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Development and Validation of Reverse Phase High Performance Chromatography Method for Determination of Fluoxetine in Rat Plasma

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Abstract

Purpose: To develop a sensitive and validated reverse phase-high performance liquid chromatographic (RP-HPLC) method for quantification of Fluoxetine in micro-sample of rat plasma using UV detection. **Methods:** A single oral dose of Fluoxetine (7 mg/kg) was given to overnight fasted rats (n = 6). Rat plasma samples containing the drug were extracted by liquid-liquid extraction using a combination of dichloromethane: n-hexane (80:20). A reverse phase chromatographic column C18 hypersil-BDS was used for chromatographic separation with a mobile phase consisting of 50 mM phosphate buffer pH 5.5, acetonitrile and methanol (50:30:20,v/v/v) pumped at a flow rate of1.2 ml/min. Fluoxetine was measured using ultraviolet (UV) detection at 235 nm. The method was validated for precision and accuracy. **Results:** Separation of compounds of interest was not affected by endogenous interference. Good linearity within the concentration range of 1 - 500 ng/ml in rat plasma was obtained with coefficient of regression (r^2) of 0.9986. Liquid-liquid extraction produced comparable recovery to solid phase extraction. Retention time of Fluoxetine and internal standard (Olanzapine) was5.0and13.4 min, respectively. Lowest limit of quantification (LLOQ) was 1 ng/ml while inter-day and intra-day precision was < 12.5 and 5.1 %, respectively. Accuracy of the method was between 94 and 105 % and the variation of results between two analysts was not significant (p = 0.626). Mean maximum plasma concentration (C_{max}) of Fluoxetine was 412.7 ng/ml, **Conclusion:** The proposed method has been successfully validated for precision and accuracy that are within the limits of U.S. Food and Drug Administration (FDA)'s guidance for bio-analytical assay validation. The method was successfully applied to preclinical pharmacokinetic analysis of Fluoxetine in rats.

Keywords

Fluoxetine, Antipsychotic, Pharmacokinetics, Rat, Plasma, Bio-analytical assay

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INTRODUCTION

Fluoxetine is a selective serotonin reuptake inhibitor (SSRI) and as the name suggests, it exerts its therapeutic effect by inhibiting the presynaptic reuptake of the neurotransmitter serotonin. As a result, levels of 5-hydroxytryptamine (5-HT) are increased in various parts of the brain. Further, fluoxetine has high affinity for 5-HT transporters, weak affinity for noradrenaline transporters and no affinity for dopamine transporters indicating that it is 5-HT selective. Fluoxetine interacts to a degree with the 5-HT_{2C} receptor, and it has been suggested that through this mechanism, it is able to increase noradrenaline and dopamine levels in the prefrontal cortex[1]. Analysis of Fluoxetine from biological samples is widely carried out by high

performance liquid chromatography (HPLC). Previous studies have reported several HPLC methods employing electrochemical, amperometry, ultraviolet and mass spectroscopic detection. The HPLC method employed in the present study utilizes ultraviolet detection for analysis and is convenient, affordable, easily available and requires less maintenance than other detection methods[2]. The method was applied to micro-sample rat plasma because minimal amount of blood can be withdrawn for a small animal like rat for repeated blood sampling. Current method was aimed to provide desired recovery and sensitivity of assay by employing liquid-liquid extraction procedure.

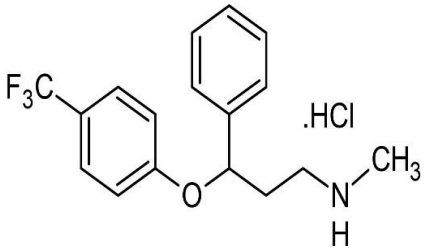

Figure1: Chemical structure of Fluoxetine

EXPERIMENTAL

Materials

Pure drug samples of FluoxetineN-methyl-3-phenyl-3-[4-(trifluoromethyl) phenoxy] propan-1-amine and Olanzapine were kindly provided by Cipla Ltd. (Hyderabad, India). HPLC-grade acetonitrile, methanol and ethanol were obtained from SD Fines (Mumbai, India) and used without further purification or filtration. All other chemicals used were of analytical grade or higher.

Chromatographic instrumentation and conditions

Chromatographic studies were performed on Agilent separation module combined with solvent delivery pump model 1100 (USA). Chromatographic data was obtained by using computerized integration software HP Chem Station.

A reverse phase chromatographic column C18 hypersil-BDS (250 - 4.6 mm; i.d., 5µm) was used. The mobile phase consisted of 50% phosphate buffer of 50mM maintained at pH 5.5-Acetonitrile-methanol (50:30:20) (v/v/v) which was run at the flow rate of 1.2 ml/min. Mobile phase was filtered through 0.45µm membrane by vacuum filtration and degassed by ultrasonicator prior to use[3]. Detection of Fluoxetine was carried out on an ultraviolet detector at 235 nm.

Sample preparation

Quantification of Fluoxetine in micro-sample rat plasma was carried out using a modified HPLC method according to previously published HPLC method by Dusci *et al*. Fluoxetine was extracted from the rat plasma samples by using liquid-liquid extraction 0.1ml of plasma was taken in a 10 ml borosilicate glass tube, 10 µl of internal standard(I.S.) solution (5µg/ml of olanzapine) was added to biological sample.300µl of 0.1 M Na_2CO_3 and 5 ml solution of hexane : dichloromethane (80:20) was added. The mixture was shaken for 5 min on a vortex mixer then centrifuged at 2000 g for 5 min. Supernatant from this mixture was transferred to a 10 ml glass tube and evaporated under the stream of nitrogen.100 µl of mobile phase was added and mixed on a vortex mixer[4]. Fifty micro liters of this sample was injected to HPLC for analysis.

Preparation of stock solutions

Stock solutions of Fluoxetine and Olanzapine were prepared at concentration of 1 mg/ml in methanol. For preparation of working standard solutions these stock solutions were further diluted with methanol[5]. For Fluoxetine, its standard working solutions were 0.01,0.05,0.1,0.5,1.0,2.0,5.0µg/ml.

Method validation

Linearity

Standard calibration curves were prepared by adding 10µl of standard working dilutions of Fluoxetine to 0.1 ml drug free rat plasma. Thus, corresponding plasma calibration standards of concentration 1, 5, 10, 50, 100, 200, 500 ng/ml were obtained. Plasma calibration standards were extracted by liquid-liquid extraction as method described above. Standard calibration curves were calculated by using peak area ratio of Fluoxetine and that of olanzapine as function of Fluoxetine concentration in plasma.

Recovery

Absolute recovery was calculated by comparing the peak areas of compounds after liquid-liquid extraction with same concentration of compounds dissolved in mobile phase[6].

To determine absolute recovery, each measurement was carried out in triplicate and computed as in Eq 1.

Recovery (%) = (Pe/Pu)100 (1)

where Pe and Pu are the peak area of extracted and unextracted standards, respectively.

Assessment of intra-day and inter-day variability

Five different rat plasma samples were tested for intra-day variability by using a single calibration curve. Inter-day variability was tested on five days by using five different rat plasma samples[7]. Standard calibration curve was constructed on each day for analysis.

Determination of LLOQ and LOD

Lower limit of quantification (LLOQ) and lower limit of detection (LOD) for plasma samples of Fluoxetine was determined according to standards of FDA's guidance for industry for bioanalytical method validation 2013. LLOQ was determined by spiking the plasma with lowest concentration of calibration curve with 20% of precision and with 80-120 % of accuracy by repeated analysis for five days[8].

Stability

Freeze and thaw stability was determined by comparing freshly prepared QC samples with freeze thawed

samples. Three concentrations (5,100,500ng/ml) were used for five cycles of freeze thaw and analyzed in triplicates[9]. Bench-top stability for 24hr at room temperature, stability for extracted sample for 24 hr. at room temperature and long-term stability for 100 days at -30 °C was measured in three concentrations (5,100,500 ng/ml) with six replicates.

Statistical analysis

The results are presented as mean ± S.D, percent relative standard deviation (% RSD) and coefficient of variation (CV). The results were analyzed for significant difference using Student's t-test and differences were considered significant at $p \leq 0.05$. All statistical analyses were performed using Microsoft Excel 2010 software[10].

RESULTS

Chromatographic separation

Different ratios of buffer, acetonitrile and methanol were used to obtain the best chromatographic conditions. Type of column and pH of buffer was also optimized to get the better sensitivity and selectivity of analysis. Chromatograms of blank plasma and drug free plasma spiked with100 ng of Fluoxetine and 500 ng of fluoxetine (I.S) is illustrated in (Figure2). Retention times of Fluoxetine and internal standard (fluoxetine) were 5.0 and 13.4 min, respectively.

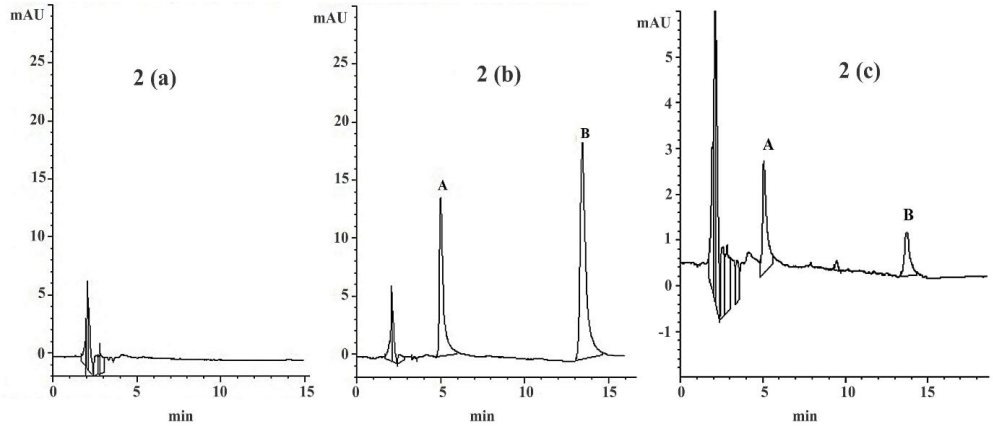

Figure 2: Chromatogram of rat plasma extract using UV detection at 235 nm. 2(a) = blank plasma; 2(b) = plasma spiked with Fluoxetine 100 ng/ml (A) and Olanzapine 500 ng/ml (B); 2(c) = plasma sample taken from rat treated with 7mg/kg after 24hr and spiked with internal standard olanzapine 50 ng/ml was 1 ng/ml. Limit of detection was 0.5 ng/ml with minimum signal to noise ratio of three.

Linearity and limit of quantification

Standard curve was established by taking ratio of peak area of Fluoxetine and that of fluoxetine (I.S) as a function of Fluoxetine plasma concentration. Excellent linearity with concentration range 1-500 ng/ml in rat plasma was obtained with coefficient of regression i.e., r^2= 0.9986. Lowest limit of quantification (LLOQ) in rat plasma with acceptable precision and accuracy (n=5, R.S.D.:12.4%, Deviation: -3%)

Precision and accuracy

Inter-day and intra-day accuracy and precision values for 1, 5, 10, 50, 100, 200, 500 ng/ml plasma concentration are shown in (Table1). Five replicates were used to determine the Inter-day and intra-day precision by calculating RSD%. Inter-day and intra-day precision were found to be lower than 12.5 and 5.1%, respectively, whereas accuracy of this method was between 94 % and 105 %. Variation between two analysts was also determined by applying t-test. The mean drug content determined by analysts 1 and 2 was 99.43 ± 0.12 and 99.37± 0.30, respectively. There was no significant difference between these two results (p=0.626).

Extraction recovery

A convenient single step liquid-liquid extraction method was employed which provided a comparable recovery too the methods employing solid phase extraction (SPE). Extraction recovery of Fluoxetine was measured at 1, 50, 500 ng/ml and 500 ng/ml for olanzapine (I.S) using triplicate samples. Extraction recoveries of Fluoxetine and internal standard are shown in (Table2).

Stability

Changes in stability for plasma samples stored at -30 °C after 5 freeze thawing cycles is shown in (Table 3). Bench-top stability and extracted sample stability were measured at room temperature and long-term stability was measured at -30 °C as shown in (Table 4). Long term stability for olanzapine in plasma sample at -30 °C was for a minimum of 100 days.

Table1: Inter-day and intra-day precision and accuracy of HPLC method for Fluoxetine in rat plasma

Concentration spiked (ng/ml)	Mean concentration (calculated, ng/ml)	SD	RSD (%) [a]	Accuracy (%)
Inter-day(n=5)				
1	1.05	0.13	12.4	105
5	4.92	0.08	1.7	98.4
50	47	3.46	7.4	94
100	97.70	4.68	4.8	97.7
200	201.1	0.85	0.4	100.6
500	484.4	28.94	6.0	96.9
Intra-day(n=5)				
1	0.96	0.02	1.6	95.7
5	5.04	0.25	5.0	100.8
50	47.97	0.71	1.5	95.9
100	100.87	2.46	2.4	100.9
200	197.23	2.95	1.5	98.6
500	485.67	5.03	1.0	97.1

[a]RSD values of normalized areas are the mean of three concentration levels of the calibration curves: SD=standard deviation

Table 2: Percentage extraction recoveries of Fluoxetine and Olanzapine (I.S) from rat plasma

Fluoxetine			
Spiked(ng/ml)	**Extracted**	**Unextracted**	**(%) [b]**
1	2.4±0.1	2.8±0.1	83
50	114.0±1.4	133.2±1.3	86
500	1177.4±3.1	1275.2±11.0	92
Olanzapine (I.S)			
500	446.2±2.9	508.2±3.0	88

Table 3: Freeze thaw stability of plasma samples after 5cycles (n=3)

Cycle	Concentration (ng/ml)	Mean stability sample(ng/ml)	CV (%)[a]	Change (%)[b]
1	5	4.93	2.1	-1.4
	100	98.4	3.5	-1.6
	500	495.1	2.8	-0.98
2	5	4.91	6.1	-1.8
	100	98.5	5.4	-1.5
	500	492.3	3.8	-1.54
3	5	4.88	1.9	-2.4
	100	91.9	6.5	-8.1
	500	491.2	3.9	-1.76
4	5	4.81	2.4	-3.8
	100	90.7	1.5	-9.3
	500	486.4	4.1	-2.72
5	5	4.7	1.9	-6
	100	90.3	3.4	-9.7
	500	485.1	2.9	-2.98

[a]CV=coefficient of variation; [b] percent change in concentration at the end of the stability study

Table4: Stability parameters of Fluoxetine plasma samples at different storage conditions(n=6)

Stability variable	Storage condition	Concentration (ng/ml)	Mean stability sample (ng/ml)	CV (%) [a]	Change (%) [b]
Bench top stability	Room temperature	5	4.87	1.3	-2.6
	For 24h	100	98	3.2	-2
		500	493.5	0.9	-1.3
Long term stability	At-30°Cfor100	5	4.81	5.3	-3.8
	days	100	90.7	2.8	-9.3
		500	485.6	3.7	-2.88
Extracted sample	Room temperature	5	4.95	0.5	-1.0
stability	For 48h	100	98.8	1.8	-1.2
		500	498.9	2.8	-0.22

[a]CV=coefficient of variation; [b]percent change in concentration at the end of the stability study

DISCUSSION

Using the current method, the chromatographic separation for micro-sample rat plasma was made with reasonable retention times. Method validation was performed, and precision and accuracy of the developed method for determining Fluoxetine in plasma is within the limits established by FDA for bioanalytical method. Results showed that extraction recovery was > 82 % for all observed concentration in rat plasma which is comparable with SPE method. Ascorbic acid was not added to the plasma samples as addition of ascorbic acid does not affect the stability of Fluoxetine during storage and extraction. All the stability parameters were found to meet the acceptance criteria of FDA's Guidance for Industry for Bioanalytical Method Validation 2013.

CONCLUSION

A validated, precise, and accurate isocratic RP-HPLC method for determining Fluoxetine in micro-sample rat plasma has been developed in this study. Single-step liquid-liquid extraction provides sufficient recovery to quantify 1 - 500 ng/ml of Fluoxetine in rat plasma. The method has been successfully applied to preclinical pharmacokinetic studies of Fluoxetine in rats.

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