Rapid Determination of Fluoxetine Concentration in Human Plasma by Ultra Performance Liquid Chromatography

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Abstract: A simple, precise, and rapid ultra-performance liquid chromatography (UPLC) method for the determination of fluoxetine level in human plasma using clomipramine as an internal standard (IS) was developed and validated. Plasma samples containing fluoxetine were spiked with the IS and extracted with hexane and iso-amyl alcohol (98:2,v:v) under alkaline condition. Extracted samples were kept at –80ºC for 10 minute then organic layers were transferred to clean tubes and dried under gentle steam of nitrogen at 40 °C. Residues were reconstituted in 200 µl of 0.05% phosphoric acid and 10 µl were injected in to the UPLC system. The compounds of interest were efficiently separated on 2.1 x 100 mm, Acuity UPLC, C18, steel column (at 30°C) and detected with a photodiode array detector set at 230 nm. The mobile phase consisted of water (pH= 2.4, adjusted with phosphoric acid) and acetonitrile (68:32, v:v) and was delivered at a flow rate of 0.3 ml/minute. No interference in blank plasma or by norfluoxetine or commonly used drugs was observed, and the detection limit of fluoxetine was 0.005 µg/ml. The relationship between fluoxetine concentration in plasma and peak height ratio of fluoxetine /IS was linear ($R^2 \geq 0.985$) in the range of 0.01 –1 µg/ml. Inter-day coefficient of variation and bias $\leq$ 13.0%, and < 9.4, respectively. Extraction recovery of fluoxetine and IS from plasma samples was $\geq$ 87%. Using the method, fluoxetine was found to be $\geq$ 85% and $\geq$ 89% stable in processed (24 hours at room temperature or 48 hours at -20 °C) and unprocessed (24 hours at room temperature or 23 weeks at -20 °C) samples, respectively.

Keywords: Fluoxetine, Norfluoxetine, Clomipramine, Human plasma, UPLC.

INTRODUCTION

Fluoxetine (CAS No. 54910-89-3), N-methyl-3-phenyl-3-[4-(trifluoromethyl) phenoxy] propan-1-amine, is a selective serotonin reuptake inhibitor that is widely used as an antidepressant. It is also used in the treatment of obsessive-compulsive, anxiety, panic disorders, and nociceptive pain [1, 2]. Fluoxetine is extensively metabolized in the liver to its active metabolite norfluoxetine. It is well absorbed with a peak plasma concentration of <1µg/ml within 6-8 hours after oral administration [3]. Figure-1 depicts the chemical structures of fluoxetine, norfluoxetine, and clomipramine, the internal standard (IS) used in the study.

A thorough literature search revealed that several analytical methods have been used for quantitative determination of fluoxetine level in pharmaceutical preparations [4-6] and/or in biological samples [7-18]. Fluoxetine level in human plasma or serum has been determined by using gas chromatography with electron capture detection [7, 8], gas chromatography-mass spectrometry [9], high-performance liquid chromatography (HPLC) with ultraviolet [10-13] or fluorescence detection [14, 17],

and HPLC-–mass spectrometry [18]. However, most of reported HPLC assays, the most commonly used technique, involved multiple-step sample preparation [10, 13, 15] and relatively long run time [14, 15].

In this paper, we described a simple and rapid ultra performance liquid chromatography (UPLC) assay for the quantification of fluoxetine in human plasma. The method was validated and used to determine stability of fluoxetine under various conditions encountered in the clinical laboratory.

MATERIALS AND METHODS

Apparatus

Chromatography was performed on Acquity UPLC system (Waters Associates Inc., Milford, MA, USA) consisting of a quaternary pump, autosampler, column thermostat, and photodiode array detector. Acuity UPLC 2.1 x 100 mm, C18, 1.7-µm (particle-size) steel column at 30°C, was used for the separation. Data were collected with a Pentium IV computer using Empower Chromatography Manager Software.

Chemical and reagents

All reagents were of analytical grade unless stated otherwise. Fluoxetine standard was purchased from Sigma, St. Louis, MO, USA. Clomipramine was supplied by United State Pharmacopoeia, Rockville, MD, USA. Acetonitrile, and methanol (both HPLC grade), sodium hydroxide, hexane, iso-amyl alcohol, and phosphoric acid were purchased from Fisher Scientific, Fairlawn, NJ, USA. HPLC grade water was prepared by reverse osmosis and was further purified by passing through a Synergy Water Purification System (Millipore, Bedford, MA, USA). Drug-free human plasma was obtained from the blood bank of King Faisal Specialist Hospital & Research Centre (KFSHRC) Riyadh, Saudi Arabia.

Chromatographic conditions

The mobile phase was composed of water (pH=2.4, adjusted with phosphoric acid) and acetonitrile (68:32, v:v). Before delivering into the system, the mobile phase was filtered through 0.22 μm polyethersulfone membrane and sonicated under vacuum for five minutes. The analysis was carried out under isocratic conditions with a flow rate of 0.3 ml/min at 30°C and a run time of 8 minutes. A photodiode array detector set at 230 nm was used.

Preparation of standard and quality control samples

Stock solutions (1.0 mg/ml) of fluoxetine and IS were prepared in methanol. Working solution (1μg/ml) for fluoxetine and IS were prepared by dilution in blank human plasma and mobile phase, respectively. Calibration curve standards (blank plus eight concentrations) in the range of 0.01 – 1.0 μg/ml and four quality control (QC) samples in the (0.01, 0.03, 0.5, and 0.9 μg/ml) were prepared in human plasma. Aliquots of 1 ml were transferred into teflon-lined, screw-capped, borosilicate, (13 x 100) mm glass culture tubes, and stored at –20 ºC until used.

Sample preparation

Aliquots of 1.0 ml calibration curve or QC samples were allowed to equilibrate to room temperature. 100 μl of the IS working solution were added and the mixture was vortexed for 10 seconds. After adding 200 μl of 5 M sodium hydroxide, samples were vortexed 2 minutes then 3.0 ml mixture of hexane and iso-amyl alcohol (98:2, v:v) were added. The samples were vortexed again for one minute and then centrifuged for 10 minutes at 4200 rpm. The resultant

solution was kept at –80ºC for 10 minutes. The organic layer was then carefully transferred into a clean tube and dried under gentle steam of nitrogen at 40 °C. Residues were reconstituted in 200 μl of 0.05% phosphoric acid, vortexed for two minutes and centrifuged at 13200 rpm for 10 minutes at room temperature. The supernatant was transferred into an auto-sampler vial and 10 μl were injected into the UPLC system with a run time of 8 minutes.

Stability studies

Three QC samples (concentration 0.01, 0.03, and 0.90 μg/ml) were used for stability studies: five aliquots of each QC sample were extracted and immediately analyzed (baseline), five aliquots were allowed to stand on the bench-top for 24 hours at room temperature before being processed and analyzed (counter stability, 24 hours at room temperature), five aliquots were stored at -20ºC for 23 weeks before being processed and analyzed (long term, freezer storage stability), and five aliquots were processed and stored at room temperature for 24 hours or 48 hours at -20 ºC before analysis (autosampler stability). Fifteen aliquots of each QC sample were stored at -20 ºC for 24 hours. They were then left to completely thaw unassisted at room temperature. Five aliquots of each sample were extracted and analyzed and the rest returned to -20 ºC for another 24 hours. The cycle was repeated three times (freeze-thaw stability).

Method validation

The method was validated according to standard procedures described in the US Food and Drug Administration (FDA) bioanalytical method validation guidance [19]. The validation parameter included: specificity, linearity, accuracy, precision, recovery and stability.

RESULTS AND DISCUSSION
Optimization of chromatographic conditions

The optimal experimental conditions were a mobile phase composed of water (pH = 2.4 adjusted with phosphoric acid) and acetonitrile (68:32: v:v) and a flow rate of 0.3 ml/min. Under these conditions fluoxetine, norfluoxetine, clomipramine, and components of plasma exhibited a well-defined separation within 8 minutes run. Retention times of fluoxetine, norfluoxetine, and clomipramine were around 3.1, 3.7, and 4.7 minutes, respectively (Figure-2).
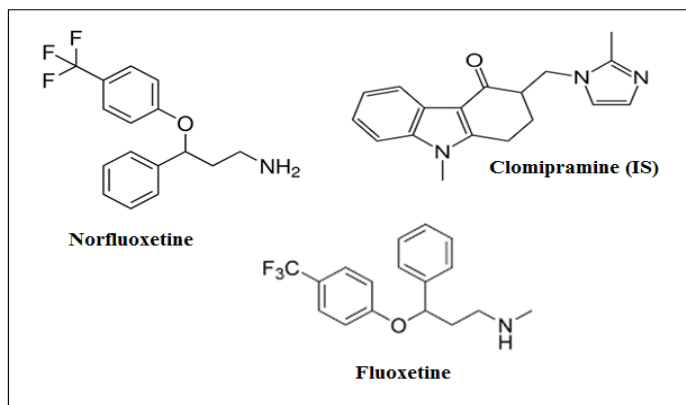
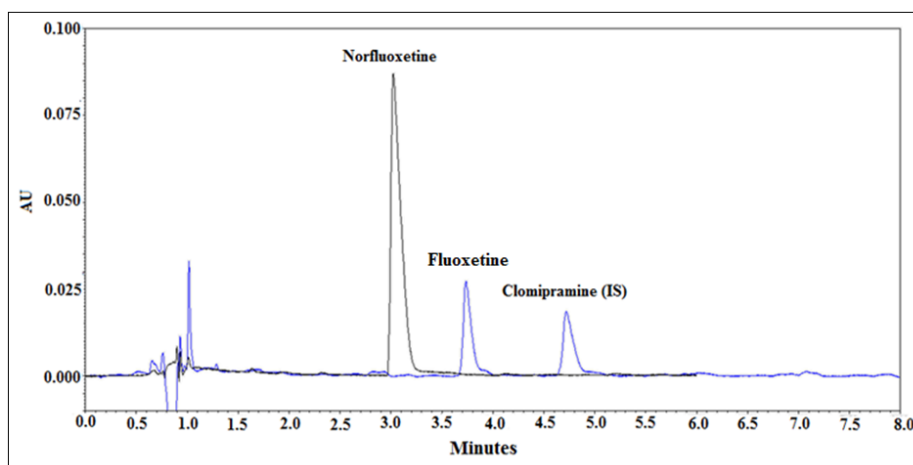**Fig-1:** **Chemical structures of fluoxetine, norfluoxetine, and clomipramine (IS)**

Fig-2: **UPLC chromatogram of norfluoxetine, fluoxetine, and clomipramine (IS)**

Specificity

We screened six batches of blank plasma and seven frequently used medications (ranitidine, acetaminophen, ibuprofen, nicotinic acid, ascorbic acid, caffeine, and diclofenac) in addition to norfluoxetine for potential interference. No interference was found in plasma and none of the drugs co-eluted with fluoxetine or the IS. Figure-3 depicts a representative chromatogram of drug free human plasma used in preparation of calibration standards and QC samples.
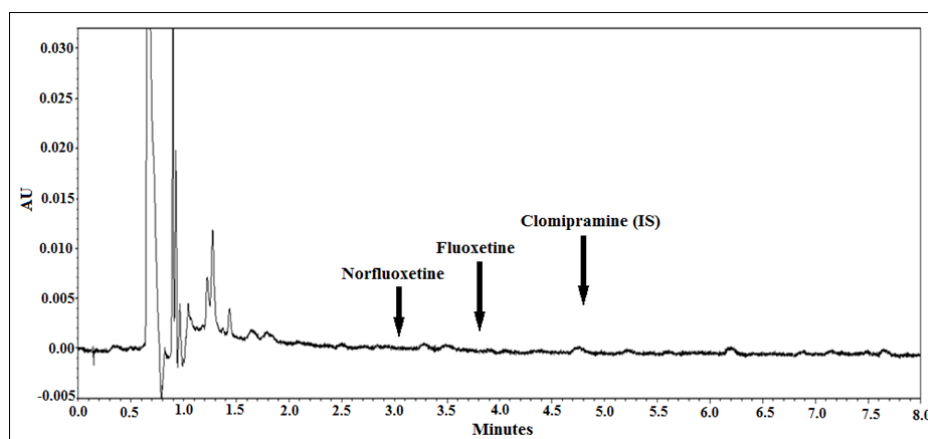

Fig-3: Representative chromatogram of drug-free human plasma

Limit of detection & quantification and Linearity

The limit of quantification, defined as the lowest concentration on the calibration curve that can be determined with acceptable precision and accuracy (i.e., coefficient of variation and bias $\leq$ 20%), was 0.01 µg/ml. The limit of detection ($\geq$3 signal/noise ratio) was 0.005 µg/ml. Linearity of fluoxetine was evaluated by analyzing ten curves prepared in human plasma.

Calibration curves were linear with an $R^2 \geq 0.985$. Mean (SD) of slope, intercept, and $(R)^2$ of the ten curves were 1.7689 (0.2450), 0.012 (0.011), and 0.986 (0.007), respectively. Figure-4 depicts a representative overlay of chromatograms of extracts of 1.0 ml human plasma spiked with the internal standard (IS) and one of eight concentrations of fluoxetine. The suitability of the calibration curves was confirmed by back-calculating concentrations of fluoxetine in human plasma from the calibration curves (Table-1). All calculated concentrations were well within the acceptable limits.

Table-1: Back calculated fluoxetine concentrations from ten calibration curves

Nominal Level (µg/ml)	Calculated Level (µg/ml)		CV (%)	†Bias (%)
	Mean	SD		
0.010	0.0104	0.0008	7.7	4.0
0.025	0.0253	0.0028	11.1	1.2
0.050	0.0514	0.0045	8.8	2.8
0.200	0.2168	0.0178	8.2	8.4
0.400	0.3830	0.0199	5.2	-4.3
0.600	0.5296	0.0174	3.3	-11.7
0.800	0.7454	0.0320	4.3	-6.8
1.000	1.0683	0.0218	2.0	6.8

SD, standard deviation CV, standard deviation divided by mean measured concentration x100

† Bias = (mean measured concentration – nominal concentration divided by nominal concentration) × 100.
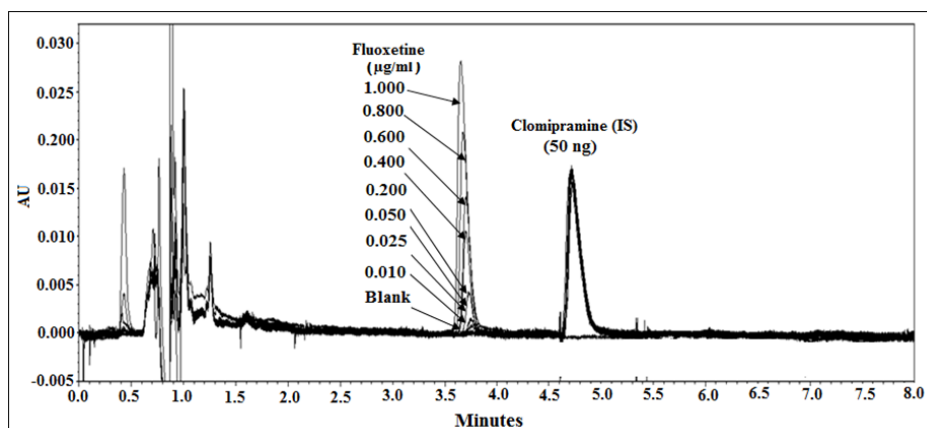

Fig-4: Overlay of chromatograms of extracts of 1.0 ml human plasma spiked with the internal standard (IS) and one of eight concentrations of fluoxetine

Precision and bias (inaccuracy)

The intra-day and inter-day precision and bias of the method were evaluated by analyzing four QC samples (0.01, 0.03, 0.5, and 0.9 µg/ml). The Intra-day precision and bias (n = 10) ranged from 5.0 % to 7.4% and from –11.6% to 9.0%, respectively. The inter-day precision and bias were determined over three different days. The inter-day precision and bias (n = 20) ranged from 4.6% to 13.0% and from –9.4% to 7.0%, respectively. The results are summarized in Table-2.

Table-2: Intra and inter-day precision and bias of fluoxetine assay

Nominal Level (µg/ml)	Measured Level (µg/ml)		CV (%)	†Bias (%)
	Mean	SD		
Intra-day (n= 10)				
0.01	0.0109	0.0008	6.9	9.0
0.03	0.0287	0.0021	7.4	-4.3
0.50	0.4560	0.0227	5.0	-8.8
0.90	0.7955	0.0517	6.5	-11.6
Inter-day (n= 20)				
0.01	0.0107	0.0014	13.0	7.0
0.03	0.0290	0.0020	8.3	-3.3
0.50	0.4652	0.0210	4.6	7.0
0.90	0.8150	0.0562	6.9	-9.4

SD, standard deviation CV, standard deviation divided by mean measured concentration x100

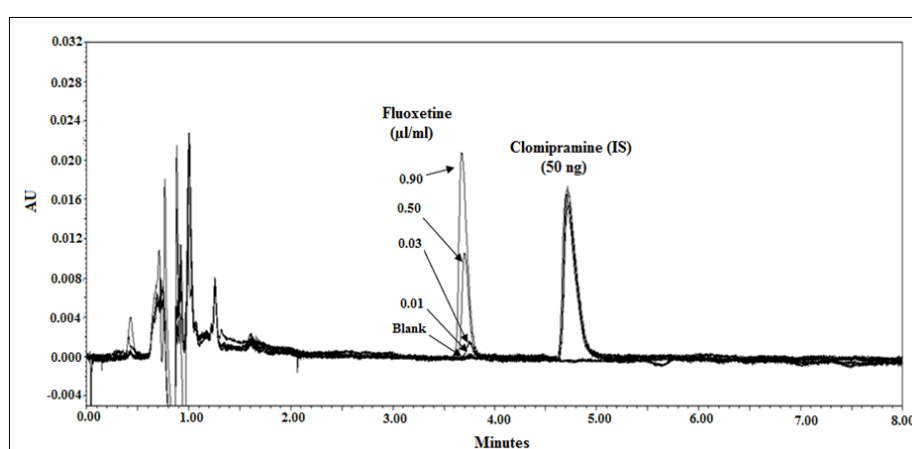† Bias = (mean measured concentration – nominal concentration divided by nominal concentration) × 100.

Fig-5: Overlay of chromatograms blank human plasma spiked with IS and one of four quality control concentrations (0.01, 0.03, 0.50, and 0.90 µg/ml)

Recovery

The absolute recovery of fluoxetine was assessed by comparing peak heights of plasma and mobile phase samples, using five replicates for each of four QC samples (0.01, 0.03, 0.50, and 0.90 µg/ml).

Similarly, the recovery of the IS was determined by comparing the peak height of the IS in 5 aliquots of human plasma spiked with 100 µl of IS (10 µg/ml) with the peak height of equivalent samples prepared in mobile phase. The results are presented in Table-3.

Table-3: Recovery of fluoxetine and the internal standard (IS) from 1.0 ml of human plasma

Concentration (µg/ml)	Human* Plasma	SD	Mobile* Phase	SD	Recovery (%)
Fluoxetine					
0.01	33920	41	37309	23	91
0.03	26633	63	29127	17	91
0.50	34142	978	39279	400	87
0.90	31639	605	34349	145	92
IS: 0.2	75433	500	83826	1109	90

* Mean peak height (SD), n = 5.

Stability

Stability of analyte in biological matrices is an important pre-analytical variable. Fluoxetine and IS stability in processed and unprocessed plasma samples was investigated. Fluoxetine in processed samples was found to be stable for 24 hours at room temperature (≥

85%) and 48 hours at −20 °C (85%). Fluoxetine in unprocessed samples was stable for at least 24 hours at room temperature (≥ 93%), 23 weeks at −20 °C (≥ 89%), and after three freeze-and thaw cycles (≥ 82%). Table-4 summarizes the results of stability studies of fluoxetine.

Table-4: Stability of fluoxetine in human plasma

Stability	Storage condition	Spiked concentration (µg/ml)	Measured concentration (µg/ml)	SD	Stability (%)
Processed/Analyzed Immediately	BL	0.01	0.0117	0.0010	100
		0.03	0.0321	0.0017	107
		0.90	1.0315	0.0223	98
Processed	24 hr. (RT)	0.01	0.0105	0.0003	90
		0.03	0.0292	0.0012	91
		0.90	0.8766	0.0319	85
	48 hr. (−20°C)	0.01	0.0101	0.0005	86
		0.03	0.0274	0.0020	85
		0.90	0.8547	0.0132	86
Un-processed	24 hr. (RT)	0.01	0.0108	0.0013	96
		0.03	0.0278	0.0026	93
		0.90	0.8880	0.0274	100
	23 wks (−20°C)	0.01	0.0116	0.0012	104
		0.03	0.0265	0.0029	89
		0.90	0.7881	0.0434	89
Un-processed	FT: Cycle-1 (−20°C)	0.01	0.0119	0.0014	101
		0.03	0.0318	0.0032	103
		0.90	1.0259	0.0362	100
	FT: Cycle-2 (−20°C)	0.01	0.0103	0.0010	87
		0.03	0.0339	0.0024	110
		0.90	1.0278	0.0267	100
	FT: Cycle-3 (−20°C)	0.01	0.0106	0.0009	90
		0.03	0.0334	0.0015	108
		0.90	0.8382	0.0317	82

BL= base line, RT = Room temperature (24°C), hr. = hours, wks = weeks, FT = Freeze-Thaw. Stability (%) = mean measured concentration (n=5) at the indicated time divided by mean measured concentration (n=5) at baseline x 100.

CONCLUSION

The described UPLC assay is simple, precise, accurate, and rapid. It was successfully used to study the stability of fluoxetine under various conditions.

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