Advances in Sample Preparation

Fabric phase sorptive extraction (FPSE) as an efficient sample preparation platform for the extraction of antidepressant drugs from biological fluids

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Dear Editor

please find enclosed the revised version of the manuscript titled "**Fabric phase sorptive extraction (FPSE) as an efficient sample preparation platform for the extraction of antidepressant drugs from biological fluids**", submitted to **Advances in Sample Preparation**, as full article paper.

We thank the Editor for His evaluations and for the suggestions that have been all accepted and reported in the R2 version. Below are reported our point-by-point response to Editor comments.

The revised version of the manuscript has been read and approved by all authors, who declare no conflict of interest. This research has not been disclosed or published and is not under consideration for publication elsewhere.

Sincerely,

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Editor

Thank you for submitting your manuscript to Advances in Sample Preparation. I have completed my evaluation of your manuscript. You and your coworkers did a very good job revising the manuscript but the text requires a final revision (see comments below) after being accepted. I invite you to resubmit your manuscript after addressing the comments below.

Dear Editor, thank you for your evaluation of the herein submitted paper.

1. The next lines in the revised manuscript requires a revision "The recovery values ranged from 54.6% to 187.6, from 54.5% to 192.9% and from 34.19% to 197.61% in saliva. Whole blood and urine samples, respectively". The relative recoveries must be in the range from 70-120 % to be acceptable. I infer that the reported values are consequence of a mistake transforming BIAS into relative recoveries. Please, check this point.

As correctly highlighted, and also considering the BIAS% values reported in supplementary material S.3, the right relative recoveries were corrected. Now the lines are: "The recovery values ranged from 86.4% to 110, from 91.5% to 114% and from 87.5% to 112% in whole blood, urine, and saliva samples, respectively". These values are in agreement with the requested percentages (in the range from 70-120 %) in order to be accepted and validated.

2. Please, check that all the acronyms used in the main text (including well known acronyms like MS) are defined.

As correctly highlighted, some acronyms were not defined in the text. This point is revised in the current version

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Declaration of Competing Interest statement (Mandatory)

Authors declare no conflict of interests

Fabric phase sorptive extraction (FPSE) as an efficient sample preparation platform for the extraction of antidepressant drugs from biological fluids

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Abstract

The quantification and interpretation of drug concentrations in biological matrices to optimize pharmacotherapy and to perform the therapeutic drug monitoring (TDM) is particularly important for compounds with narrow therapeutic ranges, known to cause adverse effects. In these cases, the biomonitoring is essential to avoid the toxicity and side effects. In this study, an innovative fabric phase sorptive extraction (FPSE) followed by high performance liquid chromatography-photodiode array detection (FPSE–HPLC–PDA) method was optimized and validated for the extraction and quantitative evaluation of seven antidepressant drugs (ADs, venlafaxine, citalopram, paroxetine, fluoxetine, sertraline, amitriptyline, and clomipramine) in human whole blood, urine, and saliva samples.

The best chromatographic separation was obtained using a reverse phase column and ammonium acetate (50 mM, pH 5.5) and acetonitrile (AcN) as mobile phases, with 0.3% of triethylamine (TEA) for the best peak shape. The used sample preparation technique, FPSE, developed in 2014, has offered numerous advantages such as low consumption of organic solvents, no sample pretreatment, and reduced overall sample preparation time. Among all tested membranes, sol-gel carbowax (CW 20 M) sorbent, coated on cellulose FPSE media, was the most efficient. The developed method provides satisfactory limit of detection of 0.06 μg/mL for all analytes except for venlafaxine that was 0.04 μg/mL. Both RSD% and BIAS% gave values below ±15%, according to current guidelines. Finally, real samples analyses were carried out, comparing the obtained data with the anamnestic data of the subjects, confirmed the validity of the method.

Keywords: antidepressant drugs, TDM, biological matrices, FPSE, real samples analysis.

1. Introduction

Antidepressants drugs (ADs) are the most widely prescribed drugs to treat major depressive disorder (MDD) [1], a disabling disease that affect around 264 million people of all ages, representing one of the most serious public health problems [2, 3]. To date, the etiology of MDD is not yet completely clear, even if there are several theories that could explain the hypothetical pathological mechanisms, above all the deficiency of neurotransmitters, in particular monoamines (serotonin and noradrenaline) [4]. Indeed, drugs for the treatment of depression act on the regulation of these neurotransmitters, even if each class acts with different mechanisms. The most common classes of antidepressants are monoamine oxidase inhibitors (MAOIs), tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRIs) and serotonin and norepinephrine reuptake inhibitors (SNRIs). Nowadays, SSRIs and SNRIs represent the most used category in the treatment of depression, as they solve the main adverse effects of tricyclic antidepressants such as cardiotoxicity, central nervous system (CNS) toxicity and dose–dependent respiratory depression. However, the typical side effects of all antidepressant drugs, such as serotonin syndrome and serotonergic drug–drug interactions remain also with other drugs [5]. The broad increase in the use of antidepressant drugs is due to their role not only in the treatment of MDD, but also in the management of other related conditions such as anxiety, obsessive compulsive disorder, nutrition and sleep disorders and as therapy for neuropathic pain and chronic inflammatory diseases [1, 6].

An inadequate treatment with antidepressant drugs (failure to use appropriate drugs, as well as the use of inadequate doses) could lead to morbidity and mortality, both for the adverse effects that characterize them, and for the high inter-patient variability in pharmacokinetic properties [5, 7]. It is therefore essential to determine the concentration to evaluate toxicity, drugs interaction and individual effects in order to obtain the therapeutic drug monitoring (TDM). Adverse drug reactions and loss of response are areas where TDM can play a key role in improving outcome. TDM, based on the analysis, assessment, and evaluation of drug concentrations, become essential to optimize the patients' drug therapy and avoid toxicity phenomena [5, 8, 9].

This study aims to develop and validate a method for the simultaneous detection and quantification of seven antidepressant drugs in whole blood, urine and saliva samples using high performance liquid chromatography coupled with a photodiode array detector (HPLC–PDA).

Although TDM on plasma and serum samples is currently considered as the gold standard, this procedure has shown several drawbacks such as invasiveness of venous blood collection, controlled temperatures for shipping, and often drug concentrations in plasma or blood do not necessarily reflect those in target tissues or cells [10]. Therefore, in recent years attention has also

been paid to unconventional matrices, in order to reduce invasiveness and costs or to obtain better information on drug concentrations on the active site.

In the present study, several biological matrices were considered, including whole blood (analyzed without any pretreatment), urine and saliva samples. Following our previous work [11], also in this study, the main goal is to enable whole blood analysis; in fact, the main disadvantage of converting blood into plasma or serum is the inevitable loss of analytical information. Regarding other matrices, the sampling is less invasive, increasing the patient compliance. Saliva is a more sensitive matrix with a greater possibility of quantification than urine, easily providing positive results.

The innovative and *green* extraction technique, Fabric Phase Sorptive Extraction (FPSE), represents an economical and easy method [12] that allows the extraction of several compounds from biological matrices without tedious sample pretreatment processes or matrix modifications, [13] reducing the use of toxic solvents and adhering to the principles of Green Analytical Chemistry (GAC) [14, 15]. FPSE is a particularly versatile technique applicable to different complex matrices, even to high viscous ones (whole blood), without having to perform operations such as protein precipitation [16-20]. This innovative technique, developed in 2014 by Kabir and Furton [21] combines the exhaustive extraction mechanisms of SPE and equilibrium extraction mechanism of SPME in a single device, using a flexible cellulose membrane that can be introduced directly into the sample matrix for the extraction process [21]. In literature, several sample preparation procedures for the determination and preconcentration of AD including solid–phase extraction (SPE), liquid–phase extraction (LLE) and solid–phase microextraction (SPME) have been described [22, 23]. However, these techniques may present some disadvantages such as the percolation phenomenon for SPE and the use of large volumes of organic solvents for LLE, which involve time consuming and expensive processes. Furthermore, FPSE has numerous advantages also by applying it in unconventional matrices (human saliva) [20, 24] or as an *in vivo* sampling device [25].

In **Table 1** have been reported the seven ADs considered in the present study, including their physical and chemical characteristics.

Table 1. *Chemical structure and properties of selected antidepressants*

2. Materials and methods

2.1 Chemicals and materials

The chemical standard of Venlaflaxina (VEN), Citalopram (CIT), Paroxetine (PAR), Fluoxetine (FLU), Sertraline (SER), Amitriptyline (AMIT), Clomipramine (CLO) and Internal Standard (IS,

butoconazole) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ammonium acetate and acetic acid were purchased from Honeywell (Seleze, Germany), while acetonitrile (AcN, HPLC grade) was obtained from Sigma–Aldrich (St. Louis, MO, USA). HPLC grade methanol (MeOH) and triethylamine (TEA) were purchased from Carlo Erba Reagents (Milan, Italy). Ultrapure water (18.2MΩ–cm^2 at 25°C) for HPLC analysis and sample preparation was produced using a Millipore Milli–Q® system, (Millipore Bedford Corp., Bedford, MA, USA). All the FPSE membranes evaluated in this study, sol–gel zwitterionic, sol–gel CW 20M, sol–gel poly(tetrahydrofuran) (sol–gel PTHF), sol–gel polydimethylsiloxane (sol–gel PDMS), sol–gel polyethylene glycol–polypropylene glycol–polyethylene 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 synthetized at the Department of Chemistry and Biochemistry of Florida International University, Miami, FL (USA).

2.2 Preparation of standard solutions

The stock solutions first prepared for each single antidepressants were prepared by solubilizing 1 mg of substance in 1 mL of MeOH. The stock solution of standard mix was prepared at the same concentration in MeOH. Subsequently, working solutions were obtained by further dilutions in the same solvent (0.2–20 μg/mL). Three concentration levels were selected as quality controls (QCs), 0.5 μg/mL (low QC), 2 μg/mL (medium QC) and 10 μg/mL (high QC). IS stock solution was also prepared in MeOH.

2.3 Blood, urine and saliva collection, storage, and preparation

Whole blood, urine and saliva were collected from healthy volunteers and did not undergo any deproteinization process. Regarding the whole blood samples, the collection was carried out by venous sampling and the matrix was stored at 4°C until the analysis. Urine and saliva samples, collected in falcon tube, were stored at –20°C until the analyses. All biological matrices were brought at room temperature and vortexed before being handled. The calibration curves were obtained spiking biological matrices with selected antidepressants and the internal standard (IS) at different concentration, obtaining the final solution in a range between 0.2 to 20 μg/mL.

2.4 Instrumentation and chromatographic conditions

Chromatographic separation was performed using a Waters Corporation (Milford, MA, USA) 600 controller instrument coupled to a 2996 Photodiode Array Detector (PDA). The column used for

antidepressant and internal standard (IS) separation was a C18 column GraceSmart® RP (150 mm x 4.6 mm; 5 µm), maintained at a constant temperature of 40°C using a column oven (Jetstream2 Plus).

The mobile phases used were ammonium acetate buffer (50 mM, pH 5.5) (mobile phase A) and AcN (mobile phase B), both added with 0.3% of TEA. The mobile phases were *on–line* degassed by the Biotech 4CH DEGASI Compact system (LabService, Anzola dell'Emilia, Italy). The optimized flow rate was set at 1.4 mL/min. The final injection volume, using a Rheodyne valve, was 10 µL. The aqueous mobile phase was previously filtered through a cellulose nitrate filter membrane (0.2 µm pore size) using a glass vacuum–filtration system and both phases were previously sonicated. Data processing was carried out using the Empower software. The wavelength used for the acquisition falls within a range between 200–400 nm, while the quantitative analyses were obtained at the maximum wavelength for each analyte of 226, 239, 294, 227, 276, 273, 240 and 252 nm for venlafaxine, citalopram, paroxetine, fluoxetine, IS, sertraline, amitriptyline, and clomipramine, respectively. The section related to the optimization of chromatographic conditions have been inserted in the *Supplementary Materials Section 1*.

2.5 Fabric phase membrane preparation and extraction process

Eight different membranes were selected for the preliminary 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. These membranes were evaluated considering the polarity range of the selected antidepressant drugs (*Log P* values between 2.5 and 5.5). The choice of the most suitable polymer was an essential operation, as it is responsible for the selectivity of the device, representing the first source of bonding with target analytes. Compared to other extraction techniques based on solid absorbents/supports/sorbents, fabric phase sorbent extraction exploits the material properties of a fabric substrate that retains chemically bonded sol–gel sorbent material on its surface, an organically modified sol–gel precursor that connects the sol–gel sorbent network to the substrate and an organic/inorganic polymer/carbonaceous particles which offers high selectivity towards analytes [26]. The synthesis of the membrane, based on sol–gel technology, has involved several steps: first, the most suitable support was selected. A commercial fabric consisting of 100% cellulose was preferred as substrates, a material that easily adapts to the different nature of the matrices. For the synthesis, the cleaned and chemically treated pieces of fabric were inserted into the sol–solution, consisting of a polymer (organic or inorganic), a sol–gel precursor, a solvent system, a catalyst, and water. The entire coating process was performed for 6 hours. At the end, the cleaned FPSE membranes were air dried for 1 hour and stored until use. For the sol–gel CW–20M, the membrane that led to better enrichment factors in this study, the molar ratio of sol–gel precursor,

organic/inorganic polymer, acetone, methylene chloride, TFA, and water was optimized and kept at 1:0.0071:1.94: 2.3: 0.75:3. After cutting the membrane into 1 cm diameter circular discs, the FPSE medium was immersed in a mixture of 2 mL of MeOH:AcN (50:50 *v:v*) to remove any impurities and at the same time to activate the functional groups of the device. After that, the clean tissue was rinsed in 2 ml milli-Q water for 5 minutes to remove residual organic solvents. The FPSE device was then inserted into the vial containing the sample (500 µL) for 20 minutes under stirring. After extraction, the FPSE device was removed from the vial, and for back-extraction of the retained analytes, the tissue was placed in a clean vial containing 150 µL of MeOH for 5 min. The extract was centrifuged and injected into the chromatographic system.

2.6 Method validation

The method validation was performed according to the International Guidelines [27, 28] in order to estimate the selectivity, limit of detection (LOD), limit of quantification (LOQ), linearity, intra- and inter-day trueness and precision, and recovery of AD drugs in whole blood, urine and saliva samples. The selectivity was examined by analyzing double blank, zero blank and blank spiked with IS and ADs, in order to exclude interferences in the same retention times as for the target analytes. The linearity was evaluated by applying the least–squares linear regression analysis, by plotting the peak area/IS versus analyte concentration for both standard solutions and spiked biological matrices. The slope, intercept and correlation coefficient were calculated for all the antidepressant drugs. LOD and LOQ were calculated by signal–to–noise (S/N) of 3 and 10 respectively. Three concentration levels (0.5, 2, 10 µg/mL) were selected to evaluate both intra and interday precision and trueness. 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.

3. Results and discussion

3.1 Optimization of the extraction procedure – FPSE

The optimization of the FPSE extraction process involved numerous steps, aim to the optimization of all involved parameters. To obtain the maximum extraction efficiency of the compounds, all the parameters were gradually optimized, following the One Variable at Time (OVAT) approach. In this way, each parameter is gradually optimized through experiments. The

sorbent material was first selected, evaluating eight different FPSE membranes: 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–PTHF gel and sol–gel zwitterionic. Each support was cut into 1 cm discs and activate in 2 mL of MeOH:AcN mixture (50:50, *v:v*) for 5 minutes. This step permits to eliminate materials impurities and to activate functional groups for subsequently interactions. The membranes were then rinsed in Milli–Q water to remove organic solvent residues before the insertion into the sample for the extraction process. The initial general conditions selected for the extraction process in order to test the different membranes involved an extraction in 500 µL of standard solution (analytes and IS at 10 µg/mL) for 30 minutes under stirring. Subsequently the membrane was immersed in 150 µL of MeOH for 30 minutes, for the desorption step (back–extraction). At the end, the samples were centrifuged for 10 minutes and 10 µl of supernatant were injected into the HPLC system. This procedure was performed for each membrane, under the same conditions, to select the one that provided the highest enrichment factor (%). These values were calculated as a percentage of the improvement in the peak area compared to the area of the reference standard solutions. Among all tested membranes, sol–gel CW–20M, sol–gel PTHF and sol–gel zwitterionic initially gave the best Enrichment Factors (%). At this point, other parameters have been evaluated for these membranes, starting from extraction time (5, 15, 20, 30, and 60 min). The sol–gel CW-20M support showed the best values for all antidepressant drugs with an extraction time of 20 minutes (**Table 2**). The best values have been reported in bold.

Table 2. *Enrichment factors (%) observed for three different FPSE membranes (extraction time 20 min)*

Subsequently, different solvents and solvent mixtures (MeOH, AcN and a mixture of both) were tested as elution phase. Since the FPSE supports are particularly resistant, different types of organic solvents could be select. Moreover, it is essential to optimize the back–extraction volume, as the organic solvent should ensure the analytes desorption from the FPSE device using the minimum volume. Different volumes were tested: 150 µL, 200 µL, 300 µL and 400 µL at different back–extraction times (5, 10, 15 and 20 min). The best performance in elution step was obtained using 150 µL of pure MeOH for 5 minutes. After selecting the optimal conditions using standard aqueous solutions, the optimization was further performed on the biological matrices (whole blood, urine and saliva) spiked with antidepressant drugs and IS, which confirmed the previously obtained data. All the graphs related to the optimization of the FPSE parameters have been shown ***in Supplementary materials Section 2.***

3.2 HPLC optimized condition

The best resolution of the selected antidepressants has been achieved through an isocratic separation which includes an ammonium acetate buffer (50mM, pH 5.5) as mobile phase A and AcN as mobile phase B. The chromatographic column was the GraceSmart® RP18 (150 mm × 4.6 mm; 5 μm particle size). The flow rate was set at 1.4 mL/min, maintaining the column temperature at 40 ° C. Venlafaxine was eluted at 3.532 min, Citalopram at 4.403 min, Paroxetine at 5.193 min, Fluoxetine at 5.562 min, Sertraline at 8.739 min, Amitriptyline at 12.149 min, and Clomipramine at 14.504 min. The maximum wavelength for each analyte were 226, 239, 294, 227, 276, 273, 240 and 252 nm for venlafaxine, citalopram, paroxetine, fluoxetine, IS, sertraline, amitriptyline, and clomipramine, respectively. No endogenous interference was noted in these retention times in the three matrices considered.

3.3 Method validation results and data

The reported method was validated according to international guidelines [27, 28] and selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), precision and trueness were evaluated. Least squares linear regression analysis was applied to calculate slope, intercept and correlation coefficient for both standard solutions and spiked whole blood, urine and saliva samples. Linearity (intercept, slope, coefficient of determination and variation) was evaluated by plotting the area of the analyte/IS ratio on the ordinate and the concentration of each standard solution (and matrices added with IS and analytes at different concentrations) on the abscissa and repeating the analyzes in quadruplicate for each concentration (**Figure 1**). To obtain the calibration curves, the analyzes were performed in triplicate, for the concentrations included in the range, 0.2 – 20 μg/mL. Over the range tested, the curves showed linear correlation and coefficients of determination $r^2 \geq 0.9916$ for whole blood, $r^2 \geq 0.9928$ for urine and $r^2 \geq 0.9812$ for saliva. LOD and LOQ was evaluated by the signal–to–noise ratio (S / N) of 3 and 10, respectively. The limit of quantification (LOQ) was 0.2 μg/mL for venlafaxine and 0.1 μg/mL for others. The Limit of Detection (LOD), the lowest detectable analyte concentration by the method, was 0.06 μg/mL for venlafaxine and 0.04 μg/mL for the other analytes in all considered matrices. Trueness across days was assessed by running analyzes in quadruplicate on the same day, while between-day accuracy was determined by measuring in triplicate for four consecutive days. Finally, RSD% and BIAS% gave values below ± 15%, according to current guidelines **(*Supplementary Materials Section 3*)**. The recovery values ranged from 86.4% to 110, from 91.5% to 114% and from 87.5% to 112% in whole blood, urine, and saliva samples, respectively.

All validation data were reported in **Table 3** (whole blood, urine, and saliva) and the entire method validation was performed according to the international guidelines [27, 28].

Figure 1. *Calibration curve in* ***a.*** *human whole blood;* ***b.*** *human urine;* ***c.*** *human saliva evaluated over the concentration range. The area response ratio (analytes vs. internal standard) was fitted to the nominal concentration using the simplest model through GraphPad software*

Table 3. *Mean linear calibration curve parameters performed by weighted-linear least-squares regression analysis of six independent calibrations in human whole blood, urine, and saliva*

3.4 Application on Real Samples

The established analytical method was applied to bioanalysis of real samples collected from healthy donor. This last phase was the decisive step to evaluate the effectiveness of both the analytical method and the innovative FPSE extraction technique. Real samples (whole blood, urine, and saliva) were obtained from four separate volunteers who were not taking antidepressants. The matrices were, first, stored at +4°C (whole blood) and –20°C (urine and saliva) and brought to room temperature before analysis. Subsequently, the samples were subjected to optimized FPSE extraction and by means of the subsequent HPLC–PDA analysis according to the validated method. The concentrations of these compounds were then calculated. The results obtained showed a negative result for all the samples, confirming the validity of the analytical method, since the result confirmed the absence of antidepressants in the matrices considered. In addition, some whole blood, urine and saliva samples have been spiked with AD drugs, adding concentrations that are usually found after therapeutic treatments with these drugs. The chromatograms related to real samples have been reported in ***Supplementary Materials Section 4***. The data obtained were reported in the **Table 4**.

Table 4. *Data obtained from spiked whole blood, urine and saliva*

Discussion and Conclusions

The main goal of therapeutic drug monitoring (TDM) is to maximize the therapeutic effect while minimizing the likelihood of side effects. TDM has become a common practice in clinical investigations, especially for drugs with a narrow therapeutic index such as anticancer, antiepileptic, antidepressant, etc. Unfortunately, there are still several limitations, such as the high costs related to the collection, preparation and analysis of the samples, the shipment to the few certified laboratories in controlled conditions. However, to date, for routine TDM practice, the reference matrices remain

blood and, above all, plasma given the numerous data available regarding the therapeutic ranges in these matrices. In recent years, the use of alternative and above all non–invasive matrices for TDM (and in particular for pharmacokinetic studies) such as saliva, urine and hair are starting to find different applications. In this way, several advantages could be introduced for TDM such as the low required volumes, no need for sample storage at low temperatures (−20°C/−80°C or dry ice), cheaper and simpler way to send sample and the major compliance of patients.

In the reported study, an FPSE-HPLC-PDA method was optimized for the determination of seven antidepressants in whole blood, urine, and saliva. The application of this innovative extraction method brought numerous advantages during in the sample preparation step, such as the minimal sample manipulation, avoiding protein precipitation or other purification processes, as well as the excellent recovery values. The reported method was compared with other methods reported in literature, that also have used other extraction techniques like solid phase extraction coupled to liquid chromatography-tandem mass spectrometry (SPE-LC-MS/MS), dispersive liquid-liquid microextraction coupled to gas chromatography-tandem mass spectrometry (DLLME-GC-MS/MS), or similar. In the present work, all aspects of the extraction technique were optimized to obtain the maximum extraction efficiency. The main point to underline is the reduced volume of organic solvent that was used, as the extraction procedure only has involved 150 µL of MeOH. Although the extraction times are very similar to other methods that use the FPSE as an extraction procedure [12, 26], the volume reduction in the elution phase is certainly an important point to consider as an advantage of the developed procedure. Furthermore, it must be emphasized that the present method has also extended the number of target analytes considered.

The method has been validated in terms of linearity, selectivity, accuracy and precision, and has been shown to be suitable for analysis in whole blood, urine and saliva samples, of antidepressants to assess concentration during therapeutic monitoring. An innovative a simple analytical method that reflect all the values of the Green Analytical Chemistry (GAC) have been reported. In conclusion, the use of alternative matrices is an increasingly topical topic, which presents an important potential for future applications to be introduced in clinical practice. Certainly, there is still a lot of work to be done and many data will need to be available to validate these matrices in a routine clinical setting. At the same time, these alternative matrices will find increasing interest and applicability for TDM studies, particularly when coupled with highly sensitive analytical techniques.

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Figure 1

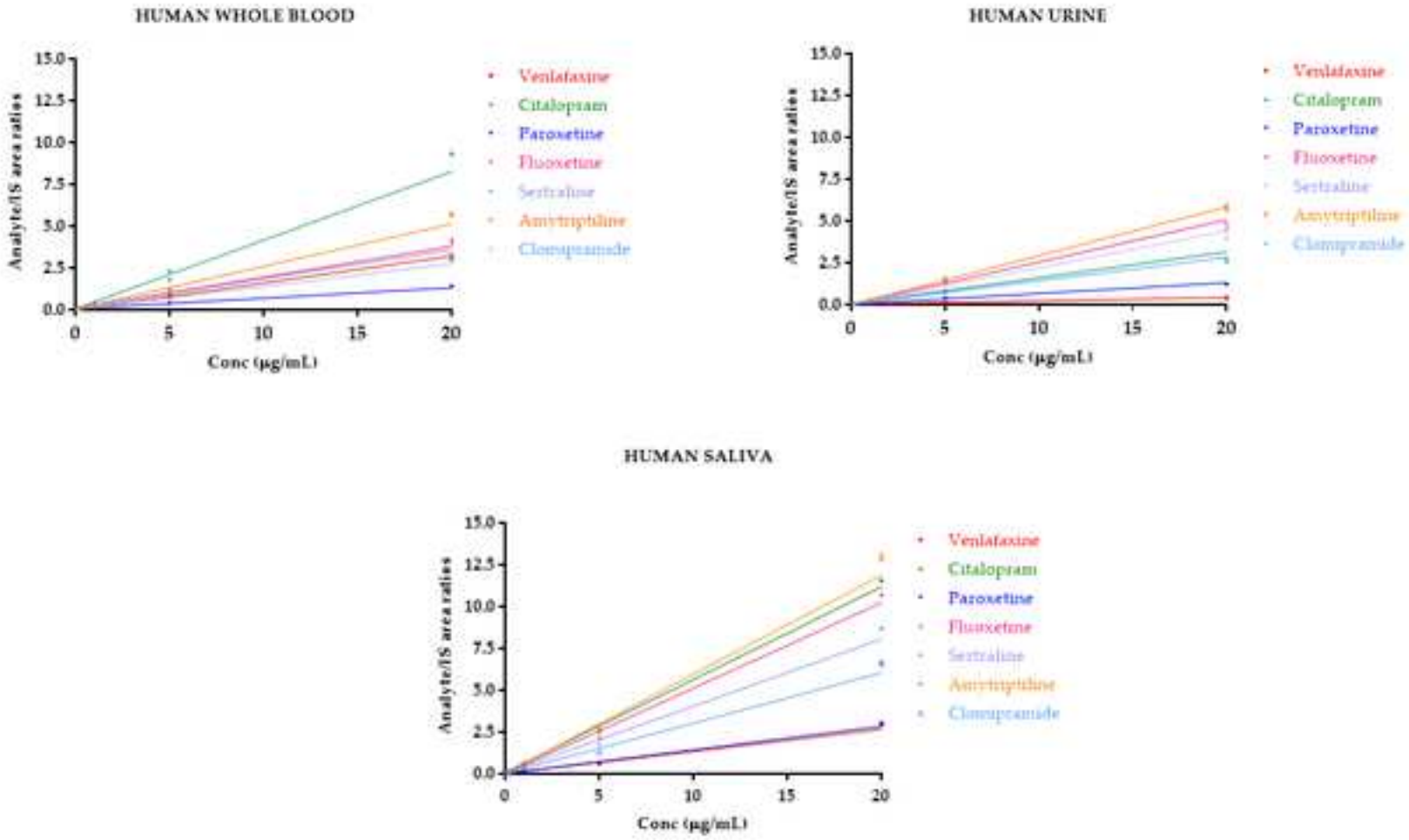
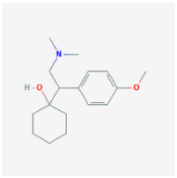
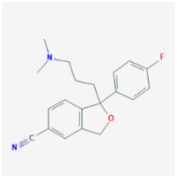
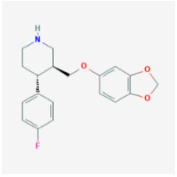
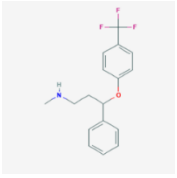
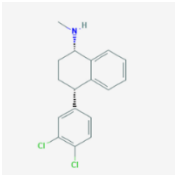
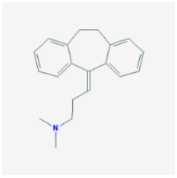
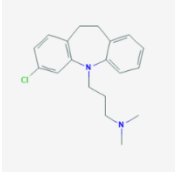


Table 1. *Chemical structure and properties of selected antidepressants*

	Chemical structure	Molecular formula	Molecular Weight (g/mol)	pKa	LogP
Venlafaxine SNRI		$C_{17}H_{27}NO_2$	277.40	8.91	2.74
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		$C_{17}H_{18}F_3NO$	309.30	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
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 20 min)*

	sol–gel ZWITTERIONIC	**sol–gel CW 20M**	**sol–gel PTHF**
Venlafaxine	11.04 (±0.55)	**17.33** (±0.87)	8.49 (±0.42)
Citalopram	28.70 (±1.72)	**44.44** (±2.67)	33.03 (±1.65)
Paroxetine	46.04 (±2.30)	**41.90** (±2.51)	37.25 (±2.24)
Fluoxetine	53.76 (±3.23)	**62.49** (±3.75)	55.78 (±3.35)
IS	78.20 (±4.69)	**96.59** (±4.83)	88.48 (±4.24)
Sertraline	110.80 (±5.54)	**88.09** (±5.28)	89.62 (±5.38)
Amytriptiline	72.66 (±5.08)	**98.54** (±4.93)	94.29 (±4.71)
Clomipramine	86.95 (±5.22)	**122.66** (±6.13)	121.33 (±6.07)
Mean	61.02 (±3.05)	**71.50** (±3.58)	66.03 (±3.96)

Table 3. *Mean linear calibration curve parameters performed by weighted-linear least-squares regression analysis of six independent calibrations in human whole blood, urine, and saliva*

Analytes	Linearity range (μg/mL)	Slope[a]		Intercept[a]		LOD (μg/mL)	LOQ (μg/mL)	r^2
		Mean	Std. Dev.	Mean	Std. Dev.			
WHOLE BLOOD								
Venlafaxine	0.2–20	0.2166	±0.008353	-0.00806	±0.003274	0.06	0.2	0.9977
Citalopram	0.1–20	0.6671	±0.02018	0.0190255	±0.004019	0.04	0.1	0.9947
Paroxetine	0.1–20	0.0978	±0.002133	-0.007773	±0.0003808	0.04	0.1	0.9974
Fluoxetine	0.1–20	0.3491	±0.00823	-0.014015	±0.001638	0.04	0.1	0.9958
Sertraline	0.1–20	0.1677	±0.00682	-0.003475	±0.001289	0.04	0.1	0.9916
Amitriptyline	0.1–20	0.4935	±0.009876	-0.014845	±0.001966	0.04	0.1	0.9937
Clomipramine	0.1–20	0.2098	±0.005833	-0.004625	±0.001041	0.04	0.1	0.9980
URINE								
Venlafaxine	0.2–20	0.02902	±0.0004916	-0.002418	±0.0001927	0.06	0.2	0.9928
Citalopram	0.1–20	0.2757	±0.006148	0.027465	±0.001162	0.04	0.1	0.9789
Paroxetine	0.1–20	0.1118	±0.003079	0.0963	±0.000578	0.04	0.1	0.9967
Fluoxetine	0.1–20	0.3977	±0.008161	-0.0061474	±0.001626	0.04	0.1	0.9956
Sertraline	0.1–20	0.3720	±0.008183	-0.0044435	±0.001629	0.04	0.1	0.9936
Amitriptyline	0.1–20	0.4437	±0.005318	-0.0063385	±0.001059	0.04	0.1	0.9965
Clomipramine	0.1–20	0.2256	±0.004515	-0.004566	±0.0008989	0.04	0.1	0.9929
SALIVA								
Venlafaxine	0.2–20	0.08247	±0.001108	-0.01709	±0.00041	0.06	0.2	0.9877
Citalopram	0.1–20	0.29860	±0.01142	0.13035	±0.002275	0.04	0.1	0.9997
Paroxetine	0.1–20	0.072255	±0.004324	0.01523	±0.00086	0.04	0.1	0.9949
Fluoxetine	0.1–20	0.27620	±0.01338	0.031705	±0.002666	0.04	0.1	0.9980
Sertraline	0.1–20	0.23460	±0.01361	0.016075	±0.002577	0.04	0.1	0.9812
Amitriptyline	0.1–20	0.33235	±0.0138	0.04845	±0.002749	0.04	0.1	0.9884
Clomipramine	0.1–20	0.16245	±0.007016	0.023275	±0.001396	0.04	0.1	0.9888

[a]Values at 95% confidence intervals on the mean of six independent calibration curves; the slope and intercept of calibration curve are expressed in μg/mL.

Table 4. *Data obtained from spiked whole blood, urine and saliva*

Analytes	Calculated conc. µg/mL	Spiked conc. µg/mL	BIAS %
Whole Blood			
Venlafaxine	0.35	0.40	-13.7
Citalopram	0.29	0.30	-4.51
Paroxetine	0.60	0.60	-0.56
Fluoxetine	0.45	0.45	0.16
Sertraline	0.17	0.15	13.3
Amitriptyline	0.35	0.40	-11.7
Clomipramine	0.40	0.45	-11.1
Urine			
Venlafaxine	0.41	0.40	2.99
Citalopram	0.29	0.30	-4.62
Paroxetine	0.69	0.60	14.6
Fluoxetine	0.27	0.30	-9.40
Sertraline	0.28	0.30	-6.10
Amitriptyline	0.42	0.40	4.33
Clomipramine	0.40	0.45	-11.5
Saliva			
Venlafaxine	0.36	0.40	-9.74
Citalopram	0.17	0.15	11.5
Paroxetine	0.14	0.15	-3.48
Fluoxetine	0.26	0.30	-12.3
Sertraline	0.29	0.30	-2.10
Amitriptyline	0.36	0.40	-9.34
Clomipramine	0.16	0.15	9.99

Table 5. *Comparison with other methods*

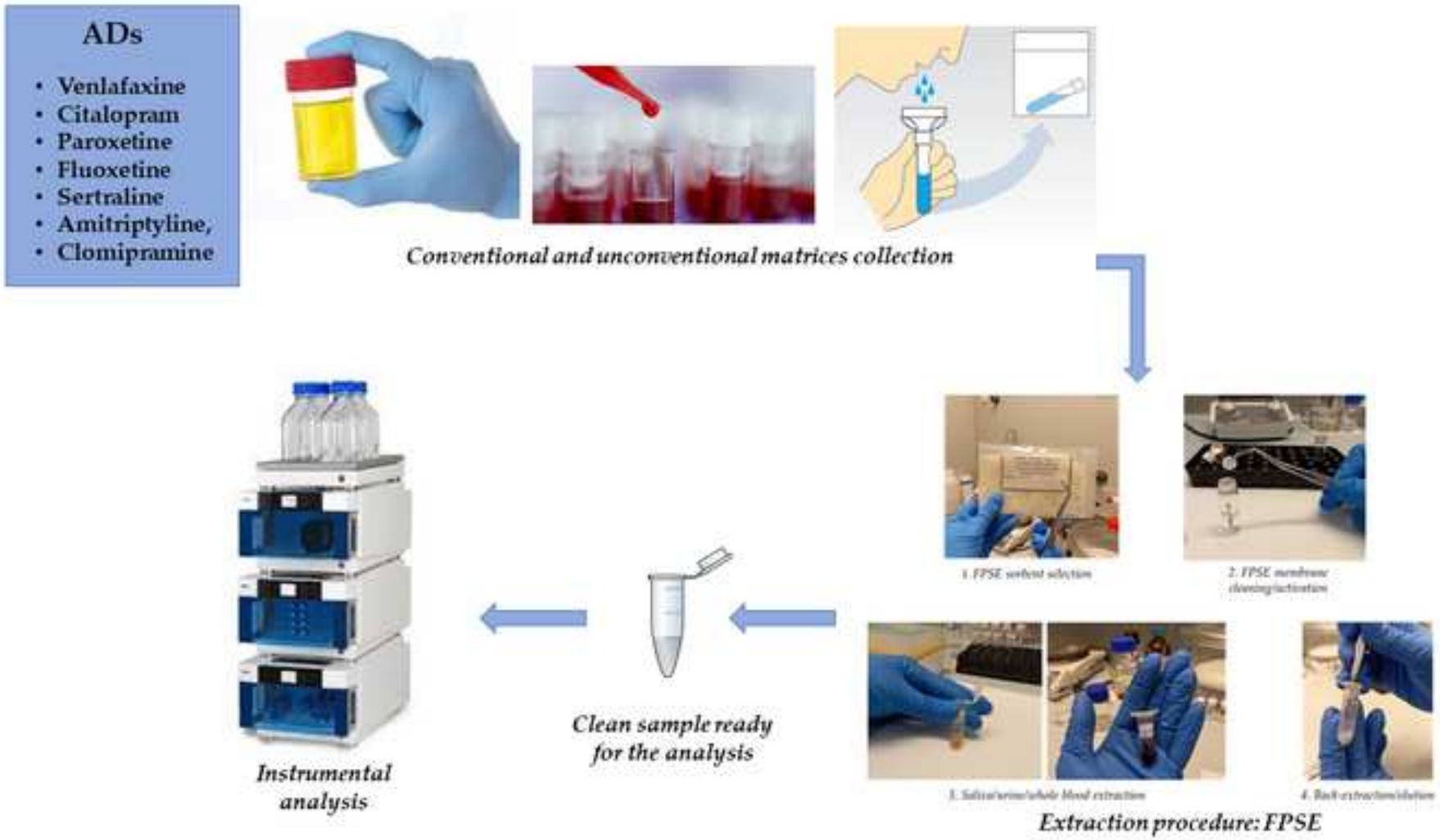
Sample	***Analytes***	***Extraction***	***Instrument***	***Run time (min)***	***LOD***	***REF***
Human serum	Venlafaxine Paroxetine Fluoxetine Amitriptyline Clomipramine	FPSE	HPLC–DAD	15	0.15 ng/μL	[12]
Human urine	Venlafaxine Proxetine Fluoxetine Amitriptyline Clomipramine	FPSE	HPLC–DAD	15	0.15 ng/μL	[26]
Oral fluid	Fluoxetine Venlafaxine *o*-desmethylvenlafaxine Citalopram Sertraline Paroxetine	DSS	GC–MS/MS	25	10–100 ng/mL	[29]
Vitreous humor	Amitriptyline Nortriptyline Citalopram Clomipramine Fluoxetine Maprotiline Mirtazapine Sertraline Venlafaxine Desmethylmaprotiline Desmethylmirtazapine	SPE followed by derivatization with Heptafluorobutyric Anhydride	GC–MS/MS	-	1.50 ng/mL	[30]

	Desmethylsertraline *o*-desmethylvenlafaxine,					
Rat plasma	Citalopram Sertraline	SPE	LC–MS/MS	-	0.12 ng/mL 0.19 ng/mL	[31]
Urine	Carbamazepine Citalopram Clomipramine Desipramine	Capillary extraction column	LC–MS/MS	10	500–20000 ng/L	[32]
Urine Plasma	Amitriptyline Nortriptyline Clomipramine Imipramin	DSPE–DES–AALLME	GC–MS	20	8–15 ng/L 32–60 ng/L	[33]
Plasma Urine	Bupropion Citalopram Amitriptyline Trazodone	BAμE	HPLC–DAD	28	50 ng/L	[34]
Urine Saliva Whole Blood	Venlafaxine Citalopram Paroxetine Fluoxetine Sertraline Amitriptyline Clomipramine	FPSE	HPLC–DAD	15	40 ng/mL	Current work

FPSE: Fabric Phase Sorptive Extraction; HPLC–DAD: High Performance Liquid Chromatography–Diode Array Detector; DSS: Dried Saliva Spot; GC–MS/MS: Gas Chromatography–Tandem Mass Spectrometry; SPE: Solid–Phase Extraction; LC–MS/MS: Liquid Chromatography–Tandem Mass Spectrometry; DSPE–DES–AALLME: dispersive solid–phase extraction–deep eutectic solvent–air–assisted liquid–liquid microextraction; BAμE: bar adsorptive microextraction.

Highlights

- ✓ Fast method for simultaneous analysis of seven ADs
- ✓ Analysis of biological matrices
- ✓ FPSE application in conventional and unconventional matrices
- ✓ Method suitable for TDM of ADs
- ✓ Performances comparable to a more complex and expensive instrumentations



Fabric phase sorptive extraction (FPSE) as an efficient sample preparation platform for the extraction of antidepressant drugs from biological fluids

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Abstract

The quantification and interpretation of drug concentrations in biological matrices to optimize pharmacotherapy and to perform the therapeutic drug monitoring (TDM) is particularly important for compounds with narrow therapeutic ranges, known to cause adverse effects. In these cases, the biomonitoring is essential to avoid the toxicity and side effects. In this study, an innovative fabric phase sorptive extraction (FPSE) followed by high performance liquid chromatography-photodiode array detection (FPSE–HPLC–PDA) method was optimized and validated for the extraction and quantitative evaluation of seven antidepressant drugs (ADs, venlafaxine, citalopram, paroxetine, fluoxetine, sertraline, amitriptyline, and clomipramine) in human whole blood, urine, and saliva samples.

The best chromatographic separation was obtained using a reverse phase column and ammonium acetate (50 mM, pH 5.5) and acetonitrile (AcN) as mobile phases, with 0.3% of triethylamine (TEA) for the best peak shape. The used sample preparation technique, FPSE, developed in 2014, has offered numerous advantages such as low consumption of organic solvents, no sample pretreatment, and reduced overall sample preparation time. Among all tested membranes, sol-gel carbowax (CW 20 M) sorbent, coated on cellulose FPSE media, was the most efficient. The developed method provides satisfactory limit of detection of 0.06 µg/mL for all analytes except for venlafaxine that was 0.04 µg/mL. Both RSD% and BIAS% gave values below ±15%, according to current guidelines. Finally, real samples analyses were carried out, comparing the obtained data with the anamnestic data of the subjects, confirmed the validity of the method.

Keywords: antidepressant drugs, TDM, biological matrices, FPSE, real samples analysis.

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1. Introduction

Antidepressants drugs (ADs) are the most widely prescribed drugs to treat major depressive disorder (MDD) [1], a disabling disease that affect around 264 million people of all ages, representing one of the most serious public health problems [2, 3]. To date, the etiology of MDD is not yet completely clear, even if there are several theories that could explain the hypothetical pathological mechanisms, above all the deficiency of neurotransmitters, in particular monoamines (serotonin and noradrenaline) [4]. Indeed, drugs for the treatment of depression act on the regulation of these neurotransmitters, even if each class acts with different mechanisms. The most common classes of antidepressants are monoamine oxidase inhibitors (MAOIs), tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRIs) and serotonin and norepinephrine reuptake inhibitors (SNRIs). Nowadays, SSRIs and SNRIs represent the most used category in the treatment of depression, as they solve the main adverse effects of tricyclic antidepressants such as cardiotoxicity, central nervous system (CNS) toxicity and dose–dependent respiratory depression. However, the typical side effects of all antidepressant drugs, such as serotonin syndrome and serotonergic drug–drug interactions remain also with other drugs [5]. The broad increase in the use of antidepressant drugs is due to their role not only in the treatment of MDD, but also in the management of other related conditions such as anxiety, obsessive compulsive disorder, nutrition and sleep disorders and as therapy for neuropathic pain and chronic inflammatory diseases [1, 6].

An inadequate treatment with antidepressant drugs (failure to use appropriate drugs, as well as the use of inadequate doses) could lead to morbidity and mortality, both for the adverse effects that characterize them, and for the high inter-patient variability in pharmacokinetic properties [5, 7]. It is therefore essential to determine the concentration to evaluate toxicity, drugs interaction and individual effects~~.~~ in order to obtain the therapeutic drug monitoring (TDM). Adverse drug reactions and loss of response are areas where TDM can play a key role in improving outcome. TDM, based on the analysis, assessment, and evaluation of drug concentrations, become essential to optimize the patients' drug therapy and avoid toxicity phenomena [5, 8, 9].

This study aims to develop and validate a method for the simultaneous detection and quantification of seven antidepressant drugs in whole blood, urine and saliva samples using high performance liquid chromatography coupled with a photodiode array detector (HPLC–PDA).

Although TDM on plasma and serum samples is currently considered as the gold standard, this procedure has shown several drawbacks such as invasiveness of venous blood collection, controlled temperatures for shipping, and often drug concentrations in plasma or blood do not necessarily reflect those in target tissues or cells [10]. Therefore, in recent years attention has also

been paid to unconventional matrices, in order to reduce invasiveness and costs or to obtain better information on drug concentrations on the active site.

In the present study, several biological matrices were considered, including whole blood (analyzed without any pretreatment), urine and saliva samples. Following our previous work [11], also in this study, the main goal is to enable whole blood analysis; in fact, the main disadvantage of converting blood into plasma or serum is the inevitable loss of analytical information. Regarding other matrices, the sampling is less invasive, increasing the patient compliance. Saliva is a more sensitive matrix with a greater possibility of quantification than urine, easily providing positive results.

The innovative and *green* extraction technique, Fabric Phase Sorptive Extraction (FPSE), represents an economical and easy method [12] that allows the extraction of several compounds from biological matrices without tedious sample pretreatment processes or matrix modifications, [13] reducing the use of toxic solvents and adhering to the principles of Green Analytical Chemistry (GAC) [14, 15]. FPSE is a particularly versatile technique applicable to different complex matrices, even to high viscous ones (whole blood), without having to perform operations such as protein precipitation [16-20]. This innovative technique, developed in 2014 by Kabir and Furton [21] combines the exhaustive extraction mechanisms of SPE and equilibrium extraction mechanism of SPME in a single device, using a flexible cellulose membrane that can be introduced directly into the sample matrix for the extraction process [21]. In literature, several sample preparation procedures for the determination and preconcentration of AD including solid–phase extraction (SPE), liquid–phase extraction (LLE) and solid–phase microextraction (SPME) have been described [22, 23]. However, these techniques may present some disadvantages such as the percolation phenomenon for SPE and the use of large volumes of organic solvents for LLE, which involve time consuming and expensive processes. Furthermore, FPSE has numerous advantages also by applying it in unconventional matrices (human saliva) [20, 24] or as an *in vivo* sampling device [25].

In **Table 1** have been reported the seven ADs considered in the present study, including their physical and chemical characteristics.

Table 1. *Chemical structure and properties of selected antidepressants*

2. Materials and methods

2.1 Chemicals and materials

The chemical standard of Venlaflaxina (VEN), Citalopram (CIT), Paroxetine (PAR), Fluoxetine (FLU), Sertraline (SER), Amitriptyline (AMIT), Clomipramine (CLO) and Internal Standard (IS,

butoconazole) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ammonium acetate and acetic acid were purchased from Honeywell (Seleze, Germany), while acetonitrile (AcN-(, HPLC grade) was obtained from Sigma–Aldrich (St. Louis, MO, USA). HPLC grade methanol (MeOH) and triethylamine (TEA) were purchased from Carlo Erba Reagents (Milan, Italy). Ultrapure water (18.2MΩ–cm^2 at 25°C) for HPLC analysis and sample preparation was produced using a Millipore Milli–Q® system, (Millipore Bedford Corp., Bedford, MA, USA). All the FPSE membranes evaluated in this study, sol–gel zwitterionic, sol–gel CW 20M, sol–gel poly(tetrahydrofuran) (sol–gel PTHF), sol–gel polydimethylsiloxane (sol–gel PDMS), sol–gel polyethylene glycol–polypropylene glycol–polyethylene 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 synthetized at the Department of Chemistry and Biochemistry of Florida International University, Miami, FL (USA).

2.2- Preparation of standard solutions

The stock solutions first prepared for each single antidepressants were prepared by solubilizing 1 mg of substance in 1 mL of MeOH. The stock solution of standard mix was prepared at the same concentration in MeOH. Subsequently, working solutions were obtained by further dilutions in the same solvent (0.2–20 µg/mL). Three concentration levels were selected as quality controls (QCs-), 0.5 µg/mL (low QC), 2 µg/mL (medium QC) and 10 µg/mL (high QC). IS stock solution was also prepared in MeOH.

2.3- Blood, urine and saliva collection, storage, and preparation

Whole blood, urine and saliva were collected from healthy volunteers and did not undergo any deproteinization process. Regarding the whole blood samples, the collection was carried out by venous sampling and the matrix was stored at 4°C until the analysis. Urine and saliva samples, collected in falcon tube, were stored at –20°C until the analyses. All biological matrices were brought at room temperature and vortexed before being handled. The calibration curves were obtained spiking biological matrices with selected antidepressants and the internal standard (IS) at different concentration, obtaining the final solution in a range between 0.2 to 20 µg/mL.

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2.4- Instrumentation and chromatographic conditions

Chromatographic separation was performed using a Waters Corporation (Milford, MA, USA) 600 controller instrument coupled to a 2996 Photodiode Array Detector (PDA). The column used for

antidepressant and internal standard (IS) separation was a C18 column GraceSmart® RP (150 mm x 4.6 mm; 5 µm), maintained at a constant temperature of 40°C using a column oven (Jetstream2 Plus).

The mobile phases used were ammonium acetate buffer (50 mM, pH 5.5) (mobile phase A) and AcN (mobile phase B), both added with 0.3% of TEA. The mobile phases were *on–line* degassed by the Biotech 4CH DEGASI Compact system (LabService, Anzola dell'Emilia, Italy). The optimized flow rate was set at 1.4 mL/min. The final injection volume, using a Rheodyne valve, was 10 µL. The aqueous mobile phase was previously filtered through a cellulose nitrate filter membrane (0.2 µm pore size) using a glass vacuum–filtration system and both phases were previously sonicated. Data processing was carried out using the Empower software. The wavelength used for the acquisition falls within a range between 200–400 nm, while the quantitative analyses were obtained at the maximum wavelength for each analyte of 226, 239, 294, 227, 276, 273, 240 and 252 nm for venlafaxine, citalopram, paroxetine, fluoxetine, IS, sertraline, amitriptyline, and clomipramine, respectively. The section related to the optimization of chromatographic conditions have been inserted in the *Supplementary Materials Section 1*.

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2.5. Fabric phase membrane preparation and extraction process

Eight different membranes were selected for the preliminary 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. These membranes were evaluated considering the polarity range of the selected antidepressant drugs (*Log P* values between 2.5 and 5.5). The choice of the most suitable polymer was an essential operation, as it is responsible for the selectivity of the device, representing the first source of bonding with target analytes. Compared to other extraction techniques based on solid absorbents/supports/sorbents, fabric phase sorbent extraction exploits the material properties of a fabric substrate that retains chemically bonded sol–gel sorbent material on its surface, an organically modified sol–gel precursor that connects the sol–gel sorbent network to the substrate and an organic/inorganic polymer/carbonaceous particles which offers high selectivity towards analytes [26]. The synthesis of the membrane, based on sol–gel technology, has involved several steps: first, the most suitable support was selected. A commercial fabric consisting of 100% cellulose was preferred as substrates, a material that easily adapts to the different nature of the matrices. For the synthesis, the cleaned and chemically treated pieces of fabric were inserted into the sol–solution, consisting of a polymer (organic or inorganic), a sol–gel precursor, a solvent system, a catalyst, and water. The entire coating process was performed for 6 hours. At the end, the cleaned FPSE membranes were air dried for 1 hour and stored until use. For the sol–gel CW–20M, the membrane that led to better enrichment factors in this study, the molar ratio of sol–gel precursor,

organic/inorganic polymer, acetone, methylene chloride, TFA, and water was optimized and kept at 1:0.0071:1.94: 2.3: 0.75:3. After cutting the membrane into 1 cm diameter circular discs, the FPSE medium was immersed in a mixture of 2 mL of MeOH:AcN (50:50 *v:v*) to remove any impurities and at the same time to activate the functional groups of the device. After that, the clean tissue was rinsed in 2 ml milli-Q water for 5 minutes to remove residual organic solvents. The FPSE device was then inserted into the vial containing the sample (500 µL) for 20 minutes under stirring. After extraction, the FPSE device was removed from the vial, and for back-extraction of the retained analytes, the tissue was placed in a clean vial containing 150 µL of MeOH for 5 min. The extract was centrifuged and injected into the chromatographic system.

2.6- Method validation

The method validation was performed according to the International Guidelines [27, 28] in order to estimate the selectivity, limit of detection (LOD), limit of quantification (LOQ), linearity, intra- and inter-day trueness and precision, and recovery of AD drugs in whole blood, urine and saliva samples. The selectivity was examined by analyzing double blank, zero blank and blank spiked with IS and ADs, in order to exclude interferences in the same retention times as for the target analytes. The linearity was evaluated by applying the least–squares linear regression analysis, by plotting the peak area/IS versus analyte concentration for both standard solutions and spiked biological matrices. The slope, intercept and correlation coefficient were calculated for all the antidepressant drugs. LOD and LOQ were calculated by signal–to–noise (S/N) of 3 and 10 respectively. Three concentration levels (0.5, 2, 10 µg/mL) were selected to evaluate both intra and interday precision and trueness. 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.

3. Results and discussion

3.1- Optimization of the extraction procedure – FPSE

The optimization of the FPSE extraction process involved numerous steps, aim to the optimization of all involved parameters. To obtain the maximum extraction efficiency of the compounds, all the parameters were gradually optimized, following the One Variable at Time

(OVAT) approach. In this way, each parameter is gradually optimized through experiments. The sorbent material was first selected, evaluating eight different FPSE membranes: 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–PTHF gel and sol–gel zwitterionic. Each support was cut into 1 cm discs and activate in 2 mL of ~~ACN:~~MeOH:AcN mixture (50:50, *v:v*) for 5 minutes. This step permits to eliminate materials impurities and to activate functional groups for subsequently interactions. The membranes were then rinsed in Milli–Q water to remove organic solvent residues before the insertion into the sample for the extraction process. The initial general conditions selected for the extraction process in order to test the different membranes involved an extraction in 500 µL of standard solution (analytes and IS at 10 µg/mL) for 30 minutes under stirring. Subsequently the membrane was immersed in 150 µL of MeOH for 30 minutes, for the desorption step (back–extraction). At the end, the samples were centrifuged for 10 minutes and 10 µl of supernatant were injected into the HPLC system. This procedure was performed for each membrane, under the same conditions, to select the one that provided the highest enrichment factor (%). These values were calculated as a percentage of the improvement in the peak area compared to the area of the reference standard solutions. Among all tested membranes, sol–gel CW–20M, sol–gel PTHF and sol–gel zwitterionic initially gave the best Enrichment Factors (%). At this point, other parameters have been evaluated for these membranes, starting from extraction time (5, 15, 20, 30, and 60 min). The sol–gel CW-20M support showed the best values for all antidepressant drugs with an extraction time of 20 minutes (**Table 2**). The best values have been reported in bold.

Table 2. *Enrichment factors (%) observed for three different FPSE membranes (extraction time 20 min)*

Subsequently, different solvents and solvent mixtures (MeOH, AcN and a mixture of both) were tested as elution phase. Since the FPSE supports are particularly resistant, different types of organic solvents could be select. Moreover, it is essential to optimize the back–extraction volume, as the organic solvent should ensure the analytes desorption from the FPSE device using the minimum volume. Different volumes were tested: 150 µL, 200 µL, 300 µL and 400 µL at different back–extraction times (5, 10, 15 and 20 min). The best performance in elution step was obtained using 150 µL of pure MeOH for 5 minutes. After selecting the optimal conditions using standard aqueous solutions, the optimization was further performed on the biological matrices (whole blood, urine and saliva) spiked with antidepressant drugs and IS, which confirmed the previously obtained data. All

the graphs related to the optimization of the FPSE parameters have been shown ***in Supplementary materials Section 2.***

3.2. HPLC optimized condition

The best resolution of the selected antidepressants has been achieved through an isocratic separation which includes an ammonium acetate buffer (50mM, pH 5.5) as mobile phase A and AcN as mobile phase B. The chromatographic column was the GraceSmart® RP18 (150 mm × 4.6 mm; 5 µm particle size). The flow rate was set at 1.4 mL/min, maintaining the column temperature at 40 ° C. Venlafaxine was eluted at 3.532 min, Citalopram at 4.403 min, Paroxetine at 5.193 min, Fluoxetine at 5.562 min, Sertraline at 8.739 min, Amitriptyline at 12.149 min, and Clomipramine at 14.504 min. The maximum wavelength for each analyte were 226, 239, 294, 227, 276, 273, 240 and 252 nm for venlafaxine, citalopram, paroxetine, fluoxetine, IS, sertraline, amitriptyline, and clomipramine, respectively. No endogenous interference was noted in these retention times in the three matrices considered.

3.3. Method validation results and data

The reported method was validated according to international guidelines [27, 28] and selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), precision and trueness were evaluated. Least squares linear regression analysis was applied to calculate slope, intercept and correlation coefficient for both standard solutions and spiked whole blood, urine and saliva samples. Linearity (intercept, slope, coefficient of determination and variation) was evaluated by plotting the area of the analyte/IS ratio on the ordinate and the concentration of each standard solution (and matrices added with IS and analytes at different concentrations) on the abscissa and repeating the analyzes in quadruplicate for each concentration (**Figure 1**). To obtain the calibration curves, the analyzes were performed in triplicate, for the concentrations included in the range, 0.2 – 20 µg/mL. Over the range tested, the curves showed linear correlation and coefficients of determination $r^2 \geq 0.9916$ for whole blood, $r^2 \geq 0.9928$ for urine and $r^2 \geq 0.9812$ for saliva. LOD and LOQ was evaluated by the signal–to–noise ratio (S / N) of 3 and 10, respectively. The limit of quantification (LOQ) was 0.2 µg/mL for venlafaxine and 0.1 µg/mL for others. The Limit of Detection (LOD), the lowest detectable analyte concentration by the method, was 0.06 µg/mL for venlafaxine and 0.04 µg/mL for the other analytes in all considered matrices. Trueness across days was assessed by running analyzes in quadruplicate on the same day, while between-day accuracy was determined by measuring in triplicate for four consecutive days. Finally, RSD% and BIAS% gave values below ± 15%, according to current guidelines **(*Supplementary Materials Section 3*)**. The recovery values ranged from

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~~54.6~~86.4% to ~~187.6~~110, from ~~54~~91.5% to ~~192.9~~114% and from ~~34.19~~87.5% to ~~197.61~~112% in ~~saliva.~~ ~~Whole~~whole blood, urine, and ~~urine~~saliva samples, respectively.

All validation data were reported in **Table 3** (whole blood, urine, and saliva) and the entire method validation was performed according to the international guidelines [27, 28].

Figure 1. *Calibration curve in* ***a.*** *human whole blood;* ***b.*** *human urine;* ***c.*** *human saliva evaluated over the concentration range. The area response ratio (~~analyes~~analytes vs. internal standard) was fitted to the nominal concentration using the simplest model through GraphPad software*

Table 3. *Mean linear calibration curve parameters performed by weighted-linear least-squares regression analysis of six independent calibrations in human whole blood, urine, and saliva*

3.4 Application on Real Samples

The established analytical method was applied to bioanalysis of real samples collected from healthy donor. This last phase was the decisive step to evaluate the effectiveness of both the analytical method and the innovative FPSE extraction technique. Real samples (whole blood, urine, and saliva) were obtained from four separate volunteers who were not taking antidepressants. The matrices were, first, stored at +-4°C (whole blood) and –20°C (urine and saliva) and brought to room temperature before analysis. Subsequently, the samples were subjected to optimized FPSE extraction and by means of the subsequent HPLC–PDA analysis according to the validated method. The concentrations of these compounds were then calculated. The results obtained showed a negative result for all the samples, confirming the validity of the analytical method, since the result confirmed the absence of antidepressants in the matrices considered. In addition, some whole blood, urine and saliva samples have been spiked with AD drugs, adding concentrations that are usually found after therapeutic treatments with these drugs. The chromatograms related to real samples have been reported in ***Supplementary Materials Section 4***. The data obtained were reported in the **Table 4**.

Table 4. *Data obtained from spiked whole blood, urine and saliva*

Discussion and Conclusions

The main goal of therapeutic drug monitoring (TDM) is to maximize the therapeutic effect while minimizing the likelihood of side effects. TDM has become a common practice in clinical investigations, especially for drugs with a narrow therapeutic index such as anticancer, antiepileptic, antidepressant, etc. Unfortunately, there are still several limitations, such as the high costs related to

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the collection, preparation and analysis of the samples, the shipment to the few certified laboratories in controlled conditions. However, to date, for routine TDM practice, the reference matrices remain blood and, above all, plasma given the numerous data available regarding the therapeutic ranges in these matrices. In recent years, the use of alternative and above all non–invasive matrices for TDM (and in particular for ~~PK~~pharmacokinetic studies) such as saliva, urine and hair are starting to find different applications. In this way, several advantages could be introduced for TDM such as the low required volumes, no need for sample storage at low temperatures (−20°C/−80°C or dry ice), cheaper and simpler way to send sample and the major compliance of patients.

In the reported study, an FPSE-HPLC-PDA method was optimized for the determination of seven antidepressants in whole blood, urine, and saliva. The application of this innovative extraction method brought numerous advantages during in the sample preparation step, such as the minimal sample manipulation, avoiding protein precipitation or other purification processes, as well as the excellent recovery values. The reported method was compared with other methods reported in literature, that also have used other extraction techniques ~~(SPE-LC-MS/MS, DLLME-GC-MS/MS, etc).~~like solid phase extraction coupled to liquid chromatography-tandem mass spectrometry (SPE-LC-MS/MS), dispersive liquid-liquid microextraction coupled to gas chromatography-tandem mass spectrometry (DLLME-GC-MS/MS), or similar. In the present work, all aspects of the extraction technique were optimized to obtain the maximum extraction efficiency. The main point to underline is the reduced volume of organic solvent that was used, as the extraction procedure only has involved 150 µL of MeOH. Although the extraction times are very similar to other methods that use the FPSE as an extraction procedure [12, 26], the volume reduction in the elution phase is certainly an important point to consider as an advantage of the developed procedure. Furthermore, it must be emphasized that the present method has also extended the number of target analytes considered.

The method has been validated in terms of linearity, selectivity, accuracy and precision, and has been shown to be suitable for analysis in whole blood, urine and saliva samples, of antidepressants to assess concentration during therapeutic monitoring. An innovative a simple analytical method that reflect all the values of the Green Analytical Chemistry (GAC) have been reported. In conclusion, the use of alternative matrices is an increasingly topical topic, which presents an important potential for future applications to be introduced in clinical practice. Certainly, there is still a lot of work to be done and many data will need to be available to validate these matrices in a routine clinical setting. At the same time, these alternative matrices will find increasing interest and applicability for TDM studies, particularly when coupled with highly sensitive analytical techniques.

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