Enantioseparation and determination of asenapine in biological fluid micromatrices by HPLC with diode array detection

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RUNNING TITLE: Asenapine chiral analysis in miniaturised biosamples

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Abbreviations: BIN, Barrel and Insert Needle; DAD, diode array detection; DBS, dried blood spots; DMS, dried matrix spots; DPS, dired plasma spots; IS, internal standard; MEPS, MicroExtraction by Packed Sorbent; RT, room temperature; SNP, asenapine; USA, United States of America; VAMS, volumetric absorptive microsampling; VAMS-B, volumetric absorptive microsamples of blood; VAMS-P, volumetric absorptive microsamples of plasma.

Keywords: Asenapine; Chiral stationary phase; Enantioseparation; Method validation; Volumetric Absorptive Microsampling.

ABSTRACT

Asenapine is a recent drug approved in the European Union for the treatment of bipolar disorder. An original approach has been developed for asenapine analysis in patients treated with the drug, including miniaturised microsampling procedures, separation and quantitation of drug enantiomers. An original enantioselective method based on HPLC with diode array detection was developed and applied to the determination of asenapine enantiomer levels in innovative haematic samples: four micromatrices have been tested, two based on dried matrix spots (dried blood spots and dried plasma spots) and two based on volumetric absorptive microsampling (from blood and plasma). Chiral separation was achieved on a cellulose-tris(3,5 dimethylphenylcarbamate) column, with a mobile phase containing bicarbonate buffer and ACN. The method was validated with satisfactory results of linearity and precision on all matrices that showed also a significant performance in terms of stability, feasibility and reliability when compared to fluid plasma sampling, handling and processing. Among micromatrices, both volumetric absorptive microsampling types were superior to dried matrix spots in terms of data reproducibility and correspondence with plasma levels. The bioanalytical approach proposed herein provides for the first time a chiral HPLC method for the determination of asenapine enantiomers, coupled to a very effective microsampling strategy.

1 INTRODUCTION

Asenapine (5-chloro-2,3,3a,12b-tetrahydro-2-methyl-1*H*-dibenz(2,3-6,7)oxepino(4,5-c)pyrrole, SNP, Figure 1) is one of the most recent antipsychotic drugs commercialised for the treatment of bipolar disorder and schizophrenia: it gained approval in both the United States of America (USA) and the European Union for the treatment of manic or mixed episodes of bipolar I disorder and also for schizophrenia in the USA [1,2]. In contrast to most traditional neuroleptics and atypical antipsychotics (which have a bicyclic or tricyclic structure), SNP is a tetracyclic molecule somewhat akin to the antidepressants mianserin and mirtazapine [3]. SNP has a very broad affinity spectrum towards central nervous system (CNS) receptors, binding with antagonistic activity to serotonin 5-HT_{2C}, 5-HT_{2A}, 5-HT_{7}, 5-HT_{2B} and 5-HT_{6} receptors, as well as to adrenergic α_{2B}, dopamine D_3, histamine H_1 and H_2 and dopamine D_{2S} and D_{2L} ones, albeit with lower affinity [4]. On the contrary, its activity toward muscarinic receptors is quite low, reducing the incidence of metabolic and cardiac side effects and sedation [5]. It is due to this peculiarly wide receptor affinity profile that SNP has proven to be effective in bipolar disorder treatment. Patients taking SNP can benefit from the analysis of its levels in the body by determining drug blood or plasma concentrations and their correlation to therapeutic activity and possible side/toxic effects [6]. SNP is a chiral compound containing two chiral centers, thus existing as four stereoisomers. Since the two *trans*-isomers, (3a*R*,12b*R*)-SNP and (3a*S*,12b*S*)-SNP, have higher binding affinity than the two *cis*-isomers, SNP is commercially available only as a racemic mixture of *R,R*- and *S,S*-enantiomers [7] (Figure 1a-b). In order to obtain more significant information and results from the analyses, the simultaneous determination of both SNP enantiomers is advisable and in this paper an original enantioselective HPLC - diode array detection (DAD) method has been developed on purpose.

Haematic analyses are normally carried out on macroscopic (a few millilitres) blood samples drawn from patients, from which plasma or serum are then obtained, pre-treated and finally analysed. We are instead proposing four

original microsampling approaches, based on dried matrix spots (DMS) of either blood (dried blood spots, DBS) or plasma (dried plasma spots, DPS) and on volumetric adsorption microsampling (VAMS) from blood (VAMS-B) or plasma (VAMS-P). The DMS strategy involves microvolumes of haematic matrix, in order to facilitate pre-treatment, handling, storage and transport procedures. In addition to these feature, VAMS are obtained using innovative samplers able to overcome certain specific disadvantages of dried matrices, such as haematocrit-dependent volumetric bias and homogeneity issues [8,9]. DMS and VAMS were applied for the monitoring of patients under therapy with SNP, and the results were compared to those obtained with classic fluid blood sampling and plasma analysis. To the best of our knowledge, just one paper has been published on SNP chiral separation, but using capillary electrophoresis (CE) with unmodified β-cyclodextrin, and no biological application [10]. On the other hand, some analytical methods have been published for the non-enantioselective determination of SNP in biological matrices [11-19] among which those designed for patient monitoring are very few [16-19] and only one dealing with micromatrices [19]. None of them was applied to real samples from psychiatric patients, or compared different and novel dried microsampling approaches.

2 MATERIALS AND METHODS

2.1 Chemicals, solutions and equipment

Asenapine maleate (racemic mixture, >98% purity), promethazine (> 98% purity, used as the internal standard, IS), HPLC-grade (> 99.8%) methanol and ACN, ammonium bicarbonate, Whatman (Maidstone, USA) 903 protein saver and Whatman FTA™ DMPK-B IND cards were purchased from Merck Italy (Milan, Italy). Ultrapure water (18.2 MΩ cm) was obtained by means of a MilliQ apparatus by Millipore (Milford, USA). Analyte and IS stock solutions were obtained by dissolving the pure standards in methanol. Stock solutions were stable for at least three months when stored at -20°C (as assessed by HPLC); standard solutions were prepared fresh every day. A Whatman Harris Uni-Core

Punch, 10 mm was used for punching the DMS discs out of the spotting cards. Mitra® VAMS™ microsamplers (20 µL) were provided by Neoteryx (Torrance, USA). For MicroExtraction by Packed Sorbent (MEPS) procedures, an SGE Analytical Science (Melbourne, Australia) 100-µL syringe with a C8 Barrel and Insert Needle (BIN) was used.

2.2 Sample collection and pre-treatment

Plasma: "blank" plasma samples were obtained from drug-free healthy volunteers. Real samples were from bipolar patients receiving the standard SNP oral daily doses of 20 mg, and were drawn 2 h after drug administration. Blood was collected in glass tubes containing sodium EDTA as the anticoagulant and then centrifuged (within 2 h from collection) at 1500×*g* for 15 min at 4°C; the supernatant (plasma) was then transferred to polypropylene tubes and stored at -20 °C until analysis. A 5-µL aliquot of analyte standard and/or ISs solution at known concentrations and 100 µL of ultrapure water were added to 100 µL of plasma; the vial was then vortex-mixed. The mixture was subjected to pre-treatment by MEPS in a C8 BIN assembly, which had been already activated by drawing and discarding 100 µL of methanol 3 times and conditioned with 100 µL of water 3 times. The sample was loaded onto the BIN with 12 draw/discharge cycles at a 5 µL/s speed; the BIN was then washed with 100 µL of water 2 times and the analyte and the IS were eluted with 500 µL of methanol (single cycle). The eluate was brought to dryness, re-dissolved in 50 µL of the mobile phase and analysed by HPLC-DAD.

*Micromatrices: f*or DBS, blood drops were collected from healthy volunteers or patients by means of a sterile, disposable finger pricker, and transferred on a Whatman 903 protein saver card. As soon as blood was completely absorbed, 5 µL of analyte standard and/or ISs solution at known concentrations were carefully transferred onto each spot. The spots were dehydrated by microwave drying at 700 W for 1.5 minutes, then stored protected from light in a plastic bag containing desiccant until analysis. To extract the analytes and the IS, a 10-mm diameter circle was punched out from the card with a punching tool

and transferred into a vial with 500 μL of methanol. The vial was then subjected to both microwave-assisted extraction at 210 W for 1 min and ultrasound-assisted extraction for 20 min, then centrifuged at 1000×*g* for 1 min. The supernatant was dried, re-constituted in 50 μL of mobile phase and injected into the HPLC system. For DPS, aliquots of 20 μL of plasma from healthy volunteers or patients were transferred onto Whatman FTA™ DMPK-B IND cards by micropipetting. All the subsequent extraction steps were the same previously described for DBS, except the whole spots were completely punched out with the punching tool before extraction. As regards VAMS, the Mitra 20-μL device is composed of a polypropylene rod with a small globous tip of a proprietary polymeric porous material. The tip size and porosity of the microsamplers are calibrated to absorb a constant volume of a fluid when exposed to it by wicking. After adding 5 μL of IS solution to 500 μL of the desired matrix (either blood or plasma), the 20-μL VAMS microsampler tip was brought into contact with the surface of the mixture with an angle of about 45°, without completely immersing it. The microsampler was left in this position for 5 s and then dried upright on the back end, at room temperature (RT), for 1h in the dedicated clamshell case. In this way, VAMS-B and VAMS-P were obtained. These samples were stored at RT, in the dark and in a dry place for 2 months at most. To extract the analyte and the IS, the microsampler tip was detached from the plastic support and subjected to ultrasound-assisted extraction for 20 min in 500 μL of methanol. The resulting solution was dried, re-dissolved with 50 μL of mobile phase and injected into the HPLC system.

2.3 HPLC-DAD analysis

All samples were analysed with an original HPLC-DAD method. The instrument was an Agilent (Palo Alto, USA) 1100 Series HPLC system, including a quaternary pump and a diode array detector. The chromatographic separation was carried out on a Phenomenex (Torrance, USA) cellulose tris(3,5-dimethylphenylcarbamate) (Lux Cellulose-1), 4.6 mm × 100 mm, 5 μm) enantioselective column, thermostatted at 25°C. Cellulose-tris(3-chloro-4-

177 methylphenylcarbamate) (Lux Cellulose-2), cellulose tris(4-chloro-3-
178 methylphenylcarbamate) (Lux Cellulose-4) and amylose tris(5-chloro-2-
179 methylphenylcarbamate) (Lux Amylose-2) columns with the same dimensions
180 and granulometries were tested in preliminary assays. The mobile phase was a
181 mixture of ACN (60%, v/v) and 50 mM sodium bicarbonate in water (40%, v/v),
182 flowing at 0.7 mL/min. Quantitative data was recorded at a wavelength of 210
183 nm and processed by means of ChemStation ver. B.04.03 software.
184
185 **2.4 Method validation**
186 *Linearity: c*alibration samples were prepared by diluting analyte and IS working
187 standard solutions with blank matrices. The resulting spiked samples were
188 subjected to the respective sample preparation procedure before injection into
189 HPLC-DAD. The analysis was carried out in triplicate for each concentration.
190 Calibration curves of both SNP enantiomers were constructed by the least-
191 square method, plotting analyte to IS peak area ratios versus the
192 corresponding nominal concentrations. LOQ and LOD values were assessed
193 by analysing seven different calibration samples and defined as the lowest
194 concentrations generating chromatographic peaks with signal-to-noise ratios
195 of 10 and 3, respectively [20,21].
196 *Absolute recovery, precision:* absolute recovery was evaluated by spiking blank
197 samples with known analyte amounts (at three different concentrations,
198 corresponding to the lower limit, a middle value and a high value of each
199 calibration curve), then carrying out sample preparation and chromatographic
200 analysis. Analyte and IS absolute peak areas were compared to those of
201 standard solutions analysed at the same concentrations and the percentage
202 absolute recovery was calculated. To test intraday and interday precision, the
203 assays described above were repeated six times within the same day and six
204 times over six different days, respectively. Both values were expressed as
205 percentage relative standard deviation (RSD%).
206 *Stability:* for plasma, blank samples were spiked at a 20 ng/mL level (10 ng/mL
207 for each enantiomer), then stored at -20°C for 2 months. At regular intervals (1

week), a different sample vial was thawed, pre-treated and analysed. For the dried micromatrices (DBS, DPS, VAMS-B, VAMS-P), blank samples were spiked at a 20 ng/mL level (10 ng/mL for each enantiomer), then stored protected from light in sealed plastic bags containing desiccant for 2 months. At regular intervals (1 week), a different sample was pre-treated and analysed. The measured analyte concentrations were compared to those of the same samples extracted and analysed immediately after biosampling (plasma), or biosampling and drying (dried matrices).

Selectivity: was tested using 6 independent blank samples from healthy volunteers not subjected to pharmacological therapy, which were individually analysed and evaluated for endogenous interferences. Standard solutions of several drugs, which are commonly co-administered with SNP to psychiatric patients, were also injected at a 500 ng/mL level to ascertain that no interference was generated. Selectivity was considered acceptable when no peak whose signal exceeded the LOD for the analyte, or 5% of the signal/noise ratio for the IS was observed.

Accuracy: was assessed by re-analysing haematic samples from bipolar patients in a further analytical session: known amounts of the analyte at three levels (lower limit, middle value and high value of the calibration curve) and of the IS at a constant level were added to the samples, pre-treated and analysed. Percentage recovery of the added analytes was calculated and the assays were repeated three times.

2.5 Quantitative data comparisons

All results obtained from SNP-treated patient samples were compared by plotting the results from each dried matrix (DBS, DPS, VAMS-B or VAMS-P) versus those obtained from plasma analysis. Then, the least-square method was applied to calculate linearity correlation coefficient and slope of each curve.

3 RESULTS AND DISCUSSION

3.1 Preliminary assays: chiral separation

At first, polymeric chiral stationary phases with a broad range of applicability were tested: cellulose-tris(3,5-dimethylphenylcarbamate), cellulose tris(3-chloro-4-methylphenylcarbamate), cellulose tris(4-chloro-3-methylphenylcarbamate) and amylose tris(5-chloro-2-methylphenylcarbamate). In these sorbents the polysaccharide chains provide a wide array of steric interaction sites: the chlorine- or methyl-substituted methylphenylcarbamate residues greatly increase the chances of interaction with the selector, both with hydrogen bonds, dipole interactions and π–π interactions. Of these sorbents, just the cellulose-tris(3,5-dimethylphenylcarbamate) sorbent (cellulose-1) provided some separation in preliminary assays. Since this is the stationary phase with the least dipole interaction-forming capability, it is possible that SNP chiral recognition relies mainly on hydrogen bonding and π–π interactions. Further assays were thus carried out on the cellulose-1 sorbent only. Initially, a mixture of ammonium bicarbonate and ACN (29:71, v/v) was tested as the mobile phase. This mixture was used to obtain a slightly basic pH and complete compatibility with MS detection, in order to allow future applications using this kind of detection. Moreover, the volatile buffer could be useful for possible adaptation of the method to preparative enantiomeric separations of SNP. This mobile phase provided complete separation (R_s = 1.8) of SNP enantiomers, but with excessive retention (run time > 60 min). Increasing the ACN percentage in the 29-70% (v/v) range decreased run times correspondingly, but with a progressive loss of resolution. The highest ACN percentage that maintained almost complete enantioresolution (R_s = 1.44) was 60% (v/v), corresponding to an acceptable run time of 10 min. Flow rate was also studied, varying it in the 0.4-1.2 mL/min range. The best efficiency was found between 0.5 and 0.7 mL/min, witj higher flow rates producing shorter analytical runs: the best compromise was found to be 0.7 mL/min. Column temperature can have drastic effects on chiral separation; for this reason, it was studied between 15 and 40°C. In this range, lower temperatures always granted better resolution, but differences were relatively small and came at the

cost of stronger retention and thus longer run times. 25°C was the chosen temperature as the best compromise that still provided good resolution. In order to obtain maximum sensitivity, the spectra of the analyte peaks provided by the HPLC-DAD system were studied. The spectrum of SNP in the described experimental conditions has two absorbance maxima, at 210 nm and at 235 nm, but the first one was 4 times more intense, thus 210 nm was chosen for quantitative purposes. Several (non-chiral) compounds were tested as possible ISs. Among them, promethazine gave the best results: It bears some structural resemblance to the analyte and has similar lipophilicity, but with lower retention; thus, it does not increase run time. Under these final chromatographic conditions, the enantiomer peaks were fully resolved, and peak asymmetry was almost negligible (data not shown).

3.2 Sample pre-treatment procedures

Plasma: although plasma is inherently a macromatrix, a reduced-volume approach was applied to its pre-treatment. For this purpose, MEPS was used: It is a small volume variant of solid phase extraction, where a few milligrams of stationary phase are enclosed in a syringe-mounted BIN, and small amounts of sample and solvents are passed through it using the syringe. Preliminary assays were carried out on spiked blank plasma (100 µL) to find the best extraction sorbent: C8, C2 and SDVB (polystyrene-divinylbenzene copolymer) were tested and best results of analyte extraction and matrix clean-up were obtained using the C8 BIN. Washing and elution solvents and volumes were also optimized; two washing steps with 100 µL of pure water were chosen as the optimal washing steps, allowing satisfactory interference elimination and optimal analyte retention. The methanol elution volume was studied in the 100-1000 µL range; best results were obtained with 500 µL. Higher elution volumes did not increase extraction yields and significantly increased the amount of eluted interference.

Micromatrices: each of the two DMS techniques has different advantages and drawbacks. Regarding DBS, although some volumetric bias could be produced

by whole blood viscosity variability, the blood volume corresponding to specific spot dimensions is relatively constant (3.5 mm^2/µL): a 20-µL volume is approximated with a 10-mm spot radius. Drying time and conditions were the first investigated parameters, taking into account both RT and microwave-assisted drying. At RT, 1h was enough to achieve complete drying for both DMS matrices; however, microwave drying significantly cut drying times: 1.5 min using a 700-W power. The extraction step is usually carried out on DMS with a suitable solvent and the resulting sample can be injected as such. Alternatively, the extract can be subjected to further clean-up steps [22-24]. In this case, solvent nature and volume were the only tested parameters, obtaining comparable results for both DMS matrices: pure methanol proved to be the most efficient extractor, while also granting satisfactory purification from matrix interference. Extraction yields did not improve beyond the 500-µL limit, thus selected as the extraction volume. As regards VAMS devices, previous papers have decisively demonstrated their good sampling volume accuracy, both for blood-based [9] and for urine- and saliva-based [25] matrices. Sampling time was tested in the 2-10 s range. Different sampling times produced small, non-significant differences in volume, and their effect on analyte quantitation was negligible. Due to the fast VAMS tip saturation and to the lack of oversampling effects observed, a 5-s contact time was set for both VAMS-B and VAMS-P. In order to optimize total pre-treatment time, drying time was also tested: complete water evaporation) was achieved in less than 1 h; this time was chosen as the drying time for all the analytical assays. Like for DMS, pre-treatment procedures were kept as simple as possible to increase throughput. Extraction solvent, volume and time were optimized: 500 µL of either pure ACN or methanol gave the best results in terms of extraction yield, although pure ACN produced stronger interference as well, thus methanol was chosen. Ultrasound helped increasing extraction yields, and 20 min of ultrasound treatment proved to be sufficient to quantitatively extract the analytes and the IS. No important differences in extraction yields and sample purification were noted between VAMS-B and VAMS-P when using the same

pre-treatment procedure. The chromatograms of a blank VAMS-P sample and a blank VAMS-B sample spiked with racemic SNP standard solution (concentration: 10 ng/mL for each enantiomer) are presented in Figure 2a-b.

3.3 Method validation

Linearity: for the setup of calibration curves, each micro- and macro-matrix was spiked with racemic standard solutions of the analyte (seven concentrations) and the IS at a constant concentration of 50 ng/mL. Method sensitivity on plasma for both enantiomers was 0.2 and 0.07 ng/mL in terms of LOQ and LOD, respectively; method linearity on the same matrix was assessed between 0.5 and 50 ng/mL. LOD and LOQ values respectively of 0.8 and 2.5 ng/mL were obtained for both SNP enantiomers on all the four considered micromatrices; the linearity range was verified between 0.5 and 50 ng/mL for DBS, DPS, VAMS-B and VAMS-P. Good linearity was obtained for both enantiomers in all matrices ($r^2 \geq 0.9989$) and the best data have been provided by the VAMS approach.

Absolute recovery, precision: blank plasma and blank micromatrices were spiked at three analyte levels. As can be surmised from Table 1, absolute recovery on all matrices was satisfactory, in the 81.7-99.8% range, for both analyte enantiomers. Precision was good, producing RSD values lower than 6.0%. Both VAMS types granted better recovery and precision than DMS.

Stability: dried micromatrices often demonstrate higher stability than "traditional" macromatrices, even when the former ones are stored at RT. In this case, the stability of the analyte in dried matrices at RT was compared to that in plasma samples under freezing (-20°C) and deep-freezing (-80°C) conditions. After 1 month, all dried matrices stored at RT consistently provided higher extraction yields than plasma (> 78%); in VAMS in particular, they were always higher than 91%. The superior stability observed in dried samples can be attributed to the water loss, which negatively impacts both chemical and enzymatic reactions. No significant stability differences were found between

the two enantiomers, indicating that they are probably configurationally stable in the conditions employed.

Selectivity: blank matrix samples (from healthy volunteers) were pre-treated and analysed. Standard solutions of several drugs, which are commonly co-administered with SNP to psychiatric patients, were also injected at a 500 ng/mL level to ascertain that no interference was created by them: carbamazepine, gabapentin, lamotrigine, valproate and vigabatrin among antiepileptics; aripiprazole, chlorpromazine, clozapine, loxapine and olanzapine among antipsychotics; citalopram, sertraline and venlafaxine among antidepressants. No interfering peak was detected in any of the matrices and for any of the tested drugs.

3.4 Analysis of real samples and accuracy

The methodology was applied to the analysis of macro- and micro-samples from patients undergoing therapy with SNP for bipolar disorder. All matrices were simultaneously obtained, either by venipuncture or by fingerpricking, to ensure that any inconsistency in results could not be attributed to sampling. The chromatogram of a VAMS-B sample from a patient is reported in Figure 2c. As can be seen, sample purification and chiral separation are both satisfactory. In this case, R_S is slightly higher than that obtained on spiked blank samples (Figure 2b); this result is probably due to the combination of different matrix and concentration effects. Table 2 reports the quantitative data obtained from the analysis of real samples from bipolar patients. Concentration differences between the two enantiomers were small and non statistically significant. This confirms what is currently known from early studies carried out by the drug manufacturer [26,27]. As a rule, raw analytical data from plasma-based matrices can be used as such for quantitation, while those obtained from blood-based matrices have to be elaborated to account for sample viscosity (related to the haematocrit), and for red blood cell / plasma partitioning [28]. A constant red blood cell / plasma concentration ratio of 1/3 was applied in all cases, according to literature evidence [26]. A mean

haematocrit value of 38% was used for samples coming from female patients and of 48% for those from male patients. Both corrections have already been included in the results of blood-based matrices (DBS, VAMS-B) in Table 2. In-line UV spectra were used to both confirm the identity of the analyte enantiomer and IS peaks, and to verify peak purity. Finally, good accuracy was reached in all matrices and the VAMS technique gave the best SNP recovery: always higher than 95.3%.

3.5 Comparison between macro- and micro-sample results

In order to evaluate the performance of each matrix approach, SNP enantiomeric concentrations obtained from patient plasma samples were compared to those obtained from each micromatrix. Linear correlation analysis between the results showed that both linearity and slope values were very close to 1 for all dried matrices, confirming that they closely mimic plasma behaviour and produce reliable data. However, the best correspondence with plasma levels was observed for VAMS matrices. These results are statistically significantly different from those obtained using DMS sampling; on the other hand, no significant difference was observed between VAMS-B and VAMS-P (nor between DBS and DPS). This, together with the better precision and recovery results, means that VAMS can be confidently rated as more reliable than DMS for SNP enantiomer analysis with this method. The better performance of VAMS could probably be attributed to the very good volume reproducibility of the sampling tips (as opposed to the intrinsic uncertainty of blotting and punching out the spots). All in all, the dried matrices produce better analyte stability and are surely more practical and more easily handled than plasma. Among micromatrices, blood-based ones (DBS and VAMS-B) are more practical than plasma-based ones (DPS and VAMS-P), since they can be obtained with a minimally invasive fingerpicking and do not require any sample manipulation prior to blotting or absorbing.

4 CONCLUDING REMARKS

Four different microsampling and pre-treatment procedures were proposed and compared for the determination of SNP enantiomers in human blood. An original enantioselective method based on HPLC-DAD was developed and validated, allowing for the first time the chromatographic separation and quantitation of SNP enantiomers in biological samples, within reasonable run times and with good reproducibility. The analytical method coupled to DBS, DPS, VAMS-B and VAMS-P demonstrated to be rapid and selective, with significant advantages over classical approaches exploiting macromatrices, and plasma in particular: the tested dried microsamples were more stable over time and more easily stored. Result comparisons have shown the four micromatrix approaches produce sound results, always strictly correlated with the corresponding data produced by classic plasma analysis. However, VAMS sampling provided consistently better results than DMS sampling in terms of precision, recovery and correspondence with plasmatic concentrations. Although all matrices are suitable for the analysis of SNP in psychiatric patients, VAMS are clearly preferable and will be proposed to clinicians as an attractive alternative to classical plasma sampling. The coupling of VAMS micromatrices and enantioselective analysis provides a solid platform that can be adapted to different needs and situations, with the added advantage of also obtaining advanced, reliable insights into chiral aspect of SNP interactions with the human body. Assays are currently in progress in order to unequivocally assign the correct absolute configuration to the chromatographic peaks of the two SNP enantiomers.

FIGURE LEGENDS

Figure 1. Chemical structures of (a) (*R,R*)-SNP, (b) (*S,S*)-SNP, (c) promethazine (IS).

Figure 2. Chromatograms of (a) a blank VAMS-P sample, (b) a blank VAMS-B sample spiked with racemic SNP standard solution (concentration: 10 ng/mL for each enantiomer), (c) a VAMS-B sample from a bipolar patient treated with 20 mg/day of SNP (patient n. 6, see Table 2). Experimental conditions: column,

Lux Cellulose-1 (4.6 mm × 100 mm, 5 µm) thermostatted at 25°C; mobile phase, ACN / 50 mM ammonium bicarbonate (60/40, v/v); flow rate, 0.7 mL/min; detection, diode array at 210 nm.

ACKNOWLEDGMENTS

This work was supported by RFO (Ricerca Fondamentale Orientata - Oriented Fundamental Research) grants from Alma Mater Studiorum - University of Bologna, Italy.

CONFLICTS OF INTEREST

All authors declare there are no conflicts of interest.

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HIGHLIGHTS

- For the first time, asenapine enantiomers have been completely resolved by HPLC using a cellulose-based sorbent.
- The original, enantioselective HPLC-DAD method has been fully validated.
- Miniaturised dried haematic matrices (DMS and VAMS) have been implemented and compared to classical plasma samples.
- Suitable extraction and pre-treatment procedures have been devised for each matrix.
- The method has been applied to real samples from bipolar patients undergoing therapy with asenapine.

Table 1. Absolute recovery and precision in spiked matrices.

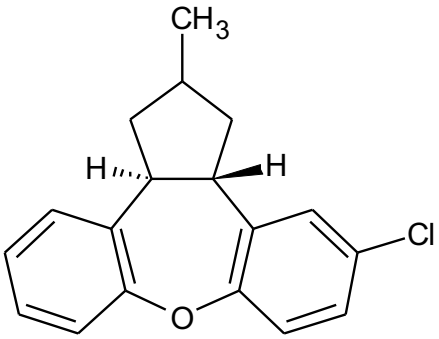
Analyte	Concentration level[a]	Matrix	Absolute recovery, %[b]	Precision, RSD%[c]	
				Intraday	Interday
SNP1	Low	Plasma	95.1	4.0	4.1
		DBS	85.1	4.6	4.8
		DPS	90.7	4.1	4.4
		VAMS-B	99.1	3.6	3.8
		VAMS-P	98.7	3.5	4.0
	Intermediate	Plasma	94.4	3.7	3.9
		DBS	83.2	4.4	4.9
		DPS	87.5	3.8	3.8
		VAMS-B	98.8	3.0	3.3
		VAMS-P	96.3	3.1	3.5
	High	Plasma	93.0	3.0	3.2
		DBS	83.1	4.7	5.9
		DPS	86.0	3.2	3.3
		VAMS-B	98.6	2.5	2.7
		VAMS-P	99.7	2.9	2.8
SNP2	Low	Plasma	95.8	3.7	4.9
		DBS	81.7	4.9	5.1
		DPS	89.3	4.1	4.4
		VAMS-B	97.2	3.2	3.6
		VAMS-P	96.8	3.1	3.9
	Intermediate	Plasma	95.0	3.2	4.1
		DBS	87.2	5.4	5.9
		DPS	85.8	3.9	4.3
		VAMS-B	95.5	2.6	3.3
		VAMS-P	93.7	3.0	3.7
	High	Plasma	93.7	3.1	3.8
		DBS	85.6	4.9	5.7
		DPS	85.5	3.2	4.1
		VAMS-B	99.6	2.0	2.2
		VAMS-P	99.8	2.5	2.6
IS	50 ng/mL	Plasma	90.9	3.5	4.6
		DBS	86.4	4.1	4.8
		DPS	87.3	4.2	4.7
		VAMS-B	98.4	2.1	2.0
		VAMS-P	99.1	2.0	2.3

[a] For each matrix, “Low” corresponds to the lower LOQ, “Intermediate”, to an intermediate point and “High” to a high value of the respective linearity curve.
[b] $n = 6$, mean value.
[c] $n = 6$.

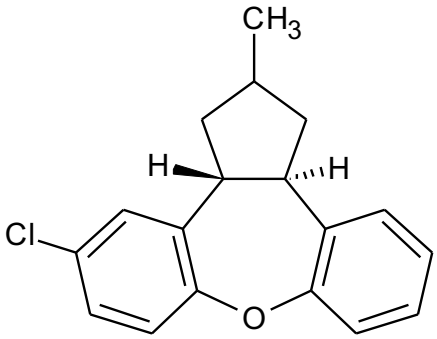
Table 2. Quantitative results from patients taking SNP.

Patient n.	Concentration found, ng/mL[a]									
	SNP1					SNP2				
	Plasma	DBS	DPS	VAMS-B	VAMS-P	Plasma	DBS	DPS	VAMS-B	VAMS-P
1	3.4	3.6	3.2	3.4	3.5	3.5	3.7	3.3	3.5	3.4
2	2.9	3.2	3.0	3.0	3.1	2.9	3.2	3.0	2.9	3.0
3	3.3	3.5	3.4	3.3	3.4	3.5	3.4	3.3	3.5	3.4
4	3.2	3.4	3.1	3.3	3.3	3.3	3.4	3.2	3.4	3.3
5	3.6	3.8	3.3	3.7	3.5	3.5	4.0	3.3	3.5	3.4
6	3.8	4.0	3.6	3.9	3.8	3.9	4.0	3.6	4.0	2.8
7	3.0	3.2	2.7	3.0	2.9	2.9	3.3	2.8	2.9	3.0

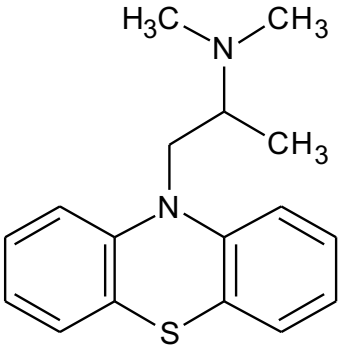
[a] $n = 3$, mean value.



a – (*R,R*)-Asenapine



b – (*S,S*)-Asenapine



c – Promethazine (IS)

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Figure 1

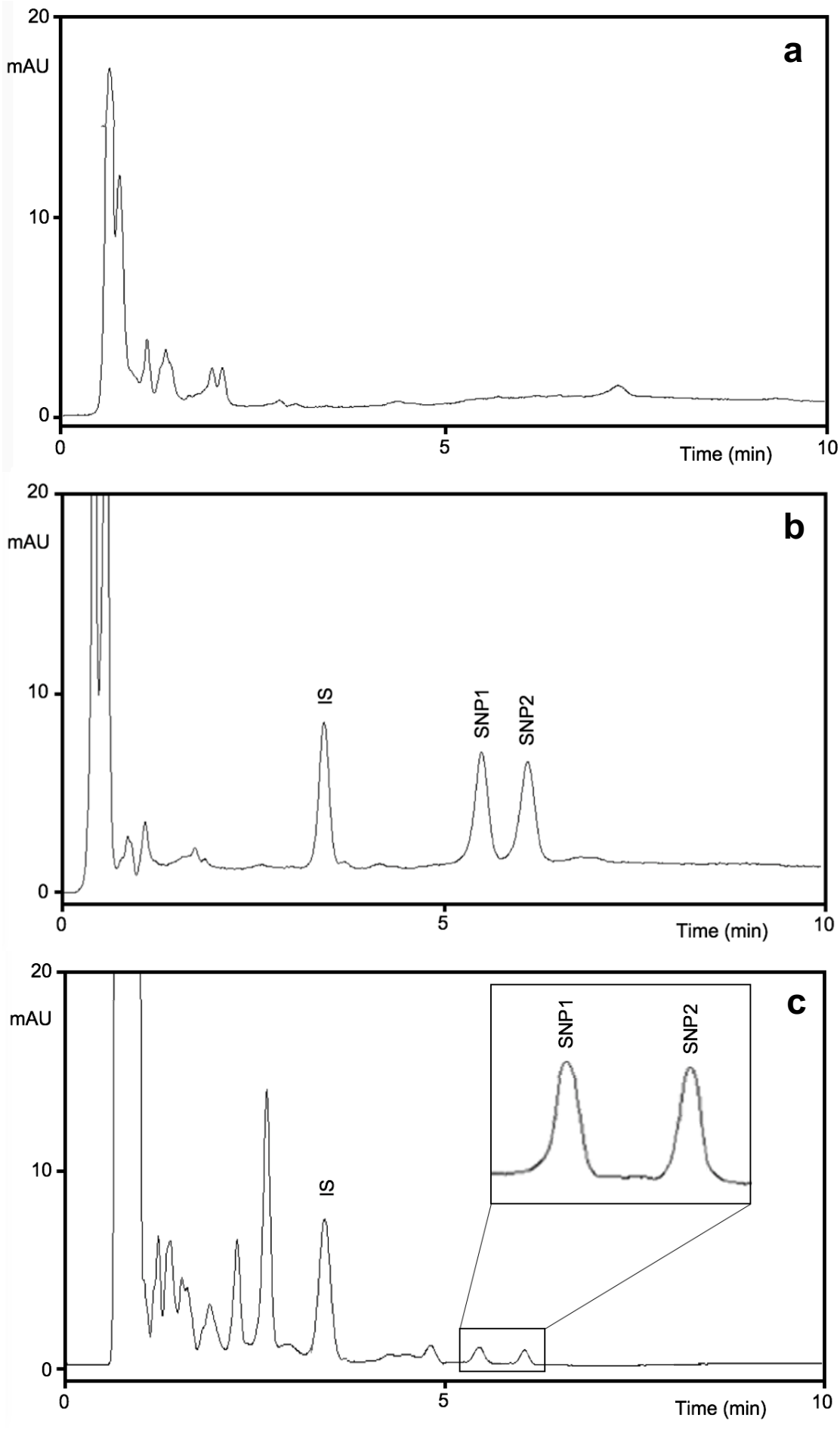

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Figure 2