

Enantioselective Analysis of Fluoxetine and Norfluoxetine in Plasma Samples by Protein Precipitation and Liquid Chromatography with Fluorescence Detection

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Neste trabalho, o método de precipitação de proteínas e cromatografia líquida de alta eficiência com detecção de fluorescência (PP/CLAE-fluorescência) foi desenvolvido para a análise enantioselectiva da fluoxetina e norfluoxetina em amostras de plasmas. Diferentes agentes precipitantes, como solventes orgânicos, ácidos e sais, em diferentes proporções, foram avaliados. Segundo testes calorimétricos de Bradford, dentre todos os agentes precipitantes avaliados, a acetonitrila apresentou maior eficiência, considerando o número de etapas requeridas e a porcentagem de precipitação de proteínas (99,7%), quando adicionado na proporção de 3:1 (agente precipitante: plasma, v/v). O método PP/CLAE-fluorescência apresentou limite de quantificação de 30 ng mL⁻¹ e intervalo linear de 30 a 1000 ng mL⁻¹ com coeficientes de regressão linear acima de 0,9973 para os quatro enantiômeros. Em conclusão, o método PP/CLAE-fluorescência padronizado e validado proposto neste estudo foi aplicado com sucesso na análise de amostras de plasma de pacientes em terapia com a fluoxetina.

In this study, the enantioselective analysis of fluoxetine and norfluoxetine in plasma samples was performed by the protein precipitation method and high performance liquid chromatography with fluorescence detection (PP/LC-FD). Different precipitating agents - organic solvents, acids, and salts - in several proportions were available. The Bradford colorimetric method employed for evaluation of the efficiency of protein precipitation, has shown that for the sake of simplicity and percentage of protein precipitation (99.7%), acetonitrile was most effective when added at a ratio of 3:1 (acetonitrile/plasma, v/v). The quantification limit of the PP/LC-FD method was 30 ng mL⁻¹ for the four enantiomers. The response of the proposed method was linear over a dynamic range from LOQ to 1000 ng mL⁻¹, with correlation coefficients higher than 0.9973. In conclusion, the PP/LC-FD method can be successfully used to analyze plasma samples from ageing patients undergoing therapy with fluoxetine.

Keywords: fluoxetine and norfluoxetine, enantioselective analysis, protein precipitation, plasma, LC-FD

Introduction

Fluoxetine (FLX) (*N*-methyl-3-phenyl-3-[(α,α,α -trifluoro-*p*-tolyl)oxy]-propylamine) is one of the most widely prescribed selective serotonin reuptake inhibitors for the treatment of depression. Factors affecting the safety and efficacy of the drug have not been fully characterized, although fluoxetine have been available for over 20 years. The lack of a suitable test to discriminate between the *R* and *S* enantiomers of fluoxetine and its active demethylated metabolite, norfluoxetine (NFLX), has hampered assessments of the potential correlation between clinical response and plasma

levels of fluoxetine and / or norfluoxetine for many years.¹ The chemical structures of the enantiomers of fluoxetine and norfluoxetine are shown in Figure 1.

In fact, while the two FLX enantiomers are approximately equipotent in blocking 5-HT (serotonin) reuptake, the NFLX enantiomers display marked differences in pharmacological activity, with the *S*-enantiomer being approximately 20 times more potent than the *R*-enantiomer as a 5-HT-reuptake inhibitor both *in vitro* and *in vivo*.^{2,3} On the basis of these results, it is evident that the concentration of individual enantiomers should be taken into account for a proper understanding of the role of pharmacokinetic variability in the explanation of intraindividual and interindividual clinical response.

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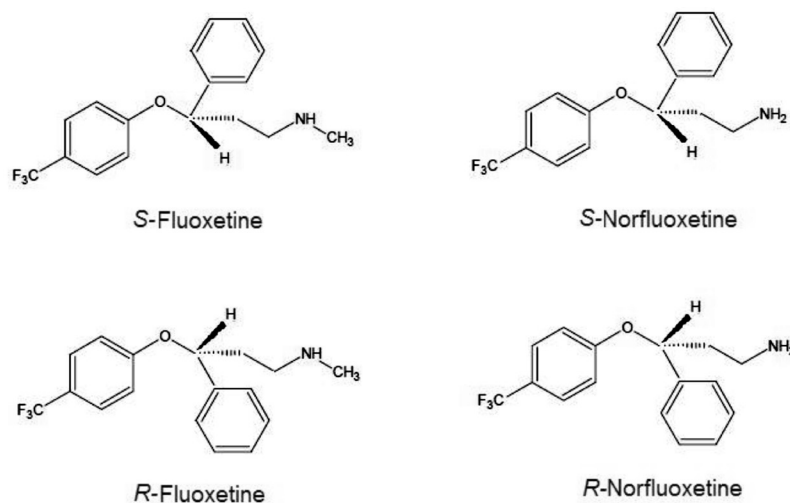


Figure 1. Chemical structures of the enantiomers of fluoxetine and norfluoxetine.

For this purpose, several chromatographic methods (both gas chromatography, GC, and liquid chromatography, LC) have been described for the determination of FLX and NFLX enantiomers, including direct (using chiral stationary phases) and indirect (after derivatization in mobile phase) methods.⁴⁻⁸ Moreover, as fast and reliable high throughput analysis of drug and metabolite concentrations in plasma samples is essential for pharmacokinetic, pharmacodynamic, and toxicokinetic studies, the protein precipitation method (PP) has been an ideal sample preparation technique of complex matrices, such as plasma, serum, and whole blood, for drug analysis.^{9,11} In protein precipitation, acids, salts, or water miscible organic solvents are used to remove the protein by denaturation and precipitation. Acids, such as trichloroacetic acid (TCA) and perchloric acid, are very efficient at precipitating proteins. Organic solvents, such as methanol and ethanol, have been widely employed in bioanalysis because of their compatibility with LC mobile phases.¹²

In order to quantify drug in a plasma sample, it is often necessary to disrupt the protein-drug binding, so that the total amount of drug can be extracted for analysis. Precipitation is useful because it can be utilized to denature the protein, destroying its drug binding ability depending on the binding mechanism.¹³ Besides being a very simple and fast procedure, the protein precipitation technique can be employed as a precursor to other methods and also to increase method precision and decrease the consumable costs, as many fully automated methods employing robotic sample processor in the 96-well plate format have been reported.^{14,15}

This study has evaluated several protein precipitants (organic solvents, acids, and salts) in human plasma samples by spectrophotometrically examining their effectiveness at protein removal, for a high throughput sample analysis of

fluoxetine, norfluoxetine, and their enantiomers by liquid chromatography - fluorescence detection (LC-FD).

Experimental

Reagents and analytical standards

Fluoxetine and norfluoxetine analytical standards were donated by Sigma-Aldrich® (São Paulo, Brazil). The working standard drug solutions, based on therapeutic interval concentrations, were prepared by diluting the stock solutions of these drugs (1 mg mL^{-1}) to a proper methanol volume. These solutions were stable for 18 months at $-20\text{ }^{\circ}\text{C}$ when stored in methanol. The water used to prepare the mobile phase had been purified by a Milli-Q system (Millipore®, São Paulo, Brazil).

Methanol, acetone and acetonitrile (HPLC grade), ethanol, hydrochloric acid, acetic acid, phosphoric acid, perchloric acid 70%, trichloroacetic acid, and concentrated sulfuric acid were purchased from J.T. Baker® (Phillipsburg, USA); potassium hexafluorophosphate 98%, bovine serum albumin (BSA), and sodium perchlorate 98% were obtained from Aldrich® (Steinheim, Germany). Sodium phosphate dibasic, sodium borate, sodium hydroxide, copper sulfate, ammonium acetate, zinc sulfate, ammonium sulfate, anhydrous sodium sulfate, sodium acetate, and Coomassie Blue G-250 dye were acquired from Merck® (Darmstadt, Germany). The saturated aqueous salt solutions were prepared at $25\text{ }^{\circ}\text{C}$ for the PP technique.

Plasma samples

Plasma from healthy volunteers that had not been subjected to any pharmacological treatment for at least

72 h (blank plasma) was supplied by Hospital das Clínicas de Ribeirão Preto, University of São Paulo, Brazil. The plasma samples were stored at $-20\text{ }^{\circ}\text{C}$ for up to 6 months. The principles embodied in the Helsinki Declaration were observed, and the study was approved by the Ethics Committee of the University of São Paulo in Ribeirão Preto, Brazil. The plasma sample was collected from elderly patients subjected to therapy with fluoxetine for at least 2 weeks. Blood samples were drawn 12 h after the last drug administration.

Instrumentation and chromatographic conditions

The LC-FD analyses were performed in a Shimadzu[®] LC-20AT chromatograph (Kyoto, Japan) equipped with a RF-10AxL detector, and a CBM-20A system controller. The enantiomers were separated in the tris-(3,5-dimethylphenyl carbamate) cellulose column (Chiralcel OD-R, $250 \times 4.6\text{ mm}$, $10\text{ }\mu\text{m}$ - Chiralcel Tech[®]) at room temperature ($25\text{ }^{\circ}\text{C}$) with a mobile phase consisting of potassium hexafluorophosphate 7.5 mmol L^{-1} and sodium phosphate 0.25 mol L^{-1} solution, pH 3.0, and acetonitrile (75:25, v/v), in the isocratic mode, at a flow rate of 1.0 mL min^{-1} . The column effluent was monitored at Ex/Em 230/290 nm. The pH of the mobile phase, adjusted with chloridric acid 0.1 mol L^{-1} , was measured in the aqueous component alone, and was filtered and degassed prior to use.

A Thermo Spectronic[®] spectrophotometer (model 4001/4, USA) was used for the spectrophotometric assays at 595 nm. Other instrumentations included a centrifuge model 206 BL from Fanem[®] (São Paulo, Brazil) and a tube shaker model MA138 (Marconi[®], São Paulo, Brazil).

Protein precipitation procedure

The following protein precipitant solutions were prepared: aqueous ammonium, sodium, copper and zinc sulfates (saturated at room temperature), aqueous ammonium acetate (saturated at room temperature), aqueous trichloroacetic acid (TCA) (10%, m/v), aqueous perchloric acid 70%, and aqueous sulfuric acid 97%. Acetonitrile, ethanol, acetone, and methanol were also used as solvent for protein precipitation. Each precipitant was added to the plasma samples at volume ratios ranging from 0.5:1 to 4:1 (precipitant/plasma, v/v), in triplicate. Solutions were shaken for 15 min and centrifuged for 25 min at 3500 rpm. The plasma sample volume was fixed at $100\text{ }\mu\text{L}$, according to previous studies.¹² After the precipitation process, the supernatant was collected and injected into the LC-FD system. In the cases of protein

precipitation using acid solutions, the supernatant pH was adjusted to 7.0 before the injection.

Protein concentration determination

The Bradford reagent was prepared according to the protocol described in the literature.¹⁶ Although the absorbance of Coomassie Blue dye at 595 nm is proportional to the amount of bound protein, it is necessary to establish a correspondence between absorbance values and known amounts of protein. For this purpose, a series of six BSA protein standards was prepared using sequential dilution of a protein solution of known concentration in water, from 0 to $10.0\text{ }\mu\text{g L}^{-1}$. A volume of $10\text{ }\mu\text{L}$ of the protein precipitant was added to $90\text{ }\mu\text{L}$ of each standard solution (0, 2, 4, 6, 8 and $10\text{ }\mu\text{g L}^{-1}$). Finally, the volume was completed with 1 mL of the Bradford reagent. These solutions were shaken for 10 s, left to stand for 2 min, and the absorbance was read on a spectrophotometer at 595 nm. Once the absorbance for all the standards had been measured, a standard curve was plotted (absorbance *versus* known protein content of each standard) for each protein precipitant. After registration of the absorbance of the unknown sample, the standard curves were used for determination of the amount of protein corresponding to the measured absorbance values. Plasma protein concentration was found by comparing absorbance values of the analytical curve and that of the plasma sample precipitation and further comparison with that of non-precipitated plasma (total), where precipitation efficiency = [(total plasma protein – protein remaining in the supernatant) / total plasma protein] $\times 100$.

Analytical validation

The analytical validation of the PP/LC-FD method was carried out with blank plasma samples spiked with drugs standard solutions at concentrations that included the therapeutic plasma levels. The linearity was evaluated by calibration curves constructed using linear regression of the drug peak area (Y) *versus* the drug nominal plasma concentration (X, ng mL^{-1}). These sample concentrations ranged from the limit of quantification (LOQ) to 1000 ng mL^{-1} .

Accuracy, recovery, and inter-day precision values were determined by calibration curves, by means of quintuplicate PP/LC-FD assays of the blank plasma samples spiked with analytes at concentrations of 100, 300, 400, 500 and 1000 ng mL^{-1} . Accuracy values were calculated by comparison between the drugs concentrations added to the plasma samples with plasma drug concentrations determined by the calibration curve. Furthermore,

the selectivity of the method was also investigated by comparison of retention times among the analytes, other drugs, and endogenous compounds.

Results and Discussion

Optimization of protein precipitation

The Bradford assay is based on the use of a dye, Coomassie Blue G-250, to which protein binds, thus altering the light absorbance properties of the dye. When the dye is prepared as an acidic solution (in 85% phosphoric acid), it maximally absorbs light with a wavelength of 465 nm. Protein addition results in a shift of the dye's absorption maximum to 595 nm. As the protein concentration increases, the absorbance of light at 595 nm rises linearly, and can be measured in a spectrophotometer.¹⁶ The BSA standard curves at 595 nm for the 12 available precipitants were found to be linear, with correlation coefficients higher than 0.9938 (Table 1).

The different protein precipitation techniques lead to different modes of protein precipitation.¹⁷ Protein solubility results from polar interactions with the aqueous solvent, ionic interactions with salts, and repulsive electrostatic forces between charged molecules. At the isoelectric point (pI), there is no net charge on a protein, so similarly the protein has minimum solubility in aqueous solvent. Above the pI, a protein has a net negative charge, while below its pI, it has a net positive charge. Precipitant effectiveness increases with volume to a maximum that is reached at a ratio of approximately 3 or 4 to 1. The selected volume ratios are listed in Table 2.

Results from protein precipitation (percentage of precipitation and remaining protein plasma concentration)

for the twelve precipitants in human plasma are shown in Table 2. Each protein precipitation efficiency value is an average of three replicates. With the exception of ammonium sulfate and sulfuric acid 97%, all precipitants were on average at least 90% effective.

Precipitants exert specific effects on proteins in order to facilitate their precipitation from solution. Organic solvent precipitants lower the dielectric constant of the plasma protein solution, which increases the attraction between charged molecules and facilitates electrostatic protein interactions. The organic solvent also displaces the ordered water molecules around the hydrophobic regions on the protein surface. Hydrophobic interactions between proteins are minimized as a result of the surrounding organic solvent, while electrostatic interactions become predominant and lead to protein aggregation. Organic solvents are the most widely employed protein precipitants in drug analysis, especially acetonitrile.^{9,15} In this work, acetonitrile was also found to be the best organic plasma protein precipitant, particularly at a 3:1 (precipitant/plasma) volume ratio. After precipitation with acetonitrile, and other organic solvents, the supernatant was easily dried, providing increased analytes concentration just before injection into the LC-FD system.

Acidic reagents form insoluble salts with the positively charged amino groups of the protein molecules at pHs below their pI. Proteins are precipitated from solutions with high salt concentrations as the salt ions become hydrated and the number of available water molecules decrease, drawing the water away from the protein hydrophobic surface regions. This results in aggregation of protein molecules via protein-protein hydrophobic interactions.^{17,18} This study has also shown that protein precipitation using strong acids (perchloric acid 70% and trichloroacetic acid)

Table 1. Equations and linear coefficients of the calibration curves

	Precipitant	Linear regression equation	Linear correlation coefficient (r^2)
Acids	Sulfuric acid 97%	$Y = 0.05166 * X$	0.99783
	Perchloric acid 70%	$Y = 0.03424 * X$	0.99499
	Trichloroacetic acid 10%	$Y = 0.03491 * X$	0.99387
Salts (saturated solution)	Ammonium acetate	$Y = 0.05174 * X$	0.99544
	Ammonium sulfate	$Y = 0.04364 * X$	0.99962
	Sodium sulfate	$Y = 0.04590 * X$	0.99402
	Copper sulfate	$Y = 0.04341 * X$	0.99842
	Zinc sulfate	$Y = 0.04389 * X$	0.99768
Organic solvents	Acetonitrile	$Y = 0.04625 * X$	0.99924
	Methanol	$Y = 0.04635 * X$	0.99619
	Acetone	$Y = 0.04715 * X$	0.99513
	Ethanol	$Y = 0.03335 * X$	0.99581

Table 2. Efficiency of proteins precipitation with different agents

Precipitant		Proportion (precipitant: plasma, v/v)	Mean absorbance / AU	Corresponding concentration / (g L ⁻¹)	Efficiency of protein precipitation / (%)
Acids	Sulfuric acid 97%	1 drop	0.413	7.995	85.7
	Perchloric acid 70%	4:1	0.011	0.032	99.9
	Trichloroacetic acid 10%	4:1	0.014	0.040	99.9
Salts (saturated solution)	Ammonium acetate	3:1	0.113	4.368	92.2
	Ammonium sulfate	2:1	0.182	8.340	85.1
	Sodium sulfate	3:1	0.097	4.226	92.5
	Copper sulfate	3:1	0.093	4.284	92.4
	Zinc sulfate	4:1	0.084	3.828	93.2
Organic solvents	Acetonitrile	3:1	0.071	0.154	99.7
	Methanol	4:1	0.336	0.725	98.7
	Acetone	2:1	0.171	0.363	99.4
	Ethanol	4:1	0.156	0.936	98.3

is effective at high volume ratios (4:1, Table 2), though there is need for an additional step in the process: dilution with basic solutions for control of pH after protein precipitation and before injection into the LC-FD system. Although salt solutions provided efficient protein precipitation by the salting-out effect, the high water volume added to the plasma sample decreased the analytes concentration in the free protein sample.

This study is in general agreement with previously reported data using the Lowry method for assessment of protein precipitation effectiveness.¹² Among the tested precipitants, acetonitrile and trichloroacetic acid provided the most efficient precipitation compared to precipitants with similar mechanisms. Thus, because of the high protein precipitation efficiency and simplicity, acetonitrile was selected as precipitant at a volume ratio 3:1 (acetonitrile/plasma) for analytical validation.

Chromatographic conditions

Due to the high solubility of FLX and its metabolite NFLX in water, separation was carried out by reversed phase chromatography. For the mobile phase, a mixture of two different salt solutions (potassium hexafluorophosphate and sodium phosphate) was necessary, to increase resolution while at the same time decreasing peak width, whereas acetonitrile provided shorter analysis time. The most suitable percentage of acetonitrile for a good separation in a reasonable analysis time was 25%.

The acid-base properties of FLX and NFLX mean that the pH of the mobile phase could influence the chromatographic separation. The studied pH range was 3 to 7, and no significant differences were observed with

regard to the chromatographic resolution. However, as the maximum excitation and emission may vary with pH, the influence of this variable on the intensity of the signal was studied. A progressive reduction could be observed from pH 3, so this was the value selected as optimum for the study.

The identification of *R* and *S* enantiomers of fluoxetine and norfluoxetine was performed according to literature results, considering the elution order of these drugs in the same chiral column and detection system.¹ The mobile phase consisted of potassium hexafluorophosphate 7.5 mmol L⁻¹ and sodium phosphate 0.25 mol L⁻¹ solution, pH 3, acetonitrile (75:25, v/v), at a flow rate of 1.0 mL min⁻¹ in the isocratic mode. Under this set conditions, the LC-FD analysis in the selected mobile phase furnished appropriate resolution and satisfactory analysis time (23 min). In passing through the stationary phase, Chiralcel OD-R, the drug can interact with groups C=O and NH groups through hydrogen bonds, in addition to the dipole-dipole interactions with the groups C=O,¹⁹ which affords good resolution ability.

Analytical validation

The validation of PP/LC-FD method was conducted to minimize errors, ensure the highest quality of analytical work and, consequently, to obtain reliable laboratory results. The specificity (selectivity) of the method is demonstrated by the following chromatograms: reference plasma blank (Figure 2a) and reference plasma sample spiked with the enantiomers at a concentration of 500 ng mL⁻¹ (Figure 2b).

No interfering peaks were found in chromatograms obtained after extraction of different sets of blank plasma

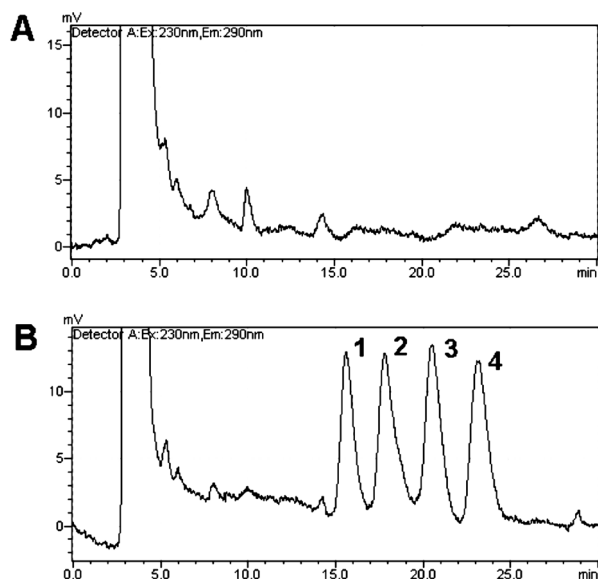


Figure 2. PP/LC-FD chromatograms. A) Blank plasma sample, B) Blank plasma sample spiked with FLX and NFLX, resulting in 500 ng mL⁻¹. 1: *S*-Norfluoxetine, 2: *R*-Norfluoxetine, 3: *S*-Fluoxetine, 4: *R*-Fluoxetine.

samples ($n = 5$) from drug-free healthy volunteers. This gives evidence of the ability of the method to unequivocally measure the drugs in the presence of endogenous plasma components. Because fluoxetine can be prescribed in combination with different psychotropic agents and/or other drugs, it is important to evaluate their co-elutions with the studied enantiomers. According to Table 3, these drugs did not co-elute with the enantiomers under study.

Fluoxetine and norfluoxetine provided a satisfactory response in the fluorescence detector. Under our LC-FD conditions, the limit of detection (LOD – $S/N = 3$) was 5 ng mL⁻¹. This determination was performed with standard drugs solutions. The limit of quantification (LOQ – $S/N = 10$) of the enantiomers in plasma was 30 ng mL⁻¹, with RSD (relative standard deviation) lower than 15%, for the four analytes studied here (Table 4).

The linearity of the method PP/LC-FD was determined in plasma samples spiked with reference antidepressants in plasma related to the LQ, 300, 400, 500, and 1000 ng mL⁻¹. This range was linear, with linear regression coefficients above 0.9973, and the coefficients of variation for all calibration points were lower than 14% (Table 5).

Table 4. Linearity and coefficient of variation (CV) for the LOQ

Drug	Linear regression	r ²	CV / (%), $n = 5$ (LOQ = 30 ng mL ⁻¹)
<i>S</i> -NFLX	$y = -27564.22 + 1083.77 x$	0.9993	13.6
<i>R</i> -NFLX	$y = -32043.92 + 1048.36 x$	0.9978	12.5
<i>S</i> -FLX	$y = -88237.23 + 1491.19 x$	0.9973	9.8
<i>R</i> -FLX	$y = -2219.33 + 1484.45 x$	0.9976	14.1

Table 3. Retention time of the drugs studied as possible interferent

Drugs	Retention time / min
4-Methyl primidone	9.343
Amitriptyline	nd
Amphetamine	nd
Caffeine	nd
Carbamazepine	nd
Cocaine	7.496
Citalopram (enantiomers)	9.460 / 11.038
Clomipramine	Nd
Desipramine	Nd
Diazepam	5.721
Phenyl hydantoin	nd
Phenytoin	nd
Phenobarbital	8.906
Imipramine	nd
Methamphetamine	nd
Mirtazapine	nd
Moclobemide	nd
Nortriptyline	nd
Paroxetine	nd
PEMA	4.894
Primidone	6.603
<i>R</i>-Fluoxetine	22.988
<i>R</i>-Norfluoxetine	17.903
Sertraline	nd
<i>S</i>-Fluoxetine	18.883
<i>S</i>-Norfluoxetine	15.664
THC	nd

*nd = not detected until 30 min of running. The drugs marked in bold are the target analytes.

The method presented adequate accuracy, with values ranging between 91.8% and 117.8%. The inter-assay precision ($n = 5$) of the standardized method was determined using plasma samples enriched with the enantiomer concentrations of 100, 300, 400, 500 and 1000 ng mL⁻¹. The coefficients of variation at the five analyzed concentrations were lower than 14% for the four

Table 5. Recovery, inter-assay accuracy, and precision of the method PP / LC-FD

Drug	[C] / (ng mL ⁻¹)	Inter-assay (n = 5)			
		Measured concentration ± σ / (ng mL ⁻¹)	CV / (%)	Accuracy / (%)	Absolute recovery / (%)
S-NFLX	100	106.1 ± 12.6	11.8	106.1	54.91
	300	304.5 ± 34.3	11.3	101.5	69.22
	400	394.5 ± 43.1	10.9	98.6	70.93
	500	489.2 ± 45.3	9.3	97.8	61.51
	1000	1005.6 ± 103.1	10.3	100.6	71.70
R-NFLX	100	116.7 ± 13.5	11.6	1116.7	68.91
	300	286.6 ± 22.8	8.0	95.5	76.48
	400	385.0 ± 43.1	11.2	96.3	66.58
	500	506.6 ± 49.1	9.7	101.3	65.50
	1000	1005.0 ± 135.8	13.5	100.5	73.18
S-FLX	100	117.8 ± 5.3	4.5	117.8	52.46
	300	283.0 ± 37.7	13.3	94.3	56.64
	400	385.3 ± 27.8	7.2	96.3	60.70
	500	509.4 ± 66.6	13.1	101.9	68.11
	1000	1004.5 ± 54.5	5.4	100.4	74.29
R-FLX	100	91.8 ± 10.1	11.0	91.8	80.02
	300	286.4 ± 30.2	10.5	95.5	81.11
	400	415.3 ± 56.8	13.7	103.8	72.35
	500	515.5 ± 63.1	12.2	103.1	79.56
	1000	991.0 ± 100.4	10.1	99.1	78.83

studied enantiomers (Table 5). These results demonstrate that the developed method allows for the quantification of antidepressants in the therapeutic interval, although no therapeutic levels have been clearly defined for selective serotonin reuptake inhibitors.²⁰

The stability of standard solutions of FLX and NFLX, as well as plasma samples, had been confirmed in previous studies.^{21,22} According to Kovacevic *et al.*,²¹ no significant loss is observed for analysis of plasma samples stored in methanol for 18 months at -20 °C or after a freeze/thaw 9-month cycle after they were analyzed for the first time. These results are proof of the stability of the plasma samples and standard solutions in conditions proposed in this work.

To evaluate the applicability of the standardized and validated method, plasma samples from elderly patients subjected to therapy with fluoxetine (Prozac®, 20 mg *per day*) were analyzed (Figure 3). The detected concentrations were 570 ng mL⁻¹ for S-NFLX, 434 ng mL⁻¹ for R-NFLX, 759 ng mL⁻¹ for S-FLX, and 58 ng mL⁻¹ for R-FLX.

The PP/LC-FD method developed here give values of accuracy and linearity close to those achieved with sample preparation methods previously described in the literature, such as liquid-liquid extraction (LLE/LC-UV),¹

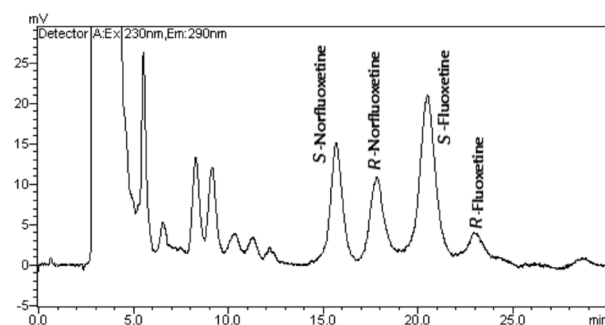


Figure 3. Chromatogram obtained after protein precipitation from plasma samples collected from elderly patient in therapy with fluoxetine in the selected mobile phase. The concentrations found in the patient sample were 570 ng mL⁻¹ for S-NFLX, 434 ng mL⁻¹ for R-NFLX, 759 ng mL⁻¹ for S-FLX, and 58 ng mL⁻¹ for R-FLX.

solid phase extraction (SPE/LC-FD),⁴ and LLE/GC-NPSD (nitrogen-phosphorus selective detector),²³ for analysis of these enantiomers in biological matrices.

Conclusions

The protein precipitation technique has been shown to be a simple and rapid preparation route of plasma samples for enantioselective analysis of the antidepressant

fluoxetine and its metabolite, norfluoxetine, by means of LC-FD. Among all the evaluated precipitants, acetonitrile was the most efficient, considering the necessary number of steps and the percentage of protein precipitation (99.7%) when it was added at a 3:1 ratio (precipitating agent/plasma, v/v).

According to the analytical validation parameters, the PP/LC-FD method is suitable for analysis of antidepressants in plasma samples, with good repeatability, inter assay precision, limit of quantification, linearity, and selectivity. The analysis time (25 min) was satisfactory for quantitative analysis.

To evaluate the applicability of the standardized and validated method, plasma samples from patients in therapy with antidepressants has been reviewed and proven the relevance of the technique for the enantioselective analysis of fluoxetine and norfluoxetine in plasma samples from patients with analyte concentrations between 100 and 1000 ng mL⁻¹.

Acknowledgments

The authors are grateful to Prof. Dr. Arthur Henrique Cavalcante de Oliveira (Departamento de Química-USP/Ribeirão Preto-SP, Brazil) for his contribution in the spectrophotometric assays and to CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) for financial support.

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Submitted: December 1, 2010

Published online: February 24, 2011

FAPESP has sponsored the publication of this article.