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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

84 original microsampling approaches, based on dried matrix spots (DMS) of
85 either blood (dried blood spots, DBS) or plasma (dried plasma spots, DPS) and
86 on volumetric adsorption microsampling (VAMS) from blood (VAMS-B) or
87 plasma (VAMS-P). The DMS strategy involves microvolumes of haematic
88 matrix, in order to facilitate pre-treatment, handling, storage and transport
89 procedures. In addition to these feature, VAMS are obtained using innovative
90 samplers able to overcome certain specific disadvantages of dried matrices,
91 such as haematocrit-dependent volumetric bias and homogeneity issues [8,9].
92 DMS and VAMS were applied for the monitoring of patients under therapy with
93 SNP, and the results were compared to those obtained with classic fluid blood
94 sampling and plasma analysis. To the best of our knowledge, just one paper
95 has been published on SNP chiral separation, but using capillary
96 electrophoresis (CE) with unmodified β-cyclodextrin, and no biological
97 application [10]. On the other hand, some analytical methods have been
98 published for the non-enantioselective determination of SNP in biological
99 matrices [11-19] among which those designed for patient monitoring are very
100 few [16-19] and only one dealing with micromatrices [19]. None of them was
101 applied to real samples from psychiatric patients, or compared different and
102 novel dried microsampling approaches.
103

104 **2 MATERIALS AND METHODS**

105 **2.1 Chemicals, solutions and equipment**

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

115 Punch, 10 mm was used for punching the DMS discs out of the spotting cards.
116 Mitra® VAMS™ microsamplers (20 µL) were provided by Neoteryx (Torrance,
117 USA). For MicroExtraction by Packed Sorbent (MEPS) procedures, an SGE
118 Analytical Science (Melbourne, Australia) 100-µL syringe with a C8 Barrel and
119 Insert Needle (BIN) was used.
120
121 **2.2 Sample collection and pre-treatment**
122 *Plasma:* "blank" plasma samples were obtained from drug-free healthy
123 volunteers. Real samples were from bipolar patients receiving the standard
124 SNP oral daily doses of 20 mg, and were drawn 2 h after drug administration.
125 Blood was collected in glass tubes containing sodium EDTA as the
126 anticoagulant and then centrifuged (within 2 h from collection) at 1500×*g* for 15
127 min at 4°C; the supernatant (plasma) was then transferred to polypropylene
128 tubes and stored at -20 °C until analysis. A 5-µL aliquot of analyte standard
129 and/or ISs solution at known concentrations and 100 µL of ultrapure water
130 were added to 100 µL of plasma; the vial was then vortex-mixed. The mixture
131 was subjected to pre-treatment by MEPS in a C8 BIN assembly, which had
132 been already activated by drawing and discarding 100 µL of methanol 3 times
133 and conditioned with 100 µL of water 3 times. The sample was loaded onto the
134 BIN with 12 draw/discharge cycles at a 5 µL/s speed; the BIN was then
135 washed with 100 µL of water 2 times and the analyte and the IS were eluted
136 with 500 µL of methanol (single cycle). The eluate was brought to dryness, re-
137 dissolved in 50 µL of the mobile phase and analysed by HPLC-DAD.
138 *Micromatrices: f*or DBS, blood drops were collected from healthy volunteers or
139 patients by means of a sterile, disposable finger pricker, and transferred on a
140 Whatman 903 protein saver card. As soon as blood was completely absorbed,
141 5 µL of analyte standard and/or ISs solution at known concentrations were
142 carefully transferred onto each spot. The spots were dehydrated by microwave
143 drying at 700 W for 1.5 minutes, then stored protected from light in a plastic
144 bag containing desiccant until analysis. To extract the analytes and the IS, a
145 10-mm diameter circle was punched out from the card with a punching tool

146 and transferred into a vial with 500 μL of methanol. The vial was then subjected
147 to both microwave-assisted extraction at 210 W for 1 min and ultrasound-
148 assisted extraction for 20 min, then centrifuged at 1000×*g* for 1 min. The
149 supernatant was dried, re-constituted in 50 μL of mobile phase and injected
150 into the HPLC system. For DPS, aliquots of 20 μL of plasma from healthy
151 volunteers or patients were transferred onto Whatman FTA™ DMPK-B IND
152 cards by micropipetting. All the subsequent extraction steps were the same
153 previously described for DBS, except the whole spots were completely
154 punched out with the punching tool before extraction. As regards VAMS, the
155 Mitra 20-μL device is composed of a polypropylene rod with a small globous
156 tip of a proprietary polymeric porous material. The tip size and porosity of the
157 microsamplers are calibrated to absorb a constant volume of a fluid when
158 exposed to it by wicking. After adding 5 μL of IS solution to 500 μL of the
159 desired matrix (either blood or plasma), the 20-μL VAMS microsampler tip was
160 brought into contact with the surface of the mixture with an angle of about 45°,
161 without completely immersing it. The microsampler was left in this position for
162 5 s and then dried upright on the back end, at room temperature (RT), for 1h in
163 the dedicated clamshell case. In this way, VAMS-B and VAMS-P were
164 obtained. These samples were stored at RT, in the dark and in a dry place for 2
165 months at most. To extract the analyte and the IS, the microsampler tip was
166 detached from the plastic support and subjected to ultrasound-assisted
167 extraction for 20 min in 500 μL of methanol. The resulting solution was dried,
168 re-dissolved with 50 μL of mobile phase and injected into the HPLC system.
169

170 **2.3 HPLC-DAD analysis**

171 All samples were analysed with an original HPLC-DAD method. The instrument
172 was an Agilent (Palo Alto, USA) 1100 Series HPLC system, including a
173 quaternary pump and a diode array detector. The chromatographic separation
174 was carried out on a Phenomenex (Torrance, USA) cellulose tris(3,5-
175 dimethylphenylcarbamate) (Lux Cellulose-1), 4.6 mm × 100 mm, 5 μm)
176 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

208 week), a different sample vial was thawed, pre-treated and analysed. For the
209 dried micromatrices (DBS, DPS, VAMS-B, VAMS-P), blank samples were
210 spiked at a 20 ng/mL level (10 ng/mL for each enantiomer), then stored
211 protected from light in sealed plastic bags containing desiccant for 2 months.
212 At regular intervals (1 week), a different sample was pre-treated and analysed.
213 The measured analyte concentrations were compared to those of the same
214 samples extracted and analysed immediately after biosampling (plasma), or
215 biosampling and drying (dried matrices).
216 *Selectivity:* was tested using 6 independent blank samples from healthy
217 volunteers not subjected to pharmacological therapy, which were individually
218 analysed and evaluated for endogenous interferences. Standard solutions of
219 several drugs, which are commonly co-administered with SNP to psychiatric
220 patients, were also injected at a 500 ng/mL level to ascertain that no
221 interference was generated. Selectivity was considered acceptable when no
222 peak whose signal exceeded the LOD for the analyte, or 5% of the signal/noise
223 ratio for the IS was observed.
224 *Accuracy:* was assessed by re-analysing haematic samples from bipolar
225 patients in a further analytical session: known amounts of the analyte at three
226 levels (lower limit, middle value and high value of the calibration curve) and of
227 the IS at a constant level were added to the samples, pre-treated and
228 analysed. Percentage recovery of the added analytes was calculated and the
229 assays were repeated three times.
230
231 **2.5 Quantitative data comparisons**
232 All results obtained from SNP-treated patient samples were compared by
233 plotting the results from each dried matrix (DBS, DPS, VAMS-B or VAMS-P)
234 versus those obtained from plasma analysis. Then, the least-square method
235 was applied to calculate linearity correlation coefficient and slope of each
236 curve.
237
238 **3 RESULTS AND DISCUSSION**

239 **3.1 Preliminary assays: chiral separation**

240 At first, polymeric chiral stationary phases with a broad range of applicability
241 were tested: cellulose-tris(3,5-dimethylphenylcarbamate), cellulose tris(3-
242 chloro-4-methylphenylcarbamate), cellulose tris(4-chloro-3-
243 methylphenylcarbamate) and amylose tris(5-chloro-2-methylphenylcarbamate).
244 In these sorbents the polysaccharide chains provide a wide array of steric
245 interaction sites: the chlorine- or methyl-substituted methylphenylcarbamate
246 residues greatly increase the chances of interaction with the selector, both with
247 hydrogen bonds, dipole interactions and π–π interactions. Of these sorbents,
248 just the cellulose-tris(3,5-dimethylphenylcarbamate) sorbent (cellulose-1)
249 provided some separation in preliminary assays. Since this is the stationary
250 phase with the least dipole interaction-forming capability, it is possible that
251 SNP chiral recognition relies mainly on hydrogen bonding and π–π interactions.
252 Further assays were thus carried out on the cellulose-1 sorbent only. Initially, a
253 mixture of ammonium bicarbonate and ACN (29:71, v/v) was tested as the
254 mobile phase. This mixture was used to obtain a slightly basic pH and
255 complete compatibility with MS detection, in order to allow future applications
256 using this kind of detection. Moreover, the volatile buffer could be useful for
257 possible adaptation of the method to preparative enantiomeric separations of
258 SNP. This mobile phase provided complete separation (R_s = 1.8) of SNP
259 enantiomers, but with excessive retention (run time > 60 min). Increasing the
260 ACN percentage in the 29-70% (v/v) range decreased run times
261 correspondingly, but with a progressive loss of resolution. The highest ACN
262 percentage that maintained almost complete enantioresolution (R_s = 1.44) was
263 60% (v/v), corresponding to an acceptable run time of 10 min. Flow rate was
264 also studied, varying it in the 0.4-1.2 mL/min range. The best efficiency was
265 found between 0.5 and 0.7 mL/min, witj higher flow rates producing shorter
266 analytical runs: the best compromise was found to be 0.7 mL/min. Column
267 temperature can have drastic effects on chiral separation; for this reason, it
268 was studied between 15 and 40°C. In this range, lower temperatures always
269 granted better resolution, but differences were relatively small and came at the

270 cost of stronger retention and thus longer run times. 25°C was the chosen
271 temperature as the best compromise that still provided good resolution. In
272 order to obtain maximum sensitivity, the spectra of the analyte peaks provided
273 by the HPLC-DAD system were studied. The spectrum of SNP in the described
274 experimental conditions has two absorbance maxima, at 210 nm and at 235
275 nm, but the first one was 4 times more intense, thus 210 nm was chosen for
276 quantitative purposes. Several (non-chiral) compounds were tested as possible
277 ISs. Among them, promethazine gave the best results: It bears some structural
278 resemblance to the analyte and has similar lipophilicity, but with lower
279 retention; thus, it does not increase run time. Under these final
280 chromatographic conditions, the enantiomer peaks were fully resolved, and
281 peak asymmetry was almost negligible (data not shown).
282
283 **3.2 Sample pre-treatment procedures**
284 *Plasma:* although plasma is inherently a macromatrix, a reduced-volume
285 approach was applied to its pre-treatment. For this purpose, MEPS was used:
286 It is a small volume variant of solid phase extraction, where a few milligrams of
287 stationary phase are enclosed in a syringe-mounted BIN, and small amounts of
288 sample and solvents are passed through it using the syringe. Preliminary
289 assays were carried out on spiked blank plasma (100 µL) to find the best
290 extraction sorbent: C8, C2 and SDVB (polystyrene-divinylbenzene copolymer)
291 were tested and best results of analyte extraction and matrix clean-up were
292 obtained using the C8 BIN. Washing and elution solvents and volumes were
293 also optimized; two washing steps with 100 µL of pure water were chosen as
294 the optimal washing steps, allowing satisfactory interference elimination and
295 optimal analyte retention. The methanol elution volume was studied in the 100-
296 1000 µL range; best results were obtained with 500 µL. Higher elution volumes
297 did not increase extraction yields and significantly increased the amount of
298 eluted interference.
299 *Micromatrices:* each of the two DMS techniques has different advantages and
300 drawbacks. Regarding DBS, although some volumetric bias could be produced

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

332 pre-treatment procedure. The chromatograms of a blank VAMS-P sample and
333 a blank VAMS-B sample spiked with racemic SNP standard solution
334 (concentration: 10 ng/mL for each enantiomer) are presented in Figure 2a-b.
335
336 **3.3 Method validation**
337 *Linearity:* for the setup of calibration curves, each micro- and macro-matrix
338 was spiked with racemic standard solutions of the analyte (seven
339 concentrations) and the IS at a constant concentration of 50 ng/mL. Method
340 sensitivity on plasma for both enantiomers was 0.2 and 0.07 ng/mL in terms of
341 LOQ and LOD, respectively; method linearity on the same matrix was assessed
342 between 0.5 and 50 ng/mL. LOD and LOQ values respectively of 0.8 and 2.5
343 ng/mL were obtained for both SNP enantiomers on all the four considered
344 micromatrices; the linearity range was verified between 0.5 and 50 ng/mL for
345 DBS, DPS, VAMS-B and VAMS-P. Good linearity was obtained for both
346 enantiomers in all matrices ($r^2 \geq 0.9989$) and the best data have been provided
347 by the VAMS approach.
348 *Absolute recovery, precision:* blank plasma and blank micromatrices were
349 spiked at three analyte levels. As can be surmised from Table 1, absolute
350 recovery on all matrices was satisfactory, in the 81.7-99.8% range, for both
351 analyte enantiomers. Precision was good, producing RSD values lower than
352 6.0%. Both VAMS types granted better recovery and precision than DMS.
353 *Stability:* dried micromatrices often demonstrate higher stability than
354 "traditional" macromatrices, even when the former ones are stored at RT. In
355 this case, the stability of the analyte in dried matrices at RT was compared to
356 that in plasma samples under freezing (-20°C) and deep-freezing (-80°C)
357 conditions. After 1 month, all dried matrices stored at RT consistently provided
358 higher extraction yields than plasma (> 78%); in VAMS in particular, they were
359 always higher than 91%. The superior stability observed in dried samples can
360 be attributed to the water loss, which negatively impacts both chemical and
361 enzymatic reactions. No significant stability differences were found between

362 the two enantiomers, indicating that they are probably configurationally stable
363 in the conditions employed.
364 *Selectivity:* blank matrix samples (from healthy volunteers) were pre-treated
365 and analysed. Standard solutions of several drugs, which are commonly co-
366 administered with SNP to psychiatric patients, were also injected at a 500
367 ng/mL level to ascertain that no interference was created by them:
368 carbamazepine, gabapentin, lamotrigine, valproate and vigabatrin among
369 antiepileptics; aripiprazole, chlorpromazine, clozapine, loxapine and olanzapine
370 among antipsychotics; citalopram, sertraline and venlafaxine among
371 antidepressants. No interfering peak was detected in any of the matrices and
372 for any of the tested drugs.
373
374 **3.4 Analysis of real samples and accuracy**
375 The methodology was applied to the analysis of macro- and micro-samples
376 from patients undergoing therapy with SNP for bipolar disorder. All matrices
377 were simultaneously obtained, either by venipuncture or by fingerpricking, to
378 ensure that any inconsistency in results could not be attributed to sampling.
379 The chromatogram of a VAMS-B sample from a patient is reported in Figure
380 2c. As can be seen, sample purification and chiral separation are both
381 satisfactory. In this case, R_S is slightly higher than that obtained on spiked
382 blank samples (Figure 2b); this result is probably due to the combination of
383 different matrix and concentration effects. Table 2 reports the quantitative data
384 obtained from the analysis of real samples from bipolar patients. Concentration
385 differences between the two enantiomers were small and non statistically
386 significant. This confirms what is currently known from early studies carried out
387 by the drug manufacturer [26,27]. As a rule, raw analytical data from plasma-
388 based matrices can be used as such for quantitation, while those obtained
389 from blood-based matrices have to be elaborated to account for sample
390 viscosity (related to the haematocrit), and for red blood cell / plasma
391 partitioning [28]. A constant red blood cell / plasma concentration ratio of 1/3
392 was applied in all cases, according to literature evidence [26]. A mean

393 haematocrit value of 38% was used for samples coming from female patients
394 and of 48% for those from male patients. Both corrections have already been
395 included in the results of blood-based matrices (DBS, VAMS-B) in Table 2. In-
396 line UV spectra were used to both confirm the identity of the analyte
397 enantiomer and IS peaks, and to verify peak purity. Finally, good accuracy was
398 reached in all matrices and the VAMS technique gave the best SNP recovery:
399 always higher than 95.3%.
400
401 **3.5 Comparison between macro- and micro-sample results**
402 In order to evaluate the performance of each matrix approach, SNP
403 enantiomeric concentrations obtained from patient plasma samples were
404 compared to those obtained from each micromatrix. Linear correlation analysis
405 between the results showed that both linearity and slope values were very
406 close to 1 for all dried matrices, confirming that they closely mimic plasma
407 behaviour and produce reliable data. However, the best correspondence with
408 plasma levels was observed for VAMS matrices. These results are statistically
409 significantly different from those obtained using DMS sampling; on the other
410 hand, no significant difference was observed between VAMS-B and VAMS-P
411 (nor between DBS and DPS). This, together with the better precision and
412 recovery results, means that VAMS can be confidently rated as more reliable
413 than DMS for SNP enantiomer analysis with this method. The better
414 performance of VAMS could probably be attributed to the very good volume
415 reproducibility of the sampling tips (as opposed to the intrinsic uncertainty of
416 blotting and punching out the spots). All in all, the dried matrices produce
417 better analyte stability and are surely more practical and more easily handled
418 than plasma. Among micromatrices, blood-based ones (DBS and VAMS-B) are
419 more practical than plasma-based ones (DPS and VAMS-P), since they can be
420 obtained with a minimally invasive fingerpicking and do not require any sample
421 manipulation prior to blotting or absorbing.
422
423 **4 CONCLUDING REMARKS**

424 Four different microsampling and pre-treatment procedures were proposed
425 and compared for the determination of SNP enantiomers in human blood. An
426 original enantioselective method based on HPLC-DAD was developed and
427 validated, allowing for the first time the chromatographic separation and
428 quantitation of SNP enantiomers in biological samples, within reasonable run
429 times and with good reproducibility. The analytical method coupled to DBS,
430 DPS, VAMS-B and VAMS-P demonstrated to be rapid and selective, with
431 significant advantages over classical approaches exploiting macromatrices,
432 and plasma in particular: the tested dried microsamples were more stable over
433 time and more easily stored. Result comparisons have shown the four
434 micromatrix approaches produce sound results, always strictly correlated with
435 the corresponding data produced by classic plasma analysis. However, VAMS
436 sampling provided consistently better results than DMS sampling in terms of
437 precision, recovery and correspondence with plasmatic concentrations.
438 Although all matrices are suitable for the analysis of SNP in psychiatric
439 patients, VAMS are clearly preferable and will be proposed to clinicians as an
440 attractive alternative to classical plasma sampling. The coupling of VAMS
441 micromatrices and enantioselective analysis provides a solid platform that can
442 be adapted to different needs and situations, with the added advantage of also
443 obtaining advanced, reliable insights into chiral aspect of SNP interactions with
444 the human body. Assays are currently in progress in order to unequivocally
445 assign the correct absolute configuration to the chromatographic peaks of the
446 two SNP enantiomers.
447
448 **FIGURE LEGENDS**
449 **Figure 1.** Chemical structures of (a) (*R,R*)-SNP, (b) (*S,S*)-SNP, (c) promethazine
450 (IS).
451 **Figure 2.** Chromatograms of (a) a blank VAMS-P sample, (b) a blank VAMS-B
452 sample spiked with racemic SNP standard solution (concentration: 10 ng/mL
453 for each enantiomer), (c) a VAMS-B sample from a bipolar patient treated with
454 20 mg/day of SNP (patient n. 6, see Table 2). Experimental conditions: column,

455 Lux Cellulose-1 (4.6 mm × 100 mm, 5 μm) thermostatted at 25°C; mobile
456 phase, ACN / 50 mM ammonium bicarbonate (60/40, v/v); flow rate, 0.7
457 mL/min; detection, diode array at 210 nm.
458
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463
464 **CONFLICTS OF INTEREST**
465 All authors declare there are no conflicts of interest.
466
467
468 **5. REFERENCES**

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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.

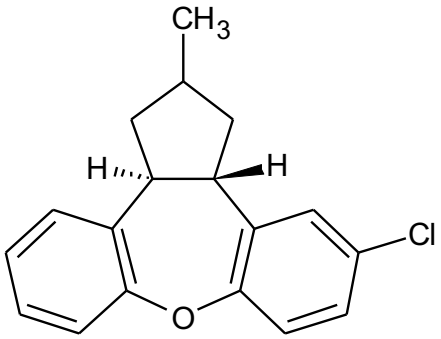
Analyte	Concentration level[a]	Matrix	Absolute recovery, %[b]	Precision, RSD%[c]	
				Intraday	Interday
SNP1	Low	Plasma	95.1	4.0	4.1
		DBS	85.1	4.6	4.8
		DPS	90.7	4.1	4.4
		VAMS-B	99.1	3.6	3.8
		VAMS-P	98.7	3.5	4.0
	Intermediate	Plasma	94.4	3.7	3.9
		DBS	83.2	4.4	4.9
		DPS	87.5	3.8	3.8
		VAMS-B	98.8	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] $n = 6$.

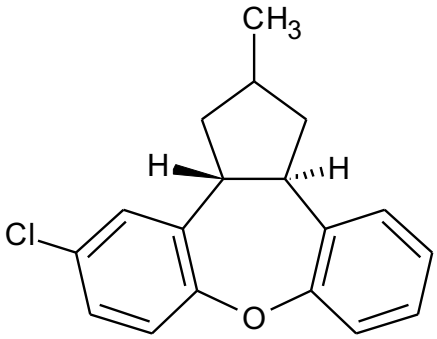
Table 2. Quantitative results from patients taking SNP.

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

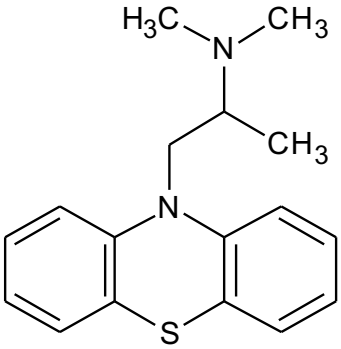
[a] $n = 3$, mean value.



a – (*R,R*)-Asenapine



b – (*S,S*)-Asenapine



c – Promethazine (IS)

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Figure 1

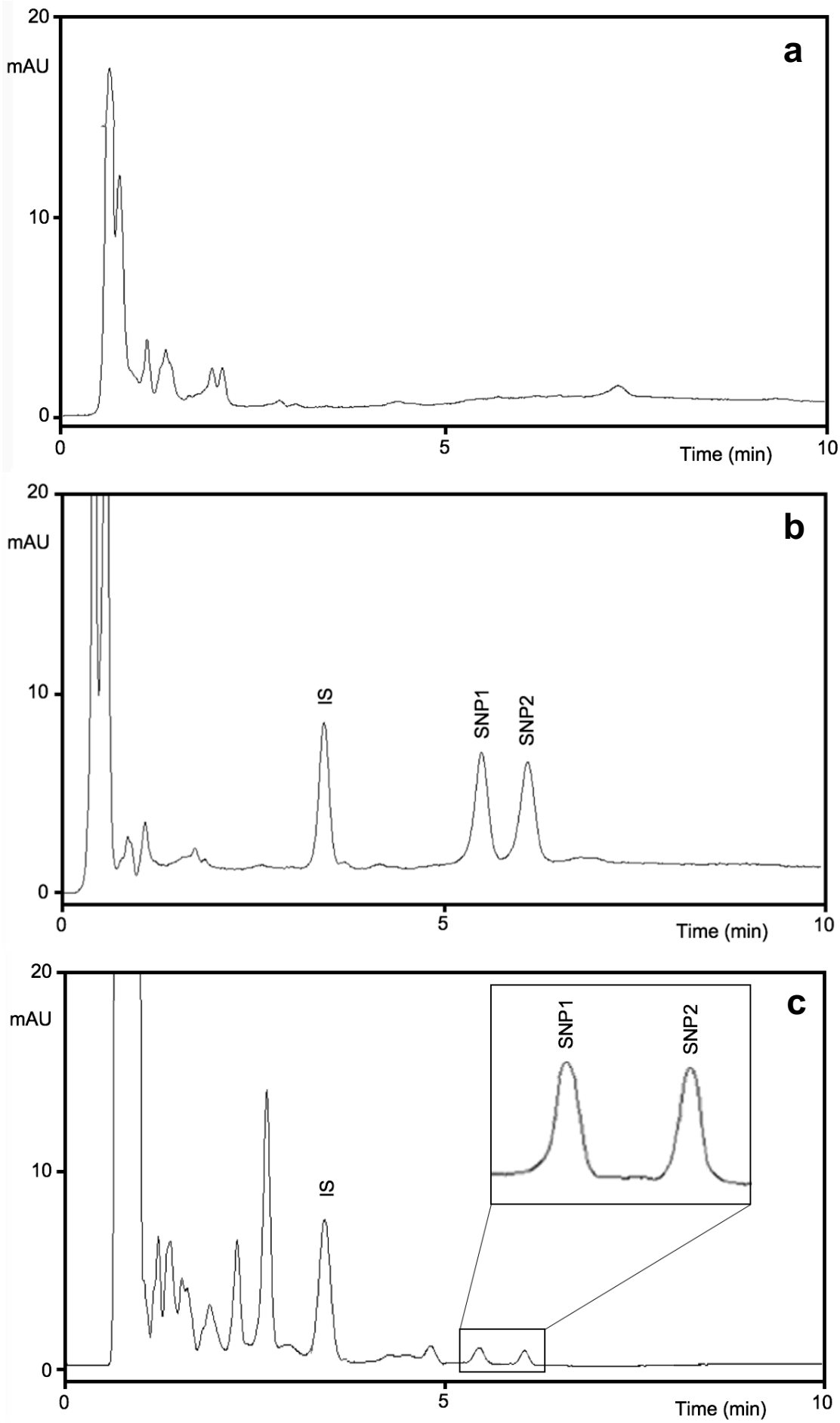

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Figure 2