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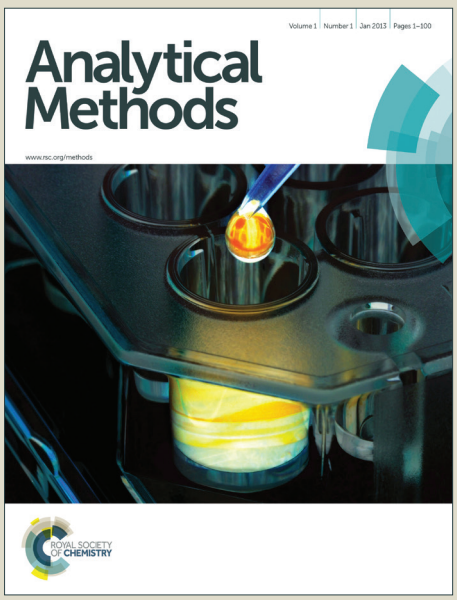

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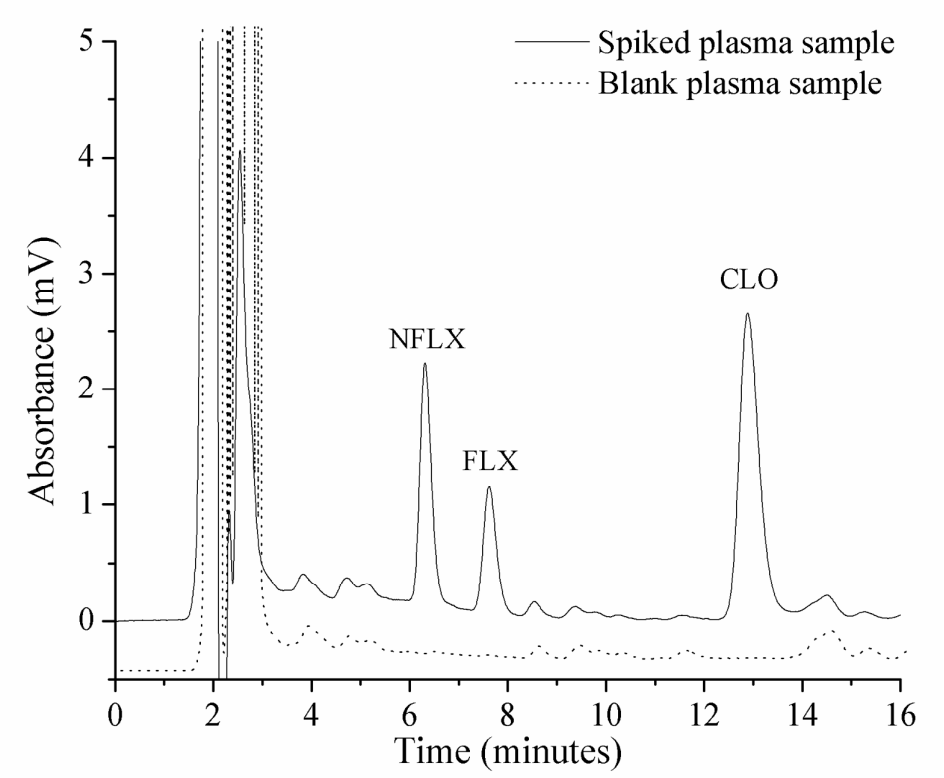
GRAPHICAL ABSTRACT



Chromatogram of blank free-drug plasma and spiked plasma sample with 250 ng mL^{-1} of FLX and NFLX and 500 ng mL^{-1} of CLO

Analysis of fluoxetine and norfluoxetine in human plasma by HPLC-UV using a high purity C18 silica-based SPE sorbent

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Abstract

This paper reports on the development and validation of a simple and sensitive method that uses solid phase extraction (SPE) and liquid chromatography with ultraviolet detection to analyze fluoxetine (FLX) and norfluoxetine (NFLX) in human plasma samples. A lab-made C18 SPE phase was synthesized by using a sol-gel process employing a low-cost silica precursor. This sorbent was fully characterized by nuclear magnetic resonance (NMR), Fourier-transformed infrared (FT-IR), and scanning electron microscopy (SEM) to check the particles' shape, size and C18 functionalization. The lab-made C18 silica was used in the sample preparation step of human plasma by SPE-HPLC-UV method. The method was validated in the 15 to 500 ng mL^{-1} range for both FLX and NFLX using a matrix matched curve. Detection limits of 4.3 and 4.2 ng mL^{-1} were obtained for FLX and NFLX, respectively. The repeatability and intermediary precision achieved varied from 7.6 to 15.0 % and accuracy ranged from -14.9 to 9.1 %. The synthesized C18 sorbent was compared to commercial C18 sorbents. The average recoveries were similar (85 – 105 %), however the lab-made C18 silica showed less interfering peaks in the chromatogram. After development and validation the method using the lab-made C18 SPE was applied to plasma samples of patients under FLX treatment (n = 6). The concentrations of FLX and NFLX found in the samples varied from 46.8 – 215.5 and 48.0 – 189.9 ng mL^{-1}, respectively.

1. Introduction

Fluoxetine (FLX) is a selective serotonin reuptake inhibitor (SSRI) antidepressant drug. [1] It is among the mostly prescribed drugs to treat major depression and related disorders, such as anxiety (panic disorder, social phobia, obsessive-compulsive disorder, post-traumatic stress disorder), eating disorders (anorexia, bulimia) and personality disorders (borderline personality disorder, attention-deficit hyperactivity disorder). [2] Norfluoxine (NFLX) is an active metabolite produced during the FLX biotransformation. [1, 2]

During depression treatment a quantity of 20 – 40 mg per day of FLX is commonly used and its therapeutic level covering the range from 50 to 300 ng mL^{-1}. To achieve these concentration levels several analytical methodologies for the analysis of FLX and NFLX in biological matrices, which are complex and require sample pretreatment, have been reported in the literature. [1, 3-7] Among the sample preparation techniques, solid phase extraction (SPE) is an attractive method due to its high selectivity and enrichment factor achieved for the analytes. [8-10] An important parameter in this technique is the extraction sorbent selection, which is based on the drug and sample polarity. Various sorbents, such as silica-based sorbents, polymeric sorbents and carbon-based sorbents are available for use in SPE. [11] Among them, porous silica is an attractive material due to some characteristics, such as high superficial area, controlled pore size, and good mechanical strength. Differently from polymeric sorbents, silica neither strains nor swells. The silica particles have a heterogeneous surface (silanol groups) able to be chemically modified by other functional groups. [9] This procedure improves silica applicability by changing the extraction selectivity.

Porous silica is also used in other fields, such as separation techniques, chemical catalysis, biotechnology and drug delivery. [12, 13] Silicate solution and silane reagents are some of the silica precursors used to synthesize the polymer. Most synthetic methods are based on the hydrolysis and condensation reactions (sol-gel process) of silica precursors prior to forming silica polymer

networks. [14] The sol-gel process may generate materials with controlled morphology, surface properties and pore structures by carefully modifying the synthesis conditions. [15, 16]

Alkoxysilane is the silica precursor most used by the sol gel process. [13, 17] Its advantage is the low presence of metals incorporated into the silica structure. [18] However, due to its high cost, the applicability of other silica precursors has been investigated. [13, 19] Sodium silicate and waterglass have become economical silica precursors for SPE sorbents. Although they have a larger quantity of metals (metals > 0.3 %), a pre-treatment with cationic resin and acid washing may diminish impurities and improve their quality. [20, 21] Surface modifications are necessary to use the silica material as an nonpolar sorbent in SPE. A mono-functionalization of silica surface with C18 group generates non-polar interactions between the silica sorbent and analytes. However, the presence of residual silanols on the silica surface promotes secondary interactions. [22] Both interaction mechanisms (nonpolar and ionic) acting on the extraction process are an interesting strategy to enhance the recovery of basic drugs using silica-based sorbents.

The present study investigates the efficiency of low-cost C18 silica sorbent for the extraction and clean up of FLX and NFLX in human plasma. The lab-made SPE sorbent was synthesized using waterglass as a silica precursor. The material was packed into a polypropylene tube for the sample preparation procedure. Afterwards, the extraction and separation parameters were evaluated, the SPE-HPLC-UV method was validated and used in the analysis of patients treated with FLX.

2. Experimental

2.1. Chemicals and reagents

A waterglass solution (28 % SiO_2) was purchased from Sigma-Aldrich (Steinheim, Germany). Hydrochloric acid and ammonium hydroxide were acquired from Fluka (Buchs, Switzerland). Formic acid and amberlite IR-120 cationic exchange resin were provided by Synth

(Diadema, Brazil). HPLC-grade acetonitrile, methanol and ethanol from Tedia (Fairfield, USA) were also used. The ultrapure water used to prepare silica, samples and solutions was purified in an Elga Purelab Ultra system (Vivendi Water, UK). The granulometric separation was performed by Granutest sieves from Telastem (Sao Paulo, Brazil). Toluene and imidazole (Mallinckrodt, Paris, USA) and chlorodimethyloctadecilsilane (ODS) (Aldrich, Oakville, USA) were applied during the silica functionalization. Triethylamine (TEA) from TEDIA (Fairfield, USA) and ammonium acetate and acetic acid from J. T. Baker (Phillipsburg, USA) were used as LC mobile phase additives.

Analytical standards of FLX, NFLX and clomipramine (CLO), with purity of 99.9 %, 97.0 % and 99.5 % respectively, were acquired from Sigma-Aldrich (Steinheim, Germany).

2.2. Preparation of C18 silica sorbent

Silica particles were prepared with waterglass as the starting material. Approximately 150 mL of waterglass solution (SiO_2 contents 8 wt%) were passed through an ion exchange column (2 cm diameter and 50 cm length) filled with cationic exchange resin in H^+ form. The eluent from the exchange column showed a pH in the 2.6 range. For the gelation process, a 1.0 M NH_4OH solution was added to the silica sol under magnetic stirring to fit the pH around 4.5. The sol solution was then transferred into polypropylene vessels for the hydrogel formation. After gelation, the hydrogel was aged for three days for the strengthening of its structure. The aged hydrogel was washed with ultrapure water and ethanol and dried in two successive steps: at 40ºC for 24 h and at 100°C for 24 h. Finally, the synthesized silica particles were classified using sieves according to their diameter. The particles of 38 - 72 μm diameters were collected and used in subsequent experiments.

For the silica surface modification, 1.7 g of imidazole was added to a suspension containing 10 g of dried silica in 50 mL of toluene under nitrogen atmosphere, magnetic stir and constant reflux. After 5 minutes, a solution of 5.4 g of ODS in 10 mL of toluene was added into the

suspension. The system was kept under reflux for 5 h. The silica was subsequently washed with toluene, methanol, methanol:water (50:50) and methanol, respectively, and dried at 120°C for 12 h.

2.3. Physico-chemical characterization

The silica morphology and its size distribution were evaluated by scanning electron microscopy (SEM) in a Zeiss-Leica model 440 apparatus (Oberkochen, Germany) operated at 20 kV. The samples were covered with a thin layer of gold. The surface area was measured by nitrogen adsorption-desorption isotherms at 77 K using a Quantachrome Nova 1000e gas adsorption analyzer (Boynton Beach, USA). The specific areas were calculated from these isotherms by using the Brunauer–Emmett–Teller (BET) method. The carbon and hydrogen contents were determined by elemental analysis on EA 1110 CHNS-O from CE Instruments (Milan, Italy). Fourier-transform infrared (FT-IR) spectra were recorded from 4000 cm^{-1} to 400 cm^{-1} in a Bomem MB-102 IR-spectrophotometer (Quebec, Canada) by a KBr pressed-disc technique. Solid-state ^{29}Si and ^{13}C NMR spectra were recorded on a Bruker Avance-III 400 MHz spectrometer (Rheinstetten, Germany). A magic angle spinning (MAS) was carried out using 4 mm double bearing zirconia rotors with spinning rate regulated at 5 kHz and magnetic field of 9.4 T. An ^{29}Si NMR analysis was conducted at 79.46 MHz under MAS conditions and cross polarization (CP) excitation with $\pi/2$ pulse widths of 5 μs, contact time of 5 ms and re-cycle delay of 5 s. The number of scans was 3600 and the chemical shifts were determined with 4,4-dimethyl-4-silapentane-1-sulfonic acid ($\delta = 0$ ppm) external standard. ^{13}C NMR spectra were acquired under CP/MAS/TOSS (total suppression of sidebands) at 100.56 MHz with $\pi/2$ pulse of 5 μs, contact time of 2 ms and pulse delay of 5 s. The number of scans was 2048 and the chemical shifts were determined with adamantane ($\delta = 38.48$ ppm for the strongest signal) as an external standard.

2.4. Instrumentation

The LC system used was a Shimadzu HPLC Prominence 20AD (Kyoto, Japan) with two LC-20AD pumps, an SIL-20A autosampler, a CTO-20A oven, an SPD-20A UV-vis detector, and a CBM-20A system controller. LC-Solution software controlled all the events in the chromatographic system.

FLX, NFLX and CLO were separated into an analytical column (2.1 mm x 150 mm x 5 µm, C18) kindly donated by NST (Sao Carlos, Brazil). An analysis flow rate of 0.2 mL min^{-1} was used under isocratic elution. The separation mobile phase was a mixture of 50:50 of acetonitrile and ammonium acetate (10 mmol L^{-1} with 3.6 mmol L^{-1} of TEA buffered at a pH of 5.4 with acetic acid). The column temperature was kept at 35°C and the UV detector was set at 226 nm.

2.5. Standard solution preparation

Stock standard solutions were prepared in methanol and maintained at -20°C. FLX and CLO concentrations were 1000 µg mL^{-1} and the NFLX concentration was 200 µg mL^{-1}. Working solutions of FLX and NFLX were achieved by diluting the intermediate solutions of FLX and NFLX (10.0 µg mL^{-1}) at concentration levels of 0.3, 1.0 and 5.0 µg mL^{-1}. The working solution for CLO was set at 10.0 µg mL^{-1}.

2.6. Sample preparation

The plasma samples were spiked by transferring an adequate volume of the working solutions of FLX, NFLX and CLO to a 1.5 mL centrifuge tube, dried under nitrogen stream and suspended in 500 µL of blank plasma. The samples were homogenized in ultrasound for 5 minutes and their final volume was increased to 1 mL by the addition of 500 µL of ultrapure water.

SPE cartridges containing 100 mg of lab-made C18 phase were used for the extraction and clean-up of FLX and NFLX. The SPE phase was conditioned with 3 mL of methanol and 3 mL of ultrapure water. Next, 1 mL of diluted plasma sample passed through the SPE cartridge followed by a clean-up step that used 3 mL of water and 3 mL of water:methanol (80:20) mixture. The SPE cartridge was dried for 3 minutes and the analytes were eluted with 3 mL of methanol containing 0.2 % of formic acid. The eluate was evaporated at 40°C under nitrogen stream and the residue was redissolved in 500 µL of mobile phase, homogenized for 20 s in a vortex and transferred to an HPLC vial. The vial was inserted in the LC autosampler and 10 µL were injected in the instrument.

2.7. Method validation

The SPE-HPLC-UV method used for the analysis of FLX and NFLX in human plasma was validated for selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), repeatability, intermediary precision, accuracy, recovery, matrix effect, and robustness, using a pool of plasma.

The method selectivity was tested through the injection of blank plasma in triplicates and the linearity was evaluated by the analysis of spiked blank plasma samples in five concentration levels in five replicates (15, 50, 100, 250 and 500 ng mL^{-1}) using a matrix matched calibration curve. The internal standard CLO was used at 500 ng mL^{-1}concentration level. The linearity was estimated based on regression curves ($y = ax + b$) and coefficient of determination (r^2). The analysis of variance (Anova) and residual plot were used to check the significance of the lack of fit to the calibration curves.

LOD was established as the lowest concentration level at which the analytes signal was three times higher than the baseline noise. LOQ was the lowest concentration level at which it was possible to quantify FLX and NFLX precisely and accurately (< 20 %) with a signal-noise ratio of 10.

The intra-assay precision (repeatability, n = 3) was evaluated at three different concentration levels of the calibration curve (low = 15 ng mL^{-1}, medium = 100 ng mL^{-1} and high = 500 ng mL^{-1}). The inter-assay precision (intermediary precision, n = 6) was evaluated at the same concentration levels, however on two different days, and the results were expressed as RSD %. The accuracy was assessed at three concentration levels on two different days (n = 6). The trueness was expressed as a percentage of bias (% bias).

The recovery was assessed by spiking blank plasma samples at three different concentration levels. The areas obtained were compared to the eluate of the blank plasma spiked at the same levels and the results were expressed in RSD %. The matrix effect was measured at three concentration levels (3 replicates) comparing the relative area obtained by an SPE extraction against the injection of the standard solution in the HPLC system.

The method robustness was checked by varying four LC parameters, namely mobile phase flow rate, column temperature, wavelength detector and percentage of organic solvent in the mobile phase, at two levels.

3. Results and Discussion

3.1. C18 silica sorbent synthesis and characterization

In the first synthesis step silica precursor purification was necessary due to the low purity of waterglass, which has a large number of sodium and also other metals. To decrease the metal contamination, a simple purification method with an open column packed with cationic exchange resin was used. The aim was to reduce both the number of metals in the silica structure and the amount of acidic silanols on the silica surface. The waterglass solution has high pH (around 13.0) and as this solution passes through the cationic resin, the eluent pH decreases to 2.6 due to the exchange of sodium atom with hydrogen atom. In this step there occurs the hydrolysis of silicate solution into silicic acid (SiO_4H_4) forming a sol solution. Subsequently, after adjusting the pH = 4.5

with 1.0 M ammonium hydroxide, the condensation reaction of silicic acids starts to form a soft gel. The mechanical strength of the material may be enhanced by an aging time step. The number of siloxane bonds (silica network) is increased by a condensation reaction between primary silica particles. The rigid gel formed is called hydrogel.

The washing process also alters the final characteristics of the silica. The hydrogel washed with an acidic solution (pH = 3.5) reduced the polymer surface area in comparison with the washing process using water at pH = 6.6. The immersion of the hydrogel in different solvents also promotes changes in the synthesized material. [16] Therefore, a washing step with ethanol was carried out to enhance the surface area, volume pore and average pore size of the silica. [23]

After the drying process, irregular silica particles, whose diameter ranged from 0.5 to 110 μm were obtained. In order to use these particles as sorbents for SPE, it was necessary to separate them according to their diameters. Sieves of different mesh sizes were applied to select silica particles between 38 - 72 μm (Electronic Supplementary Information Fig. S1). The BET analysis of silica shows a surface area of 607 m^2 g^{-1} and an average pore diameter of 4.6 nm.

The silica particles were subjected to a functionalization reaction for the formation of a chemical bond between silica silanols and the C18 monofunctional group. To improve the functionalization yield, just before the reaction the bare silica particles were dried at 120°C for 24 h so that the water molecules could be removed from the particles surface.

The elemental analysis of the bare silica indicates the absence of carbon atoms in the silica structure. After the functionalization reaction using ODS, the carbon content in the silica sorbent increased to 22 %. The amount of carbon in the silica sorbent is an important variable that directly influences the extraction efficiency of compounds.

The lab-made C18 SPE was characterized by infrared spectroscopy (Electronic Supplementary Information Fig S2). An intense characteristic band appeared in the 1300 to 1000cm^{-1} region and was attributed to an asymmetric stretching of a silicon-oxygen bond from the siloxane group. [24] Both bands around 790 and 470 cm^{-1} resulted from vibrations of the siloxane. At

3500 cm^{-1}, the band indicated an oxygen-hydrogen bond stretching due to the presence of silanol groups and physically adsorbed water, which showed another band at 1644 cm^{-1}. [25, 26] The formation of a chemical bond between silica and C18 group could be confirmed by the characteristic bands in the 2940 cm^{-1} region attributed to the carbon-hydrogen stretching and by the comparison of both spectra of FT-IR in which the silanol band decreased.

NMR spectroscopy was also used to check the structure of the silica and groups present in the synthesized material. The structure of bare silica has tetrahedral units linked by siloxane groups (Q^4 species). Its surface may have geminal silanols (Q^2 species) with two OH groups bonded to a silicon atom. Isolated and vicinal silanols (Q^3 species) are also on the silica surface and correspond to one OH group bonded to a silicon atom. [27]

The ^{29}Si CP/MASS NMR spectra of silica (Electronic Supplementary Information Fig S3a) after surface modification showed the signal at -112 ppm, assigned to Q^4 species characteristic of silica-based materials. The signals at -103 ppm and -93 ppm were related to Q^3 and Q^2 species, respectively. These two species indicated that even after the functionalization process, there were silanol groups on the silica surface. No signal around -92 ppm has confirmed the absence of Q^2 species. The signal at 12 ppm appeared just after the silica surface modification and was attributed to the monofunctional silane (M). [28]

The morphology of the alkyl chain and the endcapping process were revealed by ^{13}C CP/MAS/TOSS NMR (Electronic Supplementary Information Fig S3b). Monofunctional C18 silica has more signals in comparison to di and trifunctional C18 because the carbon group can move more freely. [29] The signal at zero ppm was assigned to methyl carbons $C_{1'}$ from the C18 group linked to silicon atoms. Due to relatively high electron densities around these nuclei, a shield against the magnetic field was formed and its chemical shift appears in the high-field (low frequency) in the spectrum. [30] The signals located at 18.50 ppm and 23.99 ppm were assigned to carbon C_1 and carbons C_2-C_{17}. Carbon atoms C_3 and C_{16} appeared in the lowest field in the spectrum, with signals at 34.68 ppm and 32.95 ppm. The strongest signal at 30.92 ppm is related to

the central carbons (C_{4-15}) along the octadecyl chain. This signal indicates that the carbon chain was in a disordered conformation, i. e. the chain exhibits mobility with a fast exchange between *gauche* and *trans* conformations. [31]

3.2. Sample preparation of FLX and NFLX

A C18 silica-based sorbent is used for the extraction of non-polar compounds in a polar matrix, as plasma. Besides the interaction with hydrophobic C18, the presence of residual silanols on the silica surface promotes secondary interactions with ionic characteristics. Therefore, compounds containing amino atoms in the structure may interact with silanols through hydrogen bonds, enhancing the interaction between analytes and sorbent.

The C18 SPE lab-made phase was used in the sample preparation step for the analysis of FLX and NFLX. The method development was performed with 100 mg of lab-made phase in the SPE cartridge. After the cartridge conditioning by 3 mL of methanol and 3 mL water, 1 mL of diluted plasma sample spiked with FLX and NFLX was applied for extraction. The plasma dilution with ultrapure water (50:50) minimized the influence of interferents on the extraction process, enhanced the FLX and NFLX extraction yield and also decreased the sample viscosity.

As a large number of interferents was present in the matrix, it was necessary to add a clean-up step to the SPE method to eliminate non-target compounds and maximize the analytes signal. First, a washing step using only water to remove interferents was evaluated; however this procedure was not efficient. A further step with a solution of water and methanol mixture was added and the result was satisfactory.

After the clean-up step, the analytes were eluted from the C18 cartridge by methanol or methanol containing 0.2 % formic acid as solvent. Methanol-acid as an elution solvent provided better recovery results than methanol. Formic acid as additive in methanol decreased the secondary interaction between the analytes and the sorbent, since silanols were not charged. The cartridge

eluent was evaporated to dryness, reconstituted in the mobile phase and analyzed by HPLC-UV. The final extract was clear; no additional filtration was necessary and the sample preparation time was slightly reduced. The chromatogram obtained for the developed sample preparation is displayed in Fig. 1. CLO (tricycle antidepressant) was used as internal standard during the analysis because its chemical proprieties (structure, molecular weight, pKa, and logP) are similar to FLX and NFLX.

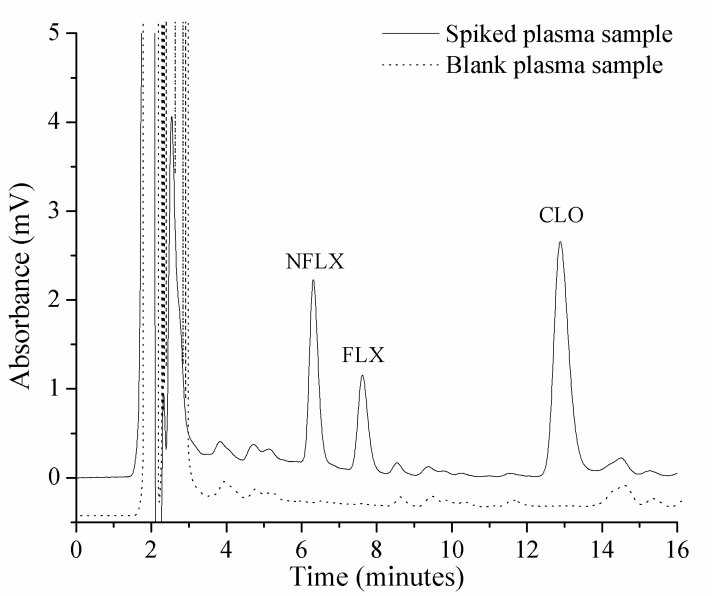

Fig. 1– Chromatogram of blank free-drug plasma and spiked plasma sample with 100 ng mL^{-1} of FLX and NFLX and 500 ng mL^{-1} of CLO.

The LC mobile phase used was already described by Santos-Neto *et al.*. [1] It consists of an isocratic elution (50:50 (v/v)) of acetonitrile and aqueous ammonium acetate (10 mmol L^{-1} with 3.6 mmol L^{-1} of TEA buffered at a pH of 5.4 with acetic acid) using a C18 analytical column. The high pKa values for FLX and NFLX (8.7 and 9.3) increase the optimum mobile phase pH above the operating range of silica-based columns (pH 2 - 8) in the reverse phase liquid chromatography. Consequently, an acidic pH was employed for the analysis of antidepressants, which generated ionic species and peak tailing in the chromatogram. To minimize this problem, TEA was added as a mobile phase additive to improve the peaks shape.

3.3. Comparison between C18 lab-made sorbent and commercial C18 sorbents

The lab-made C18 SPE phase performance was compared to the performance of other two commercially available SPE phases, namely Alltech C18 and Strata C18. The former is described an endcapped sorbent of average particle size of 50 µm and 6 % of carbon. The latter has average particle size of 55 µm, 18 % of carbon and no endcapping.

The extraction efficiency was evaluated in spiked plasma at three levels (15, 100 and 500 ng mL^{-1}) using 100 mg of each SPE sorbent. Alltech sorbent showed a high recovery range (75 – 125 %). The recovery of the Strata sorbent whose carbon percentage is similar to that of the lab-made sorbent ranged between 77 and 90 %. The recovery results for the lab-made sorbent ranged from 86 to 106 %. Under the same extraction conditions, the lab-made extraction chromatogram showed less interfering peaks than commercial phases (Fig. 2), in special around the retention time of CLO, the internal standard. Therefore the silica developed using the low-cost precursor and later purified presents advantages to be used as an SPE sorbent in the proposed method.

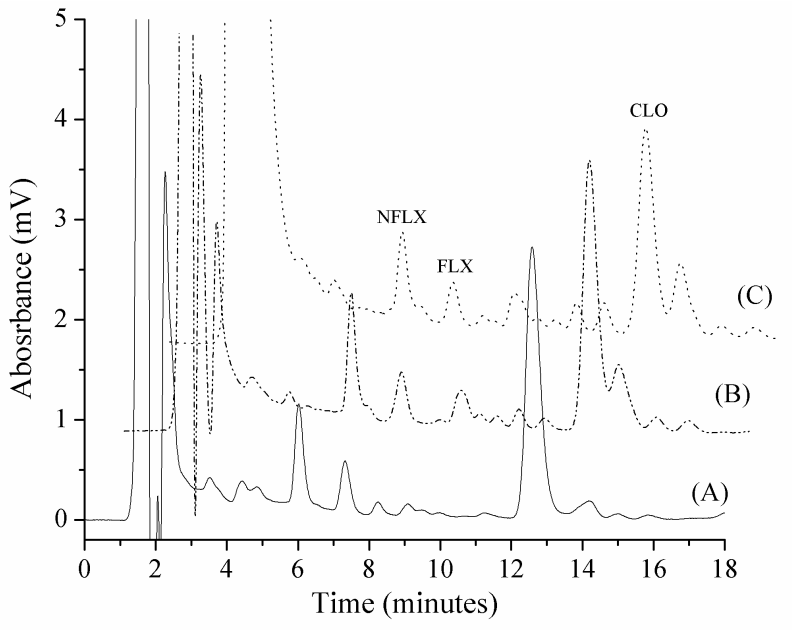

Fig. 2 – Comparative chromatographic profile of spiked plasma sample with 15 ng mL^{-1} of FLX and NFLX and 500 ng mL^{-1} of CLO after SPE procedure using (A) lab-made C18 silica-based sorbent, (B) C18 Strata sorbent and (C) C18 Alltech sorbent.

3.4. FLX and NFLX validation

A summary of the validation parameters is shown in Table 1. The method using the lab-made C18 SPE phase was validated using a human plasma pool with no FLX, NFLX and CLO. The absence of interfering peaks in the drugs retention time windows in the chromatogram demonstrated the method selectivity (Fig. 1).

Table 1 – Overall summary of the validation parameters for FLX and NFLX method in human plasma.

Drug	Fortification level (ng mL^{-1})	Repeatability (RSD %)	Intermediary Precision (RSD %)	Accuracy (%)	Recovery (%)	Matrix effect (%)
FLX	15.0	11.5	15.0	-14.9	93	-3.9
	100.0	9.0	10.8	-10.8	106	4.5
	500.0	8.2	7.6	7.4	102	-1.7
NFLX	15.0	7.8	10.5	-12.8	87	5.2
	100.0	11.3	12.1	9.1	91	-7.8
	500.0	10.9	10.2	3.9	93	4.3

The linearity of the SPE-HPLC-UV method was evaluated by the matrix matched curve, whose concentration level ranged from 15 to 500 ng mL^{-1} for both FLX and NFLX. The relative area was plotted against the fortification level to generate linear regression curves with correlation coefficients of 0.9947 and 0.9943 for FLX and NFLX, respectively. The linearity tendency was checked by a residue plot (data not shown), showing a linear behavior. The LODs were 4.3 ng mL^{-1} for FLX and 4.2 ng mL^{-1} for NFLX. The LOQs for both drugs were 15.0 ng mL^{-1}, in which the relative standard deviation and bias were lower than 20 %.

The validation results show acceptable values for repeatability and inter-assay precision with RSD lower than 11.3 % and 15.0 %, respectively. The assessment of accuracy ranged from -14.9 to 9.1 %. The recovery assay showed good agreement values, which ranged from 87 to 106 %. The comparison of the absolute area between analytes fortified in solvent and plasma revealed no significant matrix effect. The method robustness was verified varying each LC system parameter at two levels. Electronic Supplementary Information Table S1 shows the parameters chosen for the robustness test. The results are provided in terms of trueness and none of them lacked accuracy, even with small variations in the LC system.

Table 2 show the comparison of the developed method with others HPLC-UV methods published in the literature using commercially available SPE sorbents to analyze FLX and NFLX in plasma samples. Similar considerations can be derived from a comparison of the methods, however it has to be noted that our method showed better mean recovery result than two commercial apolar silica sorbents (C18 (66%) and C8 (84 %)).

Table 2 – Comparison of SPE/HPLC-UV methods for analyses of FLX and NFLX in human plasma.

Sample	SPE	Separation/ Identification	Linear Range	Mean Rec. (%)	Reference
Serum (1 mL)	Mixed mode disc	HPLC-UV	10 – 500 ng mL^{-1}	101	Frahnert *et al.* [32]
Serum (500 µL)	Micro disc mixed mode	HPLC-UV	10 – 4000 mmol L^{-1}	96	Li *et al.* [33]
Plasma (100 µL)	HLB (30 mg)	HPLC-PDA	150 – 3000 ng mL^{-1}	94	Sabbioni *et al.*[34]
Plasma	C18	HPLC-UV	20 – 600 ng mL^{-1}	93	Misztal *et al.* [35]
Plasma (500 µL)	C8 (100mg)	HPLC-UV	0.12 – 5.0 µmol L^{-1}	84	Kristoffersen *et al.* [36]
Plasma (1 mL)	C18 (Aspec system)	HPLC-UV	20 – 1000 ng mL^{-1}	66	Nichols *et al.* [37]
Plasma (500 µL)	Lab-made C18 (100 mg)	HPLC-UV	15 – 500 ng mL^{-1}	95	our paper

3.5 Application to plasma samples

In order to evaluate the proposed method for clinical use, the described protocol was applied to the analysis of plasma samples of six patients treated with FLX (Table 3). The concentrations found ranged from 46.8 – 215.5 ng mL^{-1} for FLX and 48.0 – 189.9 ng mL^{-1} for NFLX. The results are in accordance with those of other methods used for monitoring therapeutic levels of FLX in human plasma. [1, 7, 38, 39]

Table 3 – Patient plasma concentration measured with the developed method.

Sample	FLX (ng mL^{-1})	NFLX (ng mL^{-1})
1	48.2	69.5
2	109.4	81.6
3	46.8	48.0
4	61.3	64.2
5	215.5	167.9
6	129.7	181.9

4. Conclusions

A silica-based sorbent has been successfully synthesized by a sol-gel process using a low-cost precursor. The purification by cationic resin reduced the amount of contaminants present in the waterglass. The physical-chemical characterization has proved that the properties of the C18 lab-made silica sorbent are comparable to those of the commercial porous silica widely used for sample preparation. The low-cost purified sorbent eliminated plasma interferents more effectively than the commercial C18 materials.

The SPE-HPLC-UV method was validated and showed suitable selectivity, recovery, accuracy, precision, robustness, and linearity to determine FLX and NFLX in human plasma using

500 µL of sample. The method was successfully applied to the analysis of plasma of patients under FLX pharmacotherapy.

This study suggests that considerable savings can be attained by using waterglass to synthesize good purity silica sorbents.

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