Whole blood and oral fluid microsampling for the monitoring of patients under treatment with antidepressant drugs

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ABSTRACT

Patients suffering from major depression and related pathologies (feeding and eating disorders, obsessive-compulsive disorder, post-traumatic stress disorder, anxiety disorders, etc.) are usually treated with antidepressant agents belonging to several pharmacological and chemical classes; the most recent of these agents are collectively known as “new-generation antidepressants”. In these patients, therapeutic drug monitoring (TDM) with the determination of drug and metabolite blood levels is one of the most useful procedures to optimise and personalise the treatment, enhancing both effectiveness and safety. A new approach is proposed in this study, based on microsampling of both blood and oral fluid by means of volumetric absorptive microsampling (VAMS). This approach makes sampling and storage much simpler and even self- and at-home-sampling possible, while retaining reliability, vastly increasing analyte stability and reducing overall expenses. The microsamples were pretreated by means of microextraction by packed sorbent (MEPS) on C2 sorbent and analysed by liquid chromatography with sequential spectrophotometric and spectrofluorimetric detection (HPLC-UV-FL). Method validation results were satisfactory (extraction yield >84%, precision RSD < 8.9%, stability>85.0% after 3 months). Application to blood and oral fluid VAMS from patients treated with four possible different antidepressants (sertraline, fluoxetine, citalopram and vortioxetine) provided results always in good agreement with those obtained from the corresponding fluid matrices, including the levels of drug metabolites.

Keywords

Therapeutic drug monitoring (TDM); Microsampling; Volumetric absorptive microsampling (VAMS); Blood Oral fluid; Antidepressants.

1. INTRODUCTION

According to the Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (DSM-V) [1], major depression is characterised by the nearly daily presence of symptoms like feeling of loneliness, sadness, hopelessness; lack of energy; sleeping or eating disorders; thoughts of death or suicide. It is currently one of the most frequent mental disorders after anxiety disorders [2]. Currently, the most effective forms of treatment for depression involve, in addition to short-term goal-oriented psychotherapy, the use of pharmacological antidepressant agents (ADA), and in particular the so-called “new-generation” ADA. These drugs have demonstrated efficacy not only towards depression, but also against a range of related psychiatric conditions, such as feeding and eating, obsessive-compulsive, post-traumatic stress and generalised or social anxiety disorders. These drugs are divided into a plethora of different classes according to their specific mechanism of action [3,4], while their common feature is their activity on (usually potentiation of) one or more adrenergic amine pathways, and in particular those involving serotonin, norepinephrine and possibly dopamine; melatonergic (agomelatine), glutamatergic (esketamine) and opioid (tianeptine) agents have also been approved [5].Another common feature ofmostADAis their delayed onset of effect, that is usually about 6–12 weeks [6]. Unfortunately, in 20–30% of patients, current treatments are inadequate, and relapse is not uncommon. No new-generation ADA has until now demonstrated to possess better efficacy and/or safety for most patients than other agents [7], so the choice of the specific drug to be administered to each patient, is mostly leftto the specific expertise and preferences of the clinicians. In order to help psychiatrists in correctly and objectively assessing the clinical situation and their therapeutic options, one of the most useful practices is therapeutic drug monitoring (TDM) [8]. TDM provides for the periodic determination of drug and metabolite plasma levels,together with the use of chemical-clinical correlations (i.e., correlations between administered drug dose and plasma levels; between plasma levels and therapeutic efficacy; between plasma levels and side and toxic effects) [21]. TDM can also lead to reduced healthcare expenses, due to the possibility of better efficacy, increased patient compliance and enhanced safety, leading to a reduction in hospitalisations due to unwanted effects or therapy ineffectiveness [9,10]. Dried microsampling can substantially increase the feasibility and practicality of any TDM protocol. In fact, reducing the invasiveness and complication of blood sampling is a good way to increase patient compliance (especially for psychiatric patients that are often wary of any invasive procedure), possibly leading to widespread at-home self-sampling practices [11]. Moreover, the loss of water usually increases analyte stability through reduction in the rate of most degradation reactions. This in turn greatly reduces the precautions, appliances and space needed for microsample storage and shipping in comparison to the corresponding fluid matrices [12]. In the last few years, volumetric absorptive microsampling (VAMS) is attracting increasing interest, due to its

practicality, high sample volume reproducibility and, for blood-based microsamples, high independence of sampling volume from haematocrit [13]: in fact, VAMS is carried out with a device including a calibrated polymeric, porous tip that absorbs fixed volumes of matrix, according to its dimensions. It should be noted that haematocrit differences can have other effects on analysis results, including changes in extraction yields and precision, and matrix effect for mass spectrometric (MS) methods. VAMS-based analytical methods can be subject to these sources of variability, even though sampling volume is not [14]. In addition to microsampling, another attractive alternative practice is the use of non-invasive biological matrices instead of blood, for example oral fluid (OF). OF includes all fluids present in the mouth, including saliva, oral mucosal transudate, sputum from the lungs and throat, nasal drainage and others. Itis one ofthe most promising non-invasive matrices for TDM purposes, since its drug concentration can often closely mimic that of blood. Of course, this needs to be verified case by case for each drug [15]. In this study, an analytical workflow is described, based on volumetric absorptive microsampling (VAMS) of both blood and OF, followed by microextraction by packed sorbent (MEPS) [16] and liquid chromatographic (HPLC) analysis with spectrophotometric (UV) and spectrofluorimetric (FL) detection. The workflow has been validated for application to the TDM of four different new-generation ADA: sertraline (SRT, Fig. 1a), fluoxetine (FLX, Fig. 1b), citalopram (CTP, Fig. 1c), vortioxetine (VTX, Fig. 1d) and their main metabolites: norsertraline (NSR, Fig. 1e), norfluoxetine (NFL, Fig. 1f), N-desmethylcitalopram (DCT, Fig. 1g) and N,N-didesmethylcitalopram (DDC, Fig. 1h). As defined by recent Consensus Guidelines for TDM in neuropsychopharmacology, levels of recommendation for TDM are: level 1 (strongly recommended) for CTP, level 2 (recommended) for SRT and VTX, level 3 (useful) for FLX [13]. To the best of our knowledge, no scientific paper until now has been published for the simultaneous determination of these four ADA and their main metabolites in biological fluids. Regarding VTX in particular, just two papers have been published for its analysis in biological fluids [17,18], but neither included any other ADA. As for the other, less recent new-generation ADA considered herein, of course their analysis in biological fluids has been reported multiple times [19–23], but never using the microsampling approach. In the ADA field, this approach has been proposed in just two papers: one for the MS/MS screening of several psychiatric drugs in OF [24] and one for the screening of different drug classes in in dried urine spots (DUS) [25]. Neither paper includes all four considered drugs, nor application to blood-based matrix microsamples.

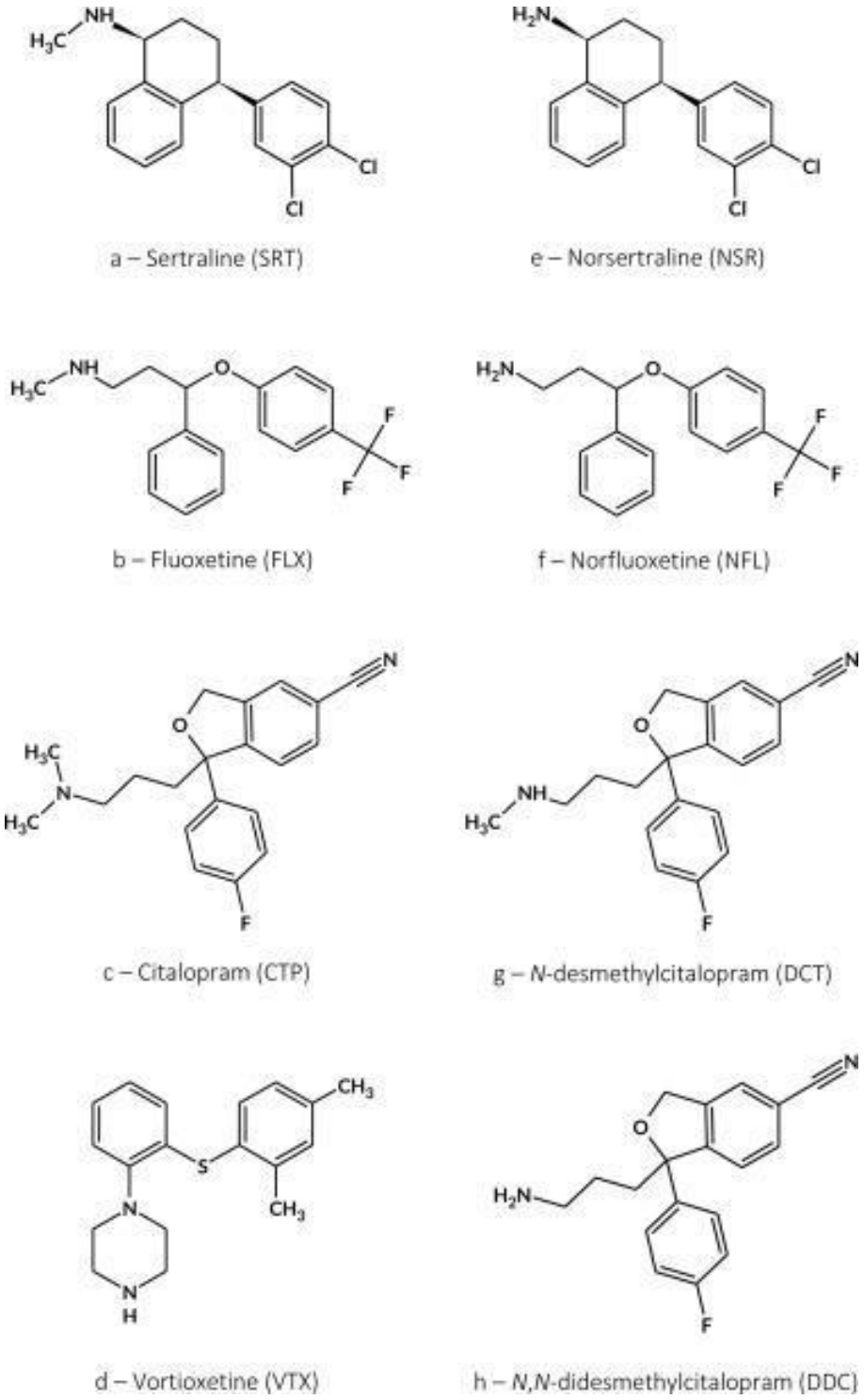


Fig. 1. Chemical structures of (a) sertraline (SRT), (b) fluoxetine (FLX), (c) citalopram (CTP), (d) vortioxetine (VTX), (e) norsertraline (NSR), (f) norfluoxetine (NFL), (g) N-desmethylcitalopram (DCT) and (h) N,N-desmethylcitalopram (DDC).

2. MATERIALS AND METHODS

2.1. Chemicals and standard solutions

SRT((1S,4S)-4-(3,4-dichlorophenyl)-N-methyl-1,2,3,4- tetrahydronaphthalen-1-amine) hydrochloride, FLX (N-methyl-3-phenyl-3-[4-(trifluoromethyl)phenoxy]propan1-amine) hydrochloride, CTP (1-[3-(dimethylamino)propyl]- 1-(4-fluorophenyl)-3H-2-benzofuran-5-carbonitrile) hydrobromide, NSR((1S,4S)-4-(3,4-dichlorophenyl)-1,2,3,4- tetrahydronaphthalen-1-amine) hydrochloride, NFL (3-phenyl-3-[4-(trifluoromethyl)phenoxy]propan-1-amine) hydrochloride, DCT (1-(4-fluorophenyl)-1-[3-(methylamino)propyl]-3H-2-benzofuran-5-carbonitrile) hydrochloride, duloxetine (IS1, used in HPLC-UV for VTX) hydrochloride and venlafaxine (IS3, used in HPLC-FL for CTP, FLX and metabolites) hydrochloride, pure powders (all >99% purity); acetonitrile, methanol and dichloromethane (for HPLC, purity: > 99.9%), monobasic potassium phosphate, triethylamine (TEA), phosphoric acid, sodium carbonate and potassium hydroxide (all pure for analysis) were purchased from Sigma Aldrich Italy (Milan, Italy). VTX (1-[2-(2,4-dimethylphenyl)sulfanylphenyl]piperazine) hydrobromide, DDC (1-(4-fluorophenyl)-1-[3-aminopropyl]-3H2-benzofuran-5-carbonitrile) hydrochloride and clotiapine (IS2, used in HPLC-UV for SRT and NSR) pure powders were purchased from LGC Standards (Teddington, Middlesex, UK). Ultrapure water (18.2 M cm) was obtained by means of a Milli-Q apparatus from Millipore (Milford, MA, USA). The analyte and IS stock solutions (1 mg/mL) were prepared by dissolving suitable amounts of pure powders in methanol and kept at −20 ◦C when not in use; the corresponding standard solutions were prepared daily by dilution with the HPLC mobile phase. All solutions were stored protected from light in amber glass vials from Phenomenex (Torrance, CA, USA).

2.2. HPLC-UV- FL instrumentation and conditions

HPLC-UV- FL analysis was performed on a Waters Corporation (Milford, MA, USA) Alliance e2695 chromatographic system with autosampler coupled to a Waters 2998 photo diode array detector and a Jasco FP-2020spectrofluorometric detector, connected in series. Separations were obtained on aWatersXBridge BEH C18 column (150 × 2.1 mm, 3.5 m) maintained at room temperature and equipped with a guard column. The mobile phase was a mixture of 33 mM, pH 3.0 aqueous phosphate buffer containing 0.3% TEA (solvent A) and acetonitrile (solvent B), flowing at a constant rate of 1.0 mL/min under gradient conditions. Gradient composition was: 0.0–3.0 min, constant 20% B; 3.1–4.0 min, linear 20%–35% B gradient; 4.1–6.5 min, constant 35% B; 6.6–7.5 min, linear 35%–55% B gradient; 7.6–14.5 min, constant 55% B; 14.6–15.5 linear 55%-20% B gradient, 15.6–17.0 constant 20% B to re-equilibrate the column. Both solvents were filtered on a polyamide filter (47 mm dimeter, 0.2 m) and degassed by ultrasonication. Injection volume was 20 L. SRT,

NSR and VTX, as well as IS1 and IS2, were detected by UV at 225 nm; FLX, CTP, NFL, DCT and DDC, as well as IS3, were detected by fluorescence at em = 235 nm, exc = 300 nm.

2.3. Real samples and compliance with ethical standards

Real blood and oral fluid samples were obtained from in- and out-patients of different Psychiatric Clinics and Hospitals of the Emilia-Romagna region in Italy, receiving antidepressants as part of their standard treatment and were collected for general needs related to the therapy; all subjects provided informed consent prior to their participation in this study. Sampling was carried out from patients in steady-state conditions and attrough time, in the morning, at least 8 h (3 times a day dosing) or 12 h (twice a day dosing) after the previous oral drug administration. In real samples, IS spiking was carried out on the VAMS tip by automatic pipetting 20 L of IS solution onto the tip before sampling; the tip was then left to dry for 2 h at RT before use.

2.4. Sample pretreatment

Mitra® VAMS microsamplers (20 L) were provided by Neoteryx (Torrance, CA, USA). A VAMS microsampler includes a polypropylene handle (about 4 cm long) topped with a small tip (about 2-mm diameter) of a proprietary polymeric porous material. B-VAMS. Blank or blank spiked B-VAMS were obtained by drawing a few millilitres of blood from volunteers, then spiking it with the analytes and the ISs and accurately sampling 20 L of matrix by VAMS. 100-L blood aliquots were spiked with 5 L of analyte standard and/or IS mixtures at known concentrations. The surface of the sample mixture was touched with a VAMS microsampler for 5 s, dried at room temperature (RT) for 1 h and stored at RT in a dedicated clamshell in order to avoid contact with any surface. VAMS microsamples were thus obtained. Clamshells were stored in sealed polyethylene bags containing desiccant. For sample pretreatment,the microsampler tip was detached from the handle and subjected ultrasound-assisted extraction (UAE) for 20 min in 1 mL of methanol. The resulting solution was quantitatively transferred into a different vial and broughtto dryness in a centrifugal evaporator. After re-dissolving with 100 L of HPLC mobile phase (a 65:35 mixture of 33 mM, pH 3.0 aqueous phosphate buffer containing 0.3% TEA / acetonitrile), the solution was subjected to MEPS pretreatment in an SGE Analytical Science (Melbourne, VIC, Australia) C2 barrel-and-needle (BIN) assembly set up in an SGE eVol XR digital analytical syringe apparatus. The BIN was activated by drawing and discarding 100 L of methanol 3 times and conditioned with 100 L of water 3 times. The sample was loaded onto the BIN with 10 draw/discharge cycles at a 5 L/s speed; the BIN was then washed twice with 100 L of water and 100 L of 10 mM, pH 9.0 carbonate buffer / methanol (90/10, V/V) mixture at 20 L/s. The analyte andthe ISs were elutedthree times with200 L ofmethanol at 5 L/s (three cycles). After merging the three eluates, they were brought to dryness, re-

dissolved in 100 L of HPLC mobile phase (a 65:35 mixture of 33 mM, pH 3.0 aqueous phosphate buffer containing 0.3% TEA / acetonitrile) and analysed by HPLC-UV-FL. OF-VAMS. Blank or blank spiked OF-VAMS were obtained by drawing about 1 mL of OF from volunteers, then spiking it with the analytes and the ISs and accurately sampling 20 L of matrix by VAMS. Oral fluid (1 mL) aliquots were centrifuged for 5 min at 6500 x g, then 100 L of supernatant were subjected to the same procedure as B-VAMS. ISs addition mode and extraction performance assays (n = 6) were carried out by comparing pooled matrix fortification (as in blank spiked samples) with VAMS tip ISs pre-soaking (as in real sample VAMS collection) in order to exclude any bias in terms of volumetric accuracy and extraction efficiency.

2.5. Method validation

The analytical method was validated according to the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) [26] guidelines. The tested parameters were linearity (including limits of detection and limit of quantitation), selectivity, extraction yield, precision, carryover and accuracy. B-VAMS and OF-VAMS samples were spiked with analyte standard solutions at seven different concentrations, containing the IS at a constant concentration, subjected to MEPS pretreatment and injected into the HPLC system. The analysis was carried out in triplicate for each concentration. The obtained analyte/IS peak area ratios were plotted against the corresponding concentrations (expressed as ng/mL) and the calibration curves were obtained by means of the least-square method (1/x weighting). LOQ and LOD were calculated as the analyte concentrations, which gave rise to peaks whose height was 10 and 3 times the baseline noise, respectively. For selectivity, six different blank matrix samples from healthy volunteers were pretreated and analysed. In addition, some common CNS drugs, such as chlorpromazine, clomipramine, clonazepam, clozapine, diphenhydramine, flurazepam, fluvoxamine, fluphenazine, haloperidol, levosulpiride, lorazepam, loxapine, lurasidone, mirtazapine, olanzapine, paroxetine, pipamperone, quetiapine, risperidone, trazodone, ziprasidone were injected in the analytical system to check for selectivity. The obtained chromatograms were checked for interferences by comparison with the peak area of the LOQ of each analyte, at their respective retention time. Selectivity was considered acceptable if any extraneous peak was ≤20% of the response of the LOQ of each analyte. Extraction yields were evaluated by repeatedly subjecting to the previously described procedure blank samples spiked with analyte standard solutions at four different, known concentrations (corresponding to the LOQ, a low, an intermediate and a high value of the linearity range). The obtained analyte peak areas were compared with those obtained by injecting standard solutions at the same theoretical concentrations in order to calculate extraction yield values. Precision assays were carried out on the same samples, which

were analysedsix times inthe sameday to obtainintradayprecision and six times over six different days to obtain interday precision, expressed as percentage relative standard deviation (RSD %). Carryover was evaluated by injecting blank matrix extracts immediately after the highest calibration sample. The acceptance criterion was no analyte peak higher than 20% of LOQ levels (5% for IS). Extraction performance assays with respect to ISs addition mode were carried out by comparing spiking offluid samples before VAMS sampling (as in blank spiked samples) with VAMS tip presoaking (as in real VAMS sampling), in order to exclude any bias in terms of extraction efficiency. To test analyte stability, microsamples were spiked with the analytes at two concentration levels (high and low concentrations with respect to the calibration curve), then stored at RT, protected from light, in sealed polyethylene bags containing desiccant for 3 months. At regular intervals (1 week), microsamples were pretreated and analysed in triplicate. The measured analyte concentrations were compared to those of the same samples extracted and analysed immediately after microsampling and drying. The stability values thus obtained were also compared to those of fluid samples stored at 4 ◦C, −20 ◦C or −80 ◦C. For autosampler processed stability, samples spiked atthe same two concentration levels were freshly pretreated in triplicate and stored in the autosampler at RT for 48 h before re-analysis, while for bench-top stability, spiked, extracted microsamples were stored for 12 h at room temperature without any further precautions. Samples were considered stable when % bias from the nominal concentrations was within ±15%. Recovery assays were carried out in order to evaluate method accuracy: 20 L of standard solutions containing known amounts of the analytes (corresponding to the LOQ, a low, an intermediate and a high value of the calibration curves) were added to VAMS tips before microsampling real samples. The obtained spiked samples were then analysed and analyte recovery was calculated by comparison with non-spiked real samples.

2.6. Quantitative data comparisons

All results obtained from real samples were compared by plotting the results from each dried matrix (B-VAMS, OF-VAMS,) versus those obtained from the corresponding fluid matrix analysis. Then, the least-square method and Passing-Bablok regression were applied to calculate linearity correlation coefficient and slope of each comparison curve, while Bland Altman plots were built to evaluate biases between results obtained from microsamples and those obtained from fluid counterparts.

3. RESULTS AND DISCUSSION

3.1. Chromatographic conditions optimization and IS choice

For the development of chromatographic conditions, the starting point was isocratic elution systems dedicated to the analysis of individual compounds together with their metabolites. A chro matographic column was then selected to be tested under these conditions leading to the best performance in terms of resolution and peak sharpness. The considered parameters were column length (50−200 mm), diameter (2.1–4.6 mm) and sorbent particle size (1.7–5 m). The best compromise was achieved with a 150 mm length, 2.1 mm diameter, 3.5 m particle C18 column. For the optimisation of the mobile phase composition gradient, several programs were carefully tested in terms of number of steps and slope, A/B solvents ratio and duration of the single steps, to reach the best compromise between complete chromatographic resolution within the two detection systems and total chromatographic run duration. Several drugs, not commonly used together with the analytes in clinical settings, were tested as possible ISs. For example, in the HPLC-UV system diphenhydramine, clotiapine, chlorpromazine, clomipramine, duloxetine, lamotrigine, loxapine and indomethacine were tested; in the HPLC-FL system, mirtazapine, fluvoxamine, paroxetine and venlafaxine were tested. Most compounds tested in the HPLC-UV system were detected within the testing run time window; among these, loxapine, diphenhydramine, clomipramine and duloxetine had retention times not overlapping with those of the analytes and not excessively long (to avoid unnecessary lengthening of total run times). Since loxapine and diphenhydramine were scarcely retained, and based on structural considerations, duloxetine (IS1) was chosen as the IS for VTX; clotiapine (IS2) was chosen as the IS for SRT and its metabolite. Most compounds tested in the HPLC-FL system had unsuitable (either too strong or too weak) retention, so the only suitable compound for both FLX and its metabolite, and CTP and its metabolites, was deemed to be venlafaxine (IS3). Although the chosen ISs are CNS drugs, and in particular two antidepressants and an antipsychotic agent, it should be noted that polypharmacy with different antidepressants of the same generation (SSRIs, SNRIs, SMSs) is quite uncommon and clotiapine use is uncommon in general, even more in association with antidepressants. Thus, interference due to coadministration of an IS should be a very rare occurrence.

3.2. VAMS procedure

The biological matrix volume absorbed by VAMS tips has been studied for both B- and OF-VAMS. Six VAMS devices for each matrix were weighed before and after sampling, then the accuracy and precision of sampling volume were calculated. B-VAMS volume testing confirmed the high-volume accuracy (100.2%) and precision (RSD = 4.7%) of this technique. 20-L OF-VAMS, on the contrary, have never been tested before, so the

following results are the first instance of suchaprocedure. OF-VAMS testingprovidedresults that were comparable to those obtainedonB-VAMS:meanaccuracy was 99.8% and RSD for precision was 5.7%. Sampling time was also tested. Both B-VAMS and OF-VAMS provided basically constant sampling volumes over the entire range of tested sampling times (1−20 s), excluding the shortest times (1, 2 and 3 s), which caused a notably higher volume variability than higher sampling times (mean sampled volume < 75.5%, RSD > 15.9%). Contact times of 10 and 20 s showed no over-sampling (mean volume accuracy 100.5%, RSD < 5.1%). Thus, 5 s was chosen as the sampling time that provided maximum speed while maintaining optimal reproducibility. Gravimetric analysis on the time required to dry 20-L B-VAMS and OF-VAMS showed that under complete ventilation at RT (25 ◦C; 55% humidity), the samples are dried within 1 h for B-VAMS and 45 min for OF-VAMS, respectively. Extraction yield and volumetric comparative assays, performed by adding ISs to pooled matrices before VAMS sampling and pre-soaking VAMS tips with ISs standard solution, provided satisfactory and overlapping results (RSD < 4.1% as regards volumetric assays and < 5.0% for extraction yield), thus demonstrating applicative suitability of both approaches.

3.3. Sample pretreatment development

VAMS can be thought as a pretreatment of sort, since the interactions between the analytes,the biological matrix and the polymeric support can be exploited to elute the analytes selectively from the VAMS tip, or to selectively eliminate matrix interferences. Several kinds of solvent extraction procedures were tested, varying the kind of solvent (methanol, acetonitrile, ethyl acetate, diethyl ether, mixtures thereof, mixtures with water or buffer), solvent volume (0.2–2.0 mL), contact time (1−10 min) and assistive technology (microwaves, ultrasounds, vortex). Methanol provided better results than all other solvents and mixtures (i.e. organic solvents provided better purification when compared to aqueous solutions, while methanol led to higher extraction yields when compared to other organic solvents), and extraction yields increased with extraction volumes up to 1 mL (e.g. mean extraction yield >84% with 1 mL and 84%). Using UAE, extraction times provided increasing yields up to 20 min, so this was chosen as the best compromise. Despite the notable interference decrease observed upon the optimised methanolic VAMS extraction, it was not sufficient to obtain satisfactorily clean chromatograms (data not shown). As a consequence, a further pretreatment step was applied, based on MEPS. MEPS is based on the same principles as SPE, but it is carried out in a BIN containing minute amounts of sorbent and included into a syringe, using very small solvent volumes. The procedure is carried out using an electronic automation device (eVol) that autonomously draws and discharges known volumes of the chosen solvent at a known speed. All the main steps of the MEPS procedure (loading, washing, elution) were optimized in order to obtain a satisfactory combination of

295 microsample purification and analyte extraction yields. For example, different solvent /cycle /speed
296 combination were tried. It was found that loading and elution provide good yields when they are carried out
297 at low speed (5 L/s) to grant strong analyte interaction with the sorbent and the eluent, respectively; on the
298 contrary, washing is best carried out at higher speed (20 L/s) to avoid unnecessary analyte loss. Similarly,
299 loading was complete after at least 10 cycles, with fewer cycles not granting high yields (mean extraction
300 yield <63% with 5 cycles); washing with hydrophilic and basic solvents (water, carbonate buffer/methanol,
301 respectively) correctly retained the analytes while eliminating most interference. Regarding elution,
302 methanol provided optimal analyte solubility and thus good yields; mixtures with either water, buffer or
303 acetonitrile, even at low ratios, decreased this parameter (mean extraction yield <72%). Under the final
304 microsampling and sample pretreatment conditions, an HPLC-UV chromatogram of a blank B-VAMS sample
305 spiked with SRT, NSR, VTX and their ISs appears as shown in Fig. 2a; an HPLC-FL chromatogram of a blank B-
306 VAMS sample spiked with FLX, NFL, CTP, DCT, DDC and their IS is shown in Fig. 2b. Both chromatograms are
307 superimposed to blank sample chromatograms, highlighting satisfactorymethod selectivity. Chromatograms
308 of OFVAMS counterparts are reported in Supplementary Material(Figures SF1a and SF1b, respectively).

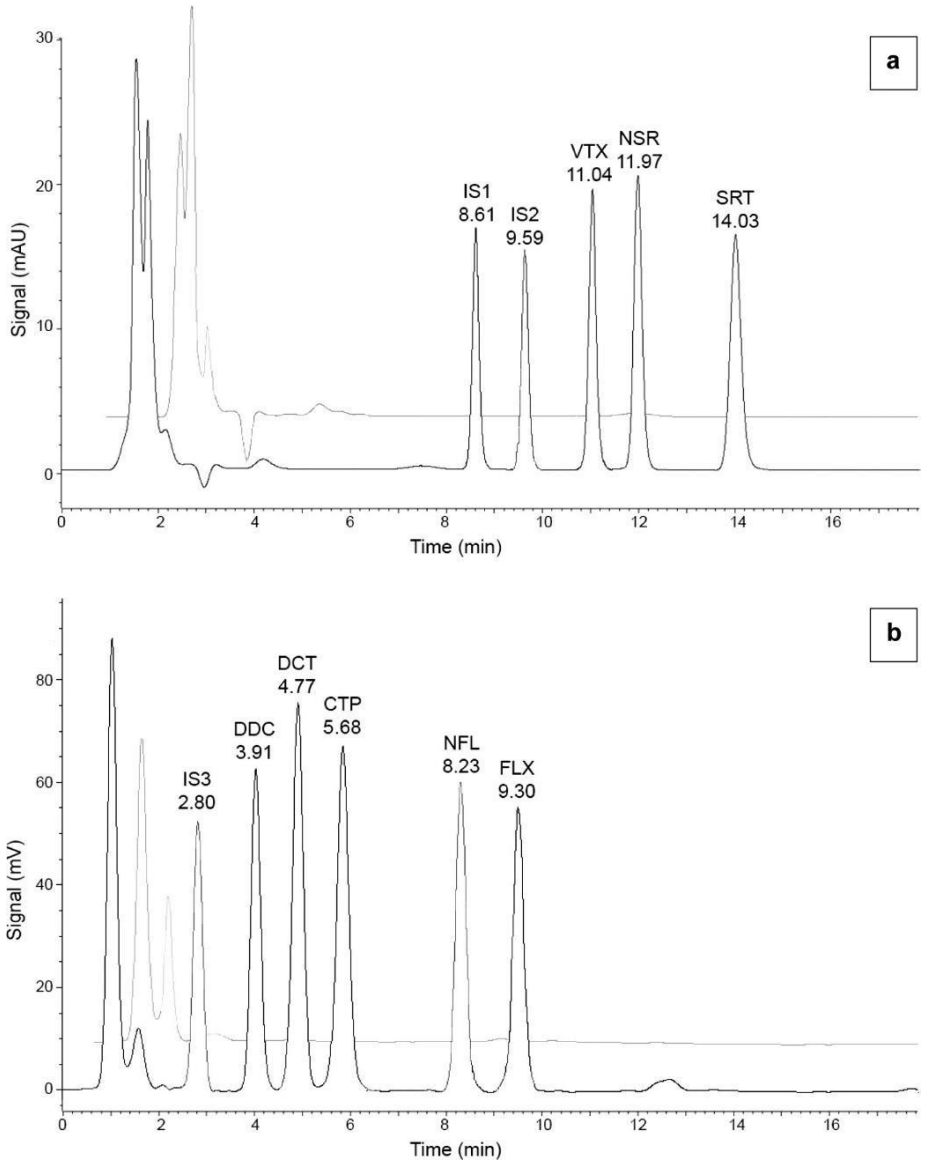


Fig. 2. (a) HPLC-UV chromatogram of a blank B-VAMS sample spiked with SRT, NSR, VTX and their ISs, superimposed to a blank B-VAMS sample and (b) HPLC-FL chromatogram of a blank B-VAMS sample spiked with FLX, NFL, CTP, DCT, DDC and IS, superimposed to a blank B-VAMS sample.

3.4. Method validation

3.4.1. Linearity

Concentration ranges for linearity assessment were selected on the basis of the expected concentration ranges and by taking into account back-calculated concentrations when performing linearity assays (acceptance criteria was defined as ±15% of the nominal value). Good linearity was obtained for both dried matrices and all analytes, with r2 values always higher than 0.9989 and up to 0.9998; neither of the two matrices produced better linearity results than the other. Sensitivity values were generally better for OF-

VAMS than B-VAMS due to slightly better signal-to-noise ratios, while LOQ results were compatible with an effective monitoring of the considered drugs and their metabolites (see Table 1).

Table 1. Linearity, LOQ, LOD on spiked samples.

Analyte	Matrix	Linearity range, ng/mL	r^2	LOQ, ng/mL	LOD, ng/mL
SRT	**B-VAMS**	7–500	0.9995	7.0	2.5
	OF-VAMS	5–500	0.9993	5.0	1.5
FLX	**B-VAMS**	10–750	0.9990	10.0	3.0
	OF-VAMS	7–750	0.9992	7.0	2.5
CTP	**B-VAMS**	1–200	0.9997	1.0	0.3
	OF-VAMS	1–200	0.9997	1.0	0.3
VTX	**B-VAMS**	5–500	0.9994	5.0	1.5
	OF-VAMS	3–500	0.9992	3.0	1.0
NSR	**B-VAMS**	7–500	0.9990	7.0	2.5
	OF-VAMS	5–500	0.9993	5.0	1.5
NFL	**B-VAMS**	10–750	0.9991	10.0	3.0
	OF-VAMS	7–750	0.9992	7.0	2.5
DCT	**B-VAMS**	1–200	0.9996	1.0	0.3
	OF-VAMS	1–200	0.9998	1.0	0.3
DDC	**B-VAMS**	1–200	0.9993	1.0	0.3
	OF-VAMS	1–200	0.9995	1.0	0.3

3.4.2. Extraction yield, precision

The results of extraction yield and precision assays are reported in Table 2. Extraction yields were quite good, in the 86–95% range for B-VAMS and in the 85–96% for OF-VAMS, while precision, expressed as RSD values, was always in the 5.4–8.8% range. Comparative extraction yield assays, performed by adding ISs to fluid matrices before VAMS sampling and pre-soaking VAMS tips with IS standard solution, provided overlapping results (extraction yield range 85–95%, RSD < 6.8%), thus demonstrating the suitability of both approaches.

335 **Table 2**. Extraction yield and precision in spiked samples.

Analyte	Concentration level[a]	Matrix	Extraction yield, %[b]	Precision, RSD%[c]	
				Intraday	Interday
SRT	LOQ	B-VAMS	89	6.7	8.1
		OF-VAMS	90	7.0	7.9
	Low	B-VAMS	91	6.5	7.9
		OF-VAMS	91	6.7	7.7
	Intermediate	B-VAMS	92	6.0	7.5
		OF-VAMS	93	6.1	7.2
	High	B-VAMS	93	5.8	7.0
		OF-VAMS	95	6.0	6.7
FLX	LOQ	B-VAMS	90	6.9	7.8
		OF-VAMS	91	7.5	8.4
	Low	B-VAMS	91	6.4	7.6
		OF-VAMS	92	7.4	8.0
	Intermediate	B-VAMS	93	5.9	7.3
		OF-VAMS	93	7.2	7.6
	High	B-VAMS	94	5.6	6.8
		OF-VAMS	96	5.8	6.9
CTP	LOQ	B-VAMS	89	6.2	7.9
		OF-VAMS	91	7.0	8.1
	Low	B-VAMS	90	5.9	7.6
		OF-VAMS	91	7.7	7.9
	Intermediate	B-VAMS	92	5.7	7.2
		OF-VAMS	93	6.0	7.5
	High	B-VAMS	95	5.4	6.6
		OF-VAMS	95	5.8	6.9
VRX	LOQ	B-VAMS	87	7.0	8.2
		OF-VAMS	89	7.2	8.4
	Low	B-VAMS	88	6.8	7.8
		OF-VAMS	90	7.2	7.6
	Intermediate	B-VAMS	90	6.1	7.6
		OF-VAMS	90	6.9	7.4
	High	B-VAMS	91	5.6	7.0
		OF-VAMS	93	6.1	7.2
NSR	LOQ	B-VAMS	87	7.3	8.6
		OF-VAMS	85	7.3	8.4
	Low	B-VAMS	88	6.9	8.4
		OF-VAMS	88	7.0	7.0
	Intermediate	B-VAMS	88	6.4	8.0
		OF-VAMS	91	6.6	7.6
	High	B-VAMS	90	5.9	7.4
		OF-VAMS	92	5.9	7.1
NFL	LOQ	B-VAMS	87	7.5	8.6
		OF-VAMS	88	7.2	8.8
	Low	B-VAMS	87	7.4	8.3
		OF-VAMS	89	6.9	8.3
	Intermediate	B-VAMS	89	7.0	7.9
		OF-VAMS	89	6.8	7.8
	High	B-VAMS	91	5.8	7.3
		OF-VAMS	91	5.6	7.4
DCT	LOQ	B-VAMS	87	7.6	8.8
		OF-VAMS	85	7.4	8.7
	Low	B-VAMS	87	7.5	8.6
		OF-VAMS	86	7.2	8.2
	Intermediate	B-VAMS	88	7.3	8.0
		OF-VAMS	87	6.9	7.7
	High	B-VAMS	89	6.0	7.5
		OF-VAMS	90	6.1	6.9
DDC	LOQ	B-VAMS	86	7.7	8.5
		OF-VAMS	85	7.7	8.6
	Low	B-VAMS	86	7.0	8.2
		OF-VAMS	88	7.5	8.4
	Intermediate	B-VAMS	88	6.6	7.6
		OF-VAMS	88	6.9	7.8
	High	B-VAMS	90	5.8	6.8
		OF-VAMS	91	6.2	7.2

336

337 [a] For each matrix, “Low”, “Intermediate” and “High” concentrations are referred to the respective linearity curve.

338 [b] n = 6, mean value. c n = 6.

339 ***3.4.3. Selectivity and carryover***

340 No interfering peak higher than 20% of the response of the LOQ of each analyte was detected in any of the
341 blank samples, and no interfering peak at the retention time of the analytes or ISs under the two detection
342 means was also observed when injecting into the HPLC system common CNS drugs. Moreover, carryover was
343 within the defined acceptance criteria: interference signals were < 20% of the LOQ (<5% for ISs).

344

345 ***3.4.4. Stability***

346 Dried microsamples are usually stored at RT while maintaining good stability, thanks to the lack of water that
347 effectively stops many chemical and enzymatic reactions, and also causes molecular immobilisation. In this
348 case, analyte stability in spiked B-VAMS and OF-VAMS after 3 months resulted to be very high: mean stability
349 was > 88.1% (range: 88.2–97.2%) for B-VAMS and >85.0% (range: 85.1–94.8%) for OF-VAMS. Moreover, all
350 analytes proved to be stable (>85.3%) also in all remaining stability assays (autosampler processed and
351 bench-top assays).

352

353 **3.5. Analysis of real samples and accuracy**

354 Having validated the microsampling, pretreatment and analysis workflow, real samples from psychiatric
355 patients undergoing treatment with new generation antidepressants were analysed. Examples of
356 chromatograms of OF-VAMS samples obtained from patients treated with SRT (75 mg/d) and FLX (50 mg/d)
357 are shown in Fig. 3a and b, respectively. As one can see, analyte separation is similar to that obtained on the
358 corresponding spiked blank samples. Table 3 shows the B-VAMS and OF-VAMS data obtained from real
359 patient samples. As can be seen, the administered drug was identifiable and quantifiable in all B-VAMS and
360 OF-VAMS samples, together with the considered metabolites. As for matrix comparison, no clear correlation
361 was found, with OF-VAMS results usually much lower than those of B-VAMS except for VTX, with OF-VAMS
362 concentrations slightly higher than those observed in B-VAMS. In order to evaluate the analytical
363 performance of the proposed dried microsampling approaches, the results obtained from VAMS on real
364 samples (Table 3) were compared to those obtained from fluid matrix counterparts (plasma and OF) by using
365 fully validated procedures (Supplementary Material S1) and after converting plasma results to whole blood
366 concentrations by using mean erythrocyte-to-plasma concentration ratios for the target analytes [27–30].
367 Sample pretreatment was based on solid phase extraction (SPE) for plasma and on liquid-liquid extraction
368 (LLE) for OF, both coupled to HPLC-UV-FL analysis. B-VAMS results are always very similar to those of the
369 original fluid matrix, i.e., plasma concentrations after conversion to whole blood values (data not shown).
370 Bland-Altman differences plots were built for B-VAMS/plasma and OF-VAMS/fluid OF correlations (Fig. 4a

and b, respectively) together with scatter plots (Supplementary Material Figure SF2). These graphs and Passing-Bablok regression, testify as to the agreement between each miniaturised matrix and its corresponding fluid matrix. Passing-Bablok regression produced for B-VAMS - plasma comparison (n = 16) a slope coefficient of 1.0054 (95% CI, 0.9652–1.0388), an intercept of 0.2512 ng/mL (95% CI, −1.3305 ng/mL–1.5911 ng/mL) and an r2 value of 0.9964; for OF-VAMS - fluid OF comparison (n = 16), slope coefficient was of 1.0000 (95% CI, 0.9135–1.0746), intercept was 0.2500 ng/mL (95% CI, −1.5500 ng/mL–2.7596 ng/mL) and r2was 0.9941. LOQ values for the microsampling methods were about twice those of the corresponding fluid matrix methods, despite the former ones diluting the samples 5 times more than the latter ones. This can be at least in part attributed to the better sample purification level achieved by VAMS coupled to MEPS pretreatment. Accuracy assays provided very high recovery values, in the 86–94% range for B-VAMS and 88–93% for OF-VAMS. In conclusion, both microsampling methods provide reliable analytical results. The results obtained from B-VAMS can be directly applied for TDM purposes. For those obtained from OF-VAMS, no clear correlation was observed with those of whole blood, with generally lower concentrations in the former one, but of course more data are needed to evaluate VAMS-OF as a useful matrix for TDM.

Table 3. Patient sample analysis.

Subject n.	Treatment, dose (mg/d)	Biological matrix	Concentration found, ng/mL[a]		
			Parent drug[b]	Metabolite 1[c]	Metabolite 2[d]
1	SRT, 75	B-VAMS	41.3	53.2	–
		OF-VAMS	32.6	27.9	–
2	SRT, 100	B-VAMS	88.6	116.1	–
		OF-VAMS	48.6	36.5	–
3	FLX, 50	B-VAMS	49.8	48.3	–
		OF-VAMS	26.8	30.4	–
4	FLX, 60	B-VAMS	86.9	127.1	–
		OF-VAMS	41.6	57.9	–
5	CTP, 20	B-VAMS	54.9	26.9	7.5
		OF-VAMS	36.4	13.4	4.8
6	CTP, 30	B-VAMS	100.6	52.1	14.7
		OF-VAMS	53.6	23.8	6.6
7	VTX, 7.5	B-VAMS	9.6	–	–
		OF-VAMS	10.4	–	–
8	VTX, 10	B-VAMS	13.3	–	–
		OF-VAMS	17.8	–	–

a n = 3.

b The same drug indicated in the “treatment” column.

c NSR for patients treated with SRT; NFL for patients treated with FLX; DCT for patients treated with CTP.

d DDC for patients treated with CTP.

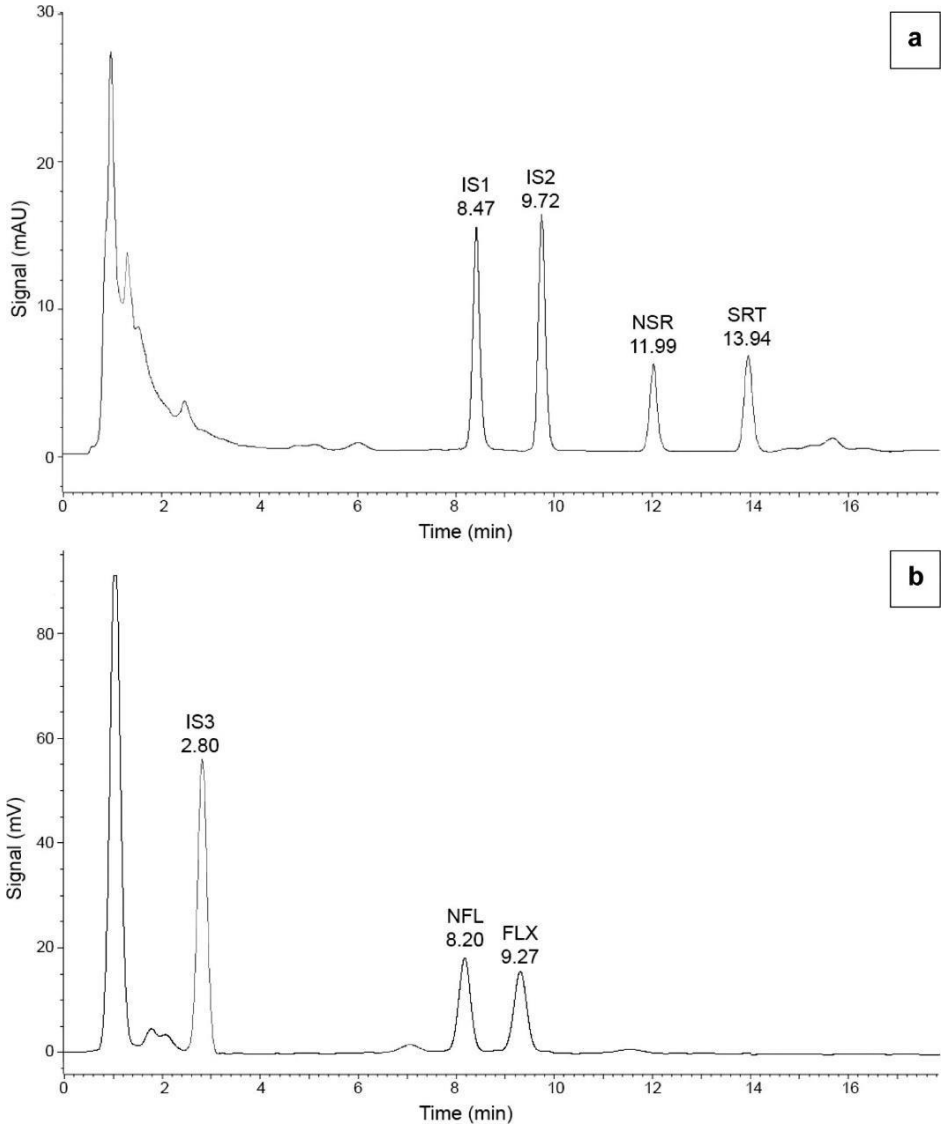
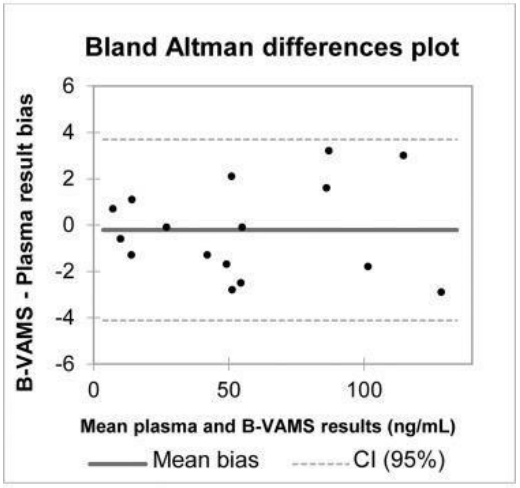


Fig. 3. (a) HPLC-UV chromatogram of an OF-VAMS sample obtained from a patient treated with SRT (75 mg/d) and (b) HPLC-FL chromatogram of an OF-VAMS sample obtained from a patient treated with FLX (50 mg/d).

a - Plasma vs. B-VAMS results



b - Fluid OF vs. OF-VAMS

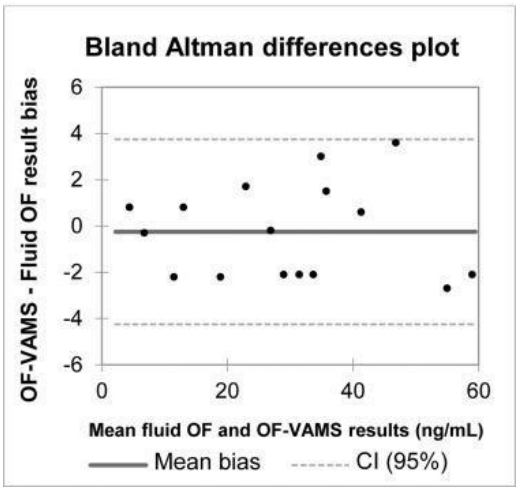


Fig. 4. Bland Altman differences plots for the following result comparison: plasma vs. B-VAMS (a) and fluid OF vs. OF-VAMS (b). Plasma results are intended after conversion to whole blood values by means of erythrocyte-to-plasma concentration ratios for each analyte.

4. CONCLUSION

An analytical workflow, based on B-VAMS or OF-VAMS, MEPS pretreatment and HPLC-UV-FL determination, has been developed, validated and applied to the TDM of patients suffering from major depression and/or related disorders, treated with SRT, FLX, CTP or VTX. Microsampling volume by VAMS for both blood and oral fluid was tested, and good data were obtained (volume accuracy 100.2%, precision RSD 4.7% for B-VAMS and volume accuracy 99.8%, precision RSD 5.7% for OF-VAMS). Validation results were very satisfactory, with high extraction yields (>85% for B-VAMS and >84% for OF-VAMS) and accuracy (>85% and >87% for B-VAMS and OFVAMS, respectively), and low RSD values for precision (85.0% for all analytes and all matrices, even though B-VAMS and OF-VAMS were stored at RT. Finally, good agreement was obtained between the data obtained from the TDM of patients when using B-VAMS as opposed to blood and OF-VAMS as opposed to oral fluid. However, no clear, convincing correlation between blood and OF levels was found for any of the considered drugs. Of course, the analysis of many more patient samples is needed for this purpose. In conclusion, both B-VAMS and OF-VAMS seem to be suitable for the reliable and feasible analysis of blood and OF levels, respectively, of antidepressants in psychiatric patients. Regarding at-home self-sampling specifically, this study represents a first proof-of-concept of the possible application of VAMS for TDM purposes. In fact, VAMS sampling can easily be carried out by most people without any particular training, let alone a specific healthcare training. Obviously, more extensive experimentation and statistical evaluations should be carried out on large numbers of volunteers, including the acceptability and preferences of patients, as well as the possible effects of self-sampling on TDM reliability and accuracy.

Declaration of Competing Interest

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

CRediT authorship contribution statement

Camilla Marasca: Methodology, Investigation, Formal analysis, Validation, Writing - original draft. Michele Protti: Methodology, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization. Roberto Mandrioli: Conceptualization, Resources, Data curation, Writing - original draft, Writing - review & editing. Anna Rita Atti: Writing - review & editing, Supervision, Resources, Funding acquisition. Andrea Armirotti: Writing - review & editing, Resources. Andrea Cavalli: Resources, Funding acquisition. Diana De Ronchi: Writing - review & editing, Resources, Funding acquisition, Project

administration. Laura Mercolini: Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Funding acquisition, Project administration.

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