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Development of a stir bar sorptive extraction based HPLC-FLD method for the quantification of serotonin reuptake inhibitors in plasma, urine and brain tissue samples

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ABSTRACT

The aim of this article is to present an analytical application of stir bar sorptive extraction (SBSE) coupled to HPLC-fluorescence detection (FLD) for the quantification of fluoxetine (FLX), citalopram (CIT) and venlafaxine (VLF) and their active metabolites – norfluoxetine (NFLX), desmethyl- (DCIT) and didesmethylcitalopram (DDCIT) and o-desmethylvenlafaxine (ODV) – in plasma, urine and brain tissue samples. All the parameters influencing adsorption (pH, ion strength, organic modifier addition, volume, extraction time and temperature) and desorption (desorption solvent composition, time, temperature and desorption mode) of the analytes on the stir bar have been optimized. For each matrix, the analytical method has been assessed by studying the linearity and the intra- and interday accuracy (89–113%) and precision (RSD < 13%). The improvement of the quantification limits (0.2–2 $\mu g\,l^{-1}$ for plasma, 2–20 $ng\,g^{-1}$ for brain tissue and 1–10 $\mu g\,l^{-1}$ for urine, depending on the respective response for analytes) and the development of a procedure for all the matrices make this method useful in clinical and forensic analysis.

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1. Introduction

Depression, a very frequent psychiatric illness, has become one of the main diseases to be studied by heath institutions. According to WHO (World Health Organization) depression was the fourth leading contributor to the global burden of disease in 2000 and it is expected to be the second in 2020 [1].

Fluoxetine (FLX) and citalopram (CIT) are important SSRI antidepressants usually used in psychiatry. Despite the SSRIs activity, some second generation antidepressant drugs also have a norepinephrine reuptake inhibition activity. These two activities make venlafaxine (VLF) an alternative for patients whose response to SSRIs has decreased. However, VLF can still cause several side effects such as nausea, somnolence, asthenia and headache [2]. FLX, CIT and VLF share the property that some of their metabolites, norfluoxetine (NFLX), desmethyl- (DCIT) and didesmethylcitalopram (DDCIT) and o-desmethylvenlafaxine (ODV), are also pharmacologically active [3] (Fig. 1).

In clinical practice, the determination of an individual optimum dose for an antidepressant drug is often based on a trial-and-error strategy. In the case of antidepressant drugs, therapeutic drug monitoring is a well-established tool for defining a more efficient and safe dose. Furthermore, in some cases the monitorization of the pharmacologically active metabolites could be interesting for the correct dose establishment.

To date, the analytical methods described in the literature for the analysis of non-tricyclic antidepressants in biological matrices usually use liquid–liquid extraction (LLE) [4–7] or solid-phase extraction [8–13] for sample preparation. In general, these procedures are laborious, time-consuming and involve multiple steps.

In the recent years, different sorptive extraction techniques have been successfully applied to analyze drugs in biological fluids. Solid-phase microextraction (SPME), where the sorbent coating is applied over a thin silica fiber mounted on a syringe needle, has been proven to be an interesting alternative for the extraction of SSRIs in urine coupled to both gas-chromatography (GC) [14] and liquid-chromatography (LC) [15].

Based on the same principles of those of SPME, stir bar sorptive extraction (SBSE) has been employed as a new sample pretreatment technique. In SBSE, a stir bar is coated with a polydimethylsiloxane (PDMS) layer (0.5–1 mm thick) and used to stir samples, thereby

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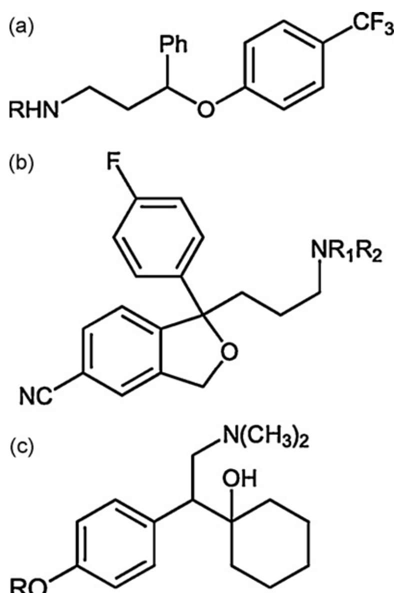

Fig. 1. Chemical structures of the drugs: (a) FLX ($R = CH_3$); NFLX (R = H), (b) CIT ($R_1 = R_2 = CH_3$); DCIT ($R_1 = CH_3$, $R_2 = H$); DDCIT ($R_1 = R_2 = H$), (c) VLF ($R = CH_3$); ODV (R = H).

extracting and enriching solutes into the PDMS layer. After extraction, the stir bar is removed and dried with a soft tissue and then, the analytes can be desorbed with thermal desorption for GC analysis. In contrast to SPME, the thermal desorption cannot be done directly in the injection port of a GC equipment and a special interface is required. As an alternative, liquid desorption can also be used coupled to LC, which is an advantage in the determination of thermolabile solutes or those with a low volatility, as well as SSRIs, that require previous derivatization in GC. Although magnetic stirring and sonication have been used, Lambert et al. observed some degradation of the stir bar coating after 30 desorptions when sonication is used [11].

The main differences between SPME and SBSE are the design of the extraction system and the much larger volume of sorbent used in the latter, increased by a factor ranging from 50 to 250 which results in higher sensitivity thus decreasing the detection limits [16].

In biomedical and life science applications, SBSE coupled to GC has been widely used to characterize chemical compounds in urine, plasma, saliva and some gland excretions, while there are fewer methods coupling SBSE to liquid-chromatography [16,17].

With regard to the extraction of antidepressants with SBSE, most of the published studies are based on the quantification of some of these compounds in plasma samples [18–20]. However, none of these studies demonstrate the applicability of the developed methods to urine and brain samples.

The aim of this work was to develop an SBSE procedure for the quantification of SSRI antidepressants and their active metabolites in plasma, brain tissue and urine samples. The proposed method should be useful at clinical levels and suitable for studies where the objective was the establishment of a more efficient and safe dose or for screening in clinical samples and in forensic analysis.

2. Experimental

2.1. Chemical and solutions

All reagents were analytical grade of the highest purity available. Fluoxetine hydrochloride, and venlafaxine hydrochloride were purchased from Sigma (St. Louis, USA). Norfluoxetine oxalate and o-desmethylvenlafaxine were supplied by Cerilliant (Texas, USA). Citalopram, demethylcitalopram and didemethylcitalopram were kindly donated by Lundbeck A/S (Copenhagen, Denmark). The ion-pair reagent tetramethylamonium chloride (TMACl) used in the mobile phase was purchased from Merck (Darmstadt, Germany) and the HPLC grade acetonitrile was obtained from Scharlab (Barcelona, Spain). All dissolutions were prepared with LC-grade water, obtained by purifying demineralized water in a Milli-Q water filtration system (Millipore, Milford, MA, USA). For the optimization of SBSE process sodium chloride, citric acid, disodium hydrogen phosphate, sodium dihydrogen phosphate, boric acid and sodium hydroxide from Merck (Darmstadt, Germany) and acetonitrile and methanol from Scharlab (Barcelona, Spain) were used.

Stock solutions containing 1 mg ml^{-1} of individual analytes were prepared in methanol from Scharlab (Barcelona, Spain) and were stored and refrigerated at −42 °C. An aqueous reference solution containing the mixture of all these compounds to a final concentration of 10 mg l^{-1} was prepared from the standard stock solution of each one. Working standard solutions were prepared by diluting the appropriate volume of the 10 mg l^{-1} reference solution up to 10 ml with water.

2.2. Instrumentation

The HPLC system consisted of a HP 1100 model quaternary pump provided with an autosampler and coupled to a fluorescence detector from Agilent Technologies (Palo Alto, CA, USA). The solvents were degassed using an on-line degasser system HP 1100 model also from Agilent Technologies.

Chromatographic separation was performed on an Extrasil ODS column (25 cm × 0.4 cm) with a particle size of 5 μm protected with an ODS guard column cartridge (1 cm × 0.4 cm) both from Tracer Analytica (Barcelona, Spain). The mobile phase consisted of a mixture of TMACl (pH 4; 0.4%)–acetonitrile (40:60, v/v) operating at room temperature and with a flow-rate of 1 ml min^{-1}. It was filtered through a 0.22 μm Millipore membrane filter type GVWP and degassed by a Selecta Ultrasound System (Selecta, Barcelona, Spain).

The detection conditions were determined from the observations made on the excitation and emission spectrum of the compounds. The detector operated at two different excitation wavelengths according to different maximums observed in the corresponding spectrums of each compound: at 228 nm for the quantification of VLF, ODV, FLX and NFLX; 240 nm for CIT, DCIT and DDCIT. Nevertheless, all the analytes have the same maximum emission wavelength at 308 nm.

Commercially available Twister™ stir bars were provided by Gerstel (Gerstel GmbH, Müllheim a/d Ruhr, Germany). It consisted of a 10 mm length glass-encapsulated magnetic stir bar coated with a 0.5 mm thick layer of PDMS that corresponds to a volume of 24 μl of polymer. 4 ml screw-cap vials supplied with a PTFE-lined septum (Kimble Glass, Vineland, NJ, USA) were used in the extraction step and a 1.5 ml glass vial with a glass vial-insert of 0.4 ml, both from Agilent Technologies, were used in the desorption step. For the stirring step a magnetic stirrer from IKA (Staufen, Germany) was used.

2.3. Sample collection and preparation

Experiments were performed using plasma and brain tissue samples of male Sprague–Dawley rats (Harlan Iberica, Barcelona, Spain) weighing 225–250 g. Animals, acclimatized to the research facility for 1 week before the study, were group-housed under standard laboratory conditions (22 ± 1 °C, 60–65% relative humidity, 12-h light:12-h dark alternate cycles with lights on at 07:00 a.m., food and water ad libitum).

FLX hydrochloride was dissolved in saline solution (0.9% NaCl) to obtain an oral dose of 10 mg kg^{-1} that was administered daily for 21 days. The same procedure was followed for VLF hydrochloride. CIT was dissolved in saline solution in a dose of 10 mg kg^{-1}, and injected intraperitoneally each day for 21 days. Control rats received only the vehicle (0.9% NaCl, daily). These doses result in plasma levels of the drug itself and of its metabolite comparable to those found in depressed patients under standard clinical treatment.

Rats were anesthetized with ether and killed by decapitation 2 h after the last injection during the light phase. Brains were removed and dissected on ice while blood samples were drawn by cardiac puncture and collected into tubes containing 0.5 ml of sodium citrate 0.129 M. The plasma was obtained by centrifugation at 13,000 rpm for 15 min. Both plasma and brain were stored at −80 °C until analysis. All the procedures involving animals and their care were conducted in conformity with the European Communities Council Directive on “Protection of Animals Used in Experimental and Other Scientific Purposes” (86/609/EEC).

Prior to extraction, plasma samples were deproteinized by adding 0.2 ml of perchloric acid to 1 ml of plasma and centrifugated for 10 min at 4500 rpm. The deproteinized plasma was put into a 4 ml screw-cap vial and NaOH solution was added until the neutralization of the acidic media.

For the brain tissue pretreatment, 100 mg of sample were put onto a 1.5 ml eppendorf microtube with 1 ml of water and homogeneized with an ultrasonic cell disruptor for approximately 10 s (Model Labsonic, B. Braun Mesulgen AG, Leinfelden, Germany). Then, the mixture was centrifuged at 13,000 rpm for 10 min at 4 °C in a refrigerated centrifuge (Model Sorval RMC 14, Sorval Instruments Inc., Lansdale, USA). The supernatant was put into a 4 ml screw-cap vial where the extraction was to be carried out.

Human urine samples were collected from three patients that were taking one of these antidepressants. Volunteer 1 took Vandral® (37.5 mg VLF) while volunteer 2 was under daily treatment with Esertia® (10 mg CIT day^{-1}). Volunteer 3 was being chronically treated with Prozac Weekly® (90 mg week^{-1}), an enteric-coated fluoxetine hydrochloride that delays release into the bloodstream. Control urine samples were taken from healthy donors. First morning urine samples were taken in all cases in sterile containers (Deltalab Eurotubo, Barcelona, Spain) and kept at −80 °C until analysis. Prior to extraction, the urine sample was centrifuged at 13,000 rpm for 10 min at room temperature and diluted to 1:5 with water. This dilution was enough to suppress the matrix effect and should not be a problem for the quantification of the analytes which are excreted usually at mg l^{-1} levels [14]. 1 ml of the diluted sample was put in the 4 ml screw-cap vial and the extraction procedure was carried out.

2.4. Optimization of SBSE procedure

Each day before analysis, in order to ensure good selectivity and sensitivity results, stir bars were conditioned by treating them with acetonitrile for 20 min at a magnetic stirrer at 1100 rpm, rinsed with distilled water and dried using lint-free tissue.

First of all, the conditions of the liquid desorption step were established to ensure effective removal of the extracted analytes from the SBSE device. The parameters studied were: solvent composition (acetonitrile, methanol and mobile phase), desorption time (5, 10, 15, 20 and 30 min), temperature (room temperature 20 ± 1 °C, 50 °C and 75 °C) and desorption mode (magnetic stirring or sonication). For this study, a provisional extraction procedure was used: 1 ml of standard solution containing 1 mg l^{-1} of each compound and 1 ml of borate buffer (pH = 11, 0.1 M) were put in a 4 ml screw-cap vial with a stir bar which was stirred at a speed of 1100 rpm for 30 min. After extraction and before desorption step, the stir bar was removed, rinsed in distilled water and cleaned with a lint-free tissue. The efficiency of the liquid desorption process was confirmed performing a second desorption under the same conditions.

Once the liquid desorption conditions were established, the most relevant parameters affecting SBSE extraction were evaluated. The first step was the study of the pH value. As is known, the pH and ionic strength of the matrix has an influence on the distribution constant and therefore on the extractability of any analyte. For these reasons, and taking into account the stability of PDMS polymer, pH values from 2 to 11 were studied. We also paid some attention to the influence of the total volume of the solution to be extracted, studying the amount of buffer that should be added between 0.5 ml and 3 ml. Once the best pH value was selected, the influence of ionic strength adding NaCl at concentration between 0 and 300 g l^{-1}, the addition of organic solvents as well as acetonitrile and methanol (0, 5, 10 and 20%) and time (5, 10, 15, 30 and 60 min) were studied. Since the temperature plays an important role in the extraction of the analytes influencing on their mass transfer rates and the partition coefficients, extraction efficiency was studied using the stir bar at room temperature (20 ± 1 °C), 50 °C and 75 °C.

After the optimization of the extraction conditions, these have changed regarding to the provisional conditions selected for the optimization of the desorption process. With the aim of ensuring that the selected liquid desorption conditions were still adequate, the absence of carry over was checked.

2.5. Applicability of the SBSE procedure to plasma, brain tissue and urine samples

Once the optimization of the SBSE procedure was completed, the applicability to real samples was evaluated. In order to detect the presence of interferences from endogenous compounds in chromatograms control plasma, brain tissue and urine samples were analyzed. These samples were processed following the optimized procedure.

Furthermore, control samples were doped with three different concentrations of the analytes. These doped samples allowed the matrix effect on the method to be evaluated comparing the recoveries obtained with standards to the ones obtained with real samples.

2.6. Analytical assessment of the method in plasma, brain tissue and urine samples

The analytical assessment of the developed SBSE/HPLC-FLD methodology was evaluated in terms of linearity, quantification limits, precision and accuracy.

In order to simulate the conditions where the analytes would be found, the linearity of the proposed method was tested. The calibration was carried out in plasma and brain tissue of control rats and in healthy volunteer urine samples which were doped resulting in the following concentrations: 0.2–2000 ng ml^{-1} in plasma; 2–50,000 ng g^{-1} in brain tissue; 1–20,000 ng ml^{-1} in urine.

The limits of quantification were calculated according to the European Union Guideline and it is defined as the lowest concentration on the calibration curve in which the coefficient of the variation was lower than 20%.

In order to check the intra- and interday precision and accuracy of the whole optimized procedure at three concentration levels, control samples were doped to reach the following concentrations: 2, 50 and 500 μg l^{-1} for plasma, 5, 500 and 10,000 ng g^{-1} for brain tissue and 10, 200 and 5000 μg l^{-1} for urine. For each sample, ten repetitive extractions were made in the same day and also at intervals over a 2-week period (n = 10) at three different levels of concentration for each matrix.

Finally, in order to evaluate the selectivity of the procedure, the presence of possible interfering compounds was studied. For

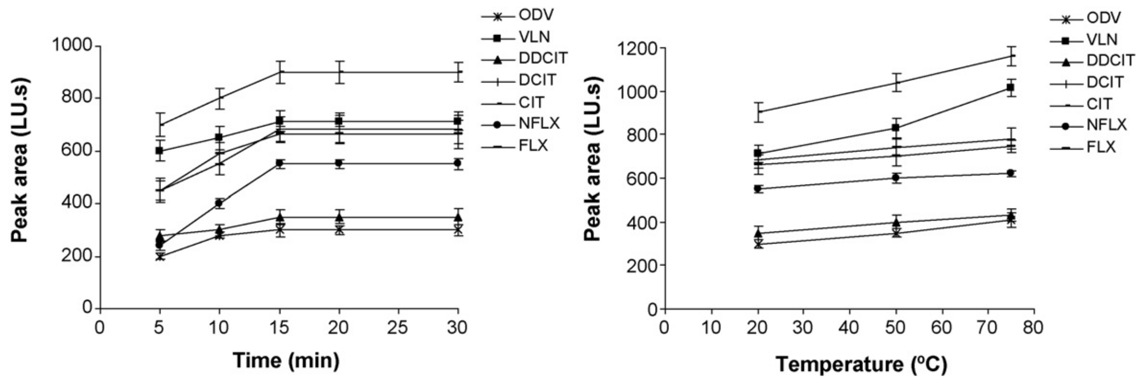

Fig. 2. Effect of desorption time and temperature on the response of the analytes. SBSE procedure: 1 ml of standard containing 1 mg l^{-1} of each compound in water; extraction pH 11; extraction temperature, 20 ± 1 °C; extraction time, 30 min at 1100 rpm; desorption mode, magnetic stirring at 1100 rpm. Each point is the average of three data points.

these aim, thirteen different antidepressants where tested with the developed methodology.

2.7. Application of the method to real samples

The effectiveness of the proposed method was tested by analyzing real plasma, urine and brain tissue samples. The analysis of FLX and NFLX in plasma and brain tissue was made in samples of rats that received daily an oral dose of 10 mg kg^{-1} of FLX. In the same way, for the analysis of VLN and ODV, plasma and brain tissue samples of rats that received daily an oral dose of 40 mg kg^{-1} day^{-1} of VLF were employed. The analysis of CIT, DCIT and DDCIT was carried out in plasma and brain tissue samples of a rat treated with an intraperitoneal dose of 10 mg kg^{-1} day^{-1} of CIT.

The analysis of these antidepressants and of their metabolites was also made in urine samples of three volunteers that were taking one of these antidepressants. Volunteer 1 took Vandral® (37.5 mg VLF), volunteer 2 Esertia® (10 mg CIT day^{-1}) and volunteer 3 Prozac Weekly® (90 mg $week^{-1}$).

3. Results and discussion

As previously stated, several parameters affecting desorption and extraction steps were evaluated. The first step was the evaluation of desorption parameters such as solvent, time and temperature (Fig. 2). The desorption solvents evaluated were acetonitrile, methanol and mobile phase varying the volume and desorption time from 5 to 30 min. The assay was performed at 20 °C, 50 °C and 75 °C for magnetic stirring and at room temperature for sonication. It was found that the best recovery values were obtained when using 300 μl acetonitrile as desorption solvent during 15 min. Besides, it was observed that magnetic stirring at 75 °C was more effective than sonication performed in the same period of time and at room temperature, because the desorption process was favored at higher temperatures. Because of the long tailed peaks

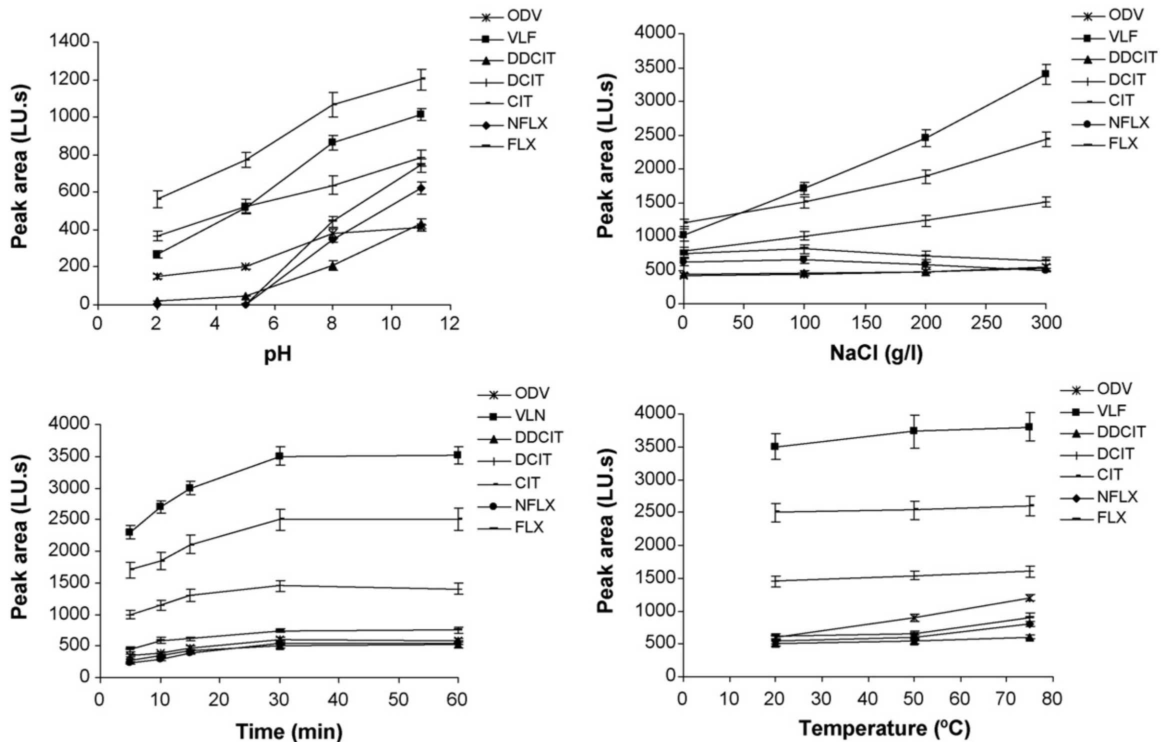

Fig. 3. Effect of extraction pH, ionic strength, time and temperature on the response of the analytes. SBSE procedure: 1 ml of standard containing 1 mg l^{-1} of each compound in water; desorption time, 15 min at 1100 rpm; desorption temperature, 20 °C; desorption mode, magnetic stirring. Each point is the average of three data points.

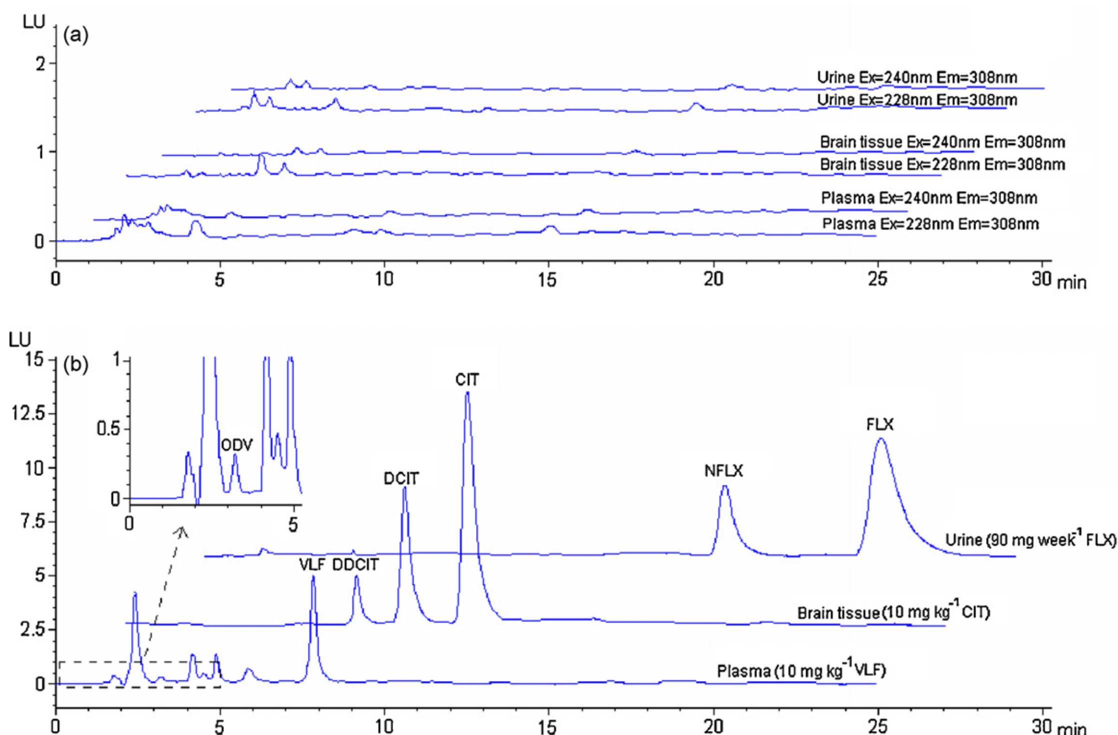

Fig. 4. (a) Chromatograms obtained for control plasma, brain tissue and urine samples at λ_{exc} = 228 nm and 240 nm and λ_{exc} = 308 nm. (b) Chromatograms obtained from: plasma of rat treated with 40 mg kg day^{-1} of VLF (ODV = 2 ng ml^{-1}; VLF = 18 ng ml^{-1}); brain tissue of rat treated with 10 mg kg day^{-1} of CIT (DDCIT = 1480 ng g^{-1}; DCIT = 1410 ng g^{-1}; CIT = 1503 ng g^{-1}); urine of human treated with 90 mg week^{-1} of FLX (NFLX = 943 ng ml^{-1}; FLX = 1635 ng ml^{-1}). The retention time of the compounds were: $t_{R(ODV)}$ = 3.1 min, $t_{R(DDCIT)}$ = 7.0 min, $t_{R(VLF)}$ = 7.8 min, $t_{R(DCIT)}$ = 8.6 min, $t_{R(CIT)}$ = 10.6 min, $t_{R(NFLX)}$ = 16.1 min, $t_{R(FLX)}$ = 21.2 min.

obtained when acetonitrile extract was injected, it was evaporated and reconstituted with 200 μl of mobile phase. It was proved that at the selected desorption conditions there was no evidence of carry-over, ensuring the effectiveness of the procedure.

Once desorption parameters were defined, the next step was the establishment of extraction parameters (Fig. 3). pH values ranged between 2 and 11 were studied by adding 1 ml of citric acid, phosphate or borate buffer solutions adjusted to yield the desired pH. The best recoveries were obtained at pH = 11, due to the basic character of the analytes (pKa = 9.2–10.5), whose non-ionic form predominates at these pH values. Besides, and with regard to the volume of the solution to be extracted, it was concluded that the best recoveries were obtained with the addition of 1 ml of buffer.

For the study of the ionic strength effect, different experiments were carried out using NaCl as ionic salt at concentrations of 0, 100, 200 and 300 g l^{-1}. As shown in Fig. 3, except for FLX and NFLX, all the analytes suffer an important increase in the extractability with the increase of the NaCl concentration. Due to the smooth decrease undergone by FLX and NFLX signals, a NaCl concentration of 300 g l^{-1} was chosen.

The evaluation of the effect of the organic solvent content of the sample showed a fall in the extractability of the compounds as the percentage of acetonitrile or methanol increased. For these reasons, the addition of any organic solvent was ruled out.

Extraction efficiency was also studied at 20 °C, 50 °C and 75 °C (Fig. 3). For all the compounds, better recoveries were observed as temperature increased, which was more marked for ODV, FLX and NFLX. Finally, the effect of the equilibrium time was estimated for a period of time ranging from 5 to 60 min, observing that all the compounds reached the equilibrium in 30 min.

Based on these data, the best SBSE experimental conditions were as follow: a previously pretreated sample was put in a 4 ml screw-cap vial and 1 ml of borate buffer (pH 11, 0.1 M) and 0.6 g of NaCl were added. A stir bar was immersed in the solution and the vial was closed with the screw-cap. The sample solution was stirred at a controlled speed of 1100 rpm for 30 min at 75 °C. After extraction, the stir bar was removed, rinsed in distilled water and cleaned with a lint-free tissue.

For liquid desorption, the stir bar was placed into a vial with a glass vial-insert of 0.4 ml filled with 0.3 ml of acetonitrile, ensuring total immersion. Desorption was performed by magnetic agitation at 75 °C at a controlled speed of 1100 rpm for 15 min. After extraction the stir bar was removed, the solvent was evaporated until dryness and the dry residue was redissolved in 200 μl of mobile phase. The vial was closed with a seal using a hand crimper and placed into the automatic liquid sampler where a 100 μl aliquot was injected. Finally, the stir bar was rinsed in distilled water and cleaned with a lint-free tissue.

In the study of the applicability of the method to real samples, there was an absence of interferences from endogenous compounds in chromatograms obtained after extraction of control samples as shown in Fig. 4.

When this method was applied to real matrices, a slight matrix effect was observed for plasma and brain tissue samples which seems to be very usual in this kind of sample. The study of the extraction recovery of the analytes with this method gives results between 50 and 92 %, which is usual with SBSE using PDMS coatings [11].

The partitioning efficiency of the analyte between the sorbent phase and water samples is defined as the distribution coefficient ($K_{PDMS/W}$) at equilibrium and it has been correlated with the octanol–water distribution coefficients ($K_{o/w}$), which gives a good indication of the extraction of a solute on the SBSE [17]. Therefore, it could be concluded that the higher the polarity of the analyte, the lower the extraction efficiency on PDMS. Previous works reported that quantitative extraction can be achieved for log $K_{o/w}$ values above 3. Nevertheless, the usefulness of SBSE for the extraction of relatively polar compounds has been also reported [16]. Taking into

Table 1
Summary of validation results in plasma, brain tissue and urine: linearity, quantification limits, precision and accuracy of the proposed analytical method for three concentration levels.

Matrix	Analyte	Linearity ($n=7$)	R^2	LOQ	Intraday ($n=10$)						Interday ($n=10$)					
					RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)
		LOQ-2000 ng ml^{-1}		ng ml^{-1}	2 ng ml^{-1}		50 ng ml^{-1}		500 ng ml^{-1}		2 ng ml^{-1}		50 ng ml^{-1}		500 ng ml^{-1}	
Plasma	ODV	$y=0.82x+5.57$	0.994	0.5	4.0	111.0	3.8	110.2	4.8	112.4	6.8	102.5	3.5	92.7	5.7	105.3
	VLN	$y=3.91x+4.40$	0.999	0.2	6.9	95.2	6.2	106.5	5.2	94.5	7.9	105.6	9.3	98.5	8.4	104.6
	DDCIT	$y=0.54x+14.88$	0.998	0.5	7.7	91.0	10.2	91.0	6.3	98.4	8.4	101.8	12.7	107.4	10.3	94.3
	DCIT	$y=1.48x+2.64$	0.999	0.5	10.3	103.5	11.8	104.3	8.6	105.0	8.3	97.4	10.2	107.9	7.4	96.8
	CIT	$y=2.13x+2.92$	0.999	0.5	10.2	110.0	12.6	108.7	8.3	103.9	9.0	99.5	11.0	96.3	8.6	104.8
	NFLX	$y=0.60x+0.75$	0.999	2	6.6	89.5	5.2	109.7	7.0	98.2	8.4	104.9	4.5	94.1	8.6	105.1
	FLX	$y=0.63x+1.46$	0.999	2	7.0	93.5	5.7	89.6	8.1	92.7	7.7	94.5	4.5	102.1	6.2	93.4
		LOQ-50,000 ng g^{-1}		ng g^{-1}	5 ng g^{-1}		500 ng g^{-1}		10,000 ng g^{-1}		5 ng g^{-1}		500 ng g^{-1}		10000 ng g^{-1}	
Brain tissue	ODV	$y=0.06x+2.82$	0.998	5.0	5.3	105.2	12.7	107.1	6.2	95.7	7.3	91.0	8.1	105.9	10.4	107.8
	VLN	$y=0.44x+1.05$	0.999	2.0	4.9	90.0	4.8	97.9	7.1	97.8	6.8	96.4	7.8	95.6	8.1	95.6
	DDCIT	$y=0.02x+5.40$	0.998	5.0	7.8	110.2	7.0	91.4	9.4	92.5	8.0	112.2	8.9	99.8	5.8	104.1
	DCIT	$y=0.10x+0.67$	0.999	5.0	5.6	98.0	8.1	95.6	9.7	102.0	6.3	99.6	10.2	96.5	8.0	107.9
	CIT	$y=0.19x+0.20$	0.999	5.0	8.2	91.0	4.9	97.9	8.3	91.8	7.9	109.0	8.1	100.7	9.6	96.8
	NFLX	$y=0.02x-1.01$	0.999	20.0	8.3	90.8	7.4	100.0	8.1	108.0	9.7	89.4	9.5	109.9	8.2	96.7
	FLX	$y=0.02x-0.99$	0.999	20.0	6.1	113.4	9.8	92.7	7.3	100.4	8.3	110.0	7.2	92.5	9.3	107.9
		LOQ-20,000 ng ml^{-1}		ng ml^{-1}	10 ng ml^{-1}		200 ng ml^{-1}		5000 ng ml^{-1}		10 ng ml^{-1}		200 ng ml^{-1}		5000 ng ml^{-1}	
Urine	ODV	$y=0.11x+0.85$	0.999	2.5	4.6	109.6	7.2	110.1	6.3	110.5	6.2	95.4	10.2	110.1	9.3	108.7
	VLN	$y=0.74x+1.71$	0.999	1.0	7.2	110.1	8.8	108.4	8.5	99.4	4.3	99.6	9.1	99.1	8.9	99.0
	DDCIT	$y=0.07x+9.77$	0.997	2.5	8.1	98.9	10.9	110.8	4.8	107.9	9.5	90.1	12.5	95.3	8.9	102.0
	DCIT	$y=0.20x+18.23$	0.998	2.5	6.4	107.6	12.2	102.8	9.2	98.8	7.8	109.6	12.6	98.7	9.1	99.0
	CIT	$y=0.45x+0.19$	0.999	2.5	8.2	89.3	11.8	99.2	5.2	99.0	6.0	110.5	11.0	105.3	9.3	98.9
	NFLX	$y=0.15x+0.45$	0.999	10	5.7	90.1	11.8	97.8	8.1	99.7	7.1	107.5	10.8	104.8	10.7	100.3
	FLX	$y=0.21x-1.35$	0.999	10	3.6	106.5	6.4	102.4	7.0	107.9	8.5	96.1	5.5	98.0	10.2	99.9

Table 2
Retention time and emission wavelengths in possible interfering compounds.

Drug	Retention time	λ_{em}
Bupropion	n.d.	
Chlomipramine	n.d.	
Chlorgiline	n.d.	
Mirtazapine	n.d.	
Maprotiline	17.9 min	300 nm
Paroxetine	12.2 min	340 nm
Sertraline	n.d.	
Trazodone	n.d.	
Duloxetine	14.2 min	340 nm
Amitryptiline	n.d.	
Desipramine	n.d.	
Imipramine	n.d.	
Nortryptiline	n.d.	

n.d. not detected.

account that except FLX (log $K_{o/w}$ = 3.27) and NFLX (log $K_{o/w}$ = 3.7) the analytes have log $K_{o/w}$ values below 3 the recoveries obtained seem to be common in these kind of samples. Moreover, the preconcentration factor p (the ratio of the peak areas obtained with SBSE and that with direct injection) obtained with this method for each compound were: $p_{ODV} = 2.5$, $p_{VLF} = 3.3$, $p_{DDCIT} = 2.5$, $p_{DCIT} = 2.5$, $p_{CIT} = 4.6$, $p_{FLX} = 3.1$, $p_{NFLX} = 2.9$.

For the quantification of real samples, the calibration was carried out in plasma and brain tissue of control rats and in healthy volunteer urine samples which were doped resulting in the following concentrations: 0.2–2000 ng ml^{-1} in plasma; 2–50,000 ng g^{-1} in brain tissue; 1–20,000 ng ml^{-1} in urine. These control samples present no analyte peaks or interferences in the chromatograms. The linear regression equations (peak area vs. concentration), obtained using the least-squares method, showed a linear response in the ranges mentioned, with correlation coefficients (R^2) above 0.994 in all cases (Table 1). The LOQ values ranged between 0.2–2 μg l^{-1}, 2–20 ng g^{-1} and 1–10 μg l^{-1} for plasma, brain tissue and urine samples respectively, depending on the response of the analytes (Table 1).

For all the analytes and for the three concentrations tested, the precision presents coefficients of variation below 13% in both intra- and interday assays. The accuracy of the assay, based on the deviation of the mean measured value from the theoretical (doped) value, ranged from 89 to 113%).

With regard to the presence of exogenous compounds, the elution of a particular interference at the same retention time as one of the analytes could be easily detected since the HPLC-FLD equipment software enables the estimation of the peak purity by means of various tests (ratiogram generation, spectral similarity curves and threshold curves). Nevertheless, some possible interfering compounds were analyzed with the developed method. As shown in Table 2, with the selected chromatographic and detection conditions and due to the selectivity of the FLD detector, most of the studied interfering compounds were not detected or were not coeluting with the target solutes.

Once the optimization and evaluation of the method was concluded, it was applied for the quantification of FLX, CIT, VLF and its metabolites in real plasma, brain tissue and urine samples. Some of the chromatograms obtained for these real samples can be seen in Fig. 3. As is shown in Table 3, the concentrations of the analytes found in these samples were in the linearity range of the calibrations. It is important to see that the values obtained for the concentrations of NLFX, DCIT and DDCIT in plasma and brain tissue were higher than the ones obtained for FLX and CIT. These results showed the importance of the quantification of the active metabolites of these antidepressants in these matrices, which share the property of blocking the serotonin transporter and consequently act as SSRI.

Table 3
Concentration of the analytes in real plasma, brain tissue and urine samples.

Sample	Compound	Plasma (ng ml^{-1})	Brain tissue (ng g^{-1})
Rat (40 mg kg^{-1} VLF)[a]	ODV	2.3 ± 0.8	5.5 ± 0.7
n = 3	VLN	17.6 ± 3.6	82.3 ± 17.3
Rat (10 mg kg^{-1} CIT)[a]	DDCIT	193.0 ± 67.8	1366.5 ± 218.2
n = 3	DCIT	123.3 ± 79.8	1377.5 ± 323.2
	CIT	126.7 ± 31.9	1487.2 ± 292.8
Rat (10 mg kg^{-1} VLF)[a]	NFLX	231.7 ± 101.5	9782.2 ± 1927.3
n = 3	FLX	137.5 ± 75.2	3517.0 ± 1434.1

Sample	Compound	Urine (ng ml^{-1})
Volunteer 1[b]	ODV	4870.1 ± 213.4
	VLN	1384.8 ± 102.5
Volunteer 2	DDCIT	1714.3 ± 158.2
	DCIT	2121.2 ± 198.2
	CIT	676.7 ± 68.1
Volunteer 3	NFLX	960.9 ± 78.4
	FLX	1779.0 ± 146.1

[a] Values are mean ± S.D of n = 3 rats.
[b] Values are mean ± S.D of n = 3 analysis of the same sample.

With regard to the results obtained in urine samples, it can be said that this method is suitable for a reliable quantification of analytes at clinical levels. Moreover, it could be useful in the detection of non-adherence to treatment, a commonly observed behaviour especially in long-term treatments, which results in suboptimal medication and poor disease control.

4. Conclusions

The developed SBSE/HPLC-FLD procedure permits the quantification of FLX, CIT and NFLX and their active metabolites ODV, DCIT, DDCIT and NFLX minimizing laborious and complicated sample preparation procedures. No additional clean up step is necessary between two SBSE extractions to assure efficient removal of interferences or analytes. Moreover, the use of HPLC makes a previous derivatization step unnecessary. The selectivity of the SBSE procedure together with the selectivity of the fluorescence detector avoids the presence of endogenous and exogenous interfering compounds.

As demonstrated in this paper, this method shows the advantage of being suitable for the analysis of plasma, brain tissue and urine samples. The high sensitivity, the proven selectivity and the good precision and accuracy obtained in each matrix corroborates this benefit. All these advantages make this method useful for the establishment of a more efficient and safe dose, for the detection of non-adherence to treatments and for forensic analysis.

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