

HPLC-FL method for fluoxetine quantification in human plasma

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Abstract

Fluoxetine, a selective serotonin reuptake inhibitor (SSRI) antidepressant is proved to be safer than tricyclic antidepressants both in therapy and in overdose, although, fluoxetine and its active metabolite norfluoxetine have long half-lives and can result in potentially serious drug interactions. Therefore, clinical monitoring is justified not only for checking patients' compliance, but also to detect the overdoses risk.

The objective of the study was to develop a HPLC method with fluorescence detection (FL) applicable for quantification of fluoxetine in human plasma.

The HPLC-FL method was chosen, as it is more specific (only 15% of the active substances have native fluorescence) and the experimental conditions were simple: stationary phase - C18 – Kromasil column, the separation was achieved by reverse phase HPLC method, using as mobile phase – methanol: acetonitrile: formic acid = 25:50:25, and the method is rapid (the analysis time was only 3 minutes). Both liquid-liquid (using different solvents, such as hexane, dichloromethane) and solid phase (on the C18 and C8 cartridges) extraction procedures have been applied.

The linearity of the method has been demonstrated in the range of concentrations of 0.1 – 1 µg/mL, corresponding to both therapeutic and toxic plasma fluoxetine levels. The method is precise, accurate, with average recovery percent between 93 - 106% and has a LOD of 0.03 and a LOQ of 0.1 µg/mL.

The proposed method can be used to assess fluoxetine levels in human plasma in pharmacokinetic studies, in clinical monitoring as well as and in overdose cases.

Keywords: *fluoxetine, HPLC, fluorescence*

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I. INTRODUCTION

Fluoxetine, (RS)-N-methyl-3-phenyl-3-[4-(trifluoromethyl) phenoxy] propan-1-amine, a selective serotonin reuptake inhibitor (SSRI) is one of the most widely prescribed antidepressant. It is considered that SSRIs are safer than tricyclic antidepressants because of the lower risk of adverse and toxic reactions. From a toxicological point of view, fluoxetine increases the risk of suicide and can cause serotonergic effects (serotonergic syndrome).

The therapeutic plasma concentrations of fluoxetine are very low (at a level $< 1\mu\text{g/mL}$). Therefore a sensitive and selective method for the assay of fluoxetine is needed. Fluoxetine (FLX) is extensively metabolized by N-demethylation in vivo (CYP2D6) to its active metabolite norfluoxetine (NFLX) [1, 2].

There have been several methods for determination of FLX and NFLX by gas chromatography with electron capture detection (GC-ECD) [3], gas chromatography-mass spectrometry (GC-MS) [4] or high-performance liquid chromatography-mass spectrometry (HPLC-MS) [5, 6]. However HPLC with fluorescence detection remains the most accessible and widely used liquid-chromatographic method for FLX quantification in biological samples.

The aim of the study was to develop and validate a simple, fast and sensitive HPLC method with fluorescence detection for quantification of fluoxetine in human plasma. Two different methods of extraction were used and compared - solid phase extraction and liquid-liquid extraction - a method which had to satisfy the requirements for bioequivalence.

II. MATERIALS AND METHODS

1. Chemicals

Reagents:

- Formic acid (Sigma Aldrich)
- Acetonitrile for chromatographic use (Sigma Aldrich®)
- Methanol for chromatographic use (Merck®)
- Water for chromatographic use (obtained with Barnstead®EasysureRoDi system)
- Chloroform (Chimopar®)
- Sodium hydrogencarbonate (Sigma Aldrich®).

Reference standards and active substances:

- Fluoxetine hydrochloride (Sigma Aldrich®)
- Materials Extraction cartridges DSC C18 (Supelco®)
- Chromatographic column: Kromasil, 150 mm x 4.6 mm, 5 μm ; C₁₈stationary phase.

Apparatus:

- Liquid-chromatograph Surveyor Plus (Thermo Electron Corporation), equipped with:
 - DAD (diode array detector), with a specialized program for the determination of the spectral purity for eluted compounds
 - Fluorescence detector
 - Quaternary pump with vacuum degasser
 - Autosampler thermostat (Peltier)
- Ultrasonic bath (Elmasonic S60/H, Elma)
- Analytical balance Ohaus
- Evaporator under stream of nitrogen (Techne Dry-Block DB-3D, Bibby scientific Inc. England)
- Refrigerated centrifuge Sigma 2-16k
- Stirrer Vortex 2 Genie

Biological sample: human plasma obtained from peripheral venous blood provided by Hematology Institute „ Prof. Dr. C.T. Nicolau”, Bucharest.

2. Chromatographic conditions

- Mobile phase: formic acid: acetonitrile: methanol (25:50:25)
- Flow rate: 1 mL/min
- Column temperature: 22°C
- Sample temperature: 20°C
- Fluorescence detector: λ_{ex} = 224 nm and λ_{em} = 292 nm
- Elution: isocratic
- Injection volume: xx μ L
- Analysis time: 3 minutes

Experimental data was analysed with Chromquest 4.2 data processing software.

3. Standard solutions

Fluoxetine stock solution: 10 mg of fluoxetine hydrochloride were quantitatively transferred into a 10 mL volumetric flask, dissolved in (solvent) and diluted to volume with the same solvent. Fluoxetine working solution (10 μ g/mL) was prepared by dilution with mobile phase, from the stock solution.

4. Control samples

The „simulated” human plasma controls were prepared at the following concentrations: 0.1 μ g/mL, 0.5 μ g/mL and 1 μ g/mL.

5. Sample preparations

The techniques used for extraction are often liquid-liquid extraction methods, but solid phase extraction methods have also been reported [7, 8, 9]. One aim of this study was also to conclude which one is more appropriate for obtaining samples from human plasma for fluoxetine analysis.

5.1. Liquid-liquid extraction

Liquid-liquid extraction was applied on the samples from tubes 1-4.

The solutions were treated with 500 μ L 1% aqueous solution of sodium hydrogen carbonate and 4 mL chloroform and then the samples were vortex-mixed for 15 minutes, then centrifuged for 10 minutes at 20°C with 3000 rpm. The lower phase (chloroform) is separated and used for further processing.

The organic phases were transferred into the evaporation vials and they were evaporated at 40°C under nitrogen stream. After evaporation, the residue was sampled with 500 μ L of mobile phase, sonicated for xx minutes, and then the samples obtained were injected into the HPLC system.

5.2. Solid phase extraction

Solid phase extraction was applied on the samples from tubes 5-8. The C₁₈ cartridges were treated with 1 mL 1M HCl, 1 mL methanol, 1 mL distilled water and 1 mL 1% aqueous solution of sodium hydrogen carbonate, for maintaining the basic pH.

Then, the content of the four tubes were passed through the cartridges, which were then washed with 1 mL of distilled water and 1 mL of acetonitrile. The compounds were eluted with 1 mL mixture dichloromethane: isopropanol: ammonium hydroxide 25% (78:20:2).

The extract was evaporated at 40°C under nitrogen stream, and then the residue was sampled with 500 μ L mobile phase, sonicated for 1-2 minute and then the samples obtained were injected into the HPLC system.

6. Analytical method validation

The method validation was carried out in accordance with FDA Guidance for Industry - Bioanalytical Method Validation, DRAFT guidance, 2013.

III. RESULTS AND DISSCUTION

A HPLC method with fluorescence detection for the determination of fluoxetine from human plasma was developed and validated. The biological samples were processed using solid phase extraction. Liquid-liquid extraction was also used for comparison.

The HPLC-FL method was chosen because:

- The fluorescence detection method is less costly than the MS detection, with no loss of selectivity or sensibility.
- The HPLC technique is easily applicable, whereas for the GC technique derivatisation steps are necessary.

Method validation

1. Linearity and range

For the calibration curve, solutions of fluoxetine hydrochloride at 5 concentration levels were used: 0.1 µg/mL; 0.25 µg/mL; 0.5 µg/mL; 0.75 µg/mL; 1.0 µg/mL, concerning the concentration domain of 0.1 – 1 µg/mL. Three replicate injections from each solution were performed. Results are presented in Figures 1 and 2.

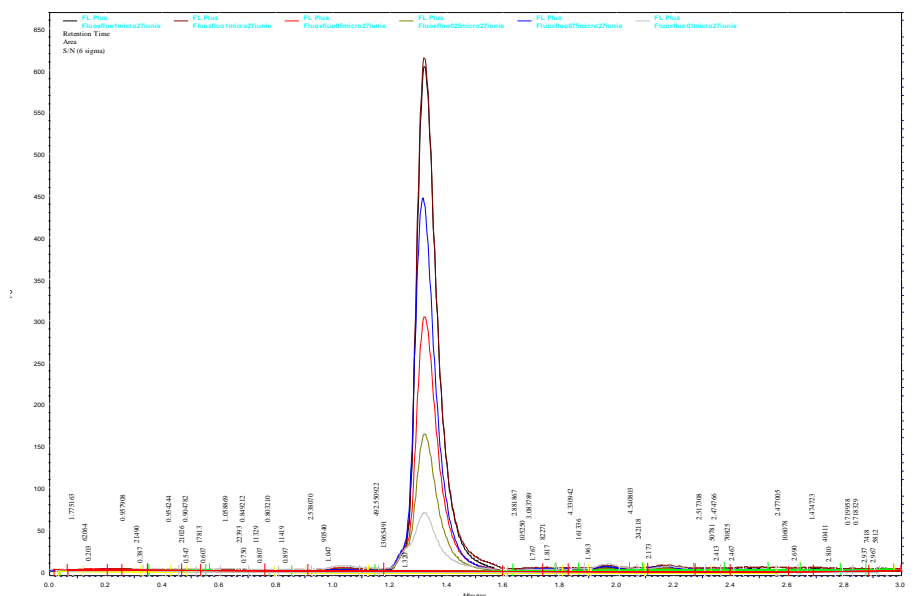


Figure 1. Chromatograms of the 5 solutions of fluoxetine hydrochloride

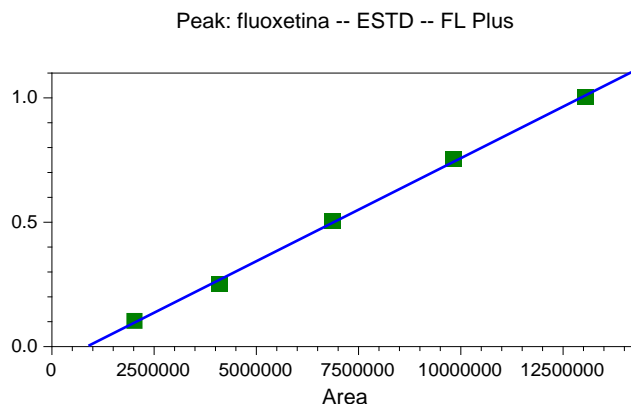


Figure 2. Calibration curve for the HPLC-FL method of assay for fluoxetine hydrochloride – every point from the calibration curve represents an average of 3 determinations

The experimental data fit the linear model, the regression equation $y=8.28558e-008x - 0.0758373$, having a correlation coefficient of 0.9991.

2. Precision of the method

The precision of the method was evaluated as repeatability at five concentration levels. The method is precise and the repeatability is satisfactory with and $RSD < 2\%$, with results presented in Table 1.

Table 1. The precision parameters for the assay of fluoxetine hydrochloride

	Level 1	Level 2	Level 3	Level 4	Level 5
Concentration $\mu\text{g/mL}$	0,10	0,25	0,50	0,75	1,00
Area	2040372	4120307	6890894	9839238	13065491
RF	4.9010670603 2037e-008	6.0675090472 6274e-008	7.2559525658 0641e-008	7.622541501 68946e-008	7.65374986 672908e-008
Last Area Residual	0.007	-0.016	0.005	0.011	-0.007
Rep StDev	53935.3	76606.2	23705.3	61517.4	194666
Rep %RSD	2.58	1.86	0.34	0.63	1.47
Rep 1 Area	2094803	4207204	6916376	9821583	13438374
Rep 2 Area	2148241	4054475	6938259	9724962	13154962
Rep 3 Area	2040372	4120307	6890894	9839238	13065491

3. The average recovery

The method accuracy is within acceptance limits according to FDA guidelines. Results are presented in Table 2.

Table 2. The results for recovery for the assay of fluoxetine hydrochloride

Concentration($\mu\text{g/mL}$)	0.10	0.25	0.50	0.75	1.00
Concentration determined ($\mu\text{g/mL}$)	0.098	0.266	0.497	0.825	0.930
Recovery (%)	98.0	106.4	99.4	110.0	93.0

Average recovery: 101.4%

4. The selectivity of the method

The two methods of extraction were used and compared. The solid phase extraction method is better regarding fluoxetine peak symmetry and separation from other plasma generated peaks.

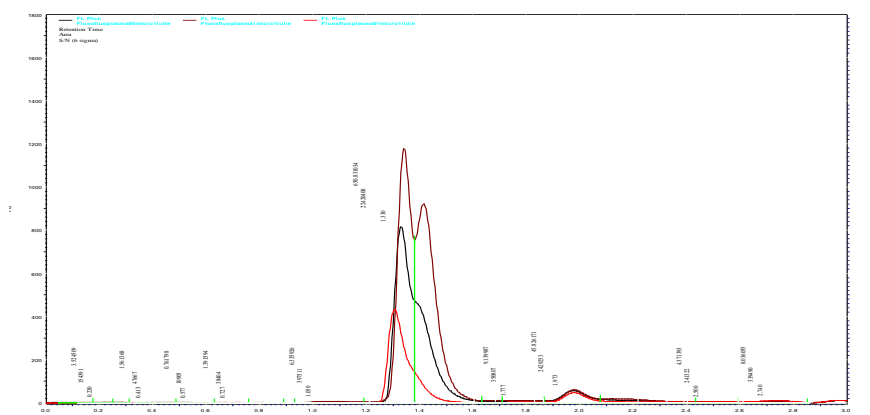


Figure 3. Chromatograms corresponding the extractive solutions of blank plasma, and plasma treated with fluoxetine hydrochloride in concentration of 0.1 $\mu\text{g/mL}$, 0.5 $\mu\text{g/mL}$, 1 $\mu\text{g/mL}$ – liquid phase extraction (LPE)

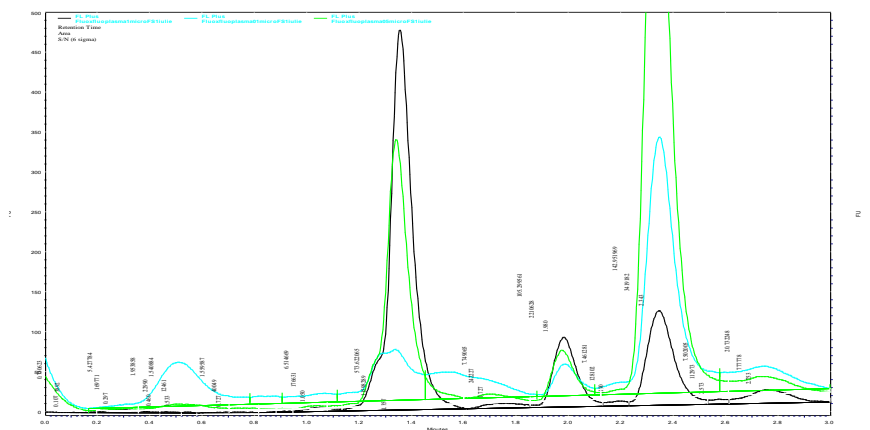


Figure 4. Chromatograms corresponding the extractive solutions of blank plasma, and plasma treated with fluoxetine hydrochloride in concentration of 0.1µg/mL, 0.5µg/mL, 1µg/mL – solid phase extraction (SPE)

It can be seen (Figure 5) that a peak from the blank plasma interferes with fluoxetine. This confirms the lack of selectivity of the liquid-liquid extraction method. The chromatogram obtained with the samples prepared using solid phase extraction (Figure 6) show that no interference with fluoxetine peak is obtained from blank plasma, therefore demonstration the specificity of this extraction method.

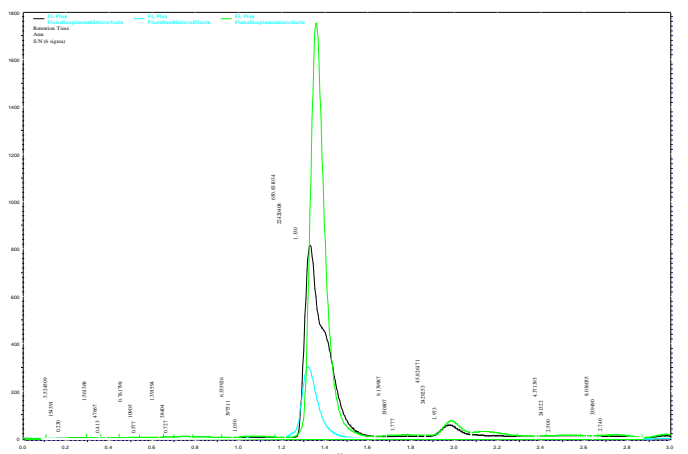


Figure 5. Chromatograms corresponding the extractive solutions of blank plasma, plasma treated with fluoxetine hydrochloride in concentration of 0.5µg/mL, and standard solution of fluoxetine 0.5µg/mL – liquid phase extraction (LPE)

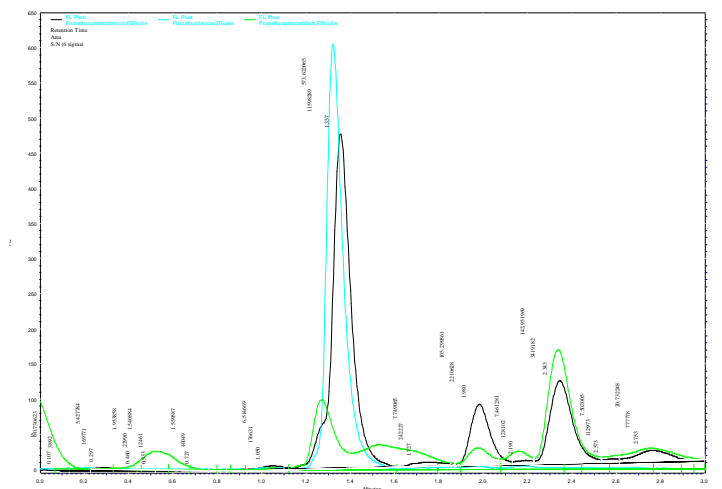


Figure 6. Chromatograms corresponding the extractive solutions of blank plasma, plasma treated with fluoxetine hydrochloride in concentration of 1 µg/mL, and standard solution of fluoxetine 1µg/mL – solid phase extraction (SPE)

5. Limit of detection (LOD) and limit of quantification (LOQ)

The detection and quantification limits were established based on the signal - noise (S/N) ratio, in accordance with the recommendations of ICH validation Q2 (R1) using the standard deviation of the response. Thus, the detection limit, LOD = 0,0110 µg/mL and the quantification limit LOQ was set at 0.1 µg/mL.

IV. CONCLUSIONS

A HPLC method with fluorescence detection for the quantification of fluoxetine in human plasma was developed and validated. The method was fully validated and all validation parameters are within acceptable limits according to bioanalytical method validation guidelines: the method is linear, accurate and precise on the domain of concentration 0,1 – 1 µg/mL, appropriate to therapeutic plasmatic concentration of fluoxetine (0,05-0,48 µg/mL). This makes the method useful for monitoring the patients under treatment. The method is simple, fast (3 minutes time of analysis) and the applicability was checked using plasma samples spiked with fluoxetine, extracted using a solid phase extraction technique. It can be considered that the method developed can be used in pharmacokinetics studies and in therapeutic drug monitoring for patients under treatment with fluoxetine.

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