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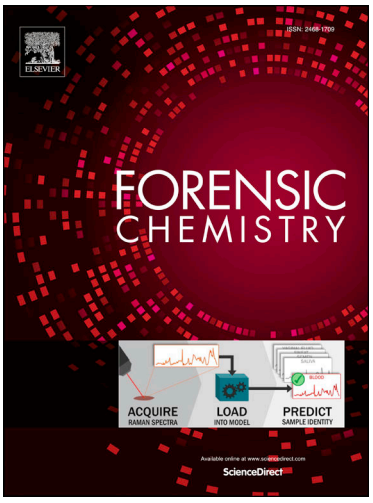

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Analysis of seven selected antidepressant drugs in post–mortem samples using fabric phase sorptive extraction followed by high performance liquid chromatography-photodiode array detection

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Abstract

Fabric phase sorptive extraction (FPSE), a recently introduced microextraction technique, was herein applied for the first time to achieve a simple and rapid simultaneous extraction of seven common antidepressant drugs (ADs, venlafaxine, citalopram, paroxetine, fluoxetine, sertraline, amitriptyline, and clomipramine) in post–mortem samples, particularly whole blood and cerebrospinal liquor collected during autopsies. By eliminating the protein precipitation step and

reducing solvent consumption, this technique resulted in sample preparation compliant with Green Analytical Chemistry (GAC) principles. FPSE uses a permeable and flexible substrate chemically coated with a sol–gel organic/inorganic sorbent as an extraction device. Among all tested FPSE membranes, the sol-gel Carbowax 20M (sol-gel CW 20M) coating on cellulose substrate showed optimal extraction efficiency for ADs. The selected drugs were analyzed and detected by the reverse phase high performance liquid chromatography (RP–HPLC) method coupled to photo diode array (PDA) detector. An isocratic elution that allows the complete separation of all analytes in only 20 min of chromatographic run time was applied using ammonium acetate buffer as aqueous mobile phase and acetonitrile (AcN) as the organic modifier. The limit of detection (LOD) ranged from 0.04 to 0.06 µg/mL, whereas limit of quantification (LOQ) was 0.1 µg/mL for all analytes except for Venlafaxine, which was 0.2 µg/mL.

Keywords: FPSE; microextraction; post–mortem samples; forensic sample preparation; antidepressant drugs; GAC.

1. Introduction

Major depressive disorder (MDD) is one of the most common psychiatric diseases in the world and is the leading cause of global disability [1]. For this reason, the prescription of antidepressant drugs (ADs) has rapidly increased in recent years, resulting in the most consumed drugs [2]. Nowadays, the etiology of depression is not fully understood, and several hypotheses have been proposed to explain its pathophysiological mechanisms. The most common theory regards the monoamine deficiency (serotonin and noradrenaline): it has been observed that low levels of these neurotransmitters in certain regions of the brain are responsible for the disorder. Other hypotheses associate MDD with hippocampal atrophy or stress system dysregulation, involving the hypothalamus–pituitary–axis of the adrenal gland [3].

The earliest treatments for depression were developed in the 1950s with the introduction of monoamine oxidase inhibitors (IMAO) and tricyclic antidepressants (TCAs). Since in the late 1980s, new generations of antidepressants have been introduced, including selective serotonin reuptake inhibitors (SSRIs), and serotonin and norepinephrine reuptake inhibitors (SNRIs) [4, 5]. The pharmacological action of these medications is precisely due to their ability to raise the level of norepinephrine and serotonin in neural synapses. However, approximately 20% of patients do not respond to current available treatments and about 33% do not achieve complete remission. Although, these drugs are used for therapeutic purposes, they are often associated with accidental deaths and suicides [2]. Indeed, numerous serious incidents have been associated with the consumption of these

drugs, mainly due to drug interactions. Overdose has also been associated with negative effects on human health, and for this reason therapeutic monitoring of concentrations in normal biological fluids is essential to ensure their safety and efficacy [6, 7].

Antidepressants are a class of drugs frequently found in forensic toxicology case. During autopsy, it is therefore important to investigate post–mortem drug concentrations to reveal whether the cause of death could be intoxication, side effects of medical treatment, or lack of compliance. Blood and urine samples are routinely collected sample matrices for toxicology analysis. Peripheral blood (PB) is considered as the gold standard for assessing post–mortem drug concentrations. The urine concentration does not necessarily reflect the concentration in the blood, as other factors such as metabolism, time elapsed since the last urination, etc. play important roles in the concentration of these drugs in urine. However, in some cases, it is not possible to collect PB, for example due to the putrefaction, charring, or large blood loss. In such cases, other matrices are usually collected, but for most drugs there is a lack of literature comparing concentrations in different matrices [8, 9]. Liquor cerebrospinalis (cerebrospinal fluid, CSF) is frequently analyzed in forensic cases, as it communicates with the extracellular space of the central nervous system (CNS) without any barriers.

In this context, analytical chemistry plays a fundamental role, based on the development of new methods for the analysis and quantification of different classes of drugs, including the analysis of ADs. Although today mass spectrometry (MS) represents the technique of choice in the forensic field, many laboratories do not have the possibility to access this type of instrumentation. Furthermore, by applying a good extraction technique and sample *clean–up*, it has been observed that it is possible to obtain good analytical performances even with less performing instruments, but available and accessible to all. Chromatographic techniques, such as high–performance liquid chromatography (HPLC) and gas chromatography (GC), coupled to different detectors, are suitable for these purposes [10, 11]. However, due to the complexity of the various biological matrices, an intensive sample preparation approach is often required, to provide an adequate sample for upstream instrumental analysis. The drug concentration in biological fluids is often low (trace and ultra–trace levels), and it is essential to choose a selective, specific, and efficient extraction method which allow to isolate and preconcentrate the target analytes [12].

Different sample preparation procedures have been reported in literature for the isolation and preconcentration of ADs from biological fluids, and the most commonly applied are solid–phase extraction (SPE), liquid–liquid extraction (LLE) and solid–phase microextraction (SPME) [13–18]. However, these techniques may have some disadvantages; in regard to the SPE, clogging and percolation problems can occur when working with biological matrices, while the LLE technique is

characterized by large volumes of solvents and complicated production processes, often expensive in terms of costs and time.

In 2014 Kabir and Furton have developed a new sample preparation technique, Fabric Phase Sorptive Extraction (FPSE) [19], arousing great interest in bioanalysis [20]. FPSE represents a sorbent-based extraction technique, i.e., those techniques that use a solid material for retaining specific compounds from a sample matrix; it is an alternative to traditional sample preparation techniques that have been widely applied for the extraction of drugs from biological fluids. This technique combines the exhaustive extraction mechanisms of SPE and equilibrium extraction mechanism of SPME in a single device, using a flexible and stable sorbent that can be directly introduced into the sample matrix for the extraction process [21, 22]. Numerous advantages have been made in sample preparation by FPSE, such as the reduction of solvents used in the extraction process, the reduction of sample volumes required and the possibility of working with complex matrices without pre–treatment steps; the FPSE has in fact been widely applied in the analysis of whole blood avoiding the preliminary protein precipitation [23–27]. Other advantages have also been demonstrated applying FPSE in non–conventional matrices (human saliva) [28, 29] or as an *in vivo* sampling device [30]. In the present study a novel method for the extraction, determination, and quantification of seven different antidepressant drugs (Venlafaxine, Citalopram, Paroxetine, Fluoxetine, Sertraline, Amitriptyline and Clomipramine) (**Table 1**) in post–mortem biological matrices, in particular blood and cerebrospinal liquor, have been described. The primary goal of this work was to determine the presence of these analytes in the selected matrices in order to verify not only the absorption of multiple antidepressants in combination, but also to assess the potential toxicity and post-death redistribution of these drugs. In the method validation, the choice of an innovative, fast, economical, and reproducible extraction procedure that respected the values of Green Analytical Chemistry (GAC) has been carried out [31, 32].

Table 1. Chemical structure and properties of antidepressants

2. Materials and methods

2.1 Chemical, solvents, and devices

Venlafaxine (VEN), Citalopram (CIT), Paroxetine (PAR), Fluoxetine (FLU), Sertraline (SER), Amitriptyline (AMIT), Clomipramine (CLO), and butoconazole (Internal Standard, IS) were purchased by Sigma–Aldrich (St. Louis, MO, USA). HPLC–grade Acetonitrile (AcN) was obtained

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from Sigma–Aldrich (St. Louis, MO, USA). HPLC–grade Methanol (MeOH) and Triethylamine (TEA) were purchased from Carlo Erba reagents (Milan, Italy). Ammonium acetate and acetic acid were found at Honeywell (Seleze, Germany). AcN, MeOH, and triethylamine were used without further purification processes. Ultrapure water (18.2 MΩ-cm2 at 25 ° C) for the HPLC analyzes was produced using a Millipore Milli–Q® system, which allows to produce highly purified and deionized water directly from tap water (Millipore Bedford Corp., Bedford, MA, USA). All FPSE membranes evaluated in this study, using 100% cellulose as substrate, sol–gel zwitterionic, sol–gel CW 20M, sol–gel poly(tetrahydrofuran) (sol–gel PTHF), sol–gel polydimethylsiloxane (sol–gel PDMS), sol–gel poly(ethylene glycol) –poly(propylene glycol) –poly(ethylene glycol) (sol–gel PEG–PPG–PEG), sol–gel poly(caprolactone–dimethylsiloxane– caprolactone) (sol–gel PCAP–PDMS–PCAP), sol–gel poly ethylene glycol 300 (sol–gel PEG 300), and sol–gel octadecyl (sol–gel C18) were kindly shipped from International Forensic Research Institute, Department of Chemistry and Biochemistry, Florida International University, Miami, FL (USA).

2.2 Preparation of standard solutions

The stock solutions of seven antidepressant drugs and IS were prepared by solubilizing 1 mg of pure substance in 1 mL of MeOH. These solutions were used for the preparation of the mix of the seven antidepressants at 1 mg/mL, with the addition of the internal standard, at the same concentration. The working solutions of each analyte, of the mix and of the IS were prepared in methanol by further dilutions at different concentrations (0.2–20 µg/mL). Matrix samples spiked with standard solutions were used for calibration and quality control samples (QCs) at three concentration levels of 0.5 (QC low), 2 (QC medium), and 10 (QC high) µg/mL.

2.3 Blood and liquor collection, storage, and preparation

Cadaveric whole blood and cadaveric cerebrospinalis liquor samples were obtained through a series of autopsies. For the collection, the judicial authority with specific assignment authorized the collection and analysis of post–mortem samples. The use of the samples was relevant to the subject of the judicial investigation and not to further research purposes, therefore, the opinion of the ethics committee and / or the consent of the representatives of the deceased was not binding.

As regarding whole blood samples, the collection was carried out through a venous sampling, while the cerebrospinal fluid was collected through a rachycentesis. The cadaveric blood was stored at + 4 °C while the liquor at –20 °C until the analysis; all the samples were brought at room temperature before being handled. Matrix–matched calibration curves were obtained using spiked samples

obtained by mixing biological matrices with analytes and Internal Standard (IS) at different concentration ranges from 0.2–20 μg/mL. No deproteinization steps were applied in the sample preparation workflow.

2.4 Instrumentation and chromatographic conditions

Chromatographic analyses were performed using a Waters corporation (Milford, MA, USA) liquid chromatograph equipped with a model 600 solvent pump and a 2996 photodiode array detector (PDA). Empower *v.2* Software (Waters Spa, Milford, MA, USA) was used for data acquisition. A C18 reversed–phase packing column (GreatSmart RP18, 150 mm × 4.6; 5 μm particle size) was used for the separation of seven antidepressant drugs and IS. The column oven (Jetstream2 Plus) was maintained at a constant temperature of 40 °C, which is designed to ensure maximum temperature stability and reproducibility. The UV/Vis acquisition wavelength was set in the range of 200–400 nm. The quantitative analyses were achieved at maximum wavelength for each compound. The sample injection was performed using a Rheodyne injection valve furnished with a 10 μL loop. Chromatographic separation was performed using ammonium acetate buffer solution (50mM, pH 5.5) and AcN (40:60, *v: v*) both with 0.3% of triethylamine in isocratic elution mode. The aqueous mobile phase was previously filtered through a cellulose nitrate filter membrane (0.2 μm pore size) using a glass vacuum–filtration system. The mobile phases were directly *on–line* degassed by using Biotech DEGASi, mod. Compact (LabService, Anzola dell'Emilia, Italy). The flow rate was set at 1.4 mL/min and all analytes and IS were eluted within 15 min. The total run time was set at 20 min.

2.5 Fabric phase membrane preparation and extraction process

The preparation of the sol–gel fabric membrane coated with inorganic/organic sorbent involves several steps: first the most suitable substrate was selected. A commercial fabric made of 100% cellulose and 100% polyester were selected as substrates. Before the sol–gel coating process, the fabric substrates need a thorough cleaning to remove residues or other unwanted dust and particles deposited on their surfaces. Additionally, this chemical treatment is useful to maximize the accessible hydroxyl functional groups involved in the sol–gel sorbent coating process. The fabric cleaning and treatment process was carried out as follow: 100% cellulose and 100% polyester fabric were individually soaked and cleaned with water and subsequently treated with a NaOH 1M for 1 hour at room temperature. The substrates were then washed again with water and subsequently treated with HCl 0.1M for 1 h. The cleaned and chemically treated pieces of fabric were then dried in an inert atmosphere for 12 h and subsequently stored in an airtight container until the coating process.

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The sol solution includes a polymer (organic or inorganic), a sol–gel precursor, a solvent system, a catalyst, and water. Since the polymer plays the most significant role in the final selectivity of the FPSE membrane, the selection of an appropriate polymer is the key to successful absorbent design. Considering the wide polarity range of the selected antidepressant drugs (*Log* $K_{o/w}$ values between 2.5 and 5.5), eight sol–gel absorbents were designed which include sol–gel zwitterionic, sol–gel CW 20M, sol–gel poly(tetrahydrofuran) (sol–gel PTHF), sol–gel polydimethylsiloxane (sol–gel PDMS), sol–gel poly(ethylene glycol)–poly(propylene glycol)–poly(ethylene glycol) (sol–gel PEG–PPG–PEG), sol–gel poly(caprolactone–dimethylsiloxane–caprolactone) (sol–gel PCAP–PDMS–PCAP), sol–gel poly ethylene glycol 300 (sol–gel PEG 300), and sol–gel octadecyl (sol–gel C18). All sol solutions were prepared using methyl trimethoxysilane (MTMS) as precursor, trifluoroacetic acid (TFA) as the acid catalyst, a mixture of acetone and methylene chloride (50:50, *v/v*) as the system solvent and deionized water as the hydrolytic agent. At the end, the coating process of the sol–gel sorbent is carried out by immersing the substrate in the sol solution in controlled reaction environments. The coating process was carried out for 6 hours. After coating, the coated fabric was air dried for 1 hour to dry residual solvents and subsequently held in a desiccator for 12 hours to harden and age the sol–gel absorbent. Finally, the FPSE membranes were rinsed with a mixture of methylene chloride: acetone (50:50, *v/v*) under sonication for 30 minutes. The cleaned FPSE membranes were air dried for 1 hour and stored until use. In particular, the system used for the synthesis of the sol–gel CW 20M membrane (used in this study for the extraction of target analytes) included: methyl trimethoxysilane (MTMS) as a sol–gel precursor, trifluoroacetic acid (TFA) as an acid catalyst, a mixture of acetone and methylene chloride (50:50, *v/v*) as the solvent system and deionized water as the hydrolytic agent. The molar ratio of sol–gel precursor, organic/inorganic polymer, acetone, methylene chloride, TFA and water was optimized and maintained at 1:0.0071:1.94:2.3:0.75:3.

One of the main advantages of this technique is the possibility to reuse the device Infact, in in several works it was observed that the membranes can be used several times and after a cleaning phase in organic solvent the membranes are totally free of residues. The FPSE device can be regenerated and reused without loss of extraction efficiency and the regeneration is easily carried out with suitable organic solvents; the device is then dried and stored under specific conditions for future reuse [11, 33-34].

To define the best sorbent among those available, the physical and chemical characteristics of these compounds were studied (in particular *LogP* values). Considering the polar and medium polar nature of these compounds, FPSE sorbents with these characteristics were preferred. Another factor to consider is certainly the biocompatibility of the device, since the analyzes are carried out in

biological matrices. Anyway, when developing an FPSE protocol, it is recommended to select at least three best FPSE membranes, and subsequently select the best sorbent experimentally (exposing all selected membranes to the preliminary conditions). Consequently, eight membranes were designated for the primary experiments: sol–gel CW 20M, sol–gel PCAP–PDMS–PCAP, sol–gel PEG–PPG–PEG, sol–gel PEG 300, sol–gel C18, sol–gel PDMS, sol–gel PTHF, and sol–gel zwitterionic. The following steps characterize the FPSE developed protocol: (*i*) the membranes were cut with a *home-made* puncher into round disks with the same surface area (diameter of 1 cm); (*ii*) activation with 2 mL of acetonitrile: methanol (50:50, *v:v*) for 5 min; (*iii*) washing in 2 mL of Milli–Q deionized water; (*iv*) extraction for 20 minutes by direct immersion of the membrane into 500 µL of sample; (*v*) back-extraction in 150 µL of MeOH for 5 min; (*vi*) centrifugation at 12000 × g for 10 min; (*vii*) injection of 10 µL of supernatant into the chromatographic system (*see* ***Supplementary Materials Section 1***). This procedure derives from an optimization process described in detail in *Section 3.2*.

3. Results and discussion

3.1 Optimization of HPLC separation

During the method development, various HPLC parameters such as chromatographic column, mobile phases solvent and composition, flow rate, and temperature were tested. The Great Smart RP18 (150 mm × 4.6 mm; 5 µm particle size) column was used, while mobile phases were ammonium acetate (0.05 M, pH 5.5) and AcN (65:35, *v:v*). The flow rate was set at 1.4 mL/min, maintaining the temperature at 40 °C. The tailing phenomena that were observed from the beginning of the optimization, was solved by adding 0.3% of TEA to both mobile phases. Venlafaxine was eluted at 3.65 ± 0.07 min, Citalopram at 4.38 ± 0.09, Paroxetine at 5.15 ± 0.13, Fluoxetine at 5.48 ± 0.12, IS at 7.03 ± 0.12, Sertraline at 8.56 ± 0.15, Amitriptyline at 11.65 ± 0.25 and Clomipramide at 13.78 ± 0.31. The peaks were fully resolved, and no interferences or overlaps were observed at the retention times. The wavelengths of 226, 239, 294, 227, 276, 230, 240, and 252 nm was selected for Venlafaxine, Citalopram, Paroxetine, Fluoxetine, IS, Sertraline, Amitriptyline, and Clomipramide, respectively, to obtain the best signal–to–noise ratio during chromatographic analysis.

3.2 Optimization of the extraction procedure – FPSE

Compared to solid–phase micro–extraction (SPME) and similar sorbent–based techniques, optimization of FPSE procedure is simple and straightforward. This technique requires no sample pretreatment process to minimize interferents, such as filtration, protein precipitation or centrifugation, and the membrane can be introduced directly into the sample, regardless of the

complexity of the matrix. However, extraction efficiency can be substantially improved following an optimization strategy where several factors were evaluated, such as sorbent chemistry and substate (FPSE membrane), sample volume, extraction time, back–extraction time, solvent, and volume. The FPSE optimization involved many steps, aimed at obtaining the best extraction efficiency for target compounds. Furthermore, the evaluation of the various parameters was carried out by gradually modifying one variable at a time (OVAT) and maintaining the best conditions. In this study, eight different FPSE membranes were evaluated: sol–gel CW 20M, sol–gel PCAP–PDMS–PCAP, sol–gel PEG–PPG–PEG, sol–gel PEG 300, sol–gel C18, sol–gel PDMS, sol–gel PTHF and sol–gel zwitterionic. First of all, the membranes were cut into 1 cm (0.785 cm^2 surface area) discs. The initial procedure chosen to test all the membranes consisted in washing the membrane in 2mL of an ACN: MeOH mixture (50:50, *v: v*) for 5 minutes, in order to eliminate any impurities. Subsequently, to remove the organic solvent, each membrane was rinsed in 2 mL of milli–Q water, and finally placed inside the sample (500 µL of the mix of 7 analytes and IS) for 30 minutes. Subsequently, the membrane was immersed in 150 µL of MeOH for 30 minutes, to favor the process of analytes desorption. The sample was then and 10 µL were injected into the HPLC system. This procedure was carried out for each membrane, under the same conditions, in order to evaluate the one that provided the higher enrichment factor (%). The enrichment factors (%) were calculated as the percentage of the peak area enhancement with respect to the area of reference standard solutions. The enrichment factor is a parameter that takes into account the concentration of the analytes in the processed sample (*cp*) and the concentration in the initial and untreated sample (*ci*).

$$EF\ \% = \frac{Cp}{Ci}\ x\ 100$$

A high enrichment factor indicates a good increase in analyte concentration during sample preparation. Based on the results obtained from this preliminary analysis, the three membranes that gave the best enrichment factors (sol–gel CW 20M, sol–gel PTHF and sol–gel zwitterionic) were tested in subsequent experiments. For these FPSE membranes different extraction times were tested: 5 min, 15 min, 20 min, 30 min and 60 min. Among them, the sol–gel CW 20 coated cellulose FPSE membrane showed the best values for all antidepressant drugs when an extraction time of 20 min was applied (**Table 2**). In bold have been reported the better values.

Table 2. Enrichment factors (%) observed for three different FPSE membranes

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Thanks to the high chemical stability of the FPSE device, resulting from the strong chemical bonds between the substrate and the coating, any organic solvent can be tested during the optimization process. The back–extraction solvent must ensure that the analytes are desorbed from the FPSE carrier, using the smallest volume possible. Extraction and elution times are directly related to the distribution coefficients of the analytes, that establish the adsorption equilibrium between the FPSE medium and the sample solution, affecting the performance of FPSE procedure. Thus, they were optimized. About that, different solvents, and mixture of solvent (MeOH, AcN, and a mixture of both) were tested to these volumes: 150 µL, 200 µL, 300 µL and 400 µL. The back-extraction time must be necessary to ensure adequate desorption of the analytes extracted from the membrane. However, during optimization protocol, it is good to test a longer time interval between 0 and 20 minutes. The back extraction time tested in this study were: 5 min, 10 min, 15 min and 20 min. The best performance in back–extraction was obtained using 150 µL of methanol for 5 min. No further improvement in drug recovery was observed after this time. After selecting the optimal conditions using standard aqueous solutions, the optimization was further performed on post–mortem samples (cadaveric blood and liquor) spiked with antidepressant drugs and internal standard, which confirmed the previously obtained data. All graphs related to FPSE parameters (sorbent type, extraction time, Back extraction solvent and volume) optimization have been showed in ***Supplementary Materials Section 2***.

3.3 Method validation

The reported method was validated according to the International Guidelines [35, 36] and linearity, selectivity, precision, and trueness (intra ed inter day), LOD, and LOQ were evaluated in aqueous standard solutions, cadaveric blood samples and encephalic cadaveric liquor. The linearity (intercept, slope, determination coefficient and variation) in the range from 0.1 to 20 µg/mL was evaluated by plotting the analyte/IS ratio area on the ordinate and the concentration of each standard solution (and matrices spiked with IS and analytes at different concentration) on the abscissas and repeating the analyzes in quadruplicate for each concentration (**Figure 1**). In the range tested, the curves showed a linear correlation and determination coefficients $r^2 \geq 0.9800$ for cadaveric liquor and $r^2 \geq 0.9797$ for cadaveric blood. LOD and LOQ were calculated by signal–to–noise (S/N) of 3 and 10 respectively. For LOQs were also considered Bias% values that must be within ± 20%. The limit of quantification (LOQ) was 0.2 µg/mL for venlafaxine and 0.1 µg/mL for other analytes in cadaveric liquor, while in cadaveric blood it was result 0.1 µg/mL for citalopram and amitriptyline and 0.2 µg/mL for other analytes. The limit of detection (LOD), the lowest concentration of analyte that can be detected by the method, was found to be: 0.06 µg/mL for venlafaxine and 0.04 µg/mL for the other

analytes in cadaveric liquor, whereas in cadaveric blood it was found to be 0.06 μg/mL for venlafaxine and clomipramine and 0.04 μg/mL for the remaining antidepressants.

Cadaveric blood and liquor were spiked at three concentration levels of AD drugs (0.5, 2, 10 μg/mL) and analyzed in order to evaluate the precision and the trueness of the method. Concentrations of each compound were calculated from the respective linear regression equation and the results were expressed by means of recovery percentage (mean concentration found/concentration*100, R%), estimating the trueness of the method. The precision was evaluated by calculating the relative standard deviation (RSD) for the repeated measurements. Within–day precision was assessed by performing four analyses at the same day whereas between–day precision was determined by triplicate measurements repeated for four consecutive days. The RSD% and Bias% were less than ± 15%, according to current guidelines (***Supplementary Materials Section 3-4***) Validation data have been reported in **Table 3.**

Figure 1. Calibration curve in cadaveric blood (left) and cadaveric liquor (right) built by plotting the analyte/IS ratio area on the ordinate and the concentration of each standard solution (and matrices spiked with IS and analytes at different concentration) on the abscissas

Table 3. Mean linear calibration curve parameters performed by weighted-linear least-squares regression analysis of six independent calibrations in cadaveric blood and liquor

The selectivity was evaluated by analyzing blank matrices (collected during autopsies) to verify the absence of interferents. Subsequently, the chromatograms of the samples fortified with the seven antidepressants and the internal standard were evaluated (**Figure 2**). The comparison between the chromatograms of the double blank and spiked blank with allowed to verify the real absence of interferents in the matrix. No interferences, either from endogenous matrix components, were observed.

Figure 2. Chromatograms of double blank and blank fortified with Is and analytes for cadaveric blood (left) and cadaveric liquor (right) where target analytes (Ads) and their related retention times have been highlighted

3.4 Application on Real Samples

Before the analysis of the different real samples, a study was done on the biological cadaveric matrices by consulting various articles in the literature. The study was focused on identifying the

points with respect to the concentrations used in the calibration (therefore falling within the range 0.1µg/mL–20µg/mL). The following concentrations were founded for cadaveric liquor: Venlafaxine 0.4 µg/mL, Citalopram 0.3 µg/mL, Paroxetine 0.15 µg/mL, Fluoxetine 0.45 µg/mL, Sertraline 0.3 µg/mL, Amitriptyline 0.4 µg/mL, Clomipramine 0.15 µg/mL, whereas for whole blood the concentrations were Venlafaxine 0.4 µg/mL, Citalopram 0.15 µg/mL, Paroxetine 0.15 µg/mL, Fluoxetine 0.45 µg/mL, Sertraline 0.3 µg/mL, Amitriptyline 0.4 µg/mL, and Clomipramine 0.15 µg/mL [2, 37–39]. As can be observed, all concentrations mostly calculated in post-mortem specimens are higher than the Limit of Quantification (0.20 µg/mL for Venlafaxine and 0.10 µg/mL for other ADs) and the Limit of Detection (0.06 µg/mL for Venlafaxine, Paroxetine, Fluoxetine, Sertraline and Clomipramine and 0.04 µg/mL for Citalopram and Amitryptiline in cadaveric blood, while 0.06 µg/mL for Venlafaxine and 0.04 µg/mL for other ADs in cadaveric liquor) of the herein reported method.

The established analytical method has been successfully applied for the bioanalysis of real samples obtained from various autopsies, in particular postmortem venous blood and cerebrospinal fluid. Unequivocally, this test was the most decisive criterion for evaluating the efficacy of the analytical method and the extraction efficiency of FPSE. The real samples (blood and cadaveric liquor) were kindly provided by the Department of Forensic Medicine of the "*G. D'Annunzio*" University. The analysis was conducted on four real samples, in particular: three venous blood samples and cerebrospinal sample, belonging to three different subjects. The matrices were stored at + 4 °C (cadaveric blood) and –20 °C (cadaveric liquor) and brought to room temperature before the analysis. Subsequently the samples were subjected to optimized FPSE extraction and by means of the subsequent HPLC–PDA analysis according to our validated method, the concentrations of these compounds were calculated. The obtained results showed a negative result for all samples. The analysis carried out and the evaluation of the anamnestic data emerging from the deposited documentation further confirmed the negativity, therefore the absence of antidepressants in the matrices considered, confirming the validity of the optimized method.

Moreover, one cadaveric blood and cadaveric liquor sample have been spiked with AD drugs, adding the concentration usually detected in these matrices after therapeutic treatments with these drugs. The obtained data have been reported in the Table below (**Table 4**).

Table 4. Data obtained from spiked cadaveric blood and cadaveric liquor

Conclusions

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Since the introduction of this new extraction platform in 2014, several studies in the literature have widely reported FPSE application in various field, immediately attracting the interest of the scientific community, since it offers many advantages, such as a reduction in time and costs, a substantial simplification of the sample preparation workflow, elimination of most of the pre–treatment processes and allows the use of minimum quantities of solvents, in full compliance with the Green Analytical Chemistry (GAC) principle while maintaining maximum extraction efficiency. In the described study, a new FPSE–HPLC–DAD method for simultaneous extraction/preconcentration and determination of seven antidepressant drugs in post–mortem samples was developed and validated. The biological fluids were handled without prior protein precipitation, avoiding loss of analytical data. Among the different FPSE membranes evaluated, the FPSE cellulose membrane coated with sol–gel CW 20 M proved to be an appropriate and effective means for the extraction procedure, presenting the highest absolute recoveries for all compounds of interest. The extraction and elution time, the elution solvent system and the sample volume have been optimized, resulting in a simple and fast analytical method. The consumption of organic solvents has been significantly minimized and, therefore, this sample preparation technique can be considered compliant with the GAC approach as well as low cost. A full validation was performed and showed good sensitivity and accuracy. However, further studies with positive samples are needed for full validation and to make the method widely accessible for forensic studies.

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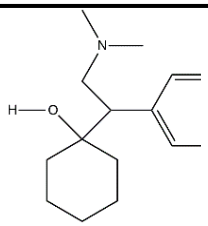
Highlights

- ✓ Fast chromatographic method for simultaneous analysis of seven ADs
- ✓ Direct analysis of post-mortem samples
- ✓ First application of FPSE in post-mortem whole blood and cerebrospinal fluid
- ✓ Method suitable for forensic purposes
- ✓ Performances comparable to a more complex and expensive instrumentations

Table 2. Chemical structure and properties of antidepressants

	Type	Chemical structure	Molecular formula	Molecular Weight (g/mol)	pKa	LogP
Venlafaxine	SNRI		$C_{17}H_{27}NO_2$	277,401	8.91	2.74

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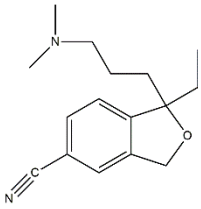
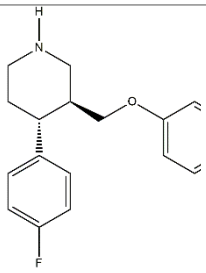
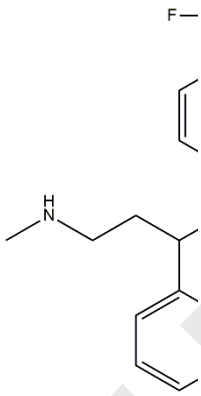
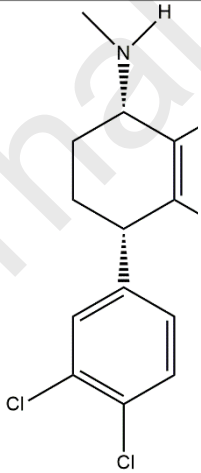
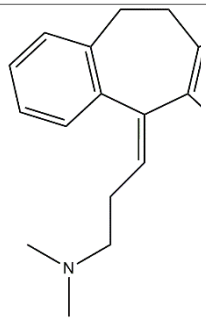
Citalopram	SSRI		$C_{20}H_{21}FN_2O$	324,39	9.78	3.76
Paroxetine	SSRI		$C_{19}H_{20}FNO_3$	329,37	9.90	2.53
Fluoxetine	SSRI		$C_{17}H_{18}F_3NO$	309,33	9.80	4.05
Sertraline	SSRI		$C_{17}H_{17}Cl_2N$	306,23	9.16	5.51
Amitriptyline	TCA		$C_{20}H_{23}N$	277,40	9.40	4.92

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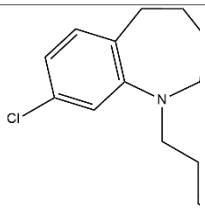
Clomipramine	TCA		$C_{19}H_{23}ClN_2$	314,90	9.20	5.19

SNRI: Serotonin and norepinephrine reuptake inhibitor; SSRI: Selective serotonin reuptake inhibitors; TCA Tricyclic antidepressants.

Table 2. Enrichment factors (%) observed for three different FPSE membranes

Extraction time 20min			
	sol–gel ZWITTERIONIC	**sol–gel CW 20M**	**sol–gel PTHF**
Venlafaxine	11.04	**17.33**	8.49
Citalopram	28.70	**44.44**	33.03
Paroxetine	46.04	**41.90**	37.25
Fluoxetine	53.76	**62.49**	55.78
IS	78.20	**96.59**	88.48
Sertraline	110.80	**88.09**	89.62
Amytriptiline	72.66	**98.54**	94.29
Clomipramine	86.95	**122.66**	121.33
Mean	61.02	**71.50**	66.03

Table 3. Mean linear calibration curve parameters performed by weighted-linear least-squares regression analysis of six independent calibrations in cadaveric blood and liquor

CADAVERIC BLOOD						
Analytes	**Linearity range (μg/mL)**	**Slope[a]**		**Intercept[a]**		**r^2**
		Mean	**Std. Dev.**	**Mean**	**Std. Dev.**	
Venlafaxine	0.2–20 (0.06 μg/mL)[b]	0.1420	0.004661	-0.003621	0.001827	0.9936
Citalopram	0.1–20 (0.04 μg/mL)[b]	0.3403	0.018430	0.201500	0.003290	0.9827
Paroxetine	0.1–20 (0.06 μg/mL)[b]	0.1297	0.004739	-0.001957	0.001738	0.9934
Fluoxetine	0.1–20 (0.06 μg/mL)[b]	0.4615	0.019800	-0.036550	0.007761	0.9891
Sertraline	0.1–20 (0.06 μg/mL)[b]	0.2702	0.015870	-0.016010	0.006220	0.9797
Amitryptiline	0.1–20 (0.04 μg/mL)[b]	0.6192	0.018490	0.0079590	0.003683	0.9929
Clomipramine	0.1–20 (0.06 μg/mL)[b]	0.2431	0.005255	0.0005762	0.002059	0.9972
CADAVERIC LIQUOR						

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Analytes	Linearity range (µg/mL)	Slope[a]		Intercept[a]		r^2
		Mean	Std. Dev.	Mean	Std. Dev.	
Venlafaxine	0.2–20 (0.06 µg/mL)[b]	0.0407	0.001824	-0.003239	0.0006691	0.9901
Citalopram	0.1–20 (0.04 µg/mL)[b]	0.2790	0.008011	0.02821	0.001595	0.9934
Paroxetine	0.1–20 (0.04 µg/mL)[b]	0.09303	0.005022	-0.0002557	0.0009999	0.9800
Fluoxetine	0.1–20 (0.04 µg/mL)[b]	0.3327	0.01181	-0.006141	0.002351	0.9913
Sertraline	0.1–20 (0.04 µg/mL)[b]	0.2367	0.01090	-0.006227	0.00217	0.9854
Amitryptiline	0.1–20 (0.04 µg/mL)[b]	0.4073	0.01766	-0.01555	0.003517	0.9852
Clomipramine	0.1–20 (0.04 µg/mL)[b]	0.1643	0.006171	-0.003844	0.001229	0.9888

Table 4. Data obtained from spiked cadaveric blood and cadaveric liquor

Cadaveric Blood			
Analytes	**Calculated conc. µg/mL**	**Spiked conc. µg/mL**	**BIAS %**
Venlafaxine	0.40	0.40	-1.14
Citalopram	0.15	0.15	-2.98
Paroxetine	0.14	0.15	-7.21
Fluoxetine	0.51	0.45	13.33
Sertraline	0.29	0.30	-3.17
Amitryptiline	0.39	0.40	-2.56
Clomipramine	0.15	0.15	1.79
Cadaveric Liquor			
Venlafaxine	0.40	0.40	-11.43
Citalopram	0.27	0.30	-8.36
Paroxetine	0.54	0.60	-9.18
Fluoxetine	0.17	0.15	12.29
Sertraline	0.31	0.30	3.10
Amitryptiline	0.41	0.40	2.78
Clomipramine	0.14	0.15	-7.16

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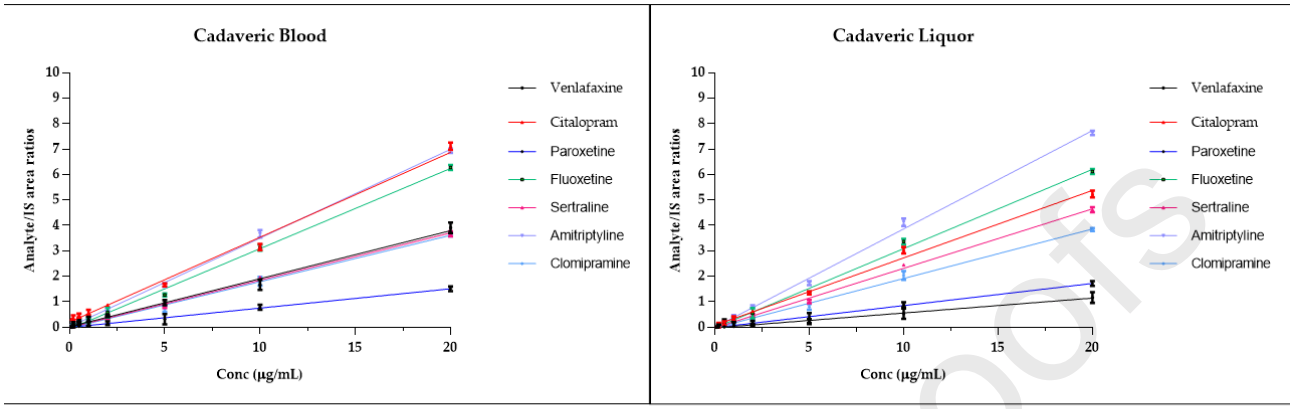

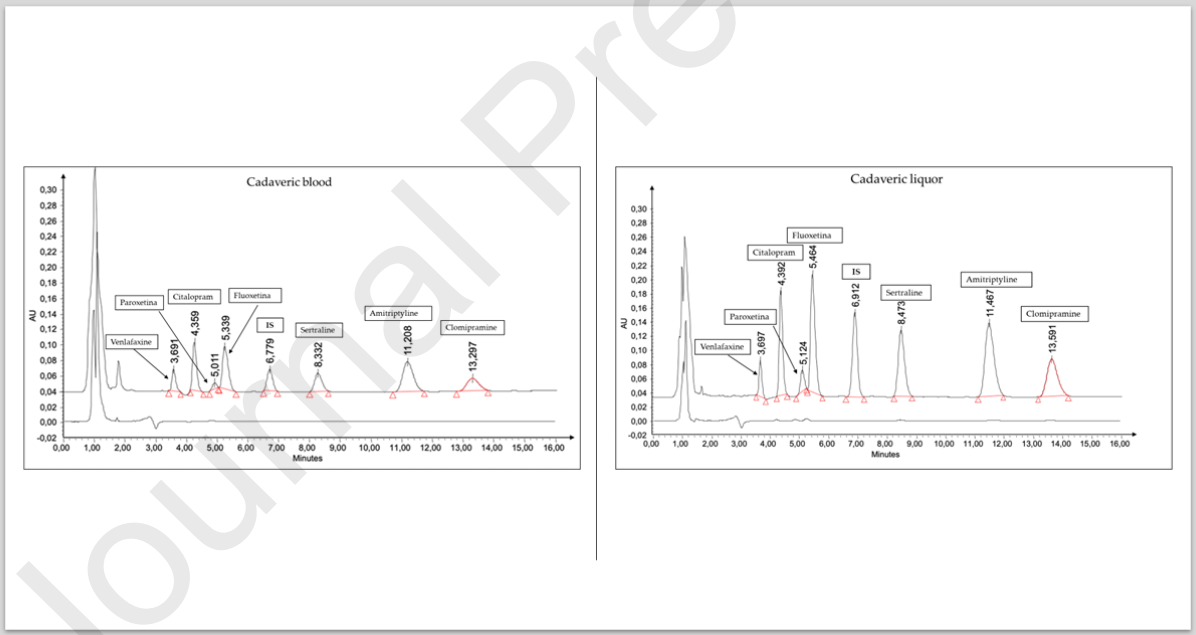

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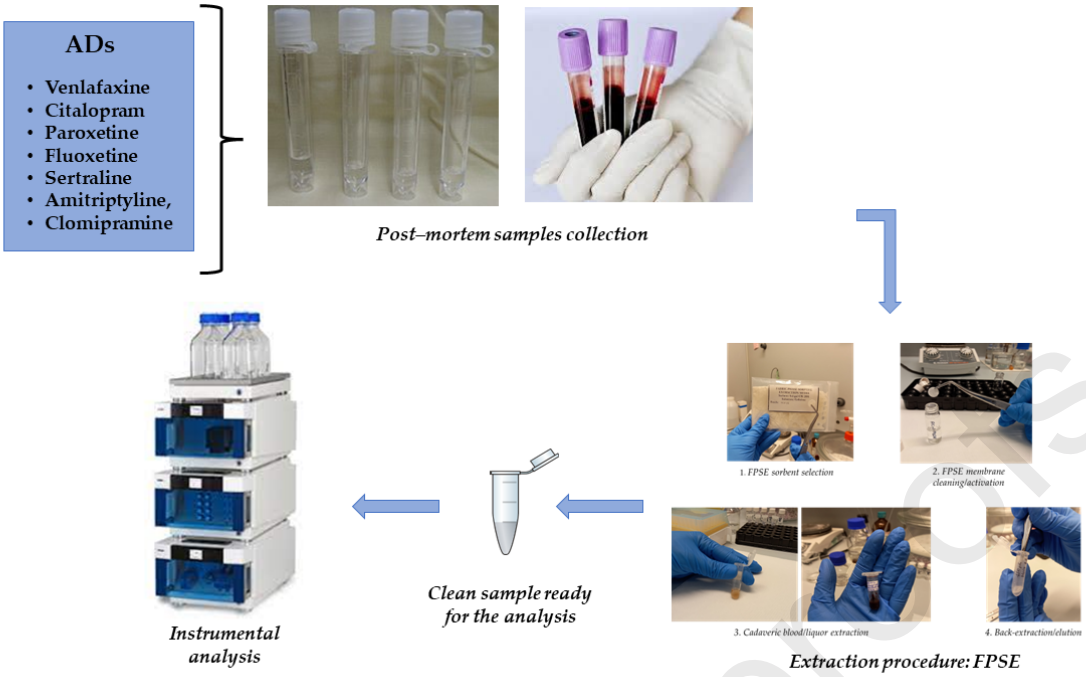

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