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Solid-phase microextraction–liquid chromatography (SPME–LC) determination of fluoxetine and norfluoxetine in plasma using a heated liquid flow through interface

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Abstract

A simple and sensitive procedure using solid-phase microextraction coupled with high performance liquid chromatography (HPLC) to analyze fluoxetine (FLU) and its metabolite norfluoxetine (nor-FLU) in plasma samples was developed and validated. SPME conditions were optimized employing a factorial design. The sampling step was performed using a PDMS-DVB fiber and desorption was carried out in a novel homemade heated interface. Fluoxetine and norfluoxetine were analyzed by HPLC, using a C18 Phase Sep column (150 mm × 4.6 mm, 3 μm) packed "in house", and acetonitrile:acetate buffer 25 mmol l^{-1} with triethylamine 25 mmol l^{-1} pH 4.6 (70:30) as the mobile phase. The developed method has shown precision, linearity, specificity, and limit of quantification (LOQ) adequate to assay fluoxetine and norfluoxetine in plasma. Furthermore, the results obtained using the homemade interface has shown an improvement in the desorption process when compared with the results obtained using the off-line mode.

Keywords: SPME; SPME–LC; Interface; Fluoxetine; Plasma

1. Introduction

Fluoxetine (D,L-*N*-methyl-3-phenyl-3-[(α,α,α-trifluoro-*p*-tolyl)oxy]propyl-amine) (Fig. 1a) is a selective serotonin (5-hydroxytryptamine; 5-HT) reuptake inhibitor (SSRI) in presynaptic neurons. It was introduced in the market in the 1980s and, since then, it is the most prescribed antidepressant drug in the world. Lately, FLU has been approved to be used in the treatment of obsessive-compulsive disorder and eating disorders, including anorexia nervosa and bulimia nervosa [1,2]. The employed dose can vary from 20 mg to 40 mg per day, depending on the treatment, and plasmatic levels from 80 ng ml^{-1} to 300 ng ml^{-1}, respectively, can be found [3,4]. FLU is extensively metabolized in the liver to a desmethyl metabolite, norfluoxetine (nor-FLU—Fig. 1b), which has activity similar to FLU. The determination of FLU in biofluids is essential in pharmacokinetic studies, in the comparison of the behavior of different formulations, in bioequivalent studies, and in therapeutic drug monitoring (TDM).

Several sample preparation techniques have been employed in order to analyze FLU in biofluids. Among them the most common are liquid–liquid extraction (LLE) [5–11] and solid-phase extraction (SPE) [12–18]. These methods, however, present some disadvantages, being laborious and time-consuming. Moreover, they employ expensive and toxic solvents. Reports of the use of column-switching, a rapid and modern technique, have been found in the literature for this analysis [19,20]. However, this technique requires more expensive and complex instrumentation to be utilized.

Solid-phase microextraction (SPME) is a microtechnique that presents almost all the necessary requirements of an ideal sample preparation technique. It uses small quantities of solvents and allows obtaining relatively clean extracts when working with complex matrices such as biofluids. SPME is based on the sorption of the analyte in a small diameter fiber, coated with a sorbent material. This technique involves two distinct steps:

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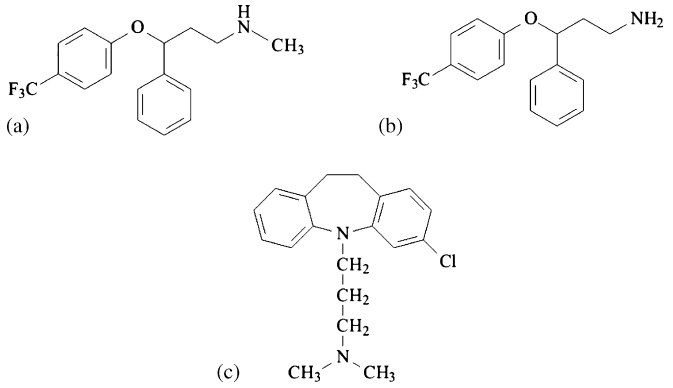

Fig. 1. Structures of fluoxetine (a); norfluoxetine (b); and clomipramine (c).

analytes partition between fiber coating and matrix, followed by desorption of the concentrated extract into the analytical instrument. After the distribution equilibrium has been reached, the amount of analyte extracted into the fiber coating is proportional to the partition coefficient and to the analyte concentration in the sample. The partition coefficient is determined by the interaction between the analyte and the matrix and between the analyte and the fiber coating. Extraction is considered complete when the analyte concentration achieves the distribution equilibrium between the fiber coating and the matrix, thus, an exhaustive extraction does not occur [21–23]. Some experimental parameters influence the analyte extraction from the matrix to the fiber coating and should be evaluated and optimized in order to obtain maximum technique performance. Parameters that influence the analyte extraction include: fiber coating type, temperature, extraction time, ionic strength in the medium, organic solvent amount, pH, and stirring speed [23].

At the start, SPME was developed to be used with gas chromatography (GC). However, it has also found more recent applications when coupled with high performance liquid chromatography (HPLC), including the analysis of drugs in biofluids. In most papers published until this moment, the desorption step has been done either using *off-line* or *on-line* mode, employing a commercial interface without a heating system [24,25]. Temperature control during desorption step allows to obtain a considerable increase in the analyte desorption and a decrease in the variation between analyses. In order to achieve these desirable characteristics, an interface with a desorption chamber of 60 μl and heating control was developed in our laboratory for coupling the SPME–LC.

Until this moment, only two papers have been found in the literature using SPME to analyze FLU. However, the determination was performed employing gas chromatography and derivatization, an additional step [26,27].

HPLC has been the most employed technique for FLU analysis in plasma, because of the structural characteristics of the analyte. Fluorescence, ultraviolet, and mass spectrometry detectors have been used with HPLC [11,16,18]. However, some studies employing GC and micellar electrokinetic capillary chromatography (MECK) have also been reported [28,29].

In this study, a method not yet described in the literature for the determination of FLU in plasma using SPME–LC–UV has been developed and validated. The use of two different types of fiber coatings – carbowax-templated resin (CW-TPR) and polidymethylsiloxane-divynilbenzene (PDMS-DVB) – was optimized and compared using a factorial design. The SPME–LC on-line coupling employed a heated interface developed in our laboratory. The developed method has shown precision, linearity, specificity, and limit of quantification (LOQ) adequate to assay FLU and nor-FLU in plasma.

2. Experimental

2.1. Chemicals and reagents

Clomipramine (CLO), employed as an internal standard (IS) (Fig. 1c), FLU, and nor-FLU analytical standards, were provided by Sigma–Aldrich (Steinhein, Germany). Ammonium acetate, acetic acid, methanol, acetonitrile from Mallinckrodt (Paris, USA), sodium tetraborate from Reagen (Rio de Janeiro, Brazil), hydrochloric acid, triethylamine (100%) from J.T. Baker (Xalostoc, Mexico), and sodium chloride from Grupo Química (Rio de Janeiro, Brazil) were also used. All solvents and reagents were of HPLC or analytical grade. The water used to prepare all the samples and solutions used in this study was purified in a Milli-Q Ultra-Pure Water System (Millipore, Bedford, USA). Drug-free plasma was kindly donated by Hospital Irmandade Santa Casa de Misericórdia de São Carlos (São Carlos, SP, Brazil) and maintained frozen at −20 °C.

Silica C18, 3 μm, "pH stable" from Phase Sep (Norwalk, USA) was used to pack the analytical column.

2.2. Instruments

A manual fiber holder for SPME, CW-TPR (50-μm coating thickness), and PDMS-DVB (60-μm coating thickness) SPME fibers were purchased from Supelco (Bellefonte, PA, USA).

A homemade interface for coupling SPME–LC was designed and built in our laboratory (Fig. 2). This new interface consists of a six-port Valco valve (Houston, USA) connecting a 60 μl (inner volume) homemade desorption chamber, a heating block, a PT 100 Type thermo couple from Casa Ferreira (São Paulo, Brazil), and a temperature controller from Thorton (RS, Brazil) [30].

The HPLC system (LC-10AVP) consisted of two pumps (LC-10ATVP), an oven (CTO-10ASVP), a fixed wavelength ultraviolet detector (SPD-10AVVP), an autoinjector (SIL-10AF), a system controller (SCL-10AVP), a degasser (DGU-14A), and an acquisition data software Class-VP, all from Shimadzu (Kyoto, Japan).

2.3. Column packing procedure

In order to analyze basic analytes (FLU, nor-FLU, and CLO), a column (150 mm × 4.6 mm) was packed in our laboratory. Modified silica was employed as the stationary phase (C18, 3 μm "pH stable"). In short, a suspension of silica in methanol at a concentration of 0.8 g ml^{-1} was prepared and the column was slurry packed during 30 min under a pressure of 7300 psi, using

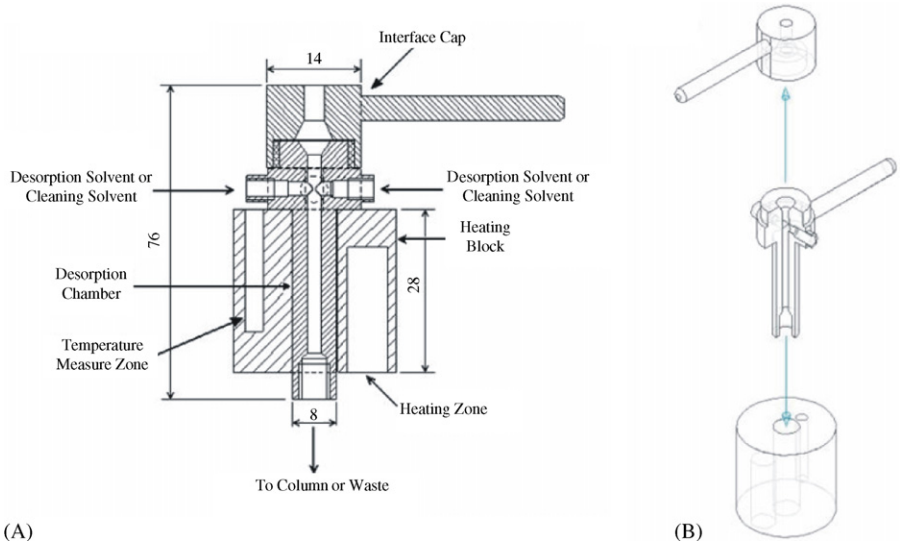

Fig. 2. (A) Two-dimensional design (2D) of heating SPME–HPLC interface. (B) Three-dimensional (3D) interface explosion view design (cross-section of the desorption chamber) [30].

a Haskel (Burbank, USA) pneumatic amplifier pump and nitrogen as pressurization gas, and using a protocol already described by us elsewhere [31]. After this period, the system was slowly and carefully depressurized for 15 h.

2.4. Chromatographic conditions

Optimized and used chromatographic conditions in the determination of FLU and nor-FLU by SPME–LC in plasma were: C18 (150 mm × 4.6 mm, 3 μm) column packed "in house"; acetonitrile: acetate buffer 25 mmol l^{-1} with triethylamine 25 mmol l^{-1} pH 4.6 (70:30) at a flow rate of 1.0 ml min^{-1} as mobile phase; temperature of 35 °C; and detection at 227 nm.

2.5. SPME procedure

The new fibers were conditioned in the mobile phase for 15 min under stirring and before every extraction step, fibers were cleaned with the same solvent for 15 min in order to avoid any carry-over effect.

Analyses were performed using 5 ml vials sealed with hole caps. Triangular magnetic stirring bars were used to agitate the samples during the extraction. One milliter of spiked plasma and 4.0 ml of borate buffer 50 mmol l^{-1} were used in all analyses with a stirring speed of 1100 rpm. After extraction, fiber was directly introduced in the interface chamber and desorption was performed in the homemade heated interface at 60 °C for 15 min in the static mode.

A fractional factorial design with eight experiments (2^{4-1}) was performed in order to optimize the extraction with the two fibers (PDMS-DVB and CW-TPR). Parameters that affect the extraction more extensively such as time (25 and 50 min), temperature (30 and 50 °C), ionic strength (0 and 10% NaCl), and pH (9.0 and 11.0) were varied and evaluated. Optimization was performed using off-line desorption mode. Statistica 6.0 software was used to build the model, evaluated by ANOVA. After optimization, extraction kinetic curves at 5, 10, 20, 30, 40, and 50 min were built in order to evaluate the necessary time to achieve the equilibrium.

Desorption using both off-line and on-line modes was also performed and compared in order to evaluate the homemade interface efficiency. Different temperatures (25, 40, 50, 60, 70, and 80 °C) were employed in order to investigate their influence on the desorption process. Water samples spiked with the analytes were employed in this evaluation.

2.6. Preparation of analytical standards

Stock solutions of FLU, nor-FLU, and CLO prepared in methanol at the concentration of 1000 μg ml^{-1} were maintained under refrigeration at 4 °C for 1 week. These solutions were diluted to working standard solutions of FLU and nor-FLU at 10.0 and 1.0 μg ml^{-1}, and CLO at 10.0 μg ml^{-1} on the day of the use.

Human plasma was centrifuged (7,100 × *g*, 15 min) and filtered through 0.45-μm membranes. In order to achieve concentrations of 25, 50, 100, 200, 300, and 500 ng ml^{-1}, adequate aliquots of working standards of FLU, nor-FLU, and CLO were transferred to 5 ml vials, dried under nitrogen, and suspended in 1.0 ml of blank plasma.

2.7. Validation procedure

Method specificity was evaluated by analyzing six different blank plasmas. The presence of any interference eluting in the same retention time of FLU, nor-FLU, or CLO was evaluated.

Linearity was verified by analyzing spiked plasma samples in the concentrations of 25, 50, 100, 200, 300, and 500 ng ml^{-1} with three replicates in each point using CLO as internal standard at a concentration of 1000 ng ml^{-1}. The linear regression equation and the correlation coefficient (r^2) were calculated by the least squares method.

Within-day ($n = 5$) precision was examined at the concentrations of 25, 100, and 500 ng ml^{-1}. Between-days ($n = 3$) precision was evaluated at the same concentrations by performing analyses in two different days. Precision was expressed as relative standard deviation (RSD%).

Limit of quantification was determined by analyzing FLU and nor-FLU in the concentration of 25 ng ml^{-1} in five replicates and verifying the RSD% that should be smaller than 20.0%. Limit of detection (LOD) was established as the concentration where analyte peak was two times higher than the baseline noise.

Recovery was evaluated through the analyses of analytical standard samples at the concentrations of 25, 100, and 500 ng ml^{-1} ($n = 3$) with the volume injection of 60 μl, the same volume injected in the SPME process, and comparison with the values obtained in the evaluation of linearity. Recovery was expressed as percentage of the extracted amount.

3. Results and discussion

3.1. Chromatographic conditions optimization

In order to achieve a good separation between FLU, nor-FLU, CLO and endogenous compounds from plasma in a short analysis time, chromatographic conditions were evaluated and optimized. The packed column showed adequate efficiency and minimum peak tailing, which is a common concern in the analysis of basic compounds, such as FLU, nor-FLU, and CLO.

Different mobile phase compositions were studied. Percentage of organic solvent and concentration of ammonium acetate buffer and triethylamine were varied. Acetonitrile:acetate buffer 25 mmol l^{-1} with triethylamine 25 mmol l^{-1} pH 4.6 (70:30) at a flow rate of 1.0 ml min^{-1} showed a good compromise between good separation and short time of analysis (9 min).

3.2. SPME optimization

After performing the eight experiments, it was observed that the use of salt had a negative effect on the extraction, decreasing the analyte recovery. Thus, this variable was removed and four new experiments were performed in order to produce a complete factorial planning (2^3).

Fig. 3 shows a Pareto diagram for PDMS-DVB fiber, where the significance of each evaluated parameter of the complete design is demonstrated. The percentage of variation explained by the designed 2^3 model was higher than 99.7, allowing an adequate evaluation of the parameters' effects. Dashed line indicates the region above which the effects were significant (with confidence limit of 95%).

Time was the parameter that presented the higher influence in the extraction. Time acted in a positive way, increasing the extracted amount. This data is in accordance with literature, once SPME is based on the partition between two phases, an adequate time being necessary to achieve the equilibrium.

Temperature also presented a positive effect on the extraction. Temperature can act in two distinct ways: increasing the analyte diffusion and, as a consequence, increasing the extraction, or decreasing the analyte partition coefficient between the fiber and the matrix, thus, decreasing the extraction yield. In these experiments, diffusion effects have stood out; so, higher temperature increased extraction.

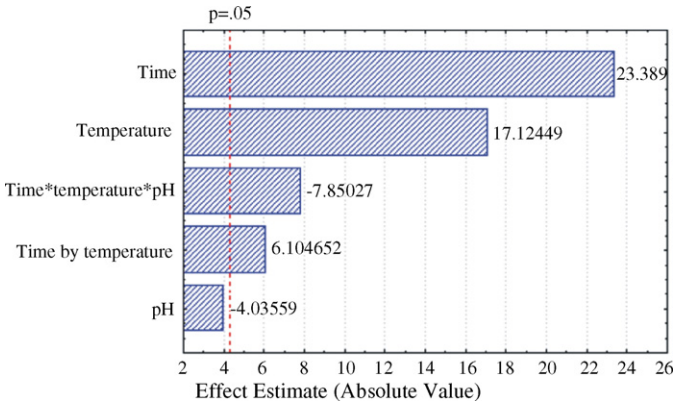

Fig. 3. Pareto diagram of the complete factorial design (2^3) for PDMS-DVB fiber coating.

The interaction between time and temperature parameters was positive. It means that extraction increases when time and temperature were increased together.

The pH presented negative effect in the extraction, but this effect was not significant. It means that when pH 9.0 was used, extraction was higher than when pH 11.0 was used. FLU is a weak base and has a pK_a of 8.7. Therefore, it is completely undissociated at pH 11.0, favoring the extraction by the fiber. However, higher extraction occurred when pH 9.0 was employed. In fiber coatings such as polydimethylsiloxane and polyacrylate, only the neutral fraction of the analyte is extracted. On the other hand, using PDMS-DVB coating some other types of interactions can occur, explaining why pH had a negative effect in the extraction.

Thus, the best condition for SPME extraction using PDMS-DVB fiber was: 50 °C, 50 min, without salt, and pH 9.0. Fig. 4 demonstrates the extraction kinetic curve for FLU under the optimized conditions. Time of 40 min was sufficient to achieve the equilibrium. However, a time of 30 min was chosen because there is a compromise between time and extracted amount; since

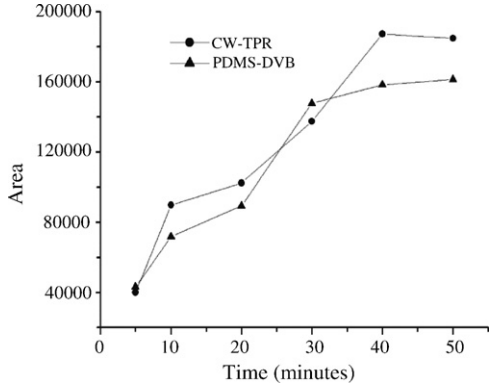

Fig. 4. Extraction kinetic curves for fluoxetine at 500 ng ml^{-1} under the following conditions: PDMS-DVB (50 °C, without salt, pH 9.0, and stirring speed of 1100 rpm) and CW-TPR (50 °C, without salt, pH 11.0, and stirring speed of 1100 rpm).

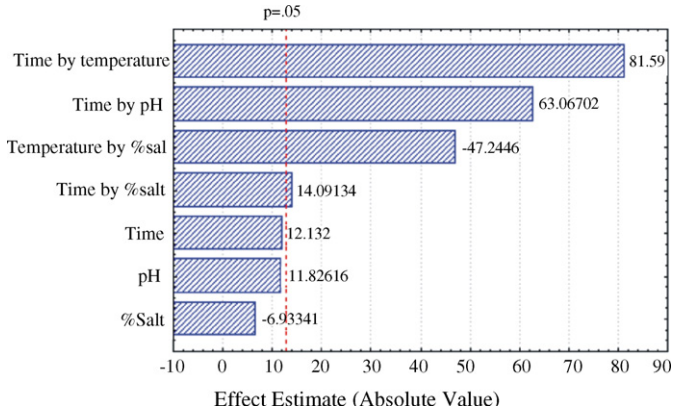

Fig. 5. Pareto diagram showing the evaluated parameters effects for CW-TPR fiber coating.

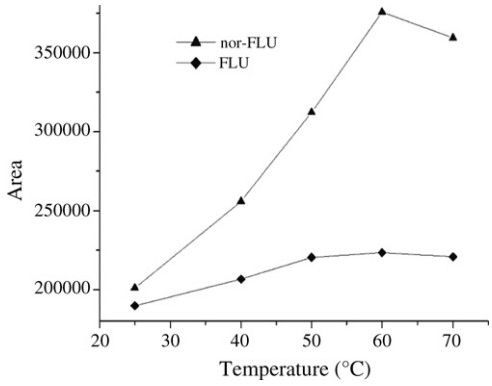

Fig. 6. Effect of temperature on desorption.

extractions were made at exactly the same time, quantitative analyses can be carried out with good reproducibility.

Higher values of time and temperature (50 min and 50 °C) increased the extracted amount in the optimization using CW-TPR fiber coating. Higher pH values (pH 11.0) also increased the extracted amount. Salt effect was not clear; so, one more experiment was carried out in order to verify salt effect in the extraction. Fig. 5 shows a Pareto diagram with one more experiment where it can be observed that higher extraction is achieved when salt is not used. Thus, the best conditions for CW-TPR fiber coating were: 50 min, 50 °C, without salt, and pH 11.0. However, high pH values, such as 11.0, are harmful for CW-TPR coating, the use of lower pH value being recommended, despite increased extraction achieved with pH 11.0.

The extraction kinetic curve for FLU under the following conditions: 50 °C, without salt, and pH 11.0 is shown in Fig. 4.

PDMS-DVB fiber coating was chosen in order to validate the SPME–LC method for FLU in plasma samples because the extracted amount was higher than that obtained with CW-TPR at pH 9.0. Furthermore, PDMS-DVB is more resistant to higher pH values than CW-TPR.

SPME interface, built in our laboratory, improved analyte desorption when compared to the off-line process in two distinct ways. First, the whole amount of desorbed analyte is injected, which is different from the off-line process, where only a part of the solution is injected. Secondly, the interface has a heated desorption chamber, which increases the analyte mass transference from the fiber coating to the extraction solvent, improving analyte desorption. Fig. 6 shows the area increasing as a result of the temperature effect observed in the homemade interface. Area values were raised from 25 °C to 60 °C. At 70 °C, the solvent seems to evaporate into the desorption chamber, the same occurring at 80 °C with a much lower area value. Acetonitrile, employed in the mobile phase, has a boiling point of 81–82 °C. As the desorption was performed in 15 min, an evaporation occurs when 70 °C and 80 °C were used. Therefore, 60 °C was chosen as the desorption temperature on the validation procedure. It is clear in Fig. 6 that the variation of temperature caused more drastic changes in the area values for nor-FLU than for FLU. Generally, increasing the temperature in liquid chromatography reduces the retention time of the analytes. In the desorption process, the same occurs with higher temperature; so, the mass transfer will increase with higher temperature. According to the data obtained, when temperature rose from 25 °C to 60 °C, the area values increased to 86% for nor-FLU, 20% for FLU, and 16% for CLOMI. Thus, the increase of temperature seems to have a more pronounced effect in the more polar compound, which is nor-FLU. The temperature affects the equilibrium kinetic of the analytes between the fiber and the mobile phase more markedly for the more polar compound. In analogy with reversed-phase liquid chromatography, temperature is known to affect the retention times of ionizable compounds more and that depends also on the pK_a of the compound and the mobile phase surrounding it. It is possible that both differences (hydrophobicity and pK_a) affect the temperature effect on the desorption of the interesting compounds at different strengths. The chromatograms obtained when desorption was carried out in off-line mode and using the homemade interface with and without heating are shown in Fig. 7. Area values obtained using the interface without heating were almost two-fold higher than that obtained in off-line mode. When the heating system was used, the area values were almost three-fold higher than that compared to the off-line mode.

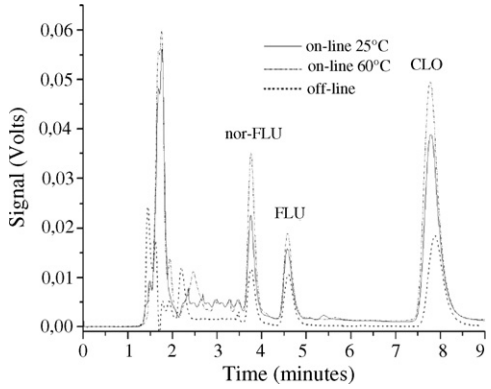

Fig. 7. Chromatograms of FLU, nor-FLU (500 ng ml^{-1}) and CLO (1000 ng ml^{-1}) in water samples extracted under the following conditions: 30 min, 50 °C, without salt, pH 9.0. Desorption was carried out in off-line mode and in on-line with (60 °C) and without heating (25 °C).

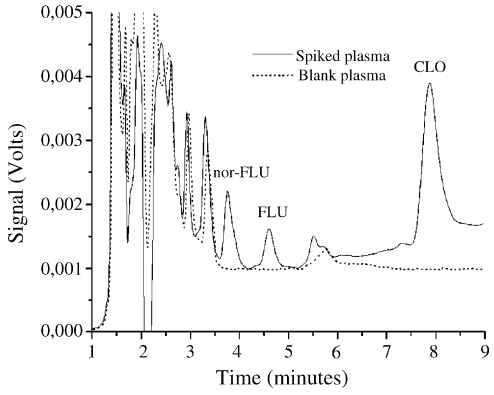

Fig. 8. Chromatograms of blank and spiked plasma (FLU and nor-FLU at 25 ng ml^{-1} and CLO at 1000 ng ml^{-1}) obtained after SPME extraction under the following conditions: 30 min, 50 °C, without salt, pH 9.0, and on-line desorption in the heated interface (60 °C).

3.3. Method validation

3.3.1. Specificity

No peak eluted in the same retention time of FLU, nor-FLU, and CLO. Fig. 8 shows chromatograms of blank and spiked plasma (FLU and nor-FLU at 25 ng ml^{-1} and CLO at 1000 ng ml^{-1}) obtained after desorption in the homemade interface.

3.3.2. Linearity and range

Range was evaluated from 25 ng ml^{-1} to 500 ng ml^{-1}. Correlation coefficients were higher than 0.991 for FLU and nor-FLU, demonstrating that there is a linear correlation between the concentration and the response obtained. The linear regression equations obtained were: $Y = -0.02126 + 0.00398X$ and $Y = 0.08179 + 0.0018X$ for FLU and nor-FLU, respectively.

3.3.3. Precision

The RSD obtained in the evaluation within day was smaller than 5.0% in all evaluated concentrations. In the evaluation of between-days RSD was smaller than 20.0% in all concentrations. These results demonstrate that the developed method has an adequate precision (Table 1).

3.3.4. Limits of quantification and detection

Limit of quantification was established at a level of 25 ng ml^{-1}. After several extractions at this concentration, the RSD obtained was smaller than 20.0%. Limits of detection for FLU and nor-FLU were established as the concentration where analyte peak was two times higher than the baseline noise. LOD was 10 ng ml^{-1} for FLU and 5 ng ml^{-1} for nor-FLU.

Table 1
Precision values obtained in the evaluation of within-day and between-days precision ($n = 3$)

Concentration (ng ml^{-1})	Within-day precision, %RSD	Between-days precision, %RSD
25	3.54	16.81
50	4.53	n.e.[a]
100	2.26	11.00
200	3.65	n.e.
300	2.75	n.e.
500	3.29	6.88

[a] n.e.: not evaluated.

Table 2
Recovery values obtained for FLU and nor-FLU in plasma in three evaluated concentrations

Concentration (ng ml^{-1})	Recovery (%)	
	FLU	Nor-FLU
25	6.81	10.54
100	3.91	4.29
500	1.94	2.67

3.3.5. Recovery

Recovery values obtained for the developed method are presented in Table 2.

When compared with some other extraction techniques, these recovery values can be considered too low. However, these values are common in SPME because it is a microscale technique based on equilibrium [24,25]. Furthermore, the extractions were performed in 30 min, when the equilibrium has not yet been achieved.

Recovery values were different for concentrations of 25, 100, and 500 ng ml^{-1} because plasma proteins progressively adsorb on the fiber coating, decreasing the extracted amount. Since the assessment had started with the lowest concentration (25 ng ml^{-1}) the recovery was lower for the highest concentration (500 ng ml^{-1}), because more plasma proteins have adsorbed on the fiber coating at this concentration. However, it does not prejudice the analysis because of the use of internal standard calibration.

Fig. 9 shows a typical chromatogram obtained after optimization of the SPME conditions and after the validation procedure. The developed method shows to be adequate to analyze FLU in plasma samples.

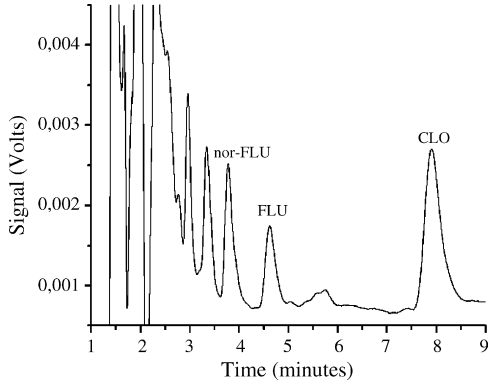

Fig. 9. Typical chromatogram of spiked plasma (FLU and nor-FLU at 100 ng ml^{-1} and CLO at 1000 ng ml^{-1}) obtained after SPME extraction under the following conditions: 30 min, 50 °C, without salt, pH 9.0, and on-line desorption with heated interface (60 °C).

4. Conclusion

Factorial design employed in this study allowed us to rationally optimize the conditions used in the extraction of FLU and nor-FLU in plasma by SPME. PDMS-DVB fiber coating, 30 min, 50 °C, without salt, pH 9.0, and stirring speed of 1100 rpm were the optimal conditions obtained. The in-house packed column showed to be adequate to analyze FLU and nor-FLU, producing good peak shapes in a shorter analysis time. The homemade interface with heating system has significantly improved the desorption process, increasing the area values (about three-fold), reducing the RSD% between different analyses, as well as the carry-over effect, which are common using off-line desorption. The developed method has shown precision, linearity, specificity, and limit of quantification adequate to assay FLU and nor-FLU in plasma.

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