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Volumetric absorptive microsampling for the therapeutic drug monitoring of psychiatric patients treated with cariprazine

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ABSTRACT

Psychiatric disorders are usually treated with antipsychotic agents belonging to different pharmacological and chemical classes, the most recent ones collectively known as "third-generation antipsychotics", such as cariprazine, approved in 2015 for the treatment of patients affected by schizophrenia. For these patients, a frequent therapeutic drug monitoring (TDM) becomes essential to assess compliance and to optimise and personalise their therapy, also due to cariprazine interindividual variability and narrow therapeutic range. In this study, a bioanalytical method featuring miniaturised sampling and pretreatment was developed, based on volumetric absorptive microsampling (VAMS) for TDM of psychiatric patients under cariprazine treatment and compared to a reference method based on fluid plasma analysis.

Minimally invasive whole blood VAMS was coupled to an original instrumental method based on ultra-high performance liquid chromatography hyphenated to mass spectrometry (UHPLC-MS). A feasible and streamlined, yet reliable VAMS pretreatment protocol was carefully optimised and the VAMS-UHPLC-MS methodology was validated with satisfactory results in terms of linearity ($r^2 > 0.9970$ in the 1.5–100 ng/mL range), precision (%RSD < 11.7), extraction yield (> 90.0 %) and matrix effect ($8.2 \leq$ %RE ≤ 10.9). Finally, the microsampling approach coupled to UHPLC-MS was successfully applied to the TDM of psychiatric patients treated with cariprazine and compared with standard fluid plasma analysis, providing reliable quali-quantitative results, and proving to be readily applicable to the clinical practice in TDM programs as a useful alternative to cariprazine plasma analysis. This is the first report of a successful microsampling application, and in particular the first report of VAMS application, for the TDM of cariprazine.

1. Introduction

Psychiatric disorders, such as schizophrenia and bipolar disorder, are a major public health problem whose onset occurs between adolescence and early adult life. These are characterised by a great heterogeneity of psychopathologic symptoms (positive, negative and/or cognitive), therefore psychiatric patients need long-term pharmacological treatment to reduce or eliminate symptoms and improve quality of life [1]. Antipsychotic drugs represent the standard therapy for the treatment of schizophrenia, and more recently also of bipolar disorder [2], and are

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divided into: classical or first-generation antipsychotics (FGAs), atypical or second-generation antipsychotics (SGAs), and finally third generation antipsychotics (TGAs) based on their specific mechanism of action, which mainly involves dopaminergic (D) and serotonergic (5-HT) receptors [3] in different roles. FGAs mainly relied on D receptor inhibition for their therapeutic activity; SGAs added 5-HT_{2A} inhibition to D inhibition, obtaining a broader spectrum of activity and lower incidence of extrapyramidal side effects; TGAs are characterised by their partial agonism at both D and 5-HT_{2A} receptors, which induces significant efficacy in the treatment of positive, negative and cognitive symptoms and an improved tolerability profile [4]. A relatively recent TGA is cariprazine (CRP, (3-[4-[2-[4-(2,3-dichlorophenyl)piperazin-1-yl]ethyl] cyclohexyl]-1,1-dimethylurea, Fig. 1), which received approval in 2015 from the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for the treatment of adult patients with schizophrenia. CRP has also been approved, only by FDA, for the acute treatment of manic illness or mixed episodes associated with bipolar I disorder [5]. In particular, this drug is a potent partial agonist of dopamine D_2/D_3 receptors (with a tenfold affinity for the D_3 over the D_2 receptors) and serotonin $5HT_{1A}$ receptors, as well as antagonist at serotonin $5HT_{2B}$ and $5HT_{2A}$ receptors and at histamine H_1 receptors. Although this pharmacological profile carries important clinical advantages over other antipsychotic agents, CRP is obviously not without adverse effects (including extrapyramidal ones) [6]. Moreover, it shows a large interindividual variability in efficacy and its beneficial effects also strongly depend on the plasma concentration of the drug remaining within a narrow therapeutic range (10–20 ng/mL) [7,8]. For these reasons, therapeutic monitoring (TDM) of CRP-treated psychiatric patients is recommended in order to evaluate patient compliance and response, optimise drug dosing and improve treatment safety [9]. Additionally, according to the Consensus Guidelines for Therapeutic Drug Monitoring in Neuropsychopharmacology, current CRP safety and efficacy data are mostly based on retrospective analyses of TDM data, single case reports or non-systematic clinical experience [8].

Thus, more extensive application of monitoring protocols is needed to further refine and establish chemical-clinical correlations (CCCs) [10].

The first step for the success of TDM is always the development and implementation of reliable and reproducible analytical methods, characterised by adequate sensitivity, sufficient precision and accuracy, and a reasonable throughput that produces results in time to facilitate clinical decisions. However, special attention must be paid to the variables, which can hinder the establishment of a smooth workflow, such as the invasive venepuncture sampling technique, in which the collection of large volumes (from 1 up to 5 mL) of blood can involve significant risks (i.e. infection, both in the patient and in the phlebotomist, excessive bleeding, difficulty in locating the right vein, damage to nerves and other blood vessels). Furthermore, both blood and plasma samples need to be stored and transported at controlled temperatures to avoid sample degradation [11]. Moreover, patient discomfort and, in particular for psychiatric patients, distrust and fear of the procedure itself for disorder-related reasons are concrete obstacles to the timely application of TDM protocols.

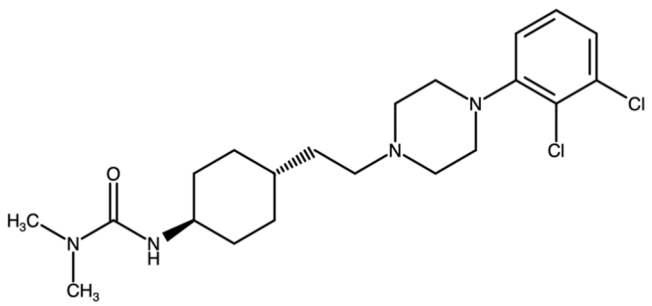

Fig. 1. Chemical structure of cariprazine.

Therefore, in order to overcome the drawbacks of the conventional sampling method, in recent years instrumental and technological advances have made it possible to provide the degree of sensitivity and automation necessary to analyse small volumes of biological samples, which seem to be promising for TDM purposes. Several minimally invasive dried microsampling strategies based on the collection of small volumes of matrix (<100 μL) by fingerprick have been developed and implemented. The ease and minimal invasiveness of this kind of sampling make it possible to simplify and incentivise TDM protocols in delicate populations, also paving the way for capillary blood at-home- and self-sampling in the near future [9]. Furthermore, dried microsamples can usually be stored and transported at room temperature (RT), thus reducing the space and equipment required for refrigerated/frozen storage, as well as reducing logistical issues and overall analysis times [12,13]. Volumetric absorptive microsampling (VAMS) technology, introduced on the market in 2014, generates dried biological microsamples at a fixed volume using a porous polymeric tip capable of collecting, depending on its format, 10, 20 or 30 μL of capillary blood regardless of the density of the fluid [14]. By this strategy, one of the most important limitations of the very well-known dried blood spots (DBS) testing, that is, the influence on the whole blood sampling by haematocrit (HCT) values (and thus density and viscosity) and other variables [15,16], is definitely overcome.

The VAMS biological microsample collection procedure can take place in two ways: by contact with the pre-collected fluid or, in the case of blood, from a fingerprick, placing the VAMS device into direct contact with the blood drop. The device is self-indicating, so when the tip turns completely red (in a few seconds) it means it has absorbed the set blood volume. Subsequently, VAMS samples are stored in a specific clamshell enclosure and left to dry at room temperature protected from light and in a low-humidity environment. Once drying is complete, the tip is removed from its holder and the desorption/extraction of the target compounds from the solid matrix is carried out [17]. Compared to fluid matrix collection and handling, VAMS shows significant advantages: the drying step can increase compound stability at RT, thus allowing more feasible sample storage and transport, also with reduced potential for sample-operator cross-contamination [18]. Furthermore, the sampling procedure is simplified and straightforward, requiring minimal skills and thus potentially allowing self- and at-home-sampling by the patients themselves. Finally, the size and geometry of VAMS devices allow their implementation in automated workflows by exploiting rapid and reliable analytical protocols despite the small volume of biological sample [19].

The aim of this work was the development and validation of a miniaturised bioanalytical approach based on VAMS coupled to UHPLC-MS for the quantitative analysis of CRP and its application for TDM purposes.

Recent reviews offer a comprehensive overview of studies on microsampling for TDM in psychiatric patients [11,20–22]. Accordingly, it is possible to find analytical methods based on different microsampling strategies, such as DBS, VAMS, or capillary volumetric microsampling for the TDM of patients treated with different classes of drugs: antipsychotics, immunosuppressants, antiepileptics, antimicrobials, antidepressants, analgesics by exploiting different biological matrices, mainly blood and urine [13,18,23–27]. Studies on CRP are still lacking and mainly include the validation of a classical analytical method for the simultaneous determination of CRP and its main metabolites in human plasma and urine, the determination of CRP in vivo effects in rodents [28,29] and methods to determine the drug in pharmaceutical products. On the contrary, to date there are no published miniaturisation studies for the quantitation of CRP for TDM purposes. The present work mostly aims to fill this gap.

2. Materials and methods

2.1. Chemicals and standard solutions

All reagents were of analytical grade or better. CRP ($\geq$ 98 %) was purchased from LGC Standards (Teddington, Middlesex, UK). CRP-D6, used as the internal standard (IS), was purchased from Cayman Chemical (Ann Harbor, MI, USA). Methanol (MeOH, < 99.9 %), acetonitrile (ACN, < 99.9 %), monobasic potassium phosphate (K_2HPO_4, $\geq$ 98 %), triethylamine (TEA, $\geq$ 99.5 %), phosphoric acid (H_3PO_4, 85–87 %, m/m), formic acid (FA, 99 %, m/m), ammonium hydroxide (25 %, m/m) were produced by Merck Italia (Milan, Italy). Ultrapure water (18.2 MΩ·cm) was obtained by means of a Millipore (Milford, MA, USA) Milli-Q apparatus. Stock solutions (1 mg/mL) were prepared in MeOH and stored at − 20 °C. Fresh working standard solutions were obtained daily by diluting stock solutions with the mobile phase.

2.2. Apparatus and chromatographic conditions

An ultra-high performance liquid chromatography system coupled to a single-quadrupole mass spectrometer (UHPLC-MS) was used. The system was composed of a Vanquish UHPLC system (pump, autosampler, column compartment) coupled to an ISQ EC single quadrupole mass spectrometer (Thermo Fisher Sci., Waltham, MA, US), while data elaboration was carried out by means of Thermo Fisher Scientific Dionex Chromeleon 7.3 Chromatography Data System software. Separations were obtained on a Waters (Milford, MA, USA) Cortecs C18 column (100 mm × 2.1 mm i.d., 2.7 μm), kept at room temperature (RT) and coupled with a VanGuard Cortecs C18 guard column (50 × 2.1 mm i.d., 2.7 μm).

The mobile phase consisted of a mixture of 0.1 % (V/V) FA in water (solvent A) and 0.1 % (V/V) FA in ACN (solvent B); gradient composition was: 0.0–1.5 min, constant 10 % B; 1.5–3.0 min, linear 10–60 % B gradient; 3.0–6.0 min, constant 60 % B; 6.0–8.0 min, linear 60–10 % B gradient; 8.0–10.0, linear 10 %B for column re-equilibration. Flow rate was 0.25 mL/min and 5 μL injections were carried out. Optimised mass spectrometric parameters were the following: positive source voltage + 3.0 kV; ion transfer tube temperature (ITT), 300 °C; vaporizer temperature (VT), 144 °C; sheath gas pressure (SGP), 32.3 psig; auxiliary gas pressure (AGP), 3.6 psig; sweep gas pressure (SWGP), 0.5 psig. Positive electrospray ionisation (ESI+) was exploited, which resulted in extracted chromatograms from their $[M+H]^+$ ions: monitored *m/z* values were 428.38 for CRP and 434.36 for the IS.

A Crison (Barcelona, Spain) Basic 20 pH-meter and a Hettich (Tuttlingen, Germany) Universal 32 R centrifuge were used.

2.3. Sample collection and pretreatment

VAMS devices (10 μL) were from Neoteryx (Torrance, CA, USA) under the Mitra® brand name. Blank blood samples for method

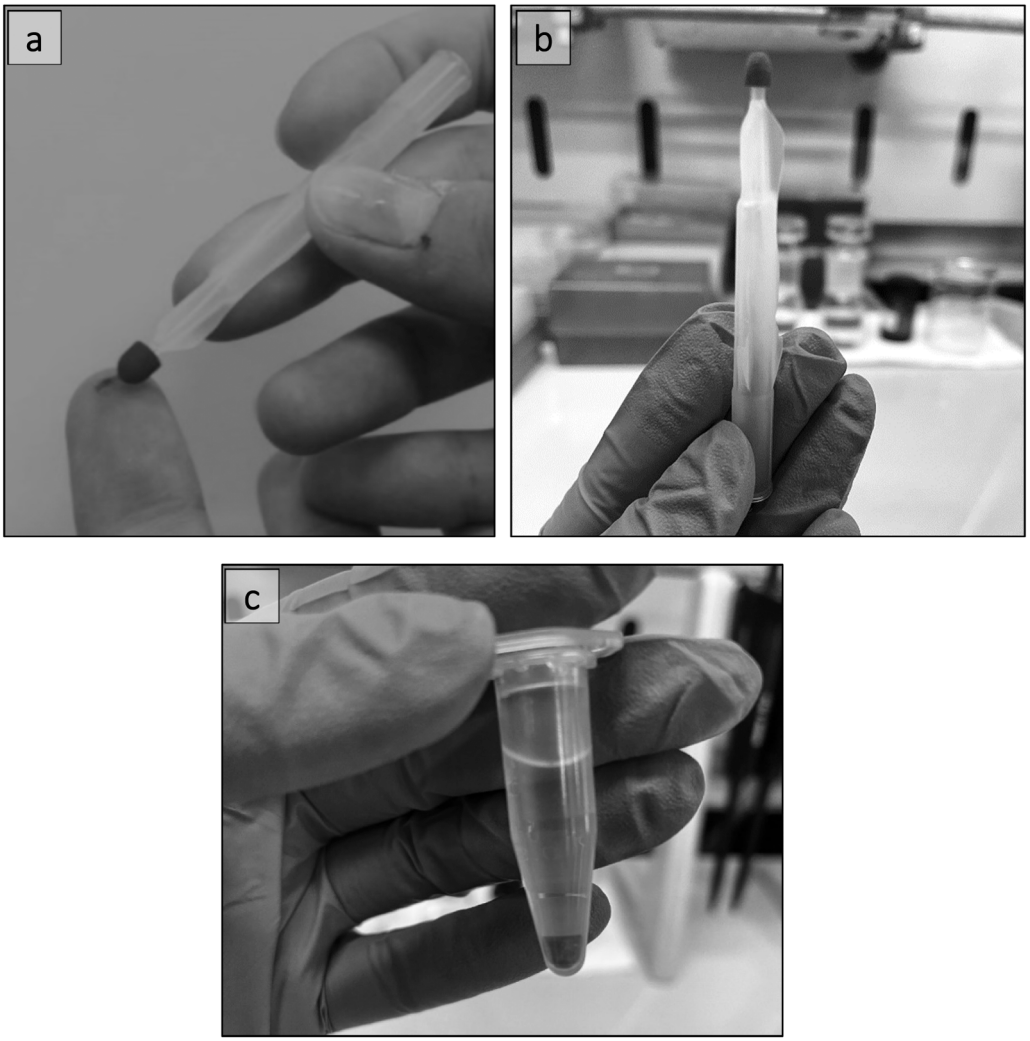

Fig. 2. VAMS sampling by fingerprick (a), whole blood VAMS sample (b) and VAMS tip extraction (c).

development and validation were collected from healthy volunteers who were not receiving any drug treatment at the time of collection. The tip of a VAMS device (10 μL) was placed into contact with the drop of blood obtained by fingerprick to allow uniform absorption and tip filling (Fig. 2a), then it was dried at RT for 70 min. Afterwards, the surface of a standard solution containing CRP and/or the IS was touched with the tip of the VAMS microsampler (Fig. 2b), dried at RT for 50 min and stored at RT in a dedicated clamshell in order to avoid contact with any surface. Thus, a CRP-fortified VAMS sample was obtained.

The tip was detached from the handle and subjected to ultrasound-assisted extraction (UAE) in 200 μL of MeOH at 150 W and RT for 15 min (Fig. 2c) and, finally, the resulting solution was directly injected into the UHPLC-MS system.

Whole blood samples for method application were obtained from patients treated with CRP, admitted to different psychiatric services and hospitals of the Emilia-Romagna Region in Italy. After VAMS collection, whole blood from the same subjects was centrifuged. After that, fluid plasma was separated and transferred to polypropylene tubes and stored at − 20 °C until sample pretreatment by solid-phase extraction (SPE), which was used as a reference plasma standard method for comparison with results obtained from whole blood VAMS.

The original procedure developed on purpose on classic plasma samples for reference was applied as follows, exploiting a SPE cartridge containing C8 sorbent (50 mg, 1 mL): Activation + conditioning, 3 × 1 mL MeOH + 3 × 1 mL H_2O; Sample loading, 250 μL plasma + 500 μL H_2O + 50 μL IS solution in mobile phase; Washing, 2 × 1 mL H_2O + 1 mL H_2O/MeOH mixture (90:10, V/V) + 50 μL MeOH; Elution, 1 mL MeOH. The extract was dried under vacuum and, finally, redissolved with 100 μL of mobile phase before being injected into the chromatographic system.

2.4. Method validation

The VAMS-UHPLC-MS method was validated according to US Food and Drug Administration (FDA) [30], International Conference on Harmonisation (ICH) M10 [31] and International Association for Therapeutic Drug Monitoring and Clinical Toxicology [32] guidelines.

2.4.1. Linearity, extraction yield, precision

Linearity was determined on standard mixtures at seven different concentrations. A calibration curve was set up using the least-squares method by plotting CRP/IS peak area ratios (pure numbers) against the corresponding analyte concentrations (expressed as ng/mL), and the LOQ was set at the lowest calibrator level (provided that the corresponding analyte peak height was at least 10 times the baseline noise). The LOD was calculated as the analyte concentration that gave rise to a peak whose height was 3 times the baseline noise.

Extraction yield and precision were assessed on blank spiked samples through UHPLC-MS analysis by adding known amounts of CRP at three different concentrations (corresponding to the lower limit, an intermediate value and the upper limit of the linearity range) and a constant amount of IS to fluid blood, collected and pretreated by means of VAMS.

Analyte peak areas were compared with those obtained by injecting standard solutions at the same theoretical concentrations to calculate extraction yield, expressed as percentage recovery. The assays were repeated five times on the same day and over five different days to obtain intra-day and inter-day precision, respectively; both were expressed as percentage relative standard deviation (%RSD).

2.4.2. Matrix effect and carryover

IS-corrected matrix effect was evaluated by analysing six blank VAMS replicates, fortified post-extraction by adding known analyte concentrations to blank VAMS extracts at the same levels as precision assays. The mean analyte/IS peak area ratios for each added concentration were compared with analyte/IS peak area ratios from standard solutions at the same theoretical concentration and the resulting percentage was calculated. Acceptability criterion: percentage relative error (%RE) $\leq$ 15 %.

Carryover was assessed by the injection of a blank VAMS extract after the highest concentration of the calibration curves (n = 3), and the absence of carryover was accepted if any resulting analyte peak at the retention time of CRP was less than 20 % of that of a LOQ calibrator.

2.4.3. Stability

The possibility that dried VAMS microsamples could increase analyte stability was verified by analysing spiked blood VAMS samples (n = 3) stored for up to 30 days at RT, in zip-lock bags with desiccant and protected from direct light. The measured analyte recovery values were compared to those of fluid blank plasma samples spiked with CRP and the IS and stored at − 20 °C for the same amounts of time.

2.5. Haematocrit effect

In order to study possible variations in method performance with respect to HCT value, spiked whole blood samples were prepared with low- (25 %), medium- (45 %), and high-HCT (65 %) blood and sampled to obtain VAMS. Extraction recoveries and matrix effect for low- and high-HCT samples were evaluated in comparison to medium-HCT samples. Recovery of the low- and high-HCT samples was considered independent from HCT if it was within ± 15 % of the recovery observed for medium-HCT ones. Similarly, if matrix effect, expressed as %RE was in the ± 15 % range with respect to medium-HCT samples, it was deemed satisfactorily independent from HCT value.

2.6. Patient samples

After development and validation, the analytical methodology involving VAMS microsampling coupled to UHPLC-MS analysis was applied to real samples from patients undergoing therapy with CRP. Whole blood samples from patients were drawn into tubes containing EDTA as an anticoagulant and the corresponding VAMS samples were obtained at the same time. Then, after whole blood centrifugation, the plasma was separated and used for the SPE-based pretreatment procedure to provide a benchmark for microsample performance.

Dried and fluid samples were pretreated and analysed according to the previously described procedures. The quali-quantitative results obtained from dried microsample analysis were compared to those from the analysis of fluid plasma to evaluate the agreement between the data sets.

Accuracy assays were performed by adding standard analyte mixtures at three concentrations (low, medium, and high levels) to VAMS patient sample replicates, whose analyte content had already been determined. Accuracy was expressed as percentage recovery of the spiked amount; the assays were carried out in triplicate.

3. Results and discussion

3.1. Development of VAMS sampling and pretreatment

Several parameters were evaluated to develop and validate the miniaturised method proposed here. As a first step, the accuracy and variability of the sampling volume were considered. Previous studies carried out on different biological matrices showed that there are no statistically significant differences between the volumes collected with the 10 μL VAMS tips and those obtained by volumetric pipettes, thus showing a good sampling accuracy by VAMS devices [23,33,34]. Sampling times were previously evaluated and it was verified that contact times of ~2 s were sufficient to completely fill the 10 μL VAMS tips [18,33]. Moreover, the level of red colour (from whole blood) in the tip can be a useful indicator of its filling. Furthermore, another sampling parameter was evaluated: the blood sample drying time. The drying test was carried out three times using a balance with a resolution of 0.01 mg.

After the VAMS tip absorbed 10 µL of capillary blood, the device was placed inside a calibrated analytical balance and the weight was evaluated every 10 min. In the perspective of point-of-care tests, self-sampling and at-home sampling, the sample drying time was measured on blank blood samples, on blank samples fortified with 10 µL of IS standard solution and on blank samples fortified with 10 µL of a standard mixture of IS and the analyte. The higher time to constant weight was 70 min, and this was taken as the complete drying time indicator (Fig. 3a). Additional drying time tests were carried out by envisaging an a posteriori sample fortification procedure, i.e. on already dried blood VAMS. After drying, the VAMS tip was made to absorb 10 µL of the fortifying working solution (containing IS or IS together with the analyte), and the time to constant weight of the CRP- and/or IS-enriched VAMS sample was again measured, which was 50 min (Fig. 3b).

Then, pretreatment parameters influencing the UAE performance of VAMS were studied: extraction solvent, volume, time, and power were optimised. Different volumes (50 µL, 100 µL, 200 µL, 500 µL, 1000 µL) of pure solvents and solvent mixtures (methanol, acetonitrile, water and their mixtures: solvent A / solvent B in 10/90, 30/70 and 50/50, V/V, ratios, where solvent A and B are all possible permutations of the three solvents), extraction times (5–30 min) and power values (50, 100, 150, 200 W) were tested. The VAMS extraction procedure consisting in exposing the tip to 200 µL of MeOH for 15 min of 150-W UAE gave the best results. Indeed, an increase in extraction yield was observed up to a volume of 200 µL; higher volumes did not bring any improvement in extraction yield while only increasing matrix effect (mean matrix effect with a volume of 200 µL was 9.6 in terms of %RE, 10.6 with a volume of 300 µL, 11.5 with a volume of 300 µL). A similar behaviour occurred for the time of UAE. The ratios of the extraction mixture have been finely optimised together with the UAE time, which was gradually reduced from the initial time of 30–15 min to avoid extensive haemolysis and, thus, contamination of the extraction mixture. Increasing ultrasound power in the 50–150 W range produced increasing extraction yields, but higher power values caused a marked decrease in yield, probably due to analyte degradation by excessive heating. As can be seen, the use of an innovative VAMS microsampling procedure allowed to significantly reduce extraction times, solvent volumes, and biological sample manipulation in comparison to common extraction procedures from fluid samples. Fig. 4 shows the obtained chromatogram of a VAMS sample enriched with CRP and the IS at known concentrations.

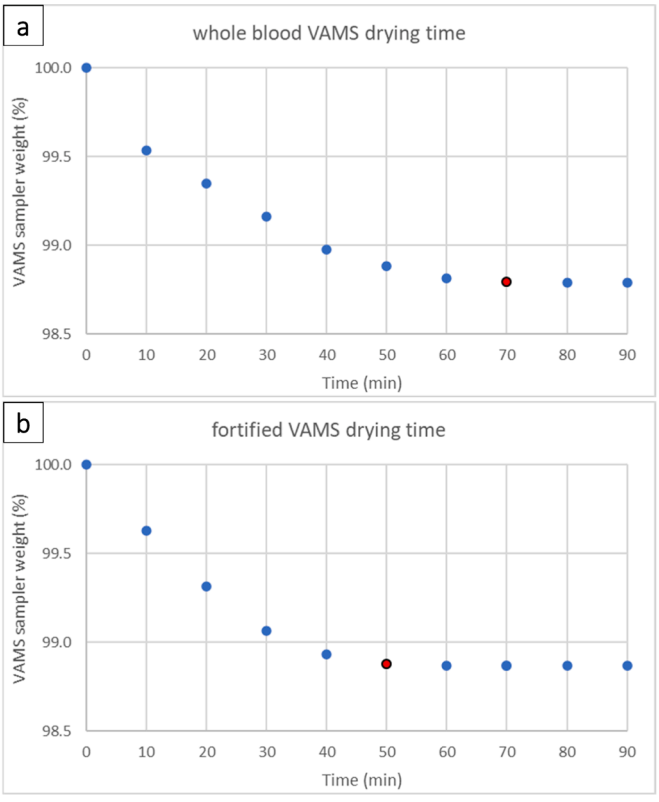

Fig. 3. Blood VAMS sample drying times (a) and VAMS drying time after fortification with a standard solution (b).

3.2. Method validation

To determine the linearity of the analytical method, CRP standard solutions were injected at seven different concentrations in a 1.5–100 ng/mL linearity range. Calibration curves were obtained and the LOQ and LOD for CRP were calculated. All linearity data are reported in Table 1.

Blank blood was fortified with three different concentrations of the analytes (LOQ, 50 ng/mL, 100 ng/mL) to evaluate extraction yield and precision. The results were satisfactory, extraction yields were higher than 90.0 % for all concentrations. Precision was also good, with %RSD values always lower than 11.7. Complete results of these assays are reported in Table 1.

Matrix effect, expressed as percentage relative error (%RE), was in the 8.2–10.9 % range. No significant carryover was observed as well, as no signal higher than the background noise was detected at the retention time and *m/z* values of the analyte and the IS, when injecting a blank solvent after analysing a sample fortified with the highest concentration of the calibration curve (n = 3).

Stability of the analyte in fluid plasma samples under controlled conditions (−20 °C) was compared to that in the dried VAMS matrix, stored at RT, in the presence of desiccating agents and protected from direct light. After 30 days, VAMS samples showed greater stability (mean analyte loss $< 3.8 \pm 0.4$ %) than plasma samples (mean analyte loss $< 5.9 \pm 0.6$ %), despite VAMS samples being stored at RT. Thus, a significant improvement in analyte stability has been achieved with micromatrices, as the higher stability of VAMS can be attributed, at least in part, to the water loss during the drying process, which slows or stops most chemical and enzymatic reactions leading to potential analyte

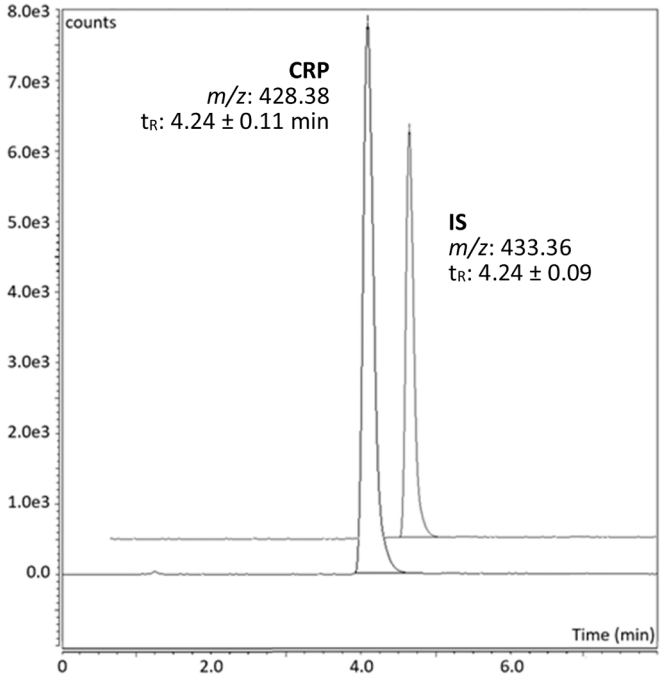

Fig. 4. UHPLC-MS chromatogram of a blank VAMS sample spiked with CRP (20 ng/mL) and the IS (25 ng/mL).

Table 1
Linearity parameters, extraction yield, precision and matrix effect in spiked VAMS samples.

CRP linearity range (ng/mL)	LOQ (ng/mL)	LOD (ng/mL)	Calibration curve (y = ax + b)		
			r^2	$a \pm$ SD	$b \pm$ SD
1.5–100	1.5	0.5	0.9971	98.294 ± 6.331	243.330 ± 73.114

Compound	Concentration level	Extraction yield, %[b]	Precision, RSD %[c]		Matrix effect, %RE[d]	Accuracy, %[d]
			Intraday	Interday		
CRP	1.5	115.7	11.5	11.6	10.9	92.2
	50	106.2	9.7	10.2	9.8	97.4
	100	90.1	4.6	5.7	8.2	101.3
IS	25	100.2	9.9	10.5	7.4	99.7

[b] $n = 5$, mean value.
[c] $n = 5$.
[d] $n = 3$.

degradation [17,19,23].

3.3. Haematocrit effect

To evaluate in depth the possible influence of HCT in terms of CRP extraction yield, whole blood prepared at three representative HCT values (25 %, 45 %, 65 %) were sampled with VAMS, extracted, and analysed. The extraction yields resulted to be HCT-independent, i.e., the recovery values from VAMS obtained from 25 % and 65 % HCT blood were always within ± 15 % of the recovery from dried VAMS prepared with 45 % HCT blood. Moreover, no significant differences in matrix effect intensity were observed when analysing VAMS prepared from low-, middle- or high-HCT blood samples (RE % in the ± 15 % range).

3.4. Analysis of blood from psychiatric patients

The validated analytical methodology was applied to the analysis of real samples from three patients undergoing CRP therapy.

The concentrations of CRP present in the VAMS were determined and the analytical performance of the miniaturised approach was evaluated by comparing the quali-quantitative results obtained with those from fluid plasma samples, subjected to the previously described SPE-based extraction procedure. Being an original protocol developed ad hoc as a reference, and one of the first examples of SPE applied to CRP plasma analysis, the optimised procedure was validated in terms of linearity and sensitivity (linearity range 0.2–100 ng/mL), extraction yield (> 90.4 %), matrix effect (10–14 %), precision (intraday %RSD < 12.0, interday % RSD < 12.5) before method application to real samples.

Plasma concentration values were converted into whole blood concentrations using a mean HCT value of 42.5 % and a tentative CRP red blood cell / plasma partitioning value of 50 %. As reported in Table 2, all results from the VAMS microsamples are in very good agreement with those from fluid plasma. The first monitoring case was applied to a well-adjusted, successful therapeutic regimen (4.5 mg/day), in which the patient did not report any side effects and responded well to therapy. The CPR level found was compatible with effective therapy, taking into account the difference between plasma and whole blood concentrations. The second case was a patient reported to the emergency ward for suspected intentional overdose (five 4.5-mg tablets added to their usual 4.5 mg/day dose). In this case, an elevated circulating CRP level was found, widely exceeding the upper limit of the reference therapeutic range, thus confirming the overdosing. The third patient received a lower dose (1.5 mg/day) and had their CRP therapy stopped 9 days before sampling, and consequently their CRP level was notably lower than that of the well-adjusted patient.

Table 2
Quantitative comparison between analysis results from fluid and dried samples obtained from patients undergoing CRP therapy.

Patient	CRP dose (mg/day)	CRP concentration (ng/mL)	
		Fluid plasma[a]	VAMS
1	4.5	7.4	7.2
2	27.0	97.0	102.5
3	1.5[b]	2.1	2.0

[a] Converted to blood concentration.
[b] Drug administration was stopped 9 days before sampling.

Accuracy assays were also carried out on VAMS obtained from real samples, analysing them after spiking with three different analyte concentrations and the IS at a constant concentration. Very good accuracy values were obtained for all concentration levels (low, middle, high), with absolute recovery values always in the 92–101 % range.

4. Conclusion

Procedures for accurate TDM require reliable analytical approaches to promote effective and frequent patient monitoring and individualisation of drug therapy. An innovative method based on miniaturised VAMS sampling coupled to UHPLC-MS has been developed and validated for the TDM of patients treated with CRP in blood micromatrices. The developed analytical method allowed to obtain satisfactory validation results in terms of linearity ($r^2 > 0.9970$ in the 1.5–100 ng/mL range), precision (%RSD < 11.7) and extraction yield (> 90.0 %). The optimised workflow was successfully applied to the TDM of psychiatric patients undergoing CRP therapy, with satisfactory results even in case of low-dose treatment interrupted days before the blood sampling.

In order to evaluate the reliability of the miniaturised methodology, classical fluid plasma analysis was also performed by means of an original, validated SPE protocol, showing satisfactory agreement between the results of conventional fluid and innovative dried matrices, therefore the results of this study confirm that the developed protocol is accurate and reliable. Dried microsampling by VAMS has significant advantages: it is easy to perform and minimally invasive, which allows to envisage use of the procedure for self-collection and at-home-collection, with clear advantages in terms of time and costs. Additionally, stability assays confirmed that biological samples can be transported and stored at RT without significant degradation, reducing the space and equipment required in comparison to fluid samples, thereby improving efficiency and logistics for routine clinical applications. In addition, the VAMS procedure was confirmed to be insensitive to HCT variability, a parameter that affects most other microsampling techniques.

In this study, microsampling, and in particular VAMS, was applied for the first time to the TDM of CRP. It has obtained satisfactory results, complying with all validation and application parameters of international guidelines, and in particular demonstrating result independence from HCT, and providing enhanced analyte stability in comparison to corresponding fluid samples. Suitability of the VAMS strategy was thus demonstrated for the purpose of developing an accurate and precise

analytical method with a rapid and feasible sample pretreatment procedure for application to the TDM of patients receiving CRP.

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.

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