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Hollow-fiber liquid phase microextraction for determination of fluoxetine in human serum by nano-liquid chromatography coupled to high resolution mass spectrometry

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ABSTRACT

Therapeutic drug monitoring (TDM) is a personalized care tool based on the determination of a target drug concentration in human serum. An antidepressant drug of interest for such investigations is fluoxetine (FXT), due to a severe impact of genetic polymorphisms on its metabolism. A bioanalytical method employed for TDM purposes must exhibit satisfactory selectivity and detectability, which becomes more difficult due to highly complex biological matrices. In this study, a highly selective bioanalytical method for the determination of FXT in human serum is proposed, which provides excellent clean-up efficiency based on a low cost hollow fiber liquid-phase microextraction (HF-LPME) sample preparation step and nano-liquid chromatography coupled to high-resolution mass spectrometry (nano-LC-HRMS). HF-LPME was performed using a two-phase "U" configuration, with 6 cm fiber, 20 μL of 1-octanol acting as supported liquid membrane, and ammonium hydroxide (pH 10) as the donor phase with NaCl (10 % m/v) and methanol (5 % v/v) as additives, requiring only 250 μL of the sample. The procedure was conducted for 30 min under a 750 rpm stirring rate. Gradient elution was carried out employing an acetonitrile–water as mobile phase, the composition of 30:70 to 100:00 (v/v) for 15 min, using formic acid 0.1 % (v/v) as an additive. MS1 was acquired in an Orbitrap mass analyzer, while MS2 was acquired in a linear trap quadrupole. Satisfactory linearity (Pearson's r = 0.99709) was obtained for a concentration range of 0.02 to 2.5 μg mL^{-1}, which is compatible with the therapeutic and toxic range for FXT. The developed method presents adequate precision (1.61 to 7.45 %) and accuracy (95 to 114 %) and allows the dilution of high concentration samples in a 1:4 ratio (v/v), enabling its application for forensic serum samples. To our knowledge, this is the first study reporting a method based on HF-LPME and nano-LC-HRMS with any analytical purpose, especially with a TDM focus.

Abbreviations: ANOVA, Analysis of variance; CID, collision induced dissociation; DLLME, Dispersive liquid–liquid microextraction; DPX, disposable pipette extraction; FDA, Food and Drug Administration; FXT, fluoxetine; FXT-D6, fluoxetine-D6; GC–MS, gas chromatography coupled to mass spectrometry; HF-LPME, hollow-fiber liquid phase microextraction; HPLC, high performance liquid chromatography; HPLC-DAD, high performance liquid chromatography with diode array detector; HPLC-FD, high performance liquid chromatography with fluorescence detector; HPLC-UV, high performance liquid chromatography with ultraviolet detector; HRMS, high resolution mass spectrometry; ID, internal diameter; IS, internal standard; LC, liquid chromatography; LC-MS, liquid chromatography coupled to mass spectrometry; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; LLE, liquid–liquid extraction; LLOQ, lower limit of quantification; LOD, limit of detection; LOQ, limit of quantification; LTQ, linear trap quadrupole; MEPS, microextraction with packed sorbent; MSPE, magnetic solid phase extraction; nano-LC, nano-liquid chromatography; nano-LC-HRMS, nano-liquid chromatography coupled to high resolution mass spectrometry; NCE, normalized collision energy; OD, outer diameter; RSD, relative standard deviation; SBSE, stir bar sorptive extraction; SLM, supported liquid membrane; SPE, solid phase extraction; SSRI, selective serotonin reuptake inhibitor; TDM, therapeutic drug monitoring.

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1. Introduction

Fluoxetine is an antidepressant that belongs to selective serotonin reuptake inhibitor (SSRI) class, commonly used to treat depressive and anxiety disorders. Usually, this pharmaceutical drug presents low toxicity and is considered a safe alternative for psychiatric treatment, however, its metabolism is severely influenced by genetic polymorphisms. Consequently, slower fluoxetine metabolism results in enhanced potential toxicity, once its half-life can double in the organism of affected patients [1].

Due to this potential toxicity, there is an interest in therapeutic drug monitoring (TDM) for fluoxetine. TDM revolves around the determination of the concentration of a specific drug in an individual's bloodstream, acting as personalized patient care tool. The drug serum concentration is a more relevant indicator of effective treatment than its administered dose. TDM allows dosage adjustment to guarantee that the drug serum concentration is compatible with its therapeutic window as well as minimizes the risk of intoxication [2–4]. Analytical methods developed for TDM purposes must respond well in the face of adversities, such as high matrix complexity, particularly high protein content characteristic of serum samples, and low analyte concentration, which can be the case for certain drugs, like fluoxetine [5,6].

When it comes to analyzing complex samples, such as biological matrices, chromatographic techniques are widely applied, due to advantages like selectivity, high precision and high accuracy, even when applied to matrices that contain various interferents. Conventional high-performance liquid chromatography, though amply used in bioanalytical applications, has certain limitations, as low analysis efficiency and a more complex coupling to mass spectrometry when compared to gas chromatography [7]. Aiming to circumvent these obstacles, efforts in miniaturizing LC started in the'60 s, culminating in the development of the first nano-liquid chromatography (nano-LC) columns in 1988 [8]. These columns were developed using silica capillaries of reduced internal diameter and packed with stationary phase particles. Its diminished internal diameter results in various advantages, such as a highly efficient analysis, facilitated coupling to MS, use of low quantities of solvents as mobile phase, small sample volume needed, as well a higher instrumental detectability. When coupled to high-resolution mass spectrometry (HRMS), this technique provides highly reliable analyte identification, a major advantage when analyzing complex matrices [9,10].

As for sample preparation, when studying complex matrices, this step acquires even more importance, once it has to combine a highly efficient sample clean-up and analyte extraction in the lesser number of steps possible. Conventional sample preparation techniques, such as solid-phase extraction and liquid–liquid extraction, present the main disadvantage of usually depending on large amounts of organic solvents considered harmful for humans and the environment, which in turn resulted in attempts of miniaturization of such techniques [11–13]. Miniaturized techniques have been successfully applied to FXT determination in biological matrices, using minimal amounts of extraction solvents or solid sorptive phases, such as dispersive liquid–liquid microextraction [14], microextraction with packed sorbent [15], disposable pipette extraction [16], stir bar sorptive extraction [17], and magnetic solid phase extraction [18].

When it comes to biological fluids, one miniaturized extraction technique that provides numerous advantages is hollow fiber liquid-phase microextraction (HF-LPME). Like its precursor, liquid-phase extraction, HF-LPME relies on the extraction of analytes based on their partition between immiscible liquids. To achieve analyte extraction, an organic solvent immiscible in water is trapped in pores of a hollow and porous polymeric fiber, resulting in a supported liquid membrane (SLM). The fiber is then filled by an acceptor phase, which can be aqueous or the same organic that composes the SLM. By partition, the analytes migrate from the aqueous sample, denominated donor phase, to the SLM and, subsequently, to the acceptor phase [13,19]. Due to the physical characteristics of the hollow fiber, macromolecules that compose the matrix, such as proteins, remain in the donor phase due to its incapability of penetrating through the fiber pores, resulting in high clean-up efficiency. Additionally, this technique requires minimal amounts of organic solvents, as well as provides advantages of high enrichment factor and low cost [20].

Bioanalytical studies or those focusing on the determination of low concentration analytes in highly complex matrices would benefit from the combination of such extraction and chromatographic techniques. To the best of our knowledge, this is the first study joining HF-LPME and nano-LC-HRMS, since it hasn't been reported in bioanalytics or analytical methods in general. Thus, the objective of this study was to develop a bioanalytical method based on said techniques for fluoxetine determination in human serum, aiming at its potential application for TDM purposes and using a lab made chromatographic nanocolumn.

2. Materials and methods

2.1. Materials and reagents

The target compound, fluoxetine hydrochloride (FXT), was obtained from USP (United States), and the isotopically labeled internal standard, fluoxetine-D6 (FXT-D6), was obtained from Cerilliant (France). Analyte stock solutions were prepared individually in methanol and stored in glass tubes, protected from light, at – 20 °C temperature, conditions in which the analyte is stable [21]. The internal standard was stored similarly.

The mobile phase was prepared using acetonitrile and water, LC-MS grade (Merck, Brazil), and formic acid, analytical standard (Merck, Brazil). Regarding the reagents (analytical grade) and solvents used in sample preparation, methanol, HPLC grade, was acquired from J. T. Baker (Brazil), ammonium hydroxide from Biotec (Brazil), 1-octanol from Sigma-Aldrich (Brazil), and sodium chloride was acquired from Alphatec (Brazil). Ultrapure water with 18.2 MΩ cm at 25 °C resistivity, purified by a Milli-Q system (Millipore®, Brazil), was used in solution preparation for the extraction procedure.

2.2. Human serum samples

Human blood samples were collected by venous puncture using 10 mL disposable syringes. These samples were transferred to serum-separating tubes (BD Vacutainer SST) and centrifuged at 4000 rpm for 10 min. The resulting serum samples were then transferred to falcon tubes for storage at −20 °C. Method development was attained employing a blank serum pool, obtained by mixing serum samples volunteered by individuals who were not in therapy with FXT. In these assays, the samples were spiked by the addition of standard solutions of FXT and FXT-D6 directly to a 10 mL vial, which was later used in the HF-LPME procedure. Methanol was dried under a nitrogen stream prior to the addition of the human serum. Similarly, the patient sample was obtained from a volunteer in regular FXT therapy and was used to verify the suitability of the developed method.

2.3. Sample preparation

Several 6 cm segments of hollow and porous polypropylene fiber (ID 600 μm, 0.2 μm porosity, wall thickness 200 μm - Q3/2 Accurel), acquired from Membrana (Wuppertal, Germany), were decontaminated by its submersion in acetone and subsequent vortex agitation for 1 min. The fibers were then removed from acetone, air-dried, and stored for posterior use. The extraction procedure took place using a single fiber segment, which was conditioned by submersion in 1-octanol and vortex agitation for 30 s, which allowed the solvent to penetrate the fiber pores, constituting the SLM.

The fiber segment was removed from the solvent, connected to the needle of two 25 μL LC microsyringes, and filled with 20 μL of 1-octanol,

which acted as the acceptor phase in a "U" configuration two-phase HF-LPME system. The filled fiber segment was completely submerged in 10.00 mL of sample, comprised of 250 μL of human serum, 1.00 g of sodium chloride, 500 μL of methanol, and 9.25 mL of ammonium hydroxide (pH 10). The extraction took place under a 750 rpm stirring rate for 30 min, after which the acceptor phase was aspirated by one of the microsyringe and transferred to a 1 mL vial. The extract was dried in a vacuum concentrator at room temperature and resolubilized with formic acid 0.1 % (v/v) for injection.

2.4. HF-LPME procedure optimization

HF-LPME parameters were univariately optimized (n = 3) according to the following ranges: system configuration (two or three-phase systems), sample pH (7 – 11), stirring rate (0 – 1250 rpm), fiber length (6 – 8 cm), extraction time (10 – 45 min), sodium chloride addition (0 – 10 % (m/v)), methanol addition (0 – 50 % (v/v)). All of the variables were statistically evaluated by analysis of variance (ANOVA), with a 95 % confidence interval, except for system configuration and sodium chloride addition, which were evaluated by F and t-tests, with a 95 % confidence interval.

2.5. Column confection

A silica capillary covered with polyimide (ID 75 μm, OD 375 μm, Polymicro Technologies) was cut into a 28 cm segment. To remove the polyimide layer, the section 5 cm away from one of its extremities was carefully burned and wiped with a cloth wet with methanol. The capillary was then fixed in the Laser-Based Micropipette Puller P-2000 (Sutter Instrument Company), equipped with a pulsating carbon dioxide laser, securing that the exposed silica was put directly in front of the laser's path. The laser was focused on the exposed silica four times, producing a conical tip.

The capillary was then affixed to a pressurization system, consisting of a pressure injection cell and a hydraulic pump. Silica C18 particles (3.0 μm diameter, 120 Å, ReproSil-Pur 120 C18-AQ, Dr. Maisch GmbH) were added to the pressure injection cell compartment under agitation to form a suspension, and methanol pressurized with nitrogen carried the particles to the interior of the silica capillary, providing a 15 cm length, 75 μm I.D. analytical column. Packing of the stationary phase was conducted in a nano-LC system (nanoLC Ultra 1D Plus, Eksigent®) through percolation cycles of water-acetonitrile, after which the packed column was filled with acetonitrile and stored until its use.

2.6. Nano-LC-MS parameters

The samples were analyzed at the mass spectrometry facility RPT02H/Carlos Chagas Institute - Fiocruz Parana. Chromatographic analysis was conducted by an EASY-nLC 1000 UHPLC coupled to LTQ Orbitrap XL ETD mass spectrometer system (Thermo Fisher Scientific®, XCalibur V.2.2 and TraceFinder V4.1), equipped with a PST-MS Thermo single column ionization source (Phoenix S&T), which allowed the positioning of the analytical column directly at the entry of the mass spectrometer, with spray voltage set as 3000 V. Autosampler temperature was maintained at 10 °C, and the injection volume was set as 2 μL.

Using a mobile phase composed of acetonitrile and water, formic acid 0.1 % (v/v) as an additive, the chromatographic method was divided into four steps: sample trapping, chromatographic separation, analytical column cleaning, and re-equilibrium. Trapping was conducted for 8 min, using a 1:99 acetonitrile and water (v/v) mobile phase, dragging the 2 μL of the resolubilized extract towards the stationary phase without significant elution. As for the chromatographic separation, the developed method applied gradient elution for 15 min, with mobile phase composition varying from 30:70 to 100:0 acetonitrile–water (v/v). Column cleaning, aiming at the removal of lower polarity substances that might have been adsorbed in the stationary phase, was then conducted using a mobile phase composition of 100:0 acetonitrile–water (v/v) for 12 min, followed by column re-equilibrium, which employed mobile phase composition of 1:99 acetonitrile–water (v/v) for 9 min. Total flow rate was fixed at 250 nL min^{-1}.

The mass spectra were acquired on positive mode employing full scan acquisition for both MS1 and MS2. MS1 acquisition was conducted in Orbitrap mass analyzer, with 30,000 resolution, 12.5 s maximum injection time, 50,000 FWHM automatic gain control, and mass range from 80 to 400 *m/z*. Exact mass acceptance criteria was set with a maximum 5 ppm mass error, while isotopic profile required a minimum 75 % match with TraceFinder library spectra. MS2 acquisition was conducted in a linear trap quadrupole (LTQ) mass analyzer employing collision-induced dissociation (CID) of the precursor ions, 316 for FXT-D6 and 310 for FXT, with 10 s maximum injection time, 30,000 FWHM automatic gain control, evaluating a product ion mass range from 75 to 330 and 75–325 *m/z*, and normalized collision energy (NCE) set as 17 and 15 % for FXT-D6 and FXT, respectively.

2.7. Method performance

Analytical curves were constructed employing the developed method, adding FXT-D6 as the internal standard (IS). The IS was added to the serum samples in a 0.025 ng mL^{-1} concentration in a final volume of 10 mL. The extraction procedure was carried out as stated, and the resulting dry extracts were resolubilized with 1.0 mL of formic acid (0.10 % v/v) in ultrapure water. FXT concentration levels were 0.02, 0.06, 0.25, 0.50, 0.75, 1.50, and 2.50 μg mL^{-1}, corresponding to a concentration range compatible with the therapeutical range and FXT toxic concentration [6].

Precision and accuracy were evaluated by calculating the relative standard deviation (RSD) and ratio between determined and theoretical concentration, respectively. With this purpose, concentrations of the analyte were the same as the ones of the analytical curve.

For selectivity (n = 5), blank serum samples were spiked with the IS, at 0.025 ng mL^{-1} in a final volume of 10 mL, to verify the response ratio between the lower limit of quantification (LLOQ) and blank serum. For specificity (n = 5), blank serum samples were spiked with FXT (0.10 μg mL^{-1}), FXT-D6 (0.025 ng mL^{-1}), and possible interferents acetylsalicylic acid, amoxicillin, caffeine, carbamazepine, cholesterol, diazepam, diclofenac, haloperidol, ibuprofen, lamivudine, lansoprazole, omeprazole, pantoprazole, paracetamol, progesterone, reserpine, sulfamethoxazole, testosterone and trimethoprim at 0.10 μg mL^{-1}, and cannabidiol, 17α-ethinylestradiol, 17β-estradiol, estriol, estrone and THC (tetrahydrocannabinol) at 0.20 μg mL^{-1}.

To evaluate the possibility of dilution of therapeutic samples and expand the application of the developed method to forensic samples, 50 μL aliquots of blank serum were spiked with 5.0 μg mL^{-1} of FXT, resulting in a 1:4 (v/v) sample dilution (n = 5). The samples were completed to a 10.0 mL volume with 9.45 mL de ammonium hydroxide solution (pH 10.0), 500 μL of methanol, and 1.0 g of sodium chloride. The extraction procedure was carried out as stated, and dry extracts were resolubilized in 1.0 mL of formic acid 0.10 % (v/v) for injection. Serum samples were spiked with the IS, at 0.025 ng mL^{-1} in a final volume of 10 mL.

For the recovery assays (n = 5), blank serum samples were spiked with the IS, at 0.025 ng mL^{-1} in a final volume of 10 mL, and extracted according to HF-LPME procedure. After the extraction and before the evaporation, the extract was spiked with 0.02 μg mL^{-1} of FXT, considering a final volume of 1.0 mL. The analytical response was compared with the results from the lowest level of the analytical curve by obtaining the ratio between calculated concentrations in the extracts spiked before and after the HF-LPME procedure and multiplying the value by 100 to obtain the percentual recovery rate. Such concentration was selected to prevent stationary phase saturation.

2.8. Application to patient serum sample

A patient serum sample was used as the incurred sample in the present study. The volunteer was under daily administration of 20 mg of FXT for at least a month, and the provided sample was processed as optimized in the developed method.

3. Results and discussion

3.1. Chromatographic method

The nano-LC method was optimized univariately. The flow rate was established by the highest value in which high column pressure, above 300 bar, wasn't observed, being set as 250 nL min^{-1}. Based on the flow rate and the 2 μL injection volume, the trapping step was defined as 8 min, corresponding to the exact time needed to drag the sample to the column head. Chromatographic separation was evaluated using different elution programs. In between isocratic and gradient elutions, best results in terms of compromise of a satisfactory number of acquisitions, resolution, and analysis time were observed for gradient elution, starting at 30:70 (v/v) and finishing at 100:0 (v/v) acetonitrile–water, with duration of 15 min.

The cleaning step was defined by the minimum time required to minimize carryover between runs, set as 12 min of 100 % acetonitrile. As for the final re-equilibrium step, the duration was set as the required time to reestablish a stable column pressure, taking place for 9 min with a mobile phase composition of 1:99 (v/v) acetonitrile–water. Extracted ion chromatograms obtained with the developed and optimized chromatographic method are presented in Fig. 1.

3.2. MS parameters

The spray voltage was set to the voltage necessary to achieve maximum ionization efficiency for the analyte. In the evaluated 2500—3500 V range, in 250 V intervals, ionization efficiency was increased until 3000 V for both analyte and internal standard, while higher spray voltages resulted in a response decline. This observation is likely due to analyte fragmentation during ionization, which would decrease $[M-H]^+$ ion response. For that reason, the spray voltage was set to 3000 V.

When analyzing MS1 and MS2 acquisition parameters, both were initially performed using an Orbitrap mass analyzer in full scan mode. However, due to the necessity of three acquisitions in each cycle (MS1, MS2 FXT, and MS2 FXT-D6), an adequate number of MS1 acquisitions couldn't be achieved when all acquisitions were conducted using an Orbitrap mass analyzer. For that reason, MS2 acquisition was then performed using a LTQ mass analyzer, employing optimized NCE of 17 and 15 % for FXT-D6 and FXT, respectively. The acquisition is performed much faster in LTQ than in Orbitrap, thus, a higher number of MS1 Orbitrap acquisitions were performed, achieving a satisfactory peak shape, containing 12 points of acquisition.

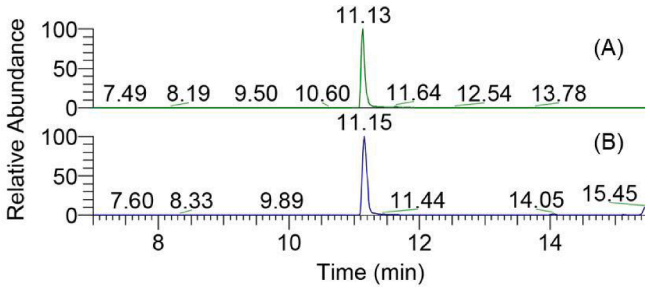

Fig. 1. Extracted ion chromatograms for (A) fluoxetine-D6 and (B) fluoxetine.

3.3. Extraction optimization

3.3.1. System configuration

Employing a "U" configuration, initially, a three-phase system was evaluated, using formic acid pH 2.0 solution as the acceptor phase and ammonium hydroxide pH 10.0 solution as the donor phase. The direct injection of the obtained extract resulted in irreversible column clogging, possibly due to the presence of 1-octanol residue, which although not detected macroscopically was an issue for the nanocolumn. Thus, an extract evaporation step was added, which allowed the evaluation of a two-phase system additionally to the three-phase system. No significant difference was observed between the two and three-phase systems, however, since the use of the two-phase system provided lower RSD value, 29.53 %, in comparison to the RSD obtained for the three-phase system, 41.31 %, the former system was selected for furthers studies.

3.3.2. Donor phase pH

Aiming to evaluate extraction efficiency according to the percentage of analyte available as its neutral species, donor phase pH was evaluated, ranging from 7 to 11. No significant difference was observed between extraction efficiencies, however, RSD varied widely, which could affect the developed method's precision. As pH 10 provided the lowest RSD, 21.72 %, such a condition was selected.

3.3.3. Stirring rate

Agitation of the sample is an important parameter to optimize, due to it being majorly responsible for mass transfer in the donor phase. Stirring rates from 0 to 1250 rpm were evaluated. A significantly lower analyte response was observed when HF-LPME was conducted in static mode, meaning applying no stirring, while there was no significant difference between stirring rates (Fig. 2). Nevertheless, when a stirring rate of 750 rpm was applied, the lowest value of RSD, 15.85 %, was obtained. Such an observation may derive from a less controlled movement by the stir bar or partial loss of SLM solvent, as it was previously stated in the literature [22,23], so such a condition was selected for further studies.

3.3.4. Fiber length

The acceptor phase was defined as 1-octanol, and due to its low vapor pressure, a small amount of extract could require hours for complete evaporation. Thus, the acceptor phase volume was set to 20 μL. Fiber lengths ranging from 6 to 8 cm, corresponding to internal volumes of approximately 20 μL, were evaluated. A significant difference between fibers of 6 and 8 cm length was observed (Fig. 3). This difference may arise from the fact that when longer fiber segments were used the fiber's lumen was not filled by 1-octanol, which in turn resulted in

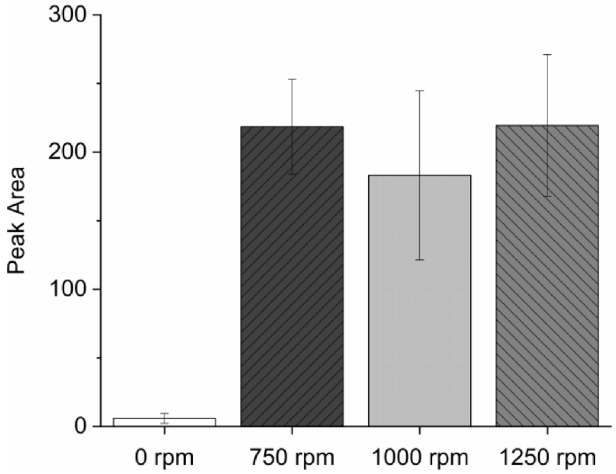

Fig. 2. Stirring rate effect on analyte extraction.

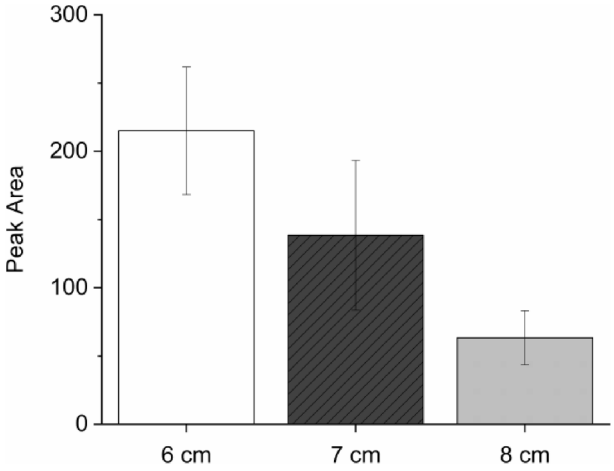

Fig. 3. Fiber length effect on analyte extraction.

bubble deposition on the surface of the fiber. The presence of bubbles may difficult analyte diffusion and cause SLM solvent evaporation [24]. Additionally, as the use of 6 cm fibers provided the lowest RSD, 21.72 %, such a condition was selected for further studies.

3.3.5. Extraction time

An equilibrium condition is not strictly necessary when using HF-LPME since achieving this state can consume long periods, especially when using higher donor phase volumes, and high extraction efficiency and adequate precision can be achieved in shorter times. The extraction time was evaluated from 10 to 45 min since 45 min is usually sufficient time to reach equilibrium when using smaller donor phase volumes [25]. It was observed that extractions conducted for 10 min had lower extraction efficiency when compared to other conditions evaluated (Fig. 4). However, no significant difference in analyte response was observed between 20, 30, and 45 min extractions. Comparing the conditions evaluated as far as precision, 30 min extraction time provided the lowest RSD, 6.89 %, compared to 39.94 % provided the 20 min extraction time condition, which in turn would provide the highest analytical frequency. Hence, 30 min extraction time was selected aiming at the highest precision possible.

3.3.6. Sodium chloride addition

Salt addition to aqueous samples is frequently done to achieve salting-out of the analyte, in this case, aiming at modifying FXT's partition coefficient between the donor phase and the SLM solvent. With this purpose, sodium chloride addition was evaluated in levels of 0 and 10 % (m/v). No significant difference was observed between analyte responses applying such conditions. However, extractions conducted with salt addition provided a lower RSD, 34.23 %, when compared to extractions without salt added, which provided an RSD of 98.22 %. Based on these results, a 10 % sodium chloride (m/v) condition was selected for further studies.

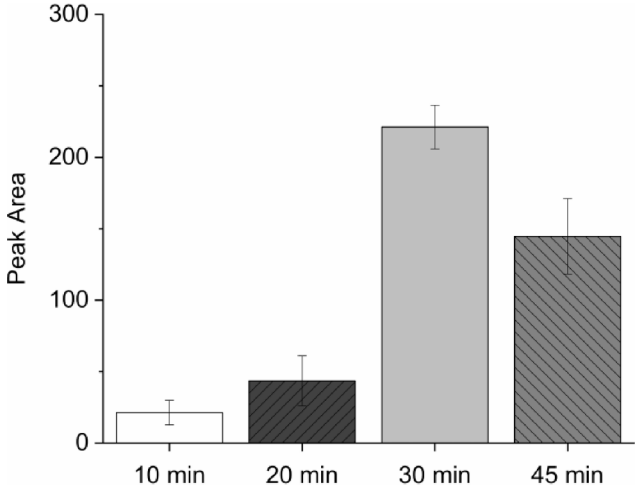

Fig. 4. Extraction time effect on analyte extraction. NCE – normalized collision energy.

3.3.7. Methanol addition

Water-miscible solvents are often employed to break existent protein-drug interactions when studying biological samples. Methanol was selected for this purpose due to it being the most commonly used solvent, and its addition ranged from 0 to 50 % (v/v). With 50 % (v/v) methanol, the sample developed turbidity as it was stirred, and evaporating the extract was possible to observe sodium chloride crystals, indicating that the organic solvent-rich donor phase resulted in considerable miscibility between the donor and acceptor phase. Due to the incompatibility of ESI and sodium chloride, as well as the possible precipitation of NaCl inside the nanocolumn and subsequent column clogging, this condition was excluded. No significant difference was observed for the analyte response obtained for the remaining conditions evaluated. Since the addition of 5 % methanol (v/v) provided the lowest RSD, 29.85 %, this condition was selected for further studies.

3.4. Method performance

After the optimization of the HF-LPME procedure, chromatographic and spectrometric parameters, the method performance was evaluated. Analytical curves were obtained in quintuplicate on different days, with concentrations ranging from 0.02 to 2.5 μg mL^{-1} of FXT, by spiking blank serum with FXT e FXT-D6 (0.025 ng mL^{-1}). Such concentration range provided satisfactory linearity (Pearson's r = 0.99709). The lower limit of quantification (LLOQ) was admitted as the lowest level of calibration, 0.02 μg mL^{-1}, following the Food and Drug Administration (FDA) guidelines for bioanalytical methods since the response ratio for such concentration and blank serum is higher than 5. Precision (1.61–7.45 %) and accuracy (95.05–114.38 %) obtained for all of the concentrations evaluated were satisfactory according to the parameters established by the FDA. Such results indicate the applicability of the developed method for therapeutic samples since the therapeutic range for FXT ranges from 0.12 to 0.5 μg mL^{-1}, and for forensic samples, such as those from accidental and purposeful intoxications, since FXT acquires considerable toxicity in concentrations above 1.0 μg mL^{-1}.

To positively identify FXT in serum samples, various mass spectrometric tools were applied to the data obtained from the 0.02 μg mL^{-1} concentration LLOQ (Fig. 5). MS1 high-resolution acquisitions were used to assess the exact mass of the [M−H] + ion, which presented an average mass error of 1.6628 ppm (n = 5). Thus, MS1 acquisitions were employed for quantification purposes, according to the standards' exact masses. As for MS2, the fragmentation pattern was compared to the m/zCloud database, with both mass spectra showing two major ions, the [M−H] + ion (*m/z* 310.14133), derived from the protonation of the FXT molecule, and the ion produced by the benzylic carbon–oxygen bond cleavage (*m/z* 148.11208). Lastly, the isotopic profile for FXT obtained for the spiked plasma was compared to the database on TraceFinder, which showed a 100 % correlation between the mass spectra. MS2 acquisition and isotopic profiles were employed for qualitative purposes only.

A satisfactory selectivity is further demonstrated by comparing the response obtained for the LLOQ and blank serum spiked with the IS, which provided a response ratio of 8.85. As for specificity, a blank serum sample was spiked with several possible interferents, such as antibiotics, and hormones, among others. It was possible to infer that those compounds evaluated do not interfere with the proposed method, since there is no coelution between the analyte and interferents.

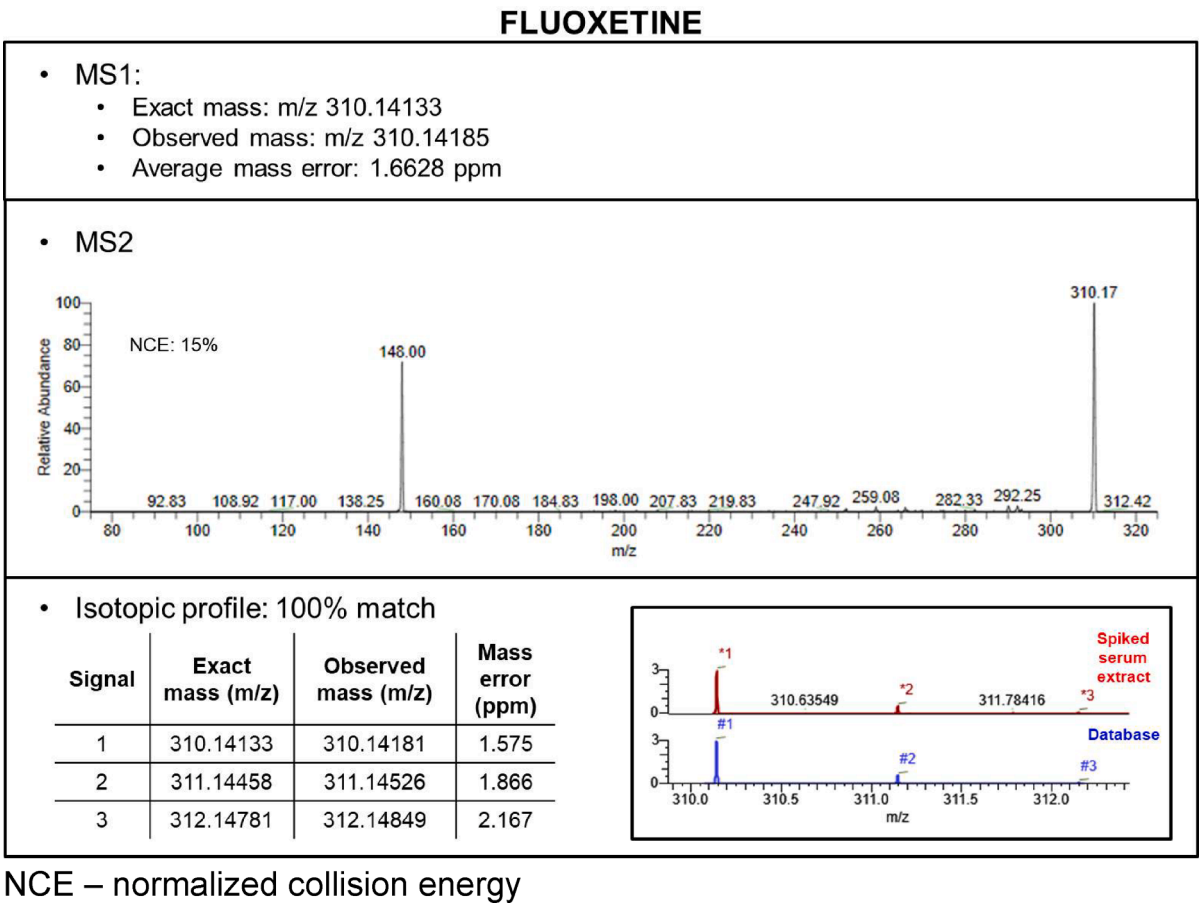

Fig. 5. Mass spectrometry tools used for fluoxetine quantification and identification.

Dilution essays were conducted to verify the possibility of diluting higher-concentration samples to fit the concentration range covered in this study. Hence, 50 μL of blank serum samples were spiked with 5 μg mL^{-1} of FXT, corresponding to a 1:4 (v/v) dilution ratio. The concentration obtained in this essay was 1.04 ± 0.03 μg mL^{-1} (n = 5), which result in a 4.48 % relative error when compared to the spiked concentration. Such results indicate that the developed method may be applied to forensic samples whose concentration exceeds the highest calibration level (2.5 μg mL^{-1}) after sample dilution.

Recovery was assessed by comparing the analyte response between extracts obtained by spiking the sample before extraction and spiking the organic extract before evaporation. The recovery rate was 1.11 %, with a RSD of 3.91 %. According to FDA guidelines, recovery may not be close to 100 % as long as the RSD remains below 15 %. Since HF-LPME corresponds to a miniaturized technique, it is not considered exhaustive, especially in the two-phase system, where extraction efficiency is solely based on the partition equilibrium between the donor and acceptor phase. Previously, Oliveira et al. [26] reported similarly low FXT recovery rates using two-phase HF-LPME system with acceptor phase of n-hexyl ether, in the range of 3.16 to 4.61 %. A summary of the quantitative performance parameters for the developed method is presented in Table 1.

Table 1
Summary of method performance parameters.

Parameters	Ranges
Concentration range (μg mL^{-1})	0.2—2.5 μg mL^{-1}
Linearity (Pearson's r)	0.99709
Lower limit of Quantification (μg mL^{-1})	0.2 μg mL^{-1}
Precision (%)	1.61–7.45 %
Accuracy (%)	95.05–114.38 %
Recovery rate (%)	1.11 %

3.5. Application to patient serum sample

To better understand the suitability of the developed bioanalytical method, an incurred serum sample was obtained from a patient undergoing FXT therapy, under daily administration of 20 mg of FXT for at least a month. The concentration observed was 110 ± 4 ng mL^{-1} (RSD = 4.11 %), which is comparable to what was found in other studies and per the pharmacokinetics curve for FXT [27]. Additionally to the concentration in accordance with the literature, the patient sample fit all the criteria provided by the mass spectrometric tools: mass error obtained was below 2.250 ppm, isotopic profile match was 75 %, and confirmation and quantification ions were observed in MS2 mass spectra.

3.6. Comparison with other bioanalytical methods

In comparison with other methods proposed for FXT determination in biological fluids (Table 2), the bioanalytical method developed in this study has a comparable detectability, in terms of LLOQ, to most bioanalytical methods that use similar sample volume [15–17,26]. However, the proposed method is applicable to a broader concentration range, even without the dilution of samples, which can be an advantage when used for forensic purposes. Additionally, the methods capable of reaching lower LLOQ or limits of quantification (LOQ) either used high sample volumes [28] or conventional extraction techniques [29,30], which typically employ higher volumes of organic solvents than its miniaturized counterparts.

Table 2
Comparison between the developed method and other bioanalytical methods for FXT determination.

Matrix	Analytical technique	Extraction technique	Concentration range (ng mL^{-1})	Sample volume (μL)	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	LLOQ (ng mL^{-1})	Source
Plasma	HPLC-FD	HF-LPME	5—500	1000			5	[31]
Plasma	GC–MS	HF-LPME	10—500	250	3	10		[26]
Plasma	LC-MS/MS	SPE	0.5—50	200			0.5	[29]
Urine and plasma	HPLC-UV	HF-LPME	2—500	1500 and 500	0.5 and 0.3			[32]
Whole blood	LC-MS/MS	DLLME	5—100	500	2	5		[14]
Plasma	LC-MS/MS	MEPS	50—850	200			50	[15]
Plasma	LC-MS/MS	DPX	50—850	200			10.0	[16]
Plasma	LC-MS/MS	LLE	0.2—25	200			0.2	[30]
Urine	HPLC-DAD	HF-SPME	1—240	12,000	0.4	1.0		[28]
Plasma	HPLC-FD	SBSE	25—250	240	9.80	32.67		[17]
Urine	HPLC-DAD	MSPE	5—500	50,000	1.58	4.82		[18]
Serum	nano-LC-HRMS	HF-LPME	20—2500	250			20	Present study

DLLME – Dispersive liquid–liquid microextraction; DPX – disposable pipette extraction; GC–MS – gas chromatography coupled to mass spectrometry; HF-LPME – hollow-fiber liquid phase microextraction; HPLC-DAD – high performance liquid chromatography with diode array detector; HPLC-FD – high performance liquid chromatography with fluorescence detector; HPLC-UV – high performance liquid chromatography with ultraviolet detector; LC-MS/MS – liquid chromatography coupled to tandem mass spectrometry; LLE – liquid–liquid extraction; LOD – limit of detection; LOQ – limit of quantification; LLOQ – lower limit of quantification; MEPS – microextraction with packed sorbent; MSPE – magnetic solid phase extraction; nano-LC-HRMS – nano-liquid chromatography coupled to high resolution mass spectrometry; SBSE – stir bar sorptive extraction; SPE – solid phase extraction.

4. Conclusion

In this study, a bioanalytical method for the determination of FXT in human serum samples was proposed. The method employs a low-cost green sample preparation technique with excellent clean-up efficiency and pre-concentration factors, HF-LPME, and an analytical technique that provides high efficiency, high detectability, and reliable analyte identification, nano-LC-HRMS. A relevant aspect of the developed method is the use of a low volume of human serum, only 250 μL, which is an advantage when it comes to biological matrices, which tend to be available in limited amounts. This method was developed aiming at its application mainly for therapeutic drug monitoring, however, its application might be extended. The concentration range in which the method performed satisfactorily in terms of linearity, precision, and accuracy, which covers concentrations above the concentration that FXT exhibits high toxicity, as well as the possibility of dilution of higher concentration samples (1:4 v/v ratio), allows its application for forensic purposes, such as accidental or purposeful intoxications. Though application on incurred samples is limited in this study, the results obtained preliminarily are positive indicators for its broader application.

CRediT authorship contribution statement

Beatriz Isabella Cestaro: . **Kelly Cavalcanti Machado:** Investigation, Writing – review & editing. **Michel Batista:** Conceptualization, Resources, Writing – original draft, Writing – review & editing. **Bruno José Gonçalves da Silva:** Conceptualization, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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