV,[7] fluorescence[8] detection, Gas chromatography-MS (GC-MS)[9] and LC-MS/MS method.[4,10,11] Similarly, several HPLC methods has been developed with electrochemical detection[12], UV[13] and LC-MS/MS[14,15] to determine other SSRIs. Some LC-MS/MS methods has also been reported which shows simultaneous quantification of fluoxetine combination with other antidepressant drugs in which Solid phase extraction (SPE) and Liquid-liquid extraction (LLE) method were used as the extraction procedure.[16] However to the best of our knowledge, there is a single article reported yet on determination of the single analyte fluoxetine by LC-MS/MS method.[17]

Since, the different article presented for determination of fluoxetine by LC-MS/MS method whether as a single analyte or in combination consists of SPE and LLE extraction method and so, this article provides benefit over them as it is based on Protein precipitation (PPT) extraction procedure. This provides an advantageous over previous approaches as the PPT method discussed in this article as it is easier, cost effective and high throughput method, also it aims at better results of recovery accuracy and precision when compared to other studies. And the mobile phase, extraction solution and the column used are also common and easily accessible. So, the main approach of this study is to develop a simple, sensitive, rapid, cost effective and reliable LC-MS/MS method for the determination of fluoxetine.

MATERIALS AND METHODS

Instrumentation and equipment

A 6460 Triple Quad/LCMS system, 1290 Infinity II HPLC system (Agilent Technologies) and Mass Hunter Workstation Software version B.08.00, Rota 4R-V/FA Refrigerated centrifuge (Plasto Crafts, India), CY 64 Analytical Digital balance (Citizen, India), Brand micro pipette (BrandTech Scientific, USA), Power sonic 410 ultrasonic bath (Power Sonic, USA), Vibramax 110 Vortexer (Heidolph, India), Deep freezer (-20±5°C) Vestfrost Solutions and (-80±15°C) Sanyo Electric Co. Ltd., Nitrogen evaporator (KeMi Concentrator), EuTech Instruments pH tutor pH meter (Aarkey Laboratories Ltd., India).

Reagents

HPLC grade acetonitrile and methanol was obtained from J. T. Baker while HPLC grade ammonium acetate, isopropyl alcohol and water was obtained from Qualigens fine chemicals, Inida. The formic acid (analytical grade) was procured from Acros Organics.

Test Compound and IS

Reference standard of Fluoxetine was purchased from Vivan Life Sciences Pvt. Ltd., Mumbai, India and Amitriptyline (IS) (shown in Figure 1) was obtained from Arbor Pharmaceuticals Pvt. Ltd., Delhi. The Fluoxetine and IS was having percentage purity of 99.72% and 99.20 % respectively.

Biological Matrix

The human plasma with K3EDTA anticoagulant was used for the preparation of calibration standards (CC) and quality control (QC) samples and the analyte was suitably extracted to remove the interfering substances.

Preparation of Buffer and Mobile Phase

Buffer-1 (10mM ammonium acetate containing 0.15% of formic acid)

Approximately 770 mg of Ammonium acetate was weighed and transferred to 1000 ml reagent bottle containing 200 ml HPLC grade/milli-Q water into it. The solution was mixed and the volume was made up to the mark. Then, 1.5 ml of Formic acid was added to it. The solution was shaken and sonicated to degas in an ultrasonic bath to get a buffer.

Mobile phase (Acetonitrile: Buffer-1: 55:45)

The mobile phase was prepared by transferring 550 ml of acetonitrile to 1000 ml reagent bottle, followed by the addition of 450 ml of Buffer-1. The mixture was mixed well, sonicated and degassed in an ultrasonic water bath. The mobile phase was used within 3 days from the date of its preparation and appropriate proportion was taken to prepare different volumes of solution and stored at room temperature.

Diluent solution (Methanol 50% v/v in water)

500 ml of HPLC grade Methanol was transferred into a 1000 ml reagent bottle, followed by the addition of 500 ml of HPLC grade water and mixed well. Sonicated and degassed in ultrasonic bath. Stored at room temperature and used within 3 days from the date of its preparation.

Experimental Method

LC-MS/MS Conditions

The liquid chromatography was carried out using an Agilent Technologies 1290 Infinity II system equipped with quaternary pump (G7104A), high performance auto sampler (G7167B) and thermostat column compartment (G7116B). The analytical column used for the separation of analyte and IS was BDS Hypersil C$_{18}$ (50 × 4.6 mm, 5 µm) from Thermo Fisher Scientific India Pvt. Ltd. The mobile phase consisted of acetonitrile and mixture of ammonium acetate containing 0.15% formic acid in the ratio 55:45 with a flow rate of 0.5 ml/min maintaining isocratic condition. The column oven and auto sampler temperature was maintained at 45°C and 6°C respectively, the injection volume was 10 µl and the total LC run time was 4.0 min.

The LC system interfaced with MS/MS model 6460 Triple quadrupole from Agilent Technologies, India was used for chromatographic analysis and mass spectral quantification of the analyte and IS in positive ion mode using the Electron spray ionization (ESI). For optimization of MS/MS parameters, the dilutions of analyte and IS solution prepared using diluent solution (Methanol: water: 50:50% v/v) were infused in the system using syringe pump. Mass spectrometer parameters were fixed such as nebulizer on 60 Psi, sheath gas heater on 400°C, gas temperature 350°C, gas flow rate on 10 l/min, capillary electrode voltage on 10 V, fragment or voltage on 100 V and ion-spray voltage on 4500 V. The multiple reaction monitoring (MRM) conditions were monitored for both the analytic and Internal Standard (IS) for data acquisition. The entire instrument (LC-MS/MS) management and data acquisition was performed using Mass Hunter Workstation Software LC/MS Data Acquisition for 6460 series Triple Quadrupole Version B.08.00.

Stock Solutions

The stock solution of Fluoxetine of concentration 1 mg/ml was prepared using its working standard dissolved in methanol. From the stock, aqueous dilutions were prepared in the diluent solution (Methanol: water: 50:50% v/v) for spiking Calibration Curve (CC) and Quality Control (QC) standards of Fluoxetine.

Amitriptyline stock solution was also prepared of concentration 1 mg/ml in the methanol and its dilution in diluent solution was prepared which was then subjected to the chromatographic analysis for interfering elements.

Calibration and Quality Control Samples Preparation

The calibration and quality control samples were prepared by spiking (2 % of the total plasma taken) with working solutions (aqueous dilutions). The spiking solutions of CCs and QCs were stored in a refrigerator (2-8°C) and used within 24.0 hrs from preparation time. The concentration of CC and QC samples was ranging between 0.25 to 50 ng/ml. Quality control samples were prepared at 0.27 ng/ml (lower limit of quantification, LLOQ), 0.73ng/ml (lower quality control, LQC), 22.14 ng/ml (medium quality control, MQC) and 36.00 ng/ml (high quality control, HQC) for fluoxetine. Aliquots of spiked plasma samples prepared in multiplicates were taken into micro-centrifuge tubes and stored at −20°C.

Sample Preparation

All CC and QC samples were withdrawn from freezer and thawed at room temperature prior to analysis. Thawed samples were then vortexed to ensure uniform mixing of contents. The protein precipitation (PPT) method was used as an extraction procedure of from plasma samples. An aliquot of 50 µl IS (approximately 250.0 ng/ml of Amitriptyline) was pipetted out into appropriately labeled polypropylene tubes (except blank). Then 250 µl aliquots of each plasma samples was pipette into these micro centrifuge tubes and vortexed. Further, 750 µl HPLC grade Acetonitrile was added to them. The samples were centrifuged for 10 min at 15000 rpm on micro centrifuge and then the samples were transferred to vials for analysis. The sample processing was carried out under the dark conditions throughout the study.

Method Validation Studies

The method was validated for selectivity, linearity, accuracy, precision, recovery, matrix effect and stability studies according to US Food and Drug administration (FDA)[18] and International conference on harmonisation (ICH) M10 guidelines.[19]

Selectivity

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. For selectivity, 10 blank plasma samples were analyzed. The selectivity should be ensured at the lower limit of quantification (LLOQ). Each blank sample were tested for checking the interference at retention time (R*t*) of analyte and

IS. Potential interfering substances in a biological matrix includes endogenous matrix components, metabolites, decomposition products and in the actual study, concomitant medication and other exogenous xenobiotic. For the acceptance, the mean peak response calculated from the analysis of LLOQ at the expected Rt of analyte must be <20% and 5% for IS.

Calibration/Standard Curve (Linearity)

A calibration (standard) curve is the relationship between instrument response and known concentrations of the analyte. A sufficient number of standards should be used to adequately define the relationship between concentration and response. A calibration curve should be constructed using a blank sample (matrix sample processed without internal standard), a zero sample (matrix sample processed with internal standard) and eight non-zero samples covering the expected range, including LLOQ.

The lowest standard on the calibration curve should be accepted as the limit of quantification if the analyte response at the LLOQ is least 5 times the response compared to blank response. Analyte peak (response) should be identifiable, discrete and reproducible with a precision of 20% and accuracy of 80-120%.

The simplest model $y=mx+b$ that adequately describes the concentration-response relationship was used, weighted by $1/x^2$, in which y is the peak area ratio of analyte to IS, m is the slope of calibration curve, b is the y-axis intercept of calibration curve and x is the analyte concentration. The calibration curve is accepted if atleast four out of six non-zero standards including the LLOQ and the calibration standard at the highest concentration should meet the criteria of ±20% deviation of the LLOQ from nominal concentration and ±15% deviation of standards other than LLOQ from nominal concentration.

Accuracy and Precision

Accuracy and Precision batch comprised of one set of calibration standards and six replicates of QC samples at four levels (LLOQ, LQC, MQC and HQC). Intra-batch accuracy and precision were evaluated within a batch and Inter-batch precision and accuracy were evaluated by running three validation batches on separate days. Precision was expressed in terms of coefficient of variation (%CV). For acceptance, the mean value of accuracy should be within 15% of the actual value except at LLOQ, where it should not deviate by more than 20% and the precision determined at each concentration level should not exceed 15% of the coefficient of variation

(CV) except for the LLOQ, where it should not exceed 20% of the CV.

Recovery

The recovery of both analyte and IS was determined by measuring the mean peak area response of six replicates of extracted QC samples against unextracted (aqueous) at three levels (LQC, MQC and HQC).

The mean response, S.D., %CV and % recovery was calculated. The percent recovery at each QC concentration can be calculated as follows:

$$\% \text{ Recovery} = \frac{\text{Mean peak response of extracted samples}}{\text{Mean peak response of unextracted samples}} \times 100 \times \text{Correction factor}$$

$$\text{Correction factor} = \frac{\text{Effective concentration of unextracted samples}}{\text{Effective concentration of extracted samples}}$$

The recovery is deemed acceptable if CV is ≤ 20% for % mean recovery between LQC, MQC and HQC concentrations.

Matrix effect

The matrix effect was determined by running the two replicates of extracted LQC and HQC from each plasma lots against direct injection of corresponding aqueous LQC and HQC. Matrix effect was determined by comparing peak area ratio of matrix sample prepared by addition of reference sample and peak area ratio of reference sample. The matrix factor can be calculated by using the following formula:

$$\text{Matrix factor} = \frac{\text{Peak area ratio of analyte to IS in presence of matrix ions}}{\text{Peak area ratio of analyte to IS in absence of matrix ions}}$$

Stability studies

Room temperature stock solution stability was carried out using six replicates of prepared stock dilution mixture left on the bench for 6 hr. The refrigerated stock solution stability was carried out to assess the stability of stored stock solutions over the period of its storage, which was at least two weeks from the date of preparation. It is accepted when the Percent comparison response should be in between 90 % to 110 %.

The following formula was used for the calculation of stability.

$$\% \text{ Stability} = \frac{\text{Mean analyte response of stored stock dilutions}}{\text{Mean analyte response of fresh stock dilutions}} \times 100 \times \text{Correction factor}$$

$$\text{Correction factor} = \frac{\text{Nominal concentration of fresh sample}}{\text{Nominal concentration of stored sample}}$$

The six replicates of QC samples of analyte at LQC and HQC level ($n=4$) was analyzed for determining the stability of fluoxetine. Different storage conditions were

maintained to check the stability of fluoxetine. Bench top stability was carried out by using four replicates of low and high concentration QC samples stored unprocessed at room temperature or as specified for a period of 6 hr. To calculate the bench top stability duration, the time difference when QC samples were processed till the samples were removed from the freezer (in hours) was noted. The freeze and thaw stability in matrix was calculated by analyzing freshly prepared calibration standard samples against four replicates of QC samples at LQC and HQC levels which were previously frozen and then thawed over multiple cycles. In Freeze and thaw multiple cycles, first freezing was performed at or below −40°C for 24 hr followed by thawing at room temperature and other freezing for a minimum of 12 hr. The freeze and thaw stability was examined at the end of first and third cycle. The mean concentration, SD, % CV and % nominal values were calculated at low and high QC levels. The bench top and freeze-thaw stability evaluation is deemed acceptable if the percent nominal is within the range of ± 15 % and % CV result is ≤ 15 % at LQC and HQC levels.

Ruggedness

Ruggedness of the proposed method was authenticated by processing one precision and accuracy batch through different analyst.

Study Design

The 20 healthy adult male volunteers were selected for the pharmacokinetic study of Fluoxetine. All the information related to the study was given to the volunteers that include aim, objective, outcome and any possible risks. To conduct the study, a written consent of all the volunteers was taken as per Helsinki declaration. After an overnight fasting, a dose of 20 mg was given to the volunteers through oral administration. Sample of blood was collected at Pre-dose (0.0) and after administration of drug, blood samples were collected at 01.00, 02.00, 03.00, 04.00, 04.50, 05.00, 05.50, 06.00, 06.33, 06.67, 07.00, 07.33, 07.67, 08.00, 08.50, 09.00, 10.00, 12.00, 16.00, 24.00, 36.00, 48.00, 60.00, 72.00 h for the quantification of plasma concentration of Fluoxetine.

RESULTS AND DISCUSSION

LC-MS/MS Method Optimization

Since, the nature of analyte and Internal Standard (IS) is basic so, they have the property of accepting the proton and form protonated species $[M+H]^+$. Therefore, the positive ion monitoring mode is used for the quantitation of fluoxetine in LC-MS assay. The fragmentor voltage

was adjusted to different values so as to obtain different base peaks. At lower fragmentor voltage, the base peak in the mass spectrum of the fluoxetine was obtained at m/z 310.1 as a protonated molecular ion $[M+H]^+$ and at higher fragmentor voltage, the intensity of daughter ion also increases and the base peak was obtained at m/z 44.2. Thus at higher voltage selecting the daughter ion at m/z 44.2 can achieve higher sensitivity. Similarly for the same fragmentor voltage, the base peak in the mass spectrum of Internal standard was obtained for the protonated molecular ion $[M+H]^+$ at 278.1 and daughter ion at m/z 233.1. The MS/MS scan of Fluoxetine and IS has been shown in Figure 2. For multiple reaction monitoring, transitions of m/z 310.1→44.2 for fluoxetine and m/z 278.1→233.1 for IS were selected as it gave excellent selectivity and sensitivity.

The different chromatographic conditions like mobile phase, column choice, flow rate, injection volume and column temperature were maintained to obtain symmetric
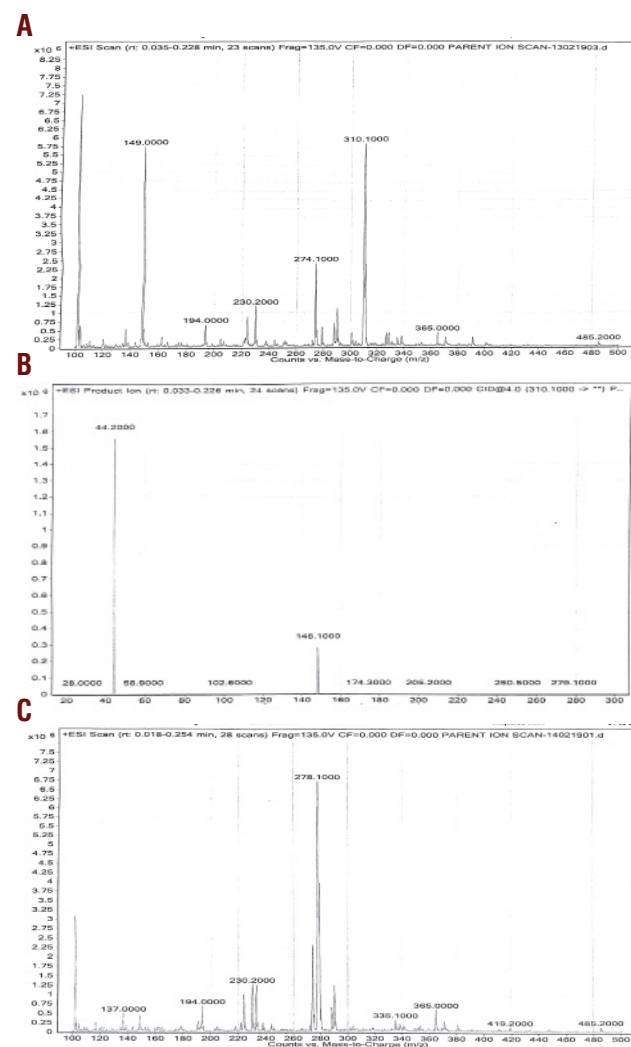

Figure 2: Mass scan of the parent ion of (A) Fluoxetine m/z 310.1, (C) IS *m/z* 278.1and Mass scan of product ion of (B) Fluoxetine 44.2 *m/z*.

peak, sharp peak shape, good resolution and a short run time for the experiment. Initially, different buffers in varying combinations with acetonitrile were used for separation with various columns like Chromolith Performance C_{18} (100 × 4.6 mm, 5 μm) and BDS Hypersil C_{18} (50 × 4.6 mm, 5 μm). The result showed that the column BDS Hypersil C_{18} (50 × 4.6 mm, 5 μm) gave good peak response and symmetric peak shape with mobile phase comprising of acetonitrile and 10 mM ammonium acetate containing 0.15% formic acid in the ration 55:45. The flow rate was maintained at 0.5 ml/min after optimization with the runtime of 4 min. The analyte and IS chromatographed at less than 3 min. Thus, this proves that the analyte elution can be achieved in shorter run time by the use of shorter column.

Sample Preparation Optimization

The reported study used PPT method in the entire study for the extraction process of analytic. The various extraction procedures available were investigated, among which PPT method gave good results whereas other methods resulted in non-reproducible recoveries and interferences from the matrix during chromatographic analysis. Thus, PPT method opts out to be the best extraction technique for the extraction of samples. The samples analysed by this method gave excellent recovery of 83.61 % for fluoxetine and 89.26 % for IS which showed the appropriacy of the developed method. This method gave symmetric chromatographic peak shape and good peak resolution.

Method Validation Results

Selectivity

The peaks obtained during performance of selectivity parameter were of good shape and were completely resolved from plasma components under the optimized chromatographic conditions. No interference was observed due to the endogenous matrix at retention times of both Fluoxetine and IS, shown in Figure 3. The retention times of fluoxetine and IS were 2.40 and 2.50 min respectively. The total chromatographic runtime was 4.0 min.

Linearity

The assay of fluoxetine showed linearity over the concentration range of 0.25 to 40.00 ng/ml. The LLOQ concentration was found to be 0.27 ng/ml, which was adequate to quantify fluoxetine in plasma samples of human (Figure 4).

Accuracy and Precision

The intra-batch and inter-batch accuracy (% nominal) of fluoxetine for quality control samples at LLOQ,
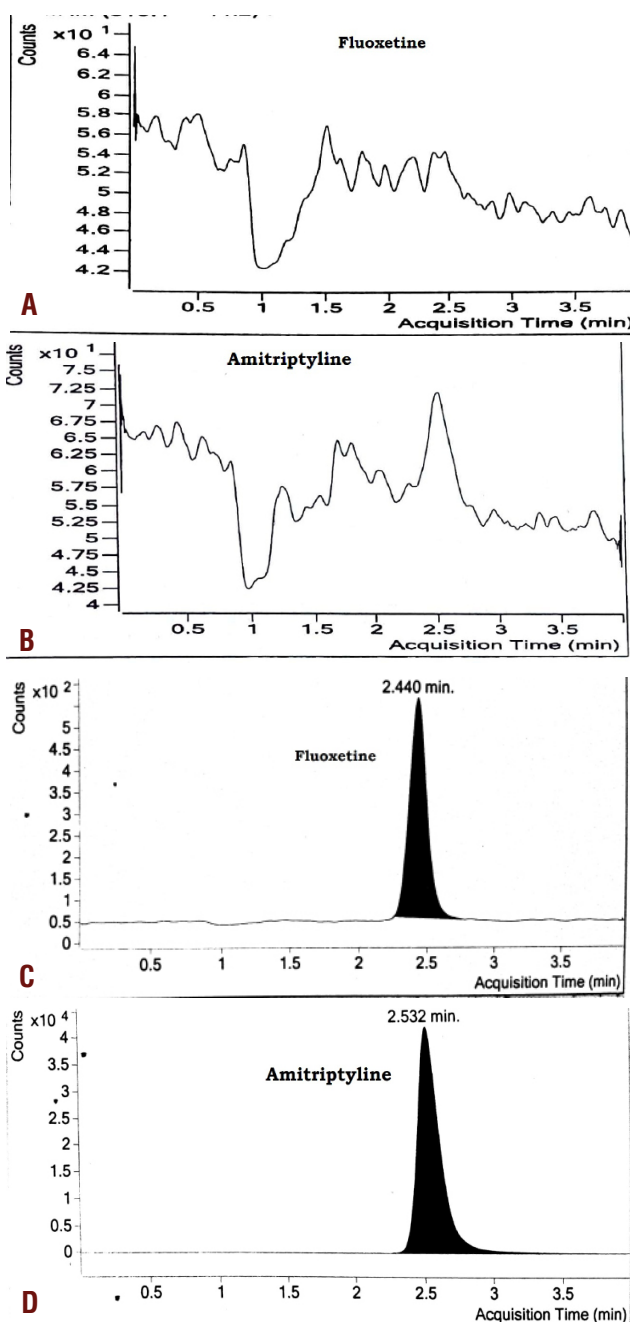

Figure 3: Representative chromatograms of Extracted blank plasma of (A) Fluoxetine, (B) Amitriptyline and Extracted LLOQ plasma samples (C) Fluoxetine, (D) Amitriptyline.

LQC, MQC and HQC levels were between 96.80% to 101.92% respectively and the intra-batch and inter-batch precision (% CV) at same 4 levels were ≤ 3.87 which are within acceptance criteria given in Table 1.

Recovery

The recovery of Fluoxetine at LQC, MQC and HQC level were 79.45%, 86.42% and 84.98% respectively and the mean recovery of Fluoxetine was found to be 83.61% with % CV of 4.40 as given in Table 2. The

Table 1: Intra-batch and Inter-batch accuracy and precision.									
Level	**QC samples (ng/ml)**	**Intra-batch**				**Inter-batch**			
		N	**Mean**	**%CV**	**Nominal (%)**	**N**	**Mean**	**%CV**	**Nominal (%)**
LLOQ	0.27	6	0.27	2.07	98.15	18	0.26	2.96	97.53
LQC	0.73	6	0.71	3.87	96.80	18	0.71	2.72	96.80
MQC	22.14	6	21.68	0.29	97.90	18	21.56	2.84	97.36
HQC	36.00	6	36.40	0.56	101.10	18	36.69	1.03	101.92

LLOQ= Lower limit of quantification
LQC= Lower quality control
MQC= Medium quality control
HQC= High quality control
%CV= Coefficient of variation in percent

Table 2: Recovery and matrix factor of Fluoxetine.				
Analyte	**QC samples (ng/ml)**	**Recovery (%)**	**Matrix Factor**	**%CV**
	0.73	79.45	1.00	0.03
Fluoxetine	22.14	86.42		
	36.00	84.98	0.98	0.03

QC= Quality control samples
%CV= Coefficient of variation in percent

Table 4: Ruggedness data of Fluoxetine.					
Level	**QC samples (ng/ml)**	**N**	**Mean**	**%CV**	**Nominal (%)**
LLOQ	0.27	6	0.26	3.10	97.53
LQC	0.73	6	0.69	2.05	94.52
MQC	22.14	6	21.35	3.29	96.42
HQC	36.00	6	36.93	0.35	102.59

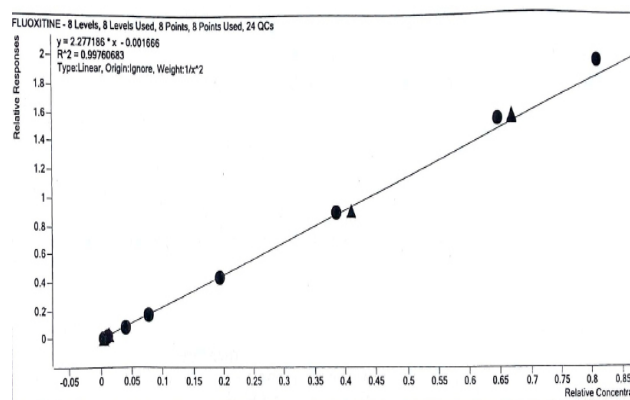*LLOQ= Lower limit of quantification*
LQC= Lower quality control
MQC= Medium quality control
HQC= High quality control
%CV= Coefficient of variation in percent
IS= Internal standard

Table 3: Stability summary of Fluoxetine.					
Stability	**Storage conditions**	**Level**	**QC samples**	**Accuracy (%)**	
				Fluoxetine	**IS**
Analyte and IS stock stability	Room temperature (6 hr)	-	-	100.11	100.12
	Refrigerator (2-8°C for atleast two weeks)	-	-	100.26	99.64
Bench-top	Room temperature (6 hr)	LQC	0.73	98.94	-
		HQC	36.00	101.57	
In-injector	Autosampler (6°C for 24 hr)	LQC	0.73	98.23	-
		HQC	36.00	99.63	
Freeze-thaw	After 4 cycles at -70°C	LQC	0.73	97.92	-
		HQC	36.00	100.33	

LQC= Lower quality control
MQC= Medium quality control
HQC= High quality control
QC= Quality control samples
IS= Internal standard

Figure 4: Representative Calibration curve for Fluoxetine in matrix.

recovery of IS was 89.26%. Thus, the method was found to be efficient in terms of recovery.

Matrix Factor

The matrix factor for Fluoxetine for the matrix sample at LQC and HQC level were found to be 1.00 and 0.98 respectively as shown in Table 2. The % CV of matrix factor for Fluoxetine was found to be 0.03%, which was within the acceptance limit of $\pm15\%$.

Stability Studies

The % Stability of stock solution of Fluoxetine and IS at room temperature were found to be 100.11% and 100.12% and in refrigerator (2-8°C) were found to be 100.26% and 99.64% respectively as shown in Table 3. There were no significant changes in concentration of analyte at Bench top and in-injector stability, so, it can be
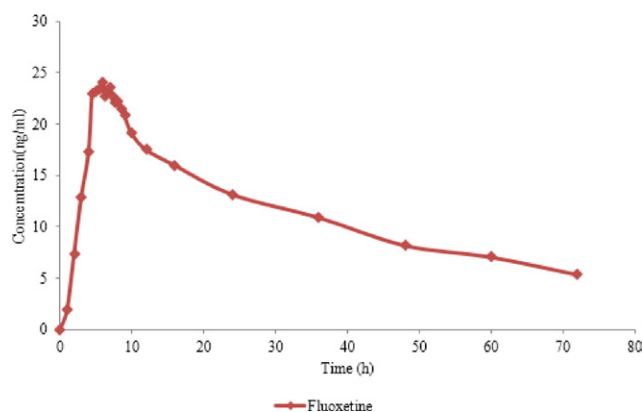
Figure 5: Mean plasma concentration-time profile of Fluoxetine in 20 healthy volunteers.

concluded that Fluoxetine is stable when stored in bench for at least 6.0 hr and in auto sampler for about 24.0 hr. It was found that Fluoxetine is stable when frozen and thawed for three cycles. The % Stability of Fluoxetine at LQC and HQC was found to be 97.92% and 100.33% respectively, which were within the acceptance range of ± 15%.

Ruggedness

The results obtained by performing ruggedness parameter for Fluoxetine was acceptable as it is within the range of ≤15% in Precision and ±15% in Accuracy and for LLOQ, it does not exceeded by 20%. The Table 4 shows the precision and accuracy results obtained at LLOQ, LQC, MQC and HQC levels for Fluoxetine during the experiment.

Application of the method in Healthy Human Subjects

An open label, randomized, two-treatment, single period, single-dose, parallel design, oral bioequivalence study of the reference and the test formulations of fluoxetine (20 mg dose) was successfully conducted for the quantification of fluoxetine in the plasma concentration upto72h after an oral administration in 20 healthy male volunteers kept under fasting condition. The study was performed as per the principles of Declaration of Helsinki and approved by the ethical committee. The mean plasma concentration-time profile curve of Fluoxetine is Figure 5. The mean time to peak plasma concentration was 6.00 h for fluoxetine. The mean pharmacokinetic parameters of Fluoxetine in 20 healthy male volunteers were calculated and C_{max} (ng/ml) of the Fluoxetine was found to be 24.08 ng/ml.

CONCLUSION

The LC-MS/MS method developed was found to be simple, precise, rapid and sensitive for the quantification

of Fluoxetine in human plasma. This method was reliable in terms of selectivity, linearity, accuracy, precision, sensitivity and was effectively validated without any matrix interferences.

Fluoxetine and Amitriptyline were extracted by protein precipitation technique. This sample preparation by the selected method yields extremely good consistent mean recoveries of 83.61% and 89.26%. This preparation technique was found to be less time consuming and also easier to perform than the existing procedure. The stability studies conducted also showed satisfactory results.

ACKNOWLEDGEMENT

CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.
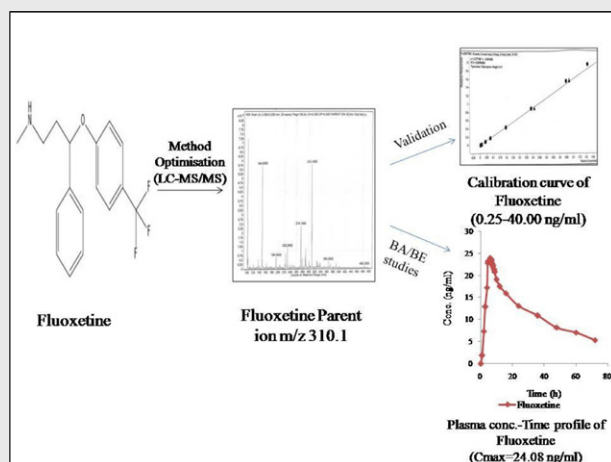
ABBREVIATIONS

LC-MS/MS: Liquid chromatography-tandem mass spectrometry; **PPT:** Protein precipitation; **SPE:** Solid Phase extraction; **LLE:** Liquid liquid extraction; **LLOQ:** Lower limit of quantification; **LOQ:** Lower Quality control; **MQC:** Middle Quality control; **HQC:** High Quality control; **QC Sample:** Quality Control Sample; **%CV:** Percentage Coefficient of Variation; **IS:** Internal Standard.

REFERENCES

1. WHO's Certified. Depression. 2020. [cited 2020 Jan 30]. [database on the Internet]Available from: https://www.who.int/news-room/fact-sheets/detail/depression

2. Fluoxetine (Prozac): Side Effects, Dosages, Treatment, Interactions, Warnings. Rx List. 2016. [cited 2016 Mar 08]. Available from: https://www.rxlist.com/fluoxetine_prozac/drugs-condition.htm

3. Drugbank CA. Educe Design and Innovation Inc. 2005. [updated 2020 July 11; cited 2005 June 13]. Fluoxetine. Available from: https://www.drugbank.ca/drugs/DB00472

4. Green R, Houghton R, Scarth J, Gregory C. Determination of fluoxetine and its major active metabolite norfluoxetine in human plasma by liquid chromatography-tandem mass spectrometry. Chromatographia. 2002;55(1):S133-6.

5. Hasnain MS, Siddiqui S, Rao S, Mohanty P, Jahan AT, Beg S. QbD-Driven Development and Validation of a Bioanalytical LC–MS Method for Quantification of Fluoxetine in Human Plasma. J Chromatogr Sci. 2016;54(5):736-43.

6. Raggi MA, Mandrioli R, Casamenti G, Volterra V, Desiderio C, Fanali S. Improved HPLC determination of fluoxetine and norfluoxetine in human plasma. Chromatographia. 1999;50(7):423-7.

7. Maya MR, Domingos C, Guerreiro MT, Morais J. Determination of the antidepressant fluoxetine in human plasma by LC with UV detection. J Pharm Biomed Anal. 2000;23(6):989-96.

8. Vlase L, Imre S, Leucuta S. Determination of fluoxetine and its N-desmethyl metabolite in human plasma by high-performance liquid chromatography. Talanta. 2005;66(3):659-63.

9. Crifasi JA, Le NX, Long C. Simultaneous identification and quantitation of fluoxetine and its metabolite, norfluoxetine, in biological samples by GC-MS. J Anal Toxicol. 1997;21(6):415-9.

10. Sutherland FCW, Badenhorst D, DeJager AD, Scanes T, Hundt HKL, Swart KJ, et al. Sensitive liquid chromatographic–tandem mass spectrometric method for the determination of fluoxetine and its primary active metabolite norfluoxetine in human plasma. J Chromatogr A. 2001;914(1):45-51.

11. Eerkes A, Weng N, King M, Du A, Shou WZ. A Sensitive and High-Throughput Lc/Ms/Ms Method Using a Silica Column and an Aqueous-Organic Mobile Phase for the Analysis of Fluoxetine and Norfluoxetine in Human Plasma. J Liq Chromatogr Relat Technol. 2002;25(8):1215-27.

12. Catlow JT, Barton RD, Clements M, Gillespie TA, Goodwin M, Swanson SP. Analysis of olanzapine in human plasma utilizing reversed-phase high-performance liquid chromatography with electrochemical detection. J Chromatogr B Biomed Sci App. 1995;668(1):85-90.

13. Boulton DW, Markowitz JS, DeVane CL. A high-performance liquid chromatography assay with ultraviolet detection for olanzapine in human plasma and urine. J Chromatogr B Biomed Sci App. 2001;759(2):319-23.

14. Bogusz MJ, Krüger KD, Maier RD, Erkwoh R, Tuchtenhagen F. Monitoring of olanzapine in serum by liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. J Chromatogr B Biomed Sci App. 1999;732(2):257-69.

15. Zhu Z, Neirinck L. High-performance liquid chromatography–mass spectrometry method for the determination of paroxetine in human plasma. J Chromatogr B. 2002;780(2):295-300.

16. Alegete P, Kancherla P, Albaseer SS, Boodida S. A fast and reliable LC-MS/MS method for simultaneous quantitation of fluoxetine and mirtazapine in human plasma. Anal Methods. 2014;6(18):7407-14.

17. Ni XJ, Wang ZZ, Shang DW, Lu HY, Zhang M, Wen YG. Simultaneous analysis of olanzapine, fluoxetine and norfluoxetine in human plasma using liquid chromatography-mass spectrometry and its application to a pharmacokinetic study. J Chromatogr B. 2018;1092:506-14.

18. FDA's certified. Center for Drug Evaluation and Research. Center for Veterinary Medicine. Bioanalytical Method Validation Guidance for Industry. U.S. Food and Drug Administration. 2018. [database on the internet]. [updated 2020 Apr 04; cited 2018 May] Available from: http://www.fda.gov/regulatory-information/search-fda-guidance-documents/bioanalytical-method-validation-guidance-industry

19. ICH's certified. Multidisciplinary Guidelines : ICH. Bioanalytical Method Validation. 2016. [cited on 2016 October]. [database on the internet]Available from: https://www.ich.org/products/guidelines/multidisciplinary/article/multidisciplinary-guidelines.html

PICTORIAL ABSTRACT

Fluoxetine

Method Optimisation (LC-MS/MS)

Fluoxetine Parent ion m/z 310.1

Validation

Calibration curve of Fluoxetine (0.25–40.00 ng/ml)

BA/BE studies

Plasma conc.-Time profile of Fluoxetine (Cmax=24.08 ng/ml)

SUMMARY

An accurate and precise LC-MS/MS method was developed for the quantification of Fluoxetine and was fully validated as per USFDA requirements. Hence, it can be concluded that the developed bioanalytical method is more reliable in terms of simplicity, cost, sensitivity and accuracy, also very beneficial for all scale industries. The method was appropriate to support the clinical pharmacokinetic studies following single dose of 20 mg fluoxetine by oral administration. Therefore, the proposed method can be successfully used for bioequivalence study to analyse the samples of Fluoxetine drug from the clinical study and to evaluate its pharmacokinetic parameters.