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The development of a new disposable pipette extraction phase based on polyaniline composites for the determination of levels of antidepressants in plasma samples

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ABSTRACT

In the present work, a new stationary phase for disposable pipette extraction (DPX) based on composites of polyaniline and a styrene–divinylbenzene (SD) copolymer was applied to the analysis of fluoxetine and norfluoxetine in plasma samples using liquid chromatography and fluorescence detector (LC–FD).

The DPX variables, such as number of draw/eject cycles, sample pH, type and volume of the desorption solvent, were optimized to established the sorption equilibrium and shorten the analysis time. Among the DPX evaluated variables, the higher extraction efficiency were obtained with 200 μL of plasma mixed with 200 μL of borate solution (pH 9), followed by liquid desorption of the drug with 200 μL acetonitrile in a single cycle. The DPX/LC–FD method demonstrated a linear response over the dynamic range from 10 to 1000 ng mL^{-1} for fluoxetine and from 80 ng mL^{-1} (LOQ) to 1000 ng mL^{-1} for norfluoxetine with r^2 = 0.997 and 0.998, respectively. The limit of quantification (LOQ) was 10 ng mL^{-1} for fluoxetine and 80 ng mL^{-1} for norfluoxetine. Based on the analytical validation results, the proposed method can be a useful tool for determining the fluoxetine and norfluoxetine levels in plasma samples from patients receiving therapeutic dosages.

1. Introduction

Therapeutic drug monitoring (TDM) of antidepressants may safeguard against drug–drug interactions, can be used to control compliance, and may increase the therapy efficacy in patients suffering from major depressive illness. Several chromatographic methods have been developed for the determination of antidepressants in plasma and serum, but they require complex isolation procedures to improve their sensitivity and specificity [1–4].

Among the sample preparation techniques, liquid–liquid extraction (LLE) [5,6] and solid-phase extraction (SPE) [7,8] are the most frequently used for drug extraction from biological fluids. However, these procedures are time-consuming and tedious and use a large amount of organic solvents.

Disposable pipette extraction (DPX) is a solid-phase extraction (SPE)-based device in which a small amount of SPE sorbent is placed inside a pipette tip fitted with a screen at the narrow bottom end and a barrier near the top of the tip [9,10]. DPX has become an essential tool for the purification and concentration of proteins and peptides in genomics, proteomics, and metabolomics [11–13]. This technique has also been successfully employed in environmental, toxicological, and drug analyses [9–11,14]. Although DPX is a technique derived from SPE, the extraction efficiency is based on the sorption equilibration time between the sample solutions and the dispersive sorbent; consequently, this process is not dependent of the sample flow-rate. Furthermore, the miniaturized format for DPX results in smaller solvent elution volumes than the conventional SPE technique [9]. The main advantages of DPX could be the adaptability to high-throughput parallel sample processing while still maintaining flexibility of sorbents and procedures [14].

The first commercially available micropipette tip was based on chromatographic media, C18 microparticulates, embedded in the scaffold of a polymer (ZipTip, Millipore, Bedford, MA, USA). Since then, different phases with different interaction modes, such as hydrophobic, ion exchange, and affinity, have been introduced [15–17]. The advantages of the developed phases include easy preparation and control of permeability and surface charge. Using

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monolith phases, frits are not necessary and often result in greater pH stability [18].

The conducting polymer coatings, such as polypyrrole, polyaniline, and polythiophene, have been used as extraction and separation phases for miniaturized techniques [1–3,19], mainly because their multifunctional properties result in assorted interactions between the analyte and phase. Polyaniline (PANI) is a conducting polymer that has attracted much interest in separation science due to its permeability (porous structure) and multifunctional properties, which result in intermolecular interactions, such as acid–base, π–π, dipole–dipole, hydrophobic, and hydrogen bonding and ion exchange between the polymer and analytes [1]. PANI is a promising alternative sorbent to ionizing compounds due to its easy synthesis from an inexpensive monomer, good conductivity, and remarkable stability under ambient conditions [20].

A conducting polymer, such as PANI, could be polymerized in the presence of supports to produce materials with defined shape. The polymerization of PANI in the presence of polystyrene (PS) latex can be carried out to produce nearly mono-dispersed PANI-PS composites with core–shell morphology, where the conducting polymer forms the shell. Moura and co-workers successfully applied PANI and a sulfonated styrene–divinylbenzene (SD) copolymer as an acid catalyst in the esterification of stearic acid with methanol [20]. Their easy synthesis and ability to control shape, size and porosity made composites of PANI and macroporous sulfonated styrene–divinylbenzene (SD) copolymer very attractive to employ as the extraction phase of DPX.

In this study, the potential of the SD copolymer/PANI composites as sorbents for the DPX technique to determine antidepressants (fluoxetine and norfluoxetine) in plasma samples by LC–FD was evaluated.

2. Experimental

2.1. Reagents, standards and samples

1,4-Dioxane UV/HPLC grade, benzoyl peroxide 65%, acetone 99.5%, aniline PA, sulfuric acid 95–98%, nitric acid 65% and hydrochloric acid 37% were obtained from VETEC (Rio de Janeiro, Brazil). The 2,6-di-tert-butyl-4-methylphenoltoluene 99.5% and heptane 98% were obtained from LABIMPEX (São Paulo, Brazil). Methyl alcohol 99.8%, ethanol 99.5%, gelatin powder and sodium hydroxide 97% were obtained from SYNTH (São Paulo, Brazil). The hydroxyethylcellulose was acquired from Polithechno (São Paulo, Brazil), sodium chloride 99%, sodium dodecyl sulfate 99% were obtained from ISOFAR (Rio de Janeiro, Brazil), phenolphthalein 1% solution was acquired from Proquimios (Rio de Janeiro, Brazil), stearic acid 95% was acquired from INDUSLAB (Paraná, Brazil). Styrene was obtained from Lyondellbasell (Dallas, TX, USA) and divinylbenzene was obtained from PARCHEN (New York, USA) both were purified by washing with NaOH solution followed by reduced pressure distillation.

The fluoxetine and norfluoxetine analytical standards were donated by Lilly (São Paulo, Brazil). Citalopram, which was used as an internal standard (IS), was donated by Roche (São Paulo, Brazil). The working standard drug solutions were prepared by diluting the stock solutions of these drugs (1 mg mL^{-1} in methanol) to a proper volume of methanol, based on their therapeutic intervals. These solutions were stable for 45 days at −20 °C. Water purified in a Milli-Q system (Millipore, São Paulo, Brazil) was used to prepare the mobile phase. Drug-free plasma samples from patients who were not exposed to any drug for at least 72 h (blank plasma) were kindly supplied by the Hospital das Clínicas de Ribeirão Preto, University of São Paulo, Brazil. The studies were performed in accordance with the World Medical Association's "Ethical principles for medical research involving human subjects". These plasma samples were spiked with IS and target drugs and used to optimized the DPX process and validate the analytical method.

2.2. Instrument and chromatographic conditions

The LC–FD analyses were performed on a Shimadzu LC-20 AT (Kyoto, Japan) equipped with a fluorescence detector (RF-10 AXL, λ_{ex} = 230 nm and λ_{em} = 290 nm) and a CBM-20 A system controller. The separation was performed in a Lichrosphere 60® RP: Select B (250 mm × 4 mm, 5 μm particle size) (MERK, Darmstadt, Germany) column at room temperature (25 °C); the mobile phase consisted of a phosphate buffer solution (0.05 mol L^{-1}, pH 3.8) and acetonitrile (55:45, v/v) in the isocratic mode, and the flow rate was 1.0 mL min^{-1}. The mobile phase was filtered and degassed prior to use.

2.3. Synthesis of the styrene–divinylbenzene (SD) copolymer

The styrene–divinylbenzene copolymer was synthesized based on the work previously published by Moura and co-workers [20]. The copolymer synthesis was carried out through aqueous suspension polymerization. The aqueous phase (AP) was composed of hydroxyethylcellulose at 0.26% (w/v), sodium chloride at 0.59% (w/v) and gelatin at 0.12% (w/v). The organic phase (OP) was prepared by dissolving 1% of the initiator, benzoyl peroxide, into a mixture containing styrene (16%, w/w) and divinylbenzene (84%, w/w) at room temperature; this copolymer was called SD84. Heptane and toluene were used as porogenic agents with a volume ratio of 85/15 and 150% dilution degree relative to monomer's volume. The organic phase was added to the aqueous phase, the system was stirred for approximately 15 min, followed by heating the solution to 70 °C while stirring at 250 rpm for 48 h. Finally, the copolymer beads were filtered and washed with water and then with ethanol. The copolymers were dried at 100 °C for 24 h, and the particles obtained were used to prepare the composites. A copolymer with styrene (71%, w/w) and divinylbenzene (29%, w/w) were prepared to compare the porosity with the extraction efficiency, this copolymer was called SD29. Except for the different proportions of styrene and divinylbenzene, the preparation procedure was the same for both copolymers.

2.4. Synthesis of the SD copolymer/PANI composites

Aniline was chemically oxidized into the copolymers. For this, 4 g of resins (SD84 and SD29) was placed into 40 mL of ethanol/aniline solution (80/20, v/v). Each system was mechanically stirred in a shaker for 3 h to swell the copolymer resin with the aniline. The reaction solution was prepared by mixing 2.3×10^{-3} mol of benzoyl peroxide in 20 mL of dioxane, 1.4×10^{-3} mol of sodium lauryl sulfate in 6 mL of water, and 0.06 mol of HCl. The swollen copolymer was filtered and added to the reaction solution.

The aniline polymerization was carried out under mechanical stirring in a temperature regulated bath at 25 °C for 24 h. Then, the composites were vacuum filtered and washed with methanol and ketone until the filtered solution became colorless. The composites were dried at 60 °C for 24 h. The procedure was repeated four times to prepare the composites from four deposition cycles.

2.5. Nitrogen adsorption measurements

The specific surface area and pore size distribution measurements were performed using a Micromeritics ASAP 2010 (Micromeritics Instrument Corporate, Norcross, GA, USA) nitrogen

sorption porosimeter. Analysis of the nitrogen sorption was carried out at 77 K. To this, 0.2 mg of each composite was added in a glass vial tube and let in the system during approximated 48 h before variables measure. The specific surface area was determined using the BET method (Brunauer–Emmett–Teller), and the pore volume and size distribution was determined using the BJH method (Barret–Joyner–Halenda) based on nitrogen desorption isotherm.

2.6. Morphology analysis

The morphology of the SD copolymers and synthesized composites were examined via SEM (scanning electron microscopy) using a JSM-6610 (JEOL Ltd., Tokyo, Japan) instrument using 10 kV as accelerating potential).

2.7. Optimization of the DPX process

Several parameters (number of draw/eject cycles, sample pH, and type and volume of the desorption solvent) were optimized to established the sorption equilibrium and shorten the analysis time. The following procedure was used to optimize the DPX method. In a glass vial (5 mL), 50 μL of internal standard (2.0 μg mL^{-1}, Citalopram) and 200 μL of buffer solution were added to 200 μL of the plasma sample spiked with the standard solutions at therapeutic levels.

The influence of the sample pH on the extraction efficiency was evaluated at different pH values ranging from 3.8 to 9.0 (0.05 mol L^{-1} buffer solutions). Different solvents (acetonitrile, methanol, and mobile phase) and the volume of these solvents were evaluated to establish the desorption conditions. After the desorption process, the sorbent was washed with 500 μL of water:methanol solution (50:50, v/v). The carryover was also evaluated.

2.8. Analytical validation

Calibration curves were constructed using linear regression of the fluoxetine and norfluoxetine peak areas (Y) against fluoxetine and norfluoxetine plasma concentration (X, ng mL^{-1}), to evaluate linearity. Accuracy and inter-assay precision were determined by means of quintuplicate assays of the blank plasma samples spiked with analytes solutions representing the entire range of the calibration curve. Accuracy values were calculated by comparing the concentrations of analytes added to the plasma samples with analytes plasma concentrations determined by the calibration curve.

3. Results

3.1. Development of the extraction phase

In the present work, eight different DPX phases were evaluated for the pre-concentration of antidepressants (fluoxetine and norfluoxetine) from plasma samples. Each copolymer SD29 and SD84 was prepared with 1 or 4 polyaniline (PANI) deposition cycles. The PANI/SD composites had a green color, characteristic of the PANI emeraldine salt form, which became darker with the increase of the polymerization cycle number.

Fig. 1 shows the extraction efficiency for the synthesized phases with 1 and 4 aniline polymerization cycles. Above 4 aniline deposition cycles, the pore blockage was observed. SD84 with 4 aniline deposition cycles showed the highest extraction efficiency (Fig. 1). Thus, this composite was select for subsequent assays.

Compared to PANI/SD29, PANI/SD84 had a higher degree of cross-linking and presented higher surface area and pore volume. This explained clearly the higher extraction efficiency of PANI/SD84 composites showed in Fig. 1.

The polymerization cycle increase could reduce the number and volume of pores or even the block pores. According to Moura and co-workers, the larger amounts of PANI filling the pores counter balance the swelling effect, such that the starting copolymer characteristics were lightly altered [20]. The PANI/SD84 with 4 aniline polymerization cycles had a specific surface area of 354 m^2 g^{-1}, a total pore volume of 0.67 cm^3 g^{-1}, and an average pore diameter of 11 nm.

Fig. 2 shows the micrographs of the external surface and inner region of the copolymer SD84 and the composite PANI/SD84 with 4 aniline polymerization cycles. According to the micrographs (Fig. 2) there is no significant morphology difference between the copolymer and composite, but they are visually different. Composite

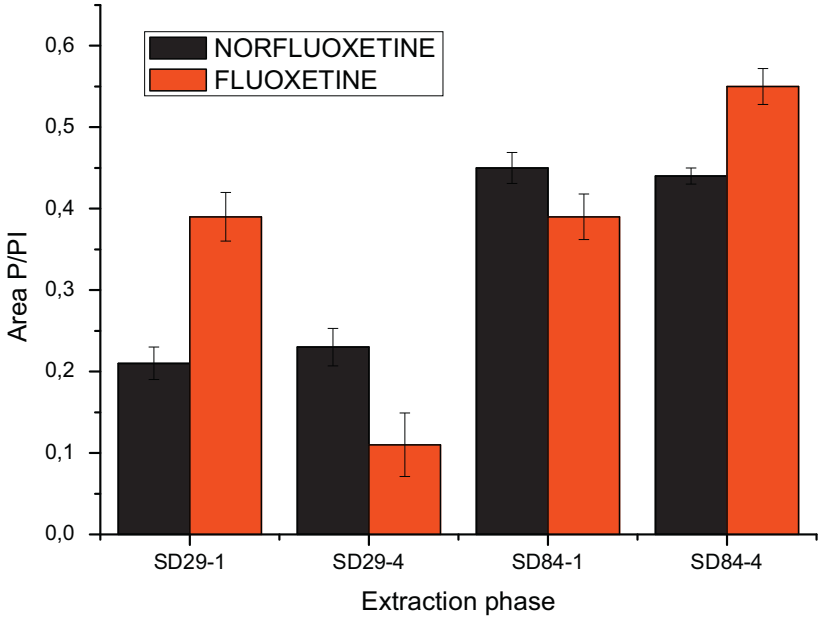

Fig. 1. Extraction efficiency for the synthesized phases SD29 and SD84 with 1 and 4 aniline deposition cycles.

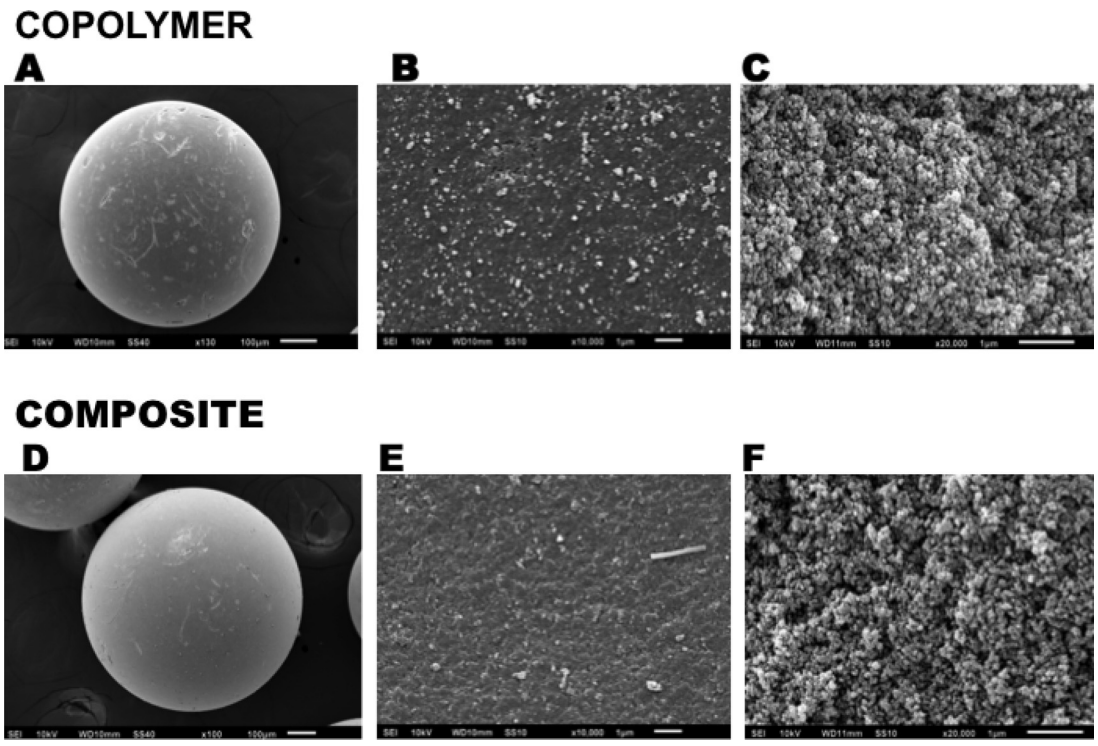

Fig. 2. Scanning electron micrographs of the copolymer SD84 and composite PANI/SD84 with 4 aniline polymerization cycles: (A) entire sphere, (B) sphere surface and, (C) the inner of sphere of the copolymer; (D) entire sphere, (E) sphere surface and (F) the inner sphere of the composite.

exhibit a dark green color, as mentioned above. Thus, the polyaniline synthesis did not modify the copolymer morphology.

It is possible to correlate the scanning electron micrographs with the nitrogen measurements, leading to the conclusion that there are polyaniline deposits over the entire copolymer surface. According to Moura and co-workers [20], this distribution of PANI on the porous matrix is interesting because it combines the chemical properties of PANI with the copolymer textural properties.

Approximately 5 mg of the PANI/SD84-4 extraction phase (75–115 μm, diameter) was introduced in pipette tips using degreased cotton at both ends to avoid sorbent loss before the extraction procedure. This amount was the minimum necessary to reach adequate extraction efficiency. The commercial DPX extraction phases contain approximately 30–60 mg of the extraction phase (size 10–90 μm). The developed DPX phase significantly reduces the amount extraction phase in the pipette tip. Each PANI/SD84-4 synthesis produces approximately 100 g of extraction phase, allowing for the production of a considerable number of pipettes tips and reducing the cost of the method.

3.2. Optimization of the DPX conditions

Sorption capacity, method sensitivity, and carryover are important parameters that should be considered during the development of new phases and methods. Using the selected phase, PANI/SD84-4, the DPX conditions were evaluated based on the extraction efficiency. The therapeutic levels of the drugs should also be considered during the optimization of the parameters DPX.

To improve method performance, Citalopram was used as an internal standard for the DPX/LC–FD analysis, due its physicochemical properties and similarity with the target drugs.

The number of draw/eject cycles is a critical parameter for extraction recovery. The effect of the number of extraction cycles (draw–eject) on the extraction efficiency was also evaluated. The recovery rate of the fluoxetine increased above 10 extraction cycles (10 × 200 μL), and the extraction efficiency for norfluoxetine clearly decreased with the number of extraction cycles (Fig. 3). The extraction cycles were performed with the same aliquot. Using three extraction cycles, the method produced precision, accuracy and LOQ values for the antidepressants levels that are adequate for therapeutic drug monitoring. Therefore, this condition (3× 200 μL), performed in less than 30 s, was selected for subsequent experiments.

The sensitivity of the DPX/LC–FD method was improved by diluting the sample with 200 μL of borate solution (pH 9.0), in which the drugs (fluoxetine pK_a = 10.05 and norfluoxetine = 9.05) [21] were partially in the charged state, favoring the sorption into PANI/SD84-4 (Fig. 4). In low pH solutions, the electrostatic repulsion between analytes and coating were observed and resulted in lower extraction efficiencies. As the sample pH increases, the positive charge on the analytes is reduced and the attractive intermolecular interactions between the drugs and the PANI/SD84-4 phase dominate [1]. It is important to note that norfluoxetine has a pK_a value close to the pK_a value for fluoxetine, and a similar behavior is expected. The adsorptive characteristic of the PANI phases is accentuated more than the acid-basic equilibrium.

The PANI/SD84-4 extraction phase was chemically stable at higher pH values, such as 9.0. The silica particles that are used in commercial DPX extraction phases are easily damaged by alkaline solutions with high pH [22].

The analytes were eluted by drawing/ejecting the solvent through the pipette tip. Methanol, acetonitrile, and the mobile phase were evaluated as desorption solvents. Among these, acetonitrile exhibited the highest desorption efficiency for one single desorption cycle (200 μL) (Fig. 5A and B). Increasing the number of aspirating/dispensing cycles above one using acetonitrile as the elution solvent did not substantially alter the results for the compounds tested (Fig. 5B). To achieve sufficient recovery, the acetonitrile was aspirated and remained in contact with the extraction phase for 10 s prior to being dispensed.

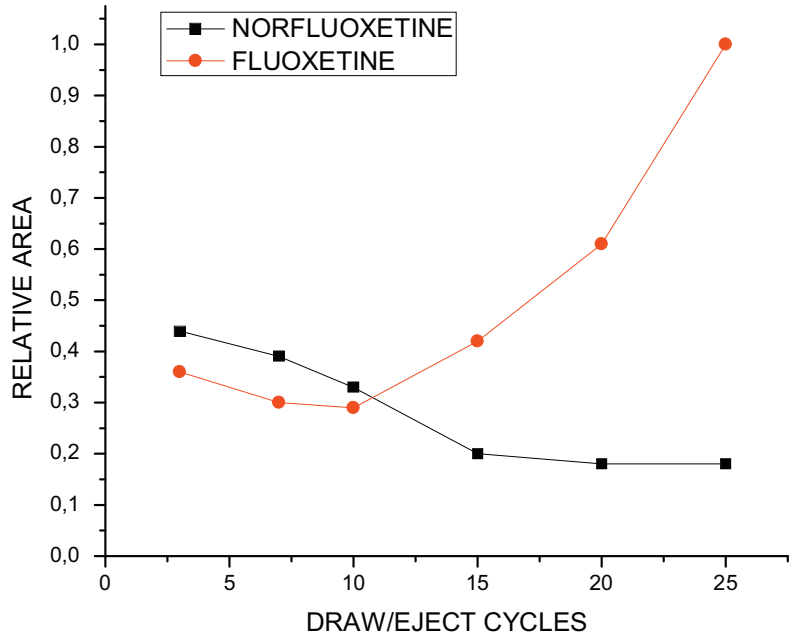

Fig. 3. Effect of the number of extraction cycles (draw–eject) on the extraction efficiency.

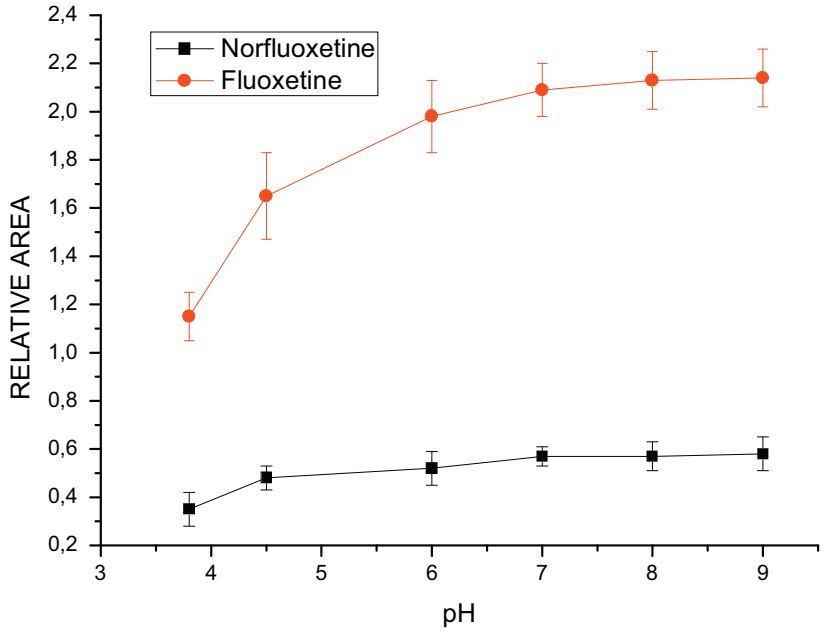

Fig. 4. The effect of the matrix pH on the DPX extraction efficiency.

Between the extractions, the DPX extraction phase was washed with methanol and water solution (50:50, v/v) for three cycles of aspirating/dispensing (500 μL) to ensure that the matrix compounds were removed. A pipette tip containing the DPX extraction phase was used over 50 times with water solutions without extraction efficiency decrease, and for the plasma samples, each pipette tips was used 10 times. After 10 uses, the pipette tips developed a fatty acid layer and were no longer used. However, no extraction efficiency decrease was observed.

Based on these data, the best DPX experimental conditions among those investigated for fluoxetine and norfluoxetine assays (Figs. 1, 3 and 4) were 200 μL of plasma mixed with 200 μL of borate solution (pH 9), followed by liquid desorption of the drug with 200 μL acetonitrile in a single cycle.

3.3. Analytical validation

The selectivity of the developed method is shown by the representative chromatograms in Fig. 6 of a drug-free human plasma sample and drug-free human plasma sample spiked with fluoxetine, norfluoxetine, and internal standard at the therapeutic concentration (300 ng mL^{-1}).

Five different blank plasma samples were analyzed and the analytical signals relative to the possible interference at the same analytes retention time were lower than 20% of the chromatographic signal of the target drug at the concentration corresponding to the quantification limit concentration.

The linearity of the DPX/LC–FD method ranged from the LOQ of 10 to 1000 ng mL^{-1} for fluoxetine and from the LOQ of 80 to

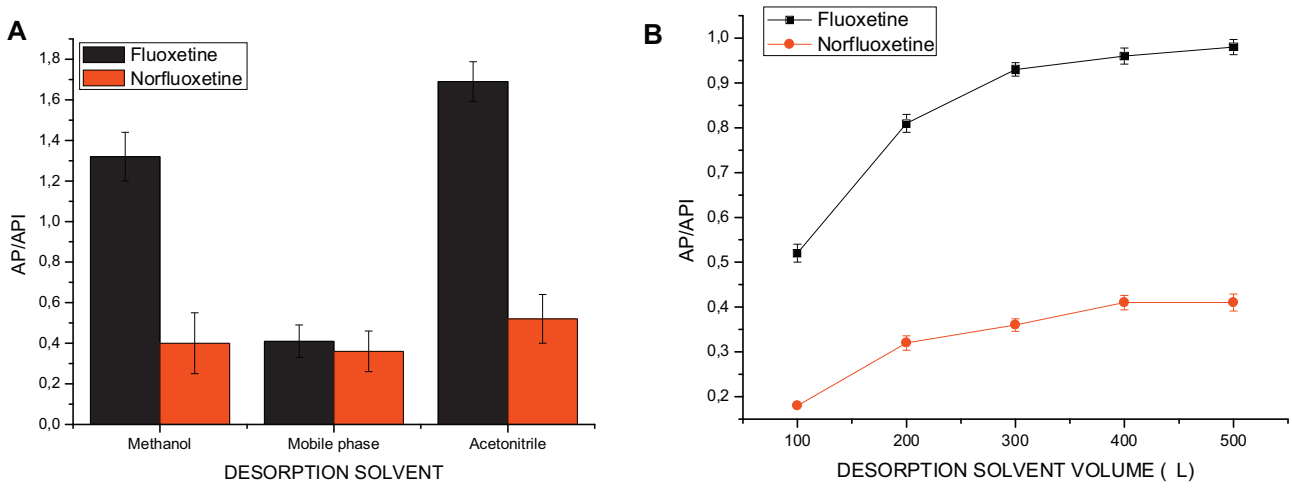

Fig. 5. Effect of the desorption solvent (A) and desorption solvent volume (B) on the DPX extraction efficiency for fluoxetine and norfluoxetine in plasma sample analysis.

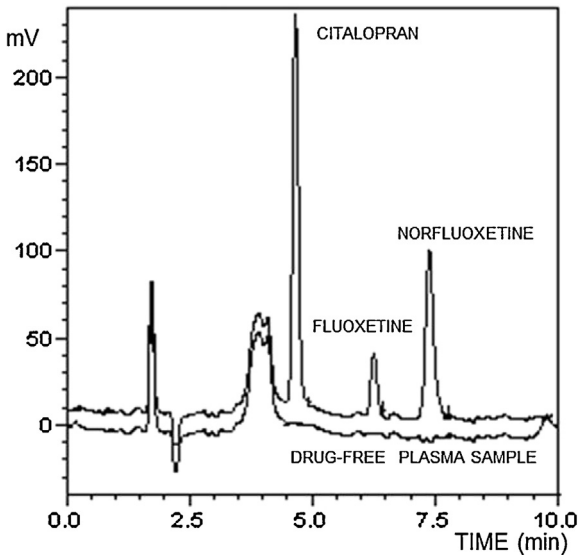

Fig. 6. DPX/LC–FD chromatogram of drug-free plasma samples and drug-free plasma sample spiked with antidepressants at 300 ng mL^{-1}. Citalopram—internal standard (500 ng mL^{-1}).

1000 ng mL^{-1} for norfluoxetine. The regression equation and the corresponding determination coefficient were $y = 0.0025x - 0.1183$ with $r^2 = 0.997$ for fluoxetine and $y = 0.0016x - 0.0329$ with $r^2 = 0.998$ for norfluoxetine, respectively. Each point of the calibration curve was performed in replicate ($n = 5$).

The limit of quantification (LOQ) was determined as the lowest concentration on the calibration curve for which the CV was less than 10% (Table 1 and 2), on the basis of a signal-to-noise ratio of approximately 10. The obtained LOQs were 10 and 80 ng mL^{-1} for fluoxetine and norfluoxetine, respectively.

The accuracy and inter-assay precision of the DPX/LC–FD method were assessed via replicate analysis ($n = 5$), using plasma samples spiked with fluoxetine and norfluoxetine standard solutions at various concentrations (Table 1). The precision was determined according to the percentage coefficient of variation (CV) (inter-assay) at three levels. The CV% values ranged from 5.4 to 9.7% for fluoxetine (Table 1) and from 7.2 to 12.1% for norfluoxetine. The relative recovery of the developed method was assessed via replicate analysis ($n = 5$) of the plasma samples spiked with

Table 1
Inter-day precision (coefficient of variation, CV), and accuracy of the DPX/LC–FD method for fluoxetine and norfluoxetine analysis.

Analyte	Concentration (ng mL^{-1})	CV (%)	Accuracy (%)	Relative recovery (%)
Fluoxetine	10 (LOQ)	9.7	76	74
	400	7.2	89	89
	1000	5.4	92	91
Norfluoxetine	80 (LOQ)	12.1	75	74
	400	8.9	79	77
	1000	7.2	87	87

standards at three different concentrations (Table 1). The recoveries were calculated by comparing the FD peak areas of the spiked samples relative to the internal standard, with the peak area of the direct injection of the standard solutions and the internal standard at equal concentrations. The recovery values obtained ranged from 74 to 91% for fluoxetine and 74 to 87% for norfluoxetine. As shown in Table 1, the relative recoveries for each analyte were quite consistent between matrices and spiking levels, and as expected, relative standard deviations (RSDs) decreased as spiking concentrations increased.

Lee and co-workers [4] analyzed tricyclic antidepressants (Amitriptyline, Amoxapine, Imipramine, and Trimipramine) in plasma samples using a commercial MonoTip C18 tip and gas chromatography with mass spectrometry detection [4]. The authors had optimized the extraction variables and found a recovery of the four antidepressants ranging from 80.2 to 92.1% and an LOQ values ranging from 0.2 to 0.5 ng/0.1 mL. However, the total extraction time required was approximately 7–8 min and the MonoTip C18 extraction phase was not reused.

To evaluate the proposed method for clinical use, the described protocol was applied to the analysis of plasma samples from elderly depressed patients (Fig. 7). The drug concentrations found in these samples were 91.0 ng mL^{-1} for fluoxetine and 45.0 ng mL^{-1} for norfluoxetine.

The entire DPX process presented here, including the conditioning, sample loading, elution, and washing steps, requires approximately 3 min. This is a significant reduction in time compared to the traditional SPE method (approximately 20 min) [23]. The low synthesis cost, high recoveries, and the ability to reuse the same phase approximately 30 times without loss in efficiency are additional advantages to this new DPX method.

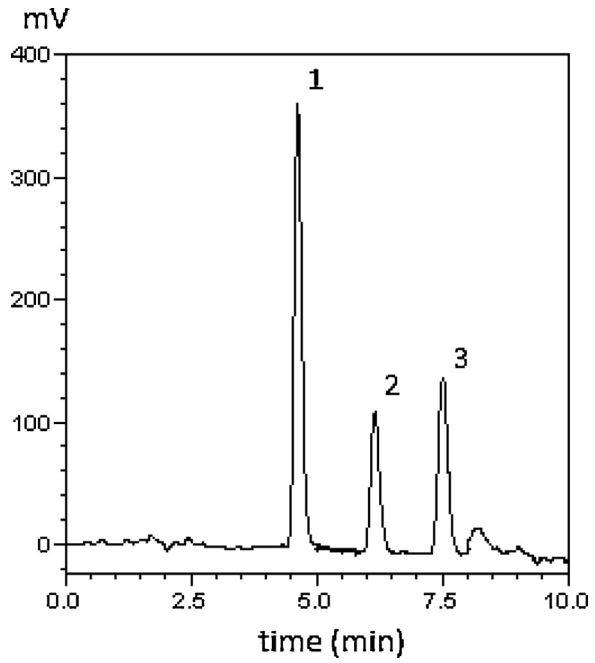

Fig. 7. DPX/LC–FD analysis of plasma samples from elderly depressed patients receiving therapeutic dosages. 1: internal standard; 2: fluoxetine and 3: norfluoxetine.

4. Conclusion

A new DPX extraction phase based on PANI composites was successfully developed and applied for the analysis of fluoxetine and norfluoxetine in plasma samples. Under the optimized conditions, good recovery, linearity, and reproducibility were obtained.

The small volumes of plasma and solvents, in addition to the reduced preparation time and cost used in this work, are significant advances for sample preparation using this miniaturized technique.

The developed method provides a useful tool for the screening and quantitative determination of fluoxetine and norfluoxetine in clinical and toxicological analyses.

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