Journal of Pharmaceutical and Biomedical Analysis 51 (2010) 170–177

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Three-phase, liquid-phase microextraction combined with high performance liquid chromatography-fluorescence detection for the simultaneous determination of fluoxetine and norfluoxetine in human plasma

Daniela Fernanda de Freitas, Carlos Eduardo Dobrovolskin Porto, Elisabeth Pizzamiglio Vieira, Maria Elisa Pereira Bastos de Siqueira*

Federal University of Alfenas, Laboratory of Toxicological Analysis, R. Gabriel Monteiro da Silva 714, Alfenas 37130-000, MG, Brazil

ARTICLE INFO

Article history:
Received 25 March 2009
Received in revised form 24 June 2009
Accepted 14 July 2009
Available online 19 July 2009

Keywords:
Liquid-phase microextraction
High-performance liquid chromatography
Fluoxetine
Norfluoxetine
Plasma

ABSTRACT

A three-phase, liquid-phase microextraction using a hollow fibre (HF-LPME) combined with high performance liquid chromatography-fluorescence detection (HPLC-FL) was developed for the analysis of fluoxetine (FLX) and its active metabolite, norfluoxetine (NFLX), in human plasma. An HF-LPME system using a disposable 7-cm polypropylene porous hollow fibre, 5 mL of alkaline plasma solution (donor phase), *n*-hexyl ether (extraction solvent) and 20 mM hydrochloric acid (acceptor phase) was used in the extraction. The method was validated after optimisation of several parameters that influence LPME efficiency. A reverse-phase LiChrospher 60 RP-Select B column (125 mm × 4 mm, 5 μm particle size) was used with 0.005 M sodium acetate buffer (pH 4.5) and acetonitrile at a 50:50 (v/v) as the mobile phase at a flow rate of 0.6 mL min^{-1}. In these conditions satisfactory chromatographic resolution and efficiency for the analytes were obtained. Fluorescence detection at 230 nm excitation wavelength and 290 nm emission wavelength was performed. Linearity over a range of 5–500 ng mL^{-1}, with determination coefficients (R^2) of 0.9999 and 0.9962 for FLX and NFLX, respectively, was established. Venlafaxine was used as the internal standard for both analytes. Extraction recoveries from plasma samples were 70.9% for FLX and 59.7% for NFLX. The intra-day coefficients of variation (CVs) were below 5.4%, and inter-day CVs were below 13.0%, for both analytes at concentrations of 20, 80 and 160 ng mL^{-1}. HF-LPME extraction followed by HPLC-FL detection for FLX and NFLX analyses demonstrated excellent sample clean-up and selectivity. This method was simple, cheap, and easy to perform, yielding substantial analytes enrichment. The method was applied to the analysis of samples from 12 patients under fluoxetine treatment and proved suitable for routine therapeutic drug monitoring for this antidepressant.

1. Introduction

Fluoxetine (FLX), *N*-methyl-3-phenyl-3-(2,2,2-trifluoro-*p*-tolyloxy) propylamine hydrochloride, is an antidepressant that selectively inhibits the reuptake of serotonin in presynaptic neurons [1]. Fluoxetine has also been used for a variety of disorders, in addition to depression, since its introduction in 1988 [2]. The FLX dosage administered to depressed patients varies between 20 and 80 mg day^{-1} [3] and its therapeutic plasma concentration is in the range of 50–500 ng mL^{-1} [4].

Fluoxetine is extensively metabolised in human beings by demethylation in the liver, via the CYP2D6 cytochrome P450 system, to its primary active metabolite norfluoxetine (NFLX). The half-lives of FLX and NFLX are approximately 1–4 days and 7–10 days, respectively [5]. FLX is rapidly absorbed after oral administration and its bioavailability is around 70%. This drug is extensively bonded to plasma proteins at levels approximately 94% [6].

Several methods have been published for the determination of FLX and NFLX in biological fluids for therapeutic drug monitoring, bioavailability studies and toxicological purposes. Determination of FLX and NFLX in biological samples involves an initial sample pretreatment step for target analyte isolation. Most procedures use liquid–liquid extraction (LLE) [7–14] and solid-phase extraction (SPE) [15–18] techniques prior to high performance liquid chromatography (HPLC) [8–11,13,14,16], gas chromatography [7,15] or capillary electrophoresis [19].

LLE is considered a tedious, time-consuming procedure, which can produce emulsions and requires large amounts of high purity organic solvents for analyte extraction [20]. SPE techniques often introduce artefacts into the sample extracts and can require lengthy processing (i.e., washing, conditioning, eluting and drying) [21].

* Corresponding author. Tel.: +55 35 32991342; fax: +55 35 32991067.
E-mail address: marelisa@unifal-mg.edu.br (M.E.P.B. de Siqueira).

0731-7085/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2009.07.017

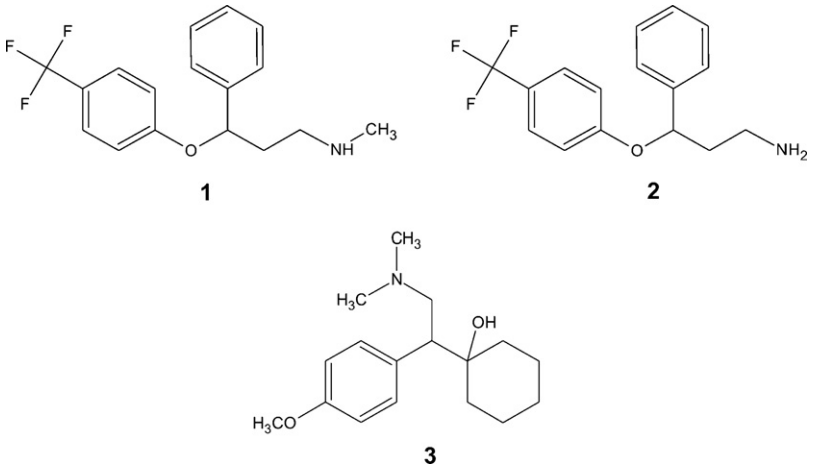

Fig. 1. Structures of fluoxetine (1), norfluoxetine (2) and internal standard, venlafaxine (3).

Recently, miniaturised techniques, such as solid-phase microextraction (SPME) using fibre [22] or stir bars (stir bar sorptive extraction, SBSE) [23,24], have been developed for sample preparation. These aim at the analysis of FLX and other antidepressants in biological fluids. SPME and SBSE require a small sample volume; however, they require a solvent desorption step when coupled to HPLC. This is necessary to extract all absorbed analytes and avoid carry-over effects.

Hollow-fibre based liquid-phase microextraction (HF-LPME) is an isolation technique that was introduced by Pedersen-Bjergaard and Rasmussen in 1999 [25]. This technique is based on the use of disposable, porous, hollow fibres made of polypropylene. HF-LPME combines extraction, concentration and sample clean-up in one step.

The basic principles of this technique have been clearly described in previous reviews [26–29]. There are two modes of HF-LPME, including two- and three-phase modes. In the two-phase mode, the sample solution is one phase (donor phase) and the organic solvent, supported by the fibre and within its lumen, is another phase (acceptor phase). In three-phase mode, analyte extraction occurs using three liquid phases, including: (1) the sample solution (donor phase), where pH is adjusted to keep compounds neutrally charged, (2) the organic extractor phase, which is immobilised in fibre pores, and (3) the receiving aqueous phase (acceptor phase), with a pH that is adjusted to ionise the analytes. Compounds in their non-ionised form are extracted into the organic solvent and are subsequently back-extracted into the acceptor phase, which can be directly analysed via HPLC [30–32].

Sample extracts in this technique do not require further concentration prior to analysis, owing to the small volume of extracting solvent used. Additional advantages of HF-LPME are its tolerance to a wide pH range, as well as its application in assays that are not suitable for SPE or SPME. Sample carry-over can be avoided because the hollow fibres used in HF-LPME are cheap, making them affordable to dispose of after a single use [33].

HF-LPME has proven to be very useful for the extraction of drugs and metabolites in biological matrices. This is achieved through the concentration and enrichment of low concentration drugs from biological samples. A recent review of the application of HF-LPME to the analysis of drugs in biological matrices was published by De Oliveira et al. [34].

The goal of this study was to develop and validate a sample preparation method using three-phase HF-LPME, coupled to HPLC-fluorescence detection, to analyse fluoxetine and its active metabolite, norfluoxetine, in plasma samples at the ng mL^{-1} level. Investigated extraction parameters included: organic solvent, extraction time, stirring rate, type and pH of acceptor solution, pH of sample solution, use of salt and organic modifier. Ultimately, the optimised and validated method was applied to determine FLX and NFLX in plasma from patients under fluoxetine treatment.

2. Experimental

2.1. Reagents and standard solutions

Fluoxetine hydrochloride and norfluoxetine hydrochloride were purchased from Sigma–Aldrich (St. Louis, USA). Venlafaxine hydrochloride was obtained from Wyeth (Steinheim, Germany) (Fig. 1). HPLC-grade acetonitrile was purchased from Vetec (Rio de Janeiro, Brazil). HPLC-grade methanol and ethanol were obtained from J.T. Baker (Philipsburg, USA). Analytical-grade solvents were utilised, including *n*-octanol and *n*-hexyl ether from Sigma–Aldrich (St. Louis, USA), *n*-hexane from Mallinckrodt (Paris, USA), and toluene from Vetec (Rio de Janeiro, Brazil). The following other chemicals were used: sodium acetate from Proquimios (Rio de Janeiro, Brazil), acetic acid and sodium chloride from Impex (Contagem, Brazil), ammonium acetate from Ecibra (São Paulo, Brazil), sodium dihydrogen phosphate from Dinâmica (Diadema, Brazil), sodium hydroxide from Labsynth (Diadema, Brazil), hydrochloric acid from Vetec (Rio de Janeiro, Brazil), and perchloric acid from Reagen (Rio de Janeiro, Brazil). All aqueous solutions were prepared with purified water, which was obtained using a MILLI-Q apparatus (Millipore Corporation, Bedford, USA).

Stock solutions of FLX, NFLX and venlafaxine (VLX) were prepared in methanol at 1 mg mL^{-1}, with working solutions at 10 μg mL^{-1}, using the appropriate dilution factor in methanol. Solutions were stored at −20 °C and protected from light, allowing them to remain stable for at least 5 weeks [35].

2.2. Chromatographic system

Sample analyses were performed on a Shimadzu model LC-10AV (Kyoto, Japan) HPLC that was equipped with a LC-10AD pump, a CTO-10AS VP column oven, Sil-10 AF automatic injector (50 μL loop), a SPD-10AVP UV detector and RF-10AXL fluorescence detector. The better chromatographic conditions were established and included a LiChrospher 60 RP-Select B reverse-phase column (125 mm × 4 mm, 5 μm particle size) from Merck (Darmstadt, Germany), operating at 25 °C. The mobile phase consisted of 0.005 M sodium acetate buffer (pH 4.5) and acetonitrile in a 50:50 (v:v) mix-

ture at 0.6 mL min^{-1} flow rate. The fluorescence detector was set at 230 nm (excitation) and 290 nm (emission).

2.3. LPME procedure

The LPME system consisted of a plasma solution (donor), extracting solvent and acceptor solution. A 1 mL sample was placed in a conventional 5 mL vial (Supelco, Bellefonte, USA) and its pH was adjusted using 100 μL of 5 M sodium hydroxide solution. The sample was diluted with ultrapure water to a total volume of 5.0 mL. All LPME experiments were performed using Accurel Q3/2 polypropylene hollow fibre membranes (600 μm I.D., 200 μm wall thickness and 0.2 μm pore size) from Membrana (Wuppertal, Germany). These were in a "U" format, as described previously [25,32,33]. The hollow fibre was manually cut to 7 cm and employed for LPME. Syringes (25 and 50 μL) with 22 s gauge, bevel tip needles (Hamilton, Reno, USA) were used to connect hollow fibre ends. One syringe served to introduce the acceptor solution, while another was used for collection of the final extract. Prior to extraction, the extracting solvent was immobilised in the pores of the hollow fibre. This was performed by dipping the fibre into *n*-hexyl ether for 10 s, followed by immersion in an ultrasonic water bath for 15 s to remove excess solvent. Subsequently, 20 μL of acceptor solution was injected into the hollow fibre and the assembly was immersed into the sample directly. During extraction, samples were stirred using 1 cm magnetic bars (Sigma–Aldrich, St. Louis, USA). After extraction, 20 μL of the acceptor solution was retracted into a 50-μL microsyringe and diluted to 100 μL with mobile phase. An aliquot of 50 μL was injected into the chromatographic system. Three samples were processed at the same time.

2.4. Method validation

To estimate the validity of the present method, human plasma samples were spiked with FLX and NFLX at concentrations of 5.0, 10.0, 30.0, 50.0, 100.0, 200.0 and 500.0 ng mL^{-1}, as well as with 50 ng mL^{-1} of the internal standard, venlafaxine (six replicates per concentration). These samples were prepared and analysed using the optimised LPME procedure. Calibration curves were plotted with peak area ratios of analyte and I.S. versus analyte concentration. The limit of quantification was determined at a signal-to-noise ratio of 10 (S/N = 10).

Intra-day precision was evaluated via the analysis of FLX- and NFLX-spiked samples at concentrations of 20.0, 80.0 and 160 ng mL^{-1} (n = 6 per concentration). Inter-day precision was carried out for the same concentrations as above, analysing the samples over three subsequent days. The results were expressed as coefficients of variation (%).

Accuracy was established by spiking plasma samples with 20, 80 and 160 ng mL^{-1} of FLX and NFLX (n = 3 per concentration). After LPME extraction and chromatographic analysis, results were compared to the theoretical added values. Recovery was calculated from plasma samples spiked with 10, 50 and 200 ng mL^{-1} of FLX and NFLX, in triplicate for each concentration. These recoveries were submitted for analysis via HF-LPME/HPLC-FL and the results were compared with those obtained by direct acceptor phase addition of the same analyte quantities.

Selectivity of the method was evaluated by analysing spiked plasma samples that were obtained from six individuals, four normal samples, one haemolysed and another lipaemic. The chromatography behaviour of other drugs (i.e., diazepam, nordiazepam, caffeine and nicotine) added to the plasma and submitted to the method was also verified. The stability of analytes in the acceptor acidic phase was studied for a period of 12 h, with chromatography being performed every 2 h.

2.5. Plasma samples

Drug-free human plasma samples used for LPME optimisation and method validation were provided by healthy volunteers. Real plasma samples were collected from 12 patients under treatment with fluoxetine at doses varying between 20 and 80 mg day^{-1}. Blood samples from these volunteers were collected immediately prior to drug administration (minimum plasma concentration). All patients signed a written and informed consent term prior to the investigation and the study was approved by the Ethics Committee of the Federal University of Alfenas (protocol number 23087.002103/2007-81).

All blood samples were collected in Vacutainer heparinised tubes (Becton Dickinson, Meylan, France). These were subsequently centrifuged for 15 min at 560 × g and the plasma samples were stored at −20 °C for no longer than 2 weeks. Stability studies of FLX in plasma samples were conducted by Binsumait et al. [35] that related good stability at least for 2 weeks when the samples are kept at −20 °C.

3. Results and discussion

Fig. 2 shows chromatogram of FLX, NFLX and VLX (internal standard) extracted from plasma by LPME, using the optimised chromatographic conditions established for this analysis. The retention time of antidepressants were: 10.5 ± 0.3 (VEN, I.S.), 15.3 ± 0.4 (NFLX) and 19.1 ± 0.3 (FLX) min. System suitability parameters of the chromatographic analysis, including adequate efficiency (theoretical plates, N, above 2000) and resolution (Rs ≥ 2), as well as tailing factor (TF ≤ 2) and capacity factor (k' > 2), were adequate according to USA-FDA recommendations [36]. A method for FLX and NFLX analyses in plasma must present high detectability to enable the detection of low analyte plasma concentrations (ng mL^{-1}). The use of a fluorescence detector in HPLC, besides increasing method detectability, results in better method selectivity.

3.1. Optimisation of the LPME procedure

In this study, parameters related to HF-LPME were optimised using a batch to batch method for plasma samples containing 50 ng mL^{-1} of fluoxetine. Optimised parameters included solvent type, extraction time, stirring rate, sample pH, acceptor phase composition and pH, salt and methanol addition.

3.1.1. Selection of the organic extraction solvent

One of the critical steps in LPME is to select an organic solvent for enrichment of the analytes [37]. In general, the chosen organic solvent must be immiscible with both the acceptor and donor phase (sample), compatible with the membrane, of low volatility, and present good affinity for the analyte to be tested [27]. One advantage of this technique is that small extraction solvent volumes are used, taking into account the fibre dimensions. Fluoxetine and norfluoxetine have a log value for the octanol–water partition coefficients (log P) of 4.47 and 4.36, respectively [38]. For this study, four solvents were chosen based on their low solubility in water. These included *n*-octanol, *n*-hexane, toluene and *n*-hexyl ether, as well as some mixtures of these in different proportions. Each solvent was tested in triplicate. According to Fig. 3, *n*-hexyl ether resulted in higher extraction efficiency and it was thus selected for subsequent experiments.

3.1.2. Optimisation of sample solution pH

Fluoxetine is a basic drug (pK_a = 10.05), as well as its metabolite, NFLX (pK_a = 9.05), and the pH of plasma sample solutions is known to play an essential role in the extraction of basic drugs.

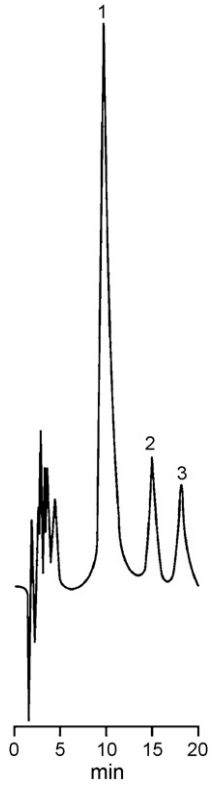

Fig. 2. Chromatogram obtained from plasma spiked with 50 ng mL^{-1} (1) venlafaxine (I.S.), (2) norfluoxetine and (3) fluoxetine.

Analytes should be in their neutral form, making them extractable by the organic solvent immobilised in the fibre pore. Four pH values were explored in this study, including: 7.0, 9.0, 12.0 and 14.0 (Fig. 4A). The results indicate that a better extraction was obtained at pH 14.0. This pH was subsequently utilised in later assays.

3.1.3. Effect of extraction time

Extraction in LPME is an equilibrium process, therefore sufficient time is needed to permit partitioning of the analyte between the two liquid phases. The rate of analyte diffusion through the pores of a hollow fibre also has an influence on the amount of time necessary for extraction. Analyte partitioning was controlled in three-phase LPME by the physicochemical properties of the analyte, sample matrix, organic phase and acceptor phase.

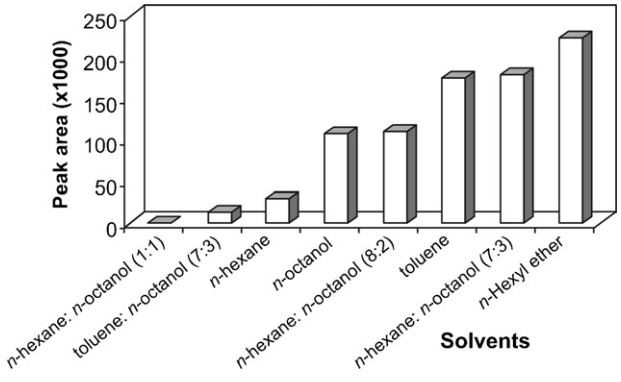

Fig. 3. Influence of organic solvent type on HF-LPME efficiency. Extraction conditions: 1 mL plasma sample; 100 μL of 5 M NaOH; 3.9 mL purified water; 10% (w/v) NaCl; extraction time = 30 min at 1000 rpm; *n*-hexyl ether as the solvent; and 20 μL of 20 mM hydrochloric acid as the acceptor phase.

To investigate the extraction rate, aliquots of the sample solution were extracted for 20, 30, 40, 60 and 90 min with constant stirring at 1000 rpm (Fig. 4B). Peak areas increased for each aliquot, up to the 90 min extraction time, without reaching equilibrium. With a more viscous sample, the system requires a longer period to reach equilibrium [39]. As LPME will be used for quantitative purposes, it is important to determine a time when reproducibility can be taken into account. On the other hand, a longer extraction time may allow the organic solvent to dissolve into the aqueous phase, especially with stirring [40]. Moreover, the excessively long exposure time necessary to reach the expected equilibrium is not considered practicable. To obtain better technique applicability, the extraction is often performed under non-equilibrium conditions [27,37]. Based on these considerations, an exposure time of 40 min was selected for later experiments. This was chosen from a practical point of view, while taking into account the potential to obtain a satisfactory sensitivity (LLOQ 5 ng mL^{-1}) and precision (CV below 20%) for FLX analysis from plasma samples.

3.1.4. Effect of stirring speed

The extraction kinetics in LPME can be accelerated by stirring, which facilitates analyte diffusion from donor phase, through the organic solvent, into the acceptor phase. The stability of the organic solvent on the outside of the hollow fibre is also affected [33]. Different stirring rates were evaluated to determine their effect on extraction efficiency (900, 1200, 1400 and 1600 rpm; Fig. 4C). The results indicate that extraction kinetics increased with increasing stirring speed, as expected. For later experiments, 1400 rpm was selected for the stirring rate. This is because, at rates above this value, the measurement imprecision also increased. Most likely, this is due to solvent layer disturbances on the outside of the hollow fibre.

3.1.5. Acceptor phase selection

Fluoxetine is a basic drug, thus the acceptor phase for trapping the analyte should be acidic to guarantee prevalence of the ionised form. This form does not diffuse back into the organic solvent. Initially three different acids were tested, including: organic (100 mM acetic acid) and mineral (10 mM perchloric acid and 20 mM hydrochloric acid) acids. The highest analyte recovery was obtained when using 20 mM HCl. Strong acids generally result in better recovery of basic analytes for the LPME system. This is because they improve the ionisation of these compounds [34,41].

3.1.6. Salt addition to the sample solution

Addition of salt to the sample can increase analyte recovery in microextraction procedures [12,42,43]. In this study, the addition of sodium chloride at concentrations of 10 and 20% (w/v) was evaluated and the response was compared with that from samples having no added salt (Fig. 4D). A clear decline in analyte extraction was observed with salt addition. A similar negative effect was reported by some authors when using LPME for the extraction of drugs from plasma [30,32]. Most likely, a high salt concentration modifies the physical properties of the diffusion film and reduces the rate of diffusion of analytes into the organic solvent [44].

3.1.7. Optimised extraction procedure

The optimum LPME conditions established for FLX extraction from plasma samples, based on results discussed above, were: (1) *n*-hexyl ether as the organic solvent; (2) addition of a 1 mL plasma sample with 100 μL of 2 M NaOH, with subsequent dilution to 5 mL with purified water; (3) 40 min extraction time with a stirring speed of 1400 rpm; and (4) 20 μL of 20 mM HCl as the acceptor phase. Under these conditions, FLX and its active metabolite (NFLX) were extracted from plasma samples.

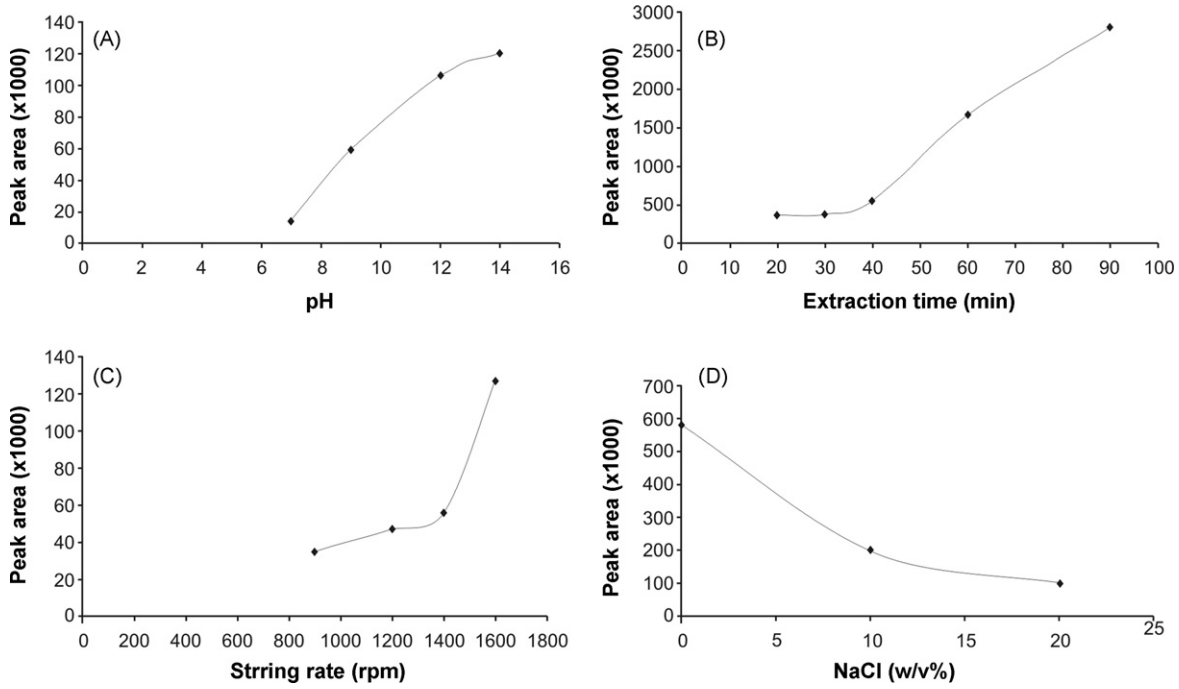

Fig. 4. Influence of different parameters on HF-LPME efficiency. (A) Sample pH [extraction conditions: stir speed = 1000 rpm, 35 min; 10% (w/v) NaCl; organic solvent = *n*-hexyl ether; and acceptor phase = 20 μL of 20 mM hydrochloric acid], (B) extraction time (extraction conditions: stir speed = 1000 rpm; sample added with 100 μL of 5 M NaOH; organic solvent = *n*-hexyl ether; and acceptor phase = 20 μL of 20 mM hydrochloric acid), (C) stirring rate (extraction conditions: sample added with 100 μL of 5 M NaOH, extraction time = 40 min; organic solvent = *n*-hexyl ether; and acceptor phase = 20 μL of 20 mM hydrochloric acid), and (D) salt addition (extraction conditions: sample added with 100 μL of 5 M NaOH; stir speed = 1400 rpm with extraction time = 40 min; organic solvent = *n*-hexyl ether; and acceptor phase = 20 μL of 20 mM hydrochloric acid).

Table 1
Precision and accuracy for the analysis of fluoxetine (FLX) and norfluoxetine (NFLX) in plasma samples.

Concentration (ng mL^{-1})	Precision[a]				Accuracy[b]	
	Intra-day[c]		Inter-day[d]			
	FLX	NFLX	FLX	NFLX	FLX	NFLX
20	4.0	5.4	1.5	4.0	−2.1	14.5
80	3.6	4.0	3.0	10.0	7.8	9.0
160	2.1	3.2	7.5	13.0	13.3	16.2

[a] Expressed as coefficient of variation.
[b] Expressed as deviation from the theoretical values.
[c] Six replicates per concentration.
[d] Two replicates per concentration per day, analysed in three subsequent days.

3.2. Method validation

Upon optimisation of the chromatography analysis and conditions for LPME extraction, the method was validated to evaluate its practical applicability.

3.2.1. Quantification limit and linearity

The lowest concentration quantified by the method (LLOQ) was 5 ng mL^{-1} for both analytes. This resulted in a signal-to-noise ratio of 10, as well as CVs of 10.6% (FLX) and 13.1% (NFLX). Linearity was determined for both FLX and NFLX using a pool of drug-free plasma that was spiked with the analytes and the internal standard. Peak area ratios (reference to I.S.) and analyte concentrations were found to be linear over the range from 5 to 500 ng mL^{-1}. A least-squares linear regression was used to determine the slope and intercept. Regression equations and determination coefficients were: $y = 0.0041x - 0.0078$ ($R^2 = 0.9999$) and $y = 0.0035x + 0.0076$ ($R^2 = 0.9962$) for FLX and NFLX, respectively. The large linear range of concentrations for this method can be satisfactorily applied to FLX and NFLX in therapeutic drug monitoring. This range can also be applied in pharmacokinetics or biodisponibility studies of the drug, even when using sub-clinical doses.

Table 2
Recovery for the analysis of fluoxetine (FLX) and norfluoxetine (NFLX) in plasma.

Plasma concentration (ng mL^{-1})	Mean recovery (%)	
	FLX	NFLX
10	76.9	52.0
50	70.6	67.5
200	65.4	59.6
Mean	70.9 ± 5.8	59.7 ± 7.8

3.2.2. Precision, accuracy and recovery

Precision and accuracy validation data are summarised in Table 1. The intra-day assay coefficients of variation (CVs) for the

Table 3
Fluoxetine (FLX) and norfluoxetine (NFLX) in plasma from patients treated with FLX.

Sample	Dose (mg day^{-1})	Plasma concentration (ng mL^{-1})	
		Fluoxetine	Norfluoxetine
1	20	42.6 ± 0.01	58.4 ± 0.01
2	20	61.7 ± 0.02	87.9 ± 0.02
3	20	48.9 ± 0.01	142.7 ± 0.01
4	20	37.1 ± 0.03	122.5 ± 0.07
5	20	53.2 ± 0.01	95.0 ± 0.01
6	25	39.2 ± 0.1	54.4 ± 0.1
7	30	149.7 ± 0.05	68.4 ± 0.06
8	40	63.3 ± 0.01	101.8 ± 0.02
9	40	157.5 ± 0.1	187.6 ± 0.1
10	40	206.0 ± 0.2	125.6 ± 0.1
11	60	199.2 ± 0.01	184.2 ± 0.02
12	80	208.7 ± 0.02	234.2 ± 0.01

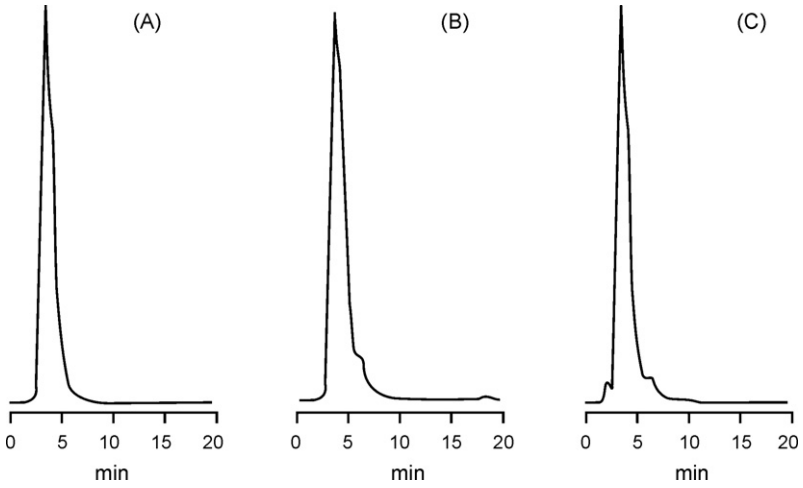

Fig. 5. Chromatograms from a (A) blank sample, (B) blank haemolysed sample, and (C) blank lipaemic sample.

two compounds were lower or equal to 5.4% and inter-day assay CVs were below 13.0%. Intra-day accuracies for FLX and NFLX were found to be between 86.7% and 116.2% for the concentrations evaluated. Mean recoveries of FLX and NFLX were, respectively, 70.9% and 59.7% (Table 2). These results may be considered relatively high in comparison with other microextraction methods reported in the literature using LPME for drugs in plasma [31,39,45]. Low recoveries in LPME, compared to LLE extraction, are a common situation due to the micro-scale characteristic of the technique [43]. Furthermore, it should be taken into account that the extraction procedure results in high values of enrichment. This method also enables a direct injection of the total amount of acceptor phase material [29]. Lower values of recovery for more polar metabolites, like norfluoxetine, are expected compared to the parent compound. These show minor affinity toward the organic solvent (*n*-hexyl ether in this study).

3.2.3. Selectivity and stability of FLX and NFLX in the acidic acceptor phase

The selectivity of the method was evaluated by analysing blank matrices collected from drug-free volunteers both before and after food ingestion (lipaemic sample). Haemolysed plasma samples

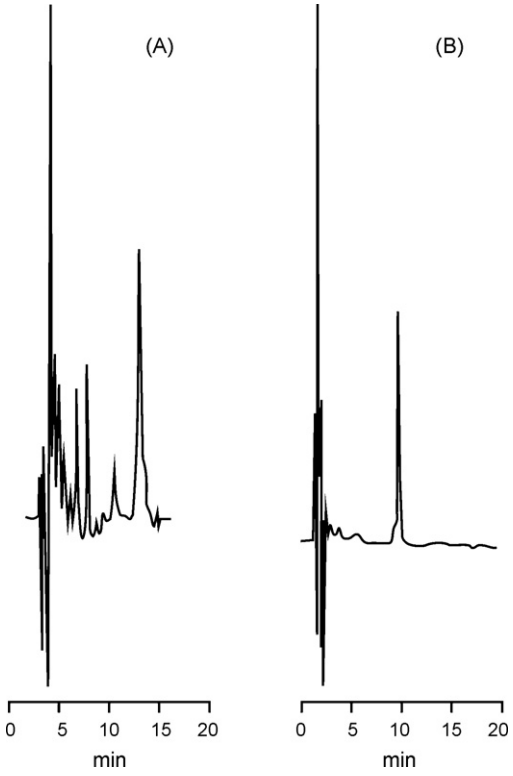

Fig. 6. Chromatograms from a sample spiked with 500 ng mL^{-1} fluoxetine and extracted by (A) LLE and (B) LPME. The HPLC was equipped with a LiChrospher 60 RP-Select B (125 mm × 4 mm × 5 μm) column operating at 25 °C. The mobile phase consisted of 0.25 M sodium acetate buffer at pH 5.5 and acetonitrile (55:45, v/v) at a flow rate of 1.0 mL min^{-1}. UV detector set at 230 nm.

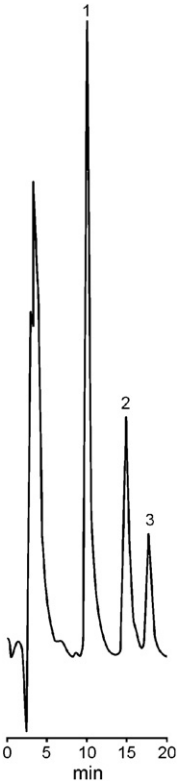

Fig. 7. Plasma sample from a patient treated with 40 mg day^{-1} fluoxetine. (1) Venlafaxine (I.S.), (2) norfluoxetine and (3) fluoxetine.

were also analysed (Fig. 5). No interfering peaks from endogenous compounds were observed in the chromatogram. Blank matrices added with caffeine, nicotine, diazepam and nordiazepam were also tested. None of these drugs presented any response to the fluorescence detector used. Plasma extracts from LPME were remarkably clean when compared with extracts from LLE. These were both from the same sample and analysed under identical HPLC-UV conditions (Fig. 6).

Fluoxetine and norfluoxetine remained stable for at least 12 h when kept in auto-injector vials in acceptor solution, mean CVs of 12.6% FLX and 11.5% NFLX.

3.3. Application of the method

The described method was employed to analyse fluoxetine and norfluoxetine in 12 human patients treated with different FLU doses (in mg day^{-1}): 20, 25, 30, 40, 60 and 80. Each sample was analysed in duplicate and the results, expressed in ng $mL^{-1} \pm$ standard deviation, are presented in Table 3. A representative chromatogram of a plasma sample is shown in Fig. 7.

4. Conclusions

The three-phase hollow-fibre liquid-phase microextraction method reported in this paper proved to be simple, cheap, and consumed minimal organic solvent. This method presented a high enrichment and enabled efficient sample clean-up, while yielding very good selectivity. HF-LPME coupled to HPLC-FL offers a large linear range, analytical precision, and low quantification limit (5 ng mL^{-1}). This method is thus suitable for routine FLX and NFLX assessment in plasma from depressed patients as a means for therapeutic drug monitoring

Acknowledgements

The authors are grateful to Dr. P.S. Bonato for her help in donating some materials used in this research and Dr. P.M. Maia for technical support. This research was supported by Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG, process number CDS-APQ-4487-4.04/07). The volunteers providing blood samples are also acknowledged.

References

[1] J.E.F. Reynolds, Martindale: The Extra Pharmacopeia, 30th ed., Pharmaceutical Press, UK, 1993, p. 243.
[2] A. Rossi, A. Barraco, P. Donda, Fluoxetine: a review on evidence based medicine, An. Gen. Hosp. Psychiatry 3 (2004) 1–8.
[3] S. Djordjevic, I. Kovacevic, B. Miljkovic, J. Vuksanovic, M. Pokrajac, Liquid chromatographic-mass spectrometric method for the determination of fluoxetine and norfluoxetine in human plasma: application to clinical study, Farmaco 60 (2005) 345–349.
[4] P. Baumann, Farmacokinetic-pharmacodynamic relationship of the selective serotonin reuptake inhibitors, Clin. Pharmacokinet. 31 (1996) 444–469.
[5] C. Hiemke, S. Härtter, Pharmacokinetics of selective serotonin reuptake inhibitors, Pharmacol. Ther. 85 (2000) 11–28.
[6] D.T. Wong, F.P. Bymaster, L.R. Reid, Norfluoxetine anantiomers as inhibitors of serotonin uptake in rat brain, Neuropsychopharmacology 8 (1993) 337–344.
[7] P. Fontanille, N. Jourdil, G.T.B. Villier, Direct analysis of fluoxetine and norfluoxetine in plasma by gas-chromatography with nitrogen-phosphorus detection, J. Chromatogr. B 692 (1997) 337–343.
[8] M.T. Maya, C.R. Domingos, M.T. Guerreiro, J.A. Morais, Determination of the antidepressant fluoxetine in human plasma by LC with UV detection, J. Pharm. Biomed. Anal. 23 (2000) 989–996.
[9] M.A. El-Dawy, M.M. Mabrouk, F.A. Elbarbary, Liquid chromatography determination of fluoxetine, J. Pharm. Biomed. Anal. 30 (2002) 561–571.
[10] G. Gatti, I. Bonomi, R. Marchiselli, C. Fatorre, E. Spina, G. Scordo, R. Pacific, E. Peruca, Improved enantioselective assay for the determination of fluoxetine and norfluoxetine enantiomers in human plasma by liquid chromatography, J. Chromatogr. B 784 (2003) 375–383.
[11] A. Llerena, P. Dorado, A. González, M.J. Noberto, A. de la Rubia, M. Cáceres, Determination of fluoxetine and norfluoxetine in human plasma by high-performance liquid chromatography with ultraviolet detection in psychiatric patients, J. Chromatogr. B 783 (2003) 25–31.
[12] S. Ulrich, Direct stereoselective assay of fluoxetine and norfluoxetine enantiomers in human plasma or serum by two-dimensional gas-liquid chromatography with nitrogen-phosphorus selective detection, J. Chromatogr. B 78 (2003) 481–490.
[13] S. Ertürk, S.M. Çetin, S. Atmaça, L. Ersoy, G.A. Baktir, A sensitive HPLC method for the determination of fluoxetine and norfluoxetine in human plasma witch fluorescence detection, Ther. Drug Monit. 27 (2005) 38–43.
[14] L. Vlase, S. Imre, S. Leucuta, Determination of fluoxetine and its *N*-desmethyl metabolite in human plasma by high-performance liquid chromatography, Talanta 66 (2005) 659–663.
[15] R.S. Addison, M.E. Franklin, W.D. Hooper, Sensitive, high-throughput gas chromatographic-mass spectrometric assay for fluoxetine and norfluoxetine in human plasma and its application to pharmacokinetic studies, J. Chromatogr. B 716 (1998) 153–160.
[16] M.A. Raggi, R. Mandrioli, G. Casamenti, F. Bugameli, V. Volerra, Determination of fluoxetine and norfluoxetine in human plasma by high-pressure liquid chromatography with fluorecence detection, J. Pharm. Biomed. Anal. 18 (1998) 193–199.
[17] P. Molander, A. thomassen, L. kristoffersen, T. greibokk, E. Ludanes, Simultaneous determination of citalopram, fluoxetine, paroxetine and their metabolites in plasma by temperature-programmed packed capillary liquid chromatography with on-column focusing of large infection volumes, J. Chromatogr. B 766 (2002) 77–87.
[18] H. Juan, Z. Zhiling, L. Huande, Simultaneous determination of fluoxetine, citalopram, paroxetine, venlafaxine in plasma by high performance liquid chromatography-electrospray ionization mass spectrometry (HPLC-MS/ESI), J. Chromatogr. B 820 (2005) 33–39.
[19] L. Labat, M. Deveaux, P. Dallet, J.P. Dubost, Separation of new antidepressants and their metabolites by micellar electro kinetic capillary chromatography, J. Chromatogr. B 773 (2002) 17–23.
[20] H. Kataoka, New trends in sample preparation for clinical and pharmaceutical analysis, Trends Anal. Chem. 22 (2003) 232–244.
[21] S.H. Gan, R. Ismail, Validation of a high performance liquid chromatography method for tramadol and *o*-desmethyltramadol in human plasma using solid-phase extraction, J. Chromatogr. B 759 (2001) 325–335.
[22] C. Fernandes, A.J.S. Neto, J.C. Rodrigues, C. Alves, F.M. Lanças, Solid-phase microextraction–liquid chromatography (SPME–LC) determination of fluoxetine and norfluoxetine in plasma using a heated liquid flow through interface, J. Chromatogr. B 847 (2007) 217–223.
[23] C. Fernandes, P. Jiayu, F.M. Lanças, Stir Bar Sorptive Extraction-LC-MS for the analysis of fluoxetine in plasma, Chromatographia 64 (2006) 517–521.
[24] L.P. Melo, A.M. Nogueira, F.M. Lanças, M.E. Queiroz, Polydimethylsiloxane/polypyrrole stir bar sorptive extraction and liquid chromatography (SBSE/LC-UV) analysis of antidepressants in plasma samples, Anal. Chim. Acta 633 (2009) 57–64.
[25] S. Pedersen-Bjegaard, K.E. Rasmussen, Liquid-liquid microextraction for sample preparation of biological fluids prior to capillary electrophoresis, Anal. Chem. 71 (1999) 2650–2656.
[26] E. Psillakis, N. Kalogerakis, Development in liquid-phase microextraction, Trends Anal. Chem. 22 (2003) 565–574.
[27] K.E. Rasmussen, S. Pedersen-Bjegaard, Developments in hollow fibre-based, liquid-phase microextraction, Trends Anal. Chem. 23 (2004) 1–10.
[28] S. Pedersen-Bjegaard, K.E. Rasmussen, Bioanalysis of drugs by liquid-phase microextraction coupled to separation techniques, J. Chromatogr. B 817 (2005) 3–12.
[29] S. Pedersen-Bjegaard, K.E. Rasmussen, Liquid-phase microextraction with porous hollow fibers, a miniaturized and highly flexible format for liquid-liquid extraction, J. Chromatogr. A 1184 (2008) 132–142.
[30] C. Yang, L. Guo, X. Liu, H. Zhang, M. Liu, Determination of tetandrine and fangchinoline in plasma samples using hollow fiber liquid-phase microextraction combined with high-performance liquid chromatography, J. Chromatogr. A 1164 (2007) 56–64.
[31] F.J.M. De Santana, P.S. Bonato, Enantioselective analysis of mirtazapine and its two major metabolites in human plasma by liquid chromatography–mass spectrometry after three-phase liquid-phase microextraction, Anal. Chim. Acta 606 (2008) 80–91.
[32] I.R.S. Magalhães, P.S. Bonato, Liquid-phase microextraction combined with high-performance liquid chromatography for the enantioselective analysis of mefloquine in plasma samples, J. Pharm. Biomed. Anal. 46 (2008) 929–936.
[33] S.M. Richoll, I. Colón, Determination of triphenylphosphine oxide in active pharmaceutical ingredients by hollow-fiber liquid-phase microextraction followed by reversed-phase liquid chromatography, J. Chromatogr. A 1127 (2006) 147–153.
[34] A.R.M. De Oliveira, I.R.S. Magalhães, F.J.M. De Santana, P.S. Bonato, Microextração em fase líquida (LPME): Fundamentos da técnica e aplicações na análise de fármacos em fluidos biológicos, Quim. Nova 15 (2008) 637–644.
[35] I.A. Binsumait, K.A. Hadidi, S. Abu-Al Rachib, Stability of fluoxetine in stored plasma, aqueous, and methanolic solutions determined by HPLC witch UV detection, Pharmazie 56 (2001) 311–313.
[36] US Food and Drug Administration (FDA), Guidance for Industry. Reviewer Guidance Validation of Chromatographic Methods, FDA Centre for Drug Evaluation and Research, Rocksville, 1994.
[37] H. Farahani, Y. Yamini, S. Shariati, M.R. Khalill-Zanjani, S. Mansour-Baghahi, Development of liquid phase microextraction method based on solidification

of floated organic drop for extraction and re-concentration of organochlorine pesticides in water sample, Anal. Chim. Acta 626 (2008) 166–173.
[38] J.P.H.T.M. Ploemen, J. Kelder, T. Hafmans, H.V. Sandt, J.A.V. Burgsteden, Use of physicochemical calcultions of pKa and ClogP to predict phospholipidosis-inducing potential. A case study with structural related piperazines, Exp. Toxicol. Pathol. 55 (2004) 347–355.
[39] T.G. Halvorsen, S. Pedersen-Bjergaard, K.E. Rasmussen, Reduction of extraction times in liquid phase microextraction, J. Chromatogr. B 760 (2001) 219–226.
[40] M. Ma, S. Kang, Q. Zhao, B. Chen, S. Yao, Liquid-phase microextraction combined with high-performance liquid chromatography for the determination of local anaesthetics in human urine, J. Pharm. Biomed. Anal. 40 (2006) 128–135.
[41] S. Pedersen-Bjergaard, K.E. Rasmussen, Liquid-phase microextraction and capillary electrophoresis of acid drugs, Electrophoresis 21 (2000) 579–585.
[42] T.S. Ho, S. Pedersen-Bjergaard, K.E. Rasmussen, Recovery, enrichment and selectivity in liquid-phase microextraction – comparison witch conventional liquid-liquid extraction, Analyst 127 (2002) 608–613.
[43] H. Lord, J. Pawliszyn, Microextraction of drugs, J. Chromatogr. A 902 (2000) 17–63.
[44] E.M. Gioti, D.C. Skalkos, Y.C. Fiamegos, C.D. Stalikas, Single-drop liquid-phase microextraction for the determination of hypericin pseudohypericin and hyperforin in biological fluids by high performance liquid chromatography, J. Chromatogr. A 1093 (2005) 1–10.
[45] F.J.M. De Santana, A.R.M. De Oliveira, P.S. Bonato, Chiral liquid chromatographic determination of mirtazapine in human plasma using two-phase liquid-phase microextraction for sample preparation, Anal. Chim. Acta 549 (2005) 96–103.