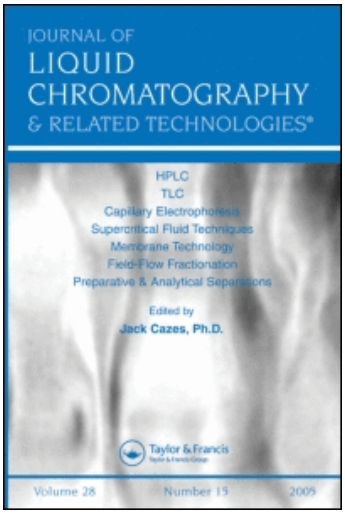

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Julia Atta-Politou[a]; P. Fraskou[a]; M. Koupparis[a]
[a] Laboratory of Analytical Chemistry, Department of Chemistry, University of Athens, Athens, Greece

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Determination of Fluoxetine and Norfluoxetine in Plasma by a Modified High Performance Reversed Phase Liquid Chromatographic Method with Fluorescence Detection

Julia Atta-Politou,* P. Fraskou, and M. Koupparis

Laboratory of Analytical Chemistry, Department of Chemistry, University of Athens, Athens, Greece

ABSTRACT

A simple and rapid reversed phase liquid chromatographic method for the determination of fluoxetine (F) and norfluoxetine (NF) in plasma has been developed and validated. Protriptyline (P) was used as internal standard. Plasma samples were extracted from alkaline pH with hexane–isoamyl alcohol 98 : 2 v/v on a rotator drive for 10 min. The organic phase was back extracted with H_3PO_4 0.1 M and the aqueous phase was injected directly onto a 5 μm Kromasil 100-C8 column (25 cm × 4 mm i.d.), using acetonitrile/0.05 M KH_2PO_4 45 : 55 v/v as the mobile phase. The fluorescence detector was set at 310 nm (λ_{em} = 231 nm). Retention times were 4.6, 5.5,

*Correspondence: Julia Atta-Politou, Laboratory of Analytical Chemistry, Department of Chemistry, University of Athens, Panepistimiopolis, Athens 15771, Greece; E-mail: politou@chem.uoa.gr.

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and 6.4 min for internal standard, NF, and F, respectively. Peak height ratios and plasma F and NF concentrations were linearly related from 8 and 40 to 800 ng/mL ($r > 0.995$ and 0.998, respectively). The detection limits for F and NF were 2.5 and 13 ng/mL, respectively. Precision and accuracy were evaluated from spiked plasma samples and were, for F: CV = 5.4–20% and $E_r = 0.6–9.4\%$ and for NF: CV = 4.7–18% and $E_r = 0.9–10\%$. The developed method is simple, accurate, and of enough detectability to be used for F and NF monitoring in plasma samples of patients under antidepressant therapy with F or in bioavailability studies.

Key Words: Fluoxetine; Norfluoxetine; Plasma; HPLC.

INTRODUCTION

Fluoxetine (F) ((±)-*N*-methyl-γ-4-(trifluoromethyl)phenoxy)benzene-propanamine) (Prozac^R-Eli Lilly), a selective serotonin uptake inhibitor,[1–3] is one of the most frequently prescribed antidepressant drugs. It is administered as tablets or oral solutions in daily doses of 20–80 mg. The major active metabolite of F is norfluoxetine (desmethylfluoxetine) (NF). Several methods[4–23] have been published for the determination of F and NF in biological fluids including gas chromatography with nitrogen–phosphorus,[4] electron capture[5] or MS detector,[6] reversed phase high performance liquid chromatography (HPLC) with fluorescence,[7–10] UV,[8,11–15] diode array,[16] or MS[17,18] detection. Recently, an LC-MS-MS [19] method has been developed for the determination of F and NF in plasma. Stereoselective determinations of F and NF enantiomers have also been achieved with HPLC using UV detection,[20,21] gas chromatography with nitrogen–phosphorus selective detection,[22] or with capillary electrophoresis.[23]

Liquid–liquid[7,10,11,13,15] or solid phase[5,10] extraction has been used for the isolation of F and NF from plasma samples before chromatographic determinations, using two[11,15] or four[10,13] extraction step procedures. The purpose of this study was to further improve the extraction recovery and the sensitivity for the liquid chromatographic determination of F and NF in plasma, by optimization of the experimental conditions.

EXPERIMENTAL

Apparatus

The chromatographic analysis was performed using an HPLC system consisting of: a JASCO (Japan Spectroscopic Co. Ltd.) Model 880 PU pump, fitted

with a Model 880-02 Ternary Gradient Unit operating under isocratic conditions on manual mode, a Model 7125 manual injector (Cotati, Rheodyne, CA) with a 50 μL sample loop; a JASCO Model FP-920 fluorescence detector set at $\lambda_{em} = 310$ nm and $\lambda_{ex} = 231$ nm, a 5 μm Kromasil 100-C8, 25 cm × 4 mm (i.d.) reversed phase column (MZ Analysentechnic D-6500 Mainz); a Hewlett Packard HP 3394A integrator for data acquisition and manipulation of chromatograms operating at peak height mode (chart speed 0.5 cm/min). A filtration system (Millipore, Bedford, MA) with type HV Millipore filters (pore size 0.45 μm) was used for degassing of the mobile phase under vacuum. A Metrohm, Model 691 pH-Meter was used for pH monitoring.

A Fisher isotherm dry bath Model 145 was used for the evaporation of extraction solvents. A vortex (Genie, Model K-550 GE, Scientific Ind., Springfield, MA) set at speed 4 was used for mixing of plasma samples and standards. A rotator drive unit (Stuart Scientific STR4, Bioline, United Kingdom) set at speed 7 was used to assist the extraction of plasma samples.

Reagents and Chemicals

Acetonitrile and water of HPLC grade (Lichrosolv®) were obtained from Merck. Analytical grade KH_2PO_4 was obtained from Serva, while conc. hydrochloric acid (12 M) and conc. phosphoric acid (14.7 M) from Merck. F and NF hydrochloride were kindly gifted from Eli Lilly (Indianapolis) and protriptyline (P) was provided from a local representative. All solutions (buffers and standards) were prepared with HPLC grade water. Phosphoric acid 0.1 M solution was prepared by diluting 680 μL of conc. phosphoric acid up to 100 mL with water.

Chromatographic Conditions

The optimized mobile phase consisted of acetonitrile/0.05 M KH_2PO_4 45 : 55 v/v. A flow rate of 1.0 mL/min was used at ambient temperature, resulting in a pressure of about 127 kg/cm^2. The mobile phase was degassed by vacuum through filtration, after mixing. The fluorescence intensity of the column effluent was monitored at 310 nm with the detector set at 0.030 or 0.060 signal response full-scale (gain = 10).

Standards for Calibration Curve

Standard stock solutions of F, NF, and P (internal standard) were prepared in methanol at a concentration of 1.00 mg/mL (expressed as base). An aqueous

reference solution containing both F and NF to final concentration 100 μg/mL was prepared from the standard stock solution of each compound. A dilute standard solution of P 100 μg/mL was prepared by diluting the standard stock solution with water. Eight working standard solutions containing 0.5–8.0 μg/mL of F and NF were prepared by diluting the appropriate volume (50–800 μL) of the 100 μg/mL aqueous reference solution of F and NF up to 10 mL with water. An aqueous reference solution of internal standard (2 μg/mL) was prepared by diluting 200 μL of the 100 μg/mL standard solution of P up to 10 mL with water.

Plasma standards for calibration curves were prepared by spiking 1.0 mL of pooled drug free plasma with 100 μL of the above-mentioned working solutions, to make plasma standards containing F and NF to a concentration range of 50–800 ng/mL.

All stock, diluted stock, and working solutions, stored at 4°C were stable for 12 months.

Calibration graphs, based on the peak height ratio of each compound to internal standard against analyte concentration, were prepared for each day of analysis to establish and check linearity and reproducibility of the method.

Extraction Procedure

In a 10-mL glass conical tube with glass stopper, 1.0 mL of plasma samples, or the prepared plasma standard and 100 μL of internal standard aqueous solution 2.0 μg/mL (200 ng), were added and mixed for 15 sec on the vortex. Consequently, 1.0 mL of sodium hydroxide 1.0 M solution was added in the tube and the whole was mixed for another 15 sec on the vortex. Each sample was extracted with 6.0 mL of hexane–isoamyl alcohol 98 : 2 v/v by gentle shaking on a rotator (speed 7) for 10 min. The sample tube was centrifuged for 5 min at 2000 rpm. After separation and transfer of the upper organic layer into a 10-mL conical glass tube with glass stopper, a back extraction procedure was performed with 200 μL of phosphoric acid 0.1 M solution by gentle shaking on the rotator for another 10 min. An aliquot of about 70 μL of the aqueous acidic phase was injected onto the HPLC system.

RESULTS AND DISCUSSION

Methodology

The HPLC method, based on the modification of previously described methods, was optimized for the experimental conditions: composition and

flow rate of mobile phase, internal standard and its concentration, procedure of extraction of plasma samples. Retention times of P, NF, and F were 4.6, 5.5, and 6.4 min, respectively, and remained constant from day to day. Endogenous plasma compounds were eluted before 4.3 min. Figure 1 shows a typical chromatogram obtained from the injection of a test solution containing 1 μg/mL of F/NF/P (injected volume 50 μL, referring to 50 ng of F, NF, P, respectively). Figure 2 shows a typical chromatogram obtained from an extracted drug free plasma, while Fig. 3 of a drug free plasma spiked with 200 ng/mL of F/NF/P. Resolution of peaks in all cases was excellent, and no interfering peaks of sample matrix were observed by analyzing a great number of plasma samples.

Extraction Procedure

Several solvent systems were tested for the extraction of F and NF from aqueous solutions and from spiked plasma samples. In order to achieve the extraction of the corresponding bases, NaOH (0.5 and 1.0 M), as well as Na_2CO_3 1.0 M solutions were used for the pH adjustment. In a preliminary study, a one step extraction was performed. After the alkalinization of the sample and the extraction with various organic solvents (Table 1), the sample was centrifuged and the organic layer was evaporated to dryness in a 50°C dry bath under a gentle stream of nitrogen. The residue was reconstituted in 200 μL of mobile phase and an aliquot of about 70 μL was injected onto the HPLC system. In the one step extraction, a solvent with a rather low boiling point, such as pentane, was tried but unfortunately resulted in low extraction recoveries. Other solvent systems were also tested but gave rather low extraction recoveries as well (Table 1). Furthermore, the one step extraction procedure, when tested in spiked plasma samples, resulted in low extraction recoveries and created noisy baselines. With the one step extraction procedure, best recoveries were received with hexane–isoamyl alcohol 98 : 2 v/v. Since both solvents of the extraction mixture have relatively high boiling points, analysis is prolonged because of the time which is required for the evaporation step. For this reason, a back extraction (reextraction of the organic solvent with an acidic aqueous phase) was then tried and HCl and H_3PO_4 solutions were used during this effort. With the two steps extraction procedure the evaporation step was avoided and, therefore, solvents with rather higher boiling points such as hexane–isoamyl alcohol 98 : 2 v/v could be used. Good chromatograms were received from spiked plasma samples with this procedure (not tailing peaks, satisfactory resolution, and not noisy baseline) although the duration of the column's life was shortened. The recoveries obtained are

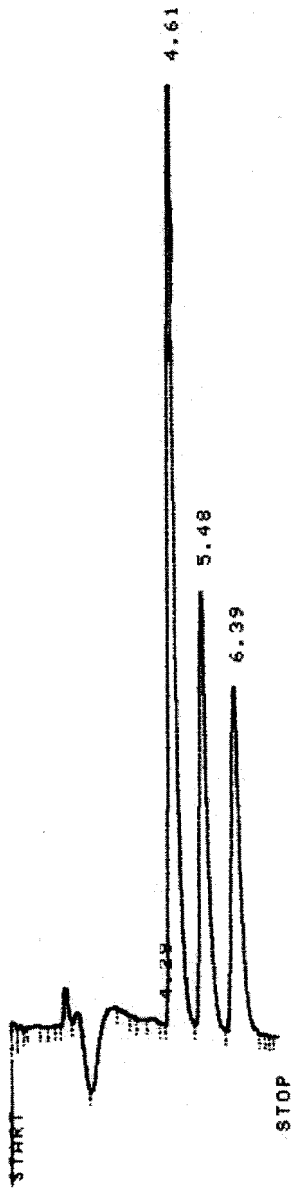

Figure 1. Typical chromatogram obtained from a direct injection of an aqueous test solution containing 1 μg/mL of F, NF, P (injected volume 50 μL, corresponding to 50 ng of each compound). Retention times: protriptyline (P) 4.61 min, norfluoxetine (NF) 5.48 min, fluoxetine (F), 6.39 min.

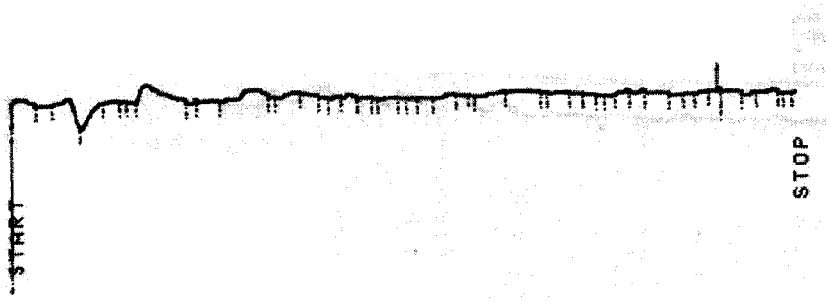

Figure 2. Typical chromatogram from an extracted drug free plasma sample.

shown in Table 2. The solvent system with the highest recovery was hexane–isoamyl alcohol 98 : 2 v/v, followed by a back extraction with 200 μL of 0.1 M H_3PO_4 and was then tested for the extraction of spiked plasma samples at several concentration levels.

Recovery

Absolute extraction recovery data and reproducibility of recovery (CV%), from plasma samples spiked with F/NF/P at 50/50/200, 300/300/200, and 800/800/200 ng/mL are presented in Tables 3 and 4, respectively, (means of five or ten experiments). For the calculation of the absolute extraction recovery (Table 3), the peak heights from extracted samples were compared with those obtained from the direct injection of the corresponding working standard in mobile phase, taking into account the 5 × preconcentration. For the calculation of the reproducibility of the extraction procedure, the ratio of peak heights of extracted analytes to that of the extracted internal standard was used (Table 4).

Linearity and Sensitivity

Calibration curves of standard solutions showed excellent linearity ($r = 0.9998 - 0.9999$) with a CV% of slopes equal to 2.4% and 2.7% for NF and F, respectively.

The peak height ratios for plasma F/internal standard and plasma NF/internal standard were linearly related to plasma concentrations of F and NF, respectively, from atleast 50–800 ng/mL.

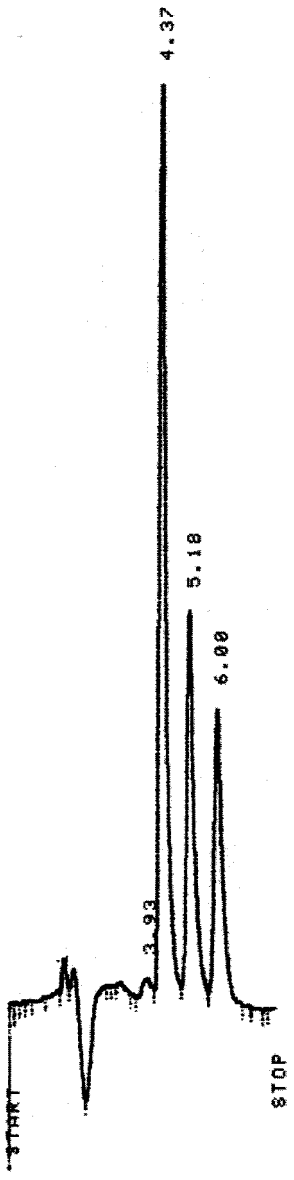

Figure 3. Typical chromatogram from an extracted plasma sample spiked with fluoxetine (F), norfluoxetine (NF), and protriptyline (P) to 200 ng/mL each (theoretically 50 ng of each compound). Retention times: protriptyline (P) 4.37 min, norfluoxetine (NF) 5.18 min, fluoxetine (F) 6.00 min.

Table 1. Absolute extraction recoveries of protriptyline (P), norfluoxetine (NF), and fluoxetine (F) from aqueous solutions (1 mL) containing 200 ng/mL of F/NF/P with various extraction solvent systems ($n = 1$).

	Solvent system (6 mL)	Alkalinization (1 mL)	Acidification (200 μL)	Recovery (%)		
				P	NF	F
1	Pentane	NaOH 0.5 M	H_3PO_4 0.1 M	58.5	28.8	32.8
2	Pentane	NaOH 0.5 M	HCl 0.1 M	92.3	77.8	89.7
3	Pentane	Na_2CO_3 1.0 M	H_3PO_4 0.1 M	59.0	46.6	93.0
4	Pentane	Na_2CO_3 1.0 M	HCl 0.1 M	67.3	51.7	96.2
5	Hexane–isoamylalcohol 98 : 2 v/v	NaOH 0.5 M	H_3PO_4 0.1 M	49.0	12.8	16.7
6	Hexane–isoamylalcohol 98 : 2 v/v	NaOH 0.5 M	HCl 0.1 M	86.4	43.2	55.3
7	Hexane–isoamylalcohol 98 : 2 v/v	Na_2CO_3 1.0 M	H_3PO_4 0.1 M	58.7	23.6	52.7
8	Hexane–isoamylalcohol 98 : 2 v/v	Na_2CO_3 1.0 M	HCl 0.1 M	68.9	77.3	59.0

(*continued*)

Table 1. Continued.

	Solvent system (6 mL)	Alkalinization (1 mL)	Acidification (200 μL)	Recovery (%)		
				P	NF	F
9	Heptane–isopropanol 95 : 5 v/v	NaOH 0.5 M	H_3PO_4 0.1 M	54.6	19.1	28.1
10	Heptane–isopropanol 95 : 5 v/v	NaOH 0.5 M	HCl 0.1 M	70.9	68.8	82.0
11	Heptane–isopropanol 95 : 5 v/v	Na_2CO_3 1.0 M	H_3PO_4 0.1 M	68.4	50.0	87.0
12	Heptane–isopropanol 95 : 5 v/v	Na_2CO_3 1.0 M	HCl 0.1 M	77.5	75.8	63.0
13	Pentane	NaOH 0.5 M	—	60.0	30.5	33.0
14	Hexane–isoamylalcohol 98 : 2 v/v	NaOH 0.5 M	—	55.0	48.0	56.0
15	Heptane–isopropanol 95 : 5 v/v	NaOH 1.0 M	—	51.0	28.6	42.0

Table 2. Absolute extraction recoveries of protriptyline (P), norfluoxetine (NF), and fluoxetine (F) from spiked plasma samples (1 mL) containing F/NF/P 600/600/200 ng/mL with various extraction solvent systems ($n = 1$).

	Solvent system	Solvent volume (mL)	Alkalinization (1 mL)	Acidification (200 μL)	Recovery (%)		
					P	NF	F
1	CH_2Cl_2–*n*-pentane 40 : 60 v/v	5	NaOH 1.5 M	—	64.0	70.0	65.5
2	Pentane	5	NaOH 1.5M	—	72.0	64.8	76.3
3	Pentane	5	NaOH 1.5M	HCl 0.1M	65.5	87.0	80.2
4	Pentane	5	NaOH 1.5M	H_3PO_4 0.1M	60.0	28.6	24.0
5	Pentane	5	NaOH 1.0M	HCl 0.1M	25.3	21.6	31.0
6	Pentane	5	NaOH 1.0M	H_3PO_4 0.1M	67.2	49.5	65.0
7	Pentane	6	NaOH 1.0M	H_3PO_4 0.1M	63.0	47.0	63.7
8	Pentane	7	NaOH 1.0M	HCl 0.1M	65.0	42.0	54.0
9	Hexane–isoamylalcohol 98 : 2 v/v	5	NaOH 1.5M	—	63.0	67.6	82.2
10	Hexane–isoamylalcohol 98 : 2 v/v	5	NaOH 1.5M	HCl 0.1M	85.6	69.5	68.4
11	Hexane–isoamylalcohol 98 : 2 v/v	6	NaOH 1.0M	H_3PO_4 0.1M	87.1	88.3	84.6
12	Hexane–isoamylalcohol 98 : 2 v/v	6	NaOH 0.5M	H_3PO_4 0.1M	68.9	77.3	59.0
13	Heptane–isopropanol 95 : 5v/v	6	NaOH 1.0M	H_3PO_4 0.1M	60.4	59.6	67.5
14	Heptane–isopropanol 95 : 5 v/v	6	NaOH 0.5M	HCl 0.1M	58.3	62.3	64.3

Table 3. Absolute extraction recoveries of protriptyline (P), norfluoxetine (NF), and fluoxetine (F) from spiked plasma samples.

Spiked plasma standards[a] (ng/mL)	Norfluoxetine (NF)		Fluoxetine (F)		Protriptyline (P)	
	% Rec. ± SD	CV (%)	% Rec. ± SD	CV (%)	% Rec. ± SD	CV (%)
50 ($n = 5$)	74 ± 13	18	79 ± 12	15	75 ± 11	15
300 ($n = 10$)	86 ± 11	12	89 ± 12	13	90 ± 10	11
800 ($n = 5$)	74.8 ± 6.8	9.1	81.2 ± 3.4	4.2	82.3 ± 5.8	7.0

[a]All spiked plasma standards contained P (internal standard) at 200 ng/mL.

The slopes of six calibration curves of F in plasma, prepared over a period of 2 months, had a CV of 4.0%. The average regression equation is $y_1 = [4.27(\pm 0.17) \times 10^{-3}]x_1 + 74.2(\pm 3.2) \times 10^{-3}$, where y_1 = peak height ratio of F/P and x_1 = plasma concentration of F in ng/mL. The correlation coefficients for each individual standard curve was greater than 0.995 and all intercepts were not statistically different from zero. The slopes of six calibration curves of NF in plasma, prepared over a period of 2 months, had a CV of 4.3%. The average regression equation is $y_2 = [4.51 (\pm 0.20) \times 10^{-3}]x_2 + 25.6(\pm 18) \times 10^{-3}$, where y_2 = peak height ratio of NF/P and x_2 = plasma concentration of NF in ng/mL. The correlation coefficients for each individual standard curve was greater than 0.998 and intercepts were not statistically different from zero. The detection and the quantification limits for the determination of F and NF in plasma were estimated from the average regression equations of the corresponding calibration curves, based on the between run standard deviation of intercepts (3.3 and 10 times the SD), respectively. The detection and the quantification limits for the

Table 4. Reproducibility of extraction procedure and %CV of norfluoxetine (NF) and fluoxetine (F) from spiked plasma samples.

Spiked plasma standards[a] (ng/mL)	Norfluoxetine (NF)		Fluoxetine (F)	
	PHR ± SD	CV (%)	PHR ± SD	CV (%)
50 ($n = 5$)	0.235 ± 0.039	17	0.286 ± 0.047	16
300 ($n = 10$)	1.39 ± 0.15	11	1.39 ± 0.18	13
800 ($n = 5$)	3.75 ± 0.18	4.8	3.41 ± 0.18	5.3

Note: PHR, peak height of extracted analyte to peak height of extracted internal standard.
[a]All spiked plasma standards contained P (internal standard) to 200 ng/mL.

determination of F in plasma, according to the described method, were 2.5 and 7.6 ng/mL, respectively, while for the determination of NF in plasma were 13 and 40 ng/mL, respectively (5 × preconcentration).

Precision and Accuracy

Precision and accuracy were evaluated by analyzing plasma samples spiked with F and NF at 50, 300, and 800 ng/mL and the results are presented in Table 5. Within-run CV% for F ranged from 5.4% to 20% over the calibration curve, while for NF from 4.7% to 18%. Between-run CV% was 16% and 14% for F and NF, respectively, at 300 ng/mL spiked plasma samples ($n = 10$) over a period of 3 months. At the low concentrations tested (50 ng/mL), the within-run precision and accuracy of the developed method, evaluated from the CV% and the relative error %, respectively, was not as high as for the median and high concentrations, but the low concentrations tested in the case of NF were very close to the quantification limit of the method.

CONCLUSIONS

The developed analytical method is simple, rapid, accurate, sensitive enough with improved extraction recovery (79–89% for F and 74–86% for NF), in comparison with previously reported methods.[10,11,13,15] It can be

Table 5. Within-run precision and accuracy data of norfluoxetine (NF) and fluoxetine (F) from spiked plasma samples.

Concentration (ng/mL)					
Added	Found, mean ± SD	CV (%)	Relative range	Relative error (E_r, %)	n
Norfluoxetine					
50.0	45.0 ± 8.3	18.4	0.391	−10.0	5
300	291 ± 32	11.1	0.337	−3.1	10
800	792 ± 38	4.7	0.113	−0.9	5
Fluoxetine					
50.0	55 ± 11	19.7	0.598	9.4	5
300	309 ± 45	14.5	0.339	3.0	10
800	805 ± 44	5.4	0.116	0.6	5

used for monitoring of F and NF plasma levels in patients under F treatment or in bioavailability studies. The developed method can be further modified to correspond to the desirable concentration range by extrapolating calibration curves at higher or lower concentrations, and appropriately choosing the amount of internal standard.

ABBREVIATIONS

F	Fluoxetine
NF	Norfluoxetine
HPLC	High performance liquid chromatography
CV	Coefficient of variation
E_r	Relative error

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