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(Article begins on next page)

1 **VAMS and StAGE as innovative tools for the enantioselective determination of**
2 **clenbuterol in urine by LC-MS/MS**

3

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Highlights

- Dried urine is obtained by volumetric absorptive microsampling (VAMS)
- The microsamples are treated by an original stop-and-go extraction (StAGE) procedure
- Five different polysaccharide-based chiral stationary phases (CSPs) are evaluated
- Enantioselective LC-MS/MS is carried out on a cellulose tris(4-methylbenzoate) column
- Successful application to real urine samples
- Future anti-doping and clinical study perspectives

28 **Abstract**

29

30 Clenbuterol is a chiral, selective β_2 -adrenergic agonist. It is administered as a racemic
31 mixture for therapeutic purposes (as a bronchodilator or prospective neuroprotective
32 agent), but also for non-therapeutic uses (athletic performance enhancement, cattle
33 growth promotion). Aim of the present study is to develop an original, enantioselective
34 workflow for the analysis of clenbuterol enantiomers in urine microsamples. An
35 innovative miniaturised sampling procedure by volumetric absorptive microsampling
36 (VAMS) and a microsample pretreatment strategy based on stop-and-go extraction
37 (StAGE) tips were developed and coupled to an original, chiral analytical method,
38 exploiting liquid chromatography with triple quadrupole detection (LC-MS/MS). The
39 method was validated, with satisfactory results: good linearity ($r^2 \geq 0.9995$) and LOQ
40 values (0.3 ng/mL) were found over suitable concentration ranges. Extraction yield
41 ($>87\%$), precision (RSD $< 4.3\%$) and matrix effect (85-90%) were all within acceptable
42 levels of confidence. After validation, the method was applied to the determination of
43 clenbuterol in dried urine VAMS from patients taking the drug for therapeutic reasons.
44 Analyte content ranged from 0.8 to 2.5 ng/mL per single enantiomer, with substantial
45 retention of the original drug racemic composition. The VAMS-StAGE-LC-MS/MS
46 workflow seems to be suitable for future application to anti-doping testing of
47 clenbuterol in urine.

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53 **Keywords:**

54 Clenbuterol; LC-MS/MS; stop-and-go extraction (StAGE); urine microsampling;

55 volumetric absorptive microsampling (VAMS).

56 **1. Introduction**

57

58 Clenbuterol (1-(4-amino-3,5-dichlorophenyl)-2-[(2-methyl-2-propanyl)amino]ethanol,
59 CBT, Figure 1) is a sympathomimetic agent with strong selectivity toward β_2 -adrenergic
60 receptors [1]. Thanks to its selectivity, it is widely used as a bronchodilator drug in
61 several pathologies, ranging from asthma to chronic obstructive pulmonary disease
62 (COPD), without soliciting strong cardiovascular effects (vasoconstriction,
63 hypertension, etc.). However, a small number of β_2 -adrenergic receptors are also present
64 in blood vessels and in the heart, thus CBT can produce negative cardiovascular events
65 despite its selectivity [2]. On the other hand, β_2 -adrenergic receptor agonism by CBT is
66 known to promote nerve growth factor (NGF) synthesis in nerve tissue both *in vitro* and
67 *in vivo*, also protecting neurons from excitotoxic and ischemic damage [3]. This has led
68 to the inclusion of CBT in clinical studies as a possible neuroprotective agent for
69 different neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS) [4].
70 CBT properties of blood pressure and oxygen transport increase, basal metabolism
71 increase, thermogenesis and fat loss, and its possible anabolic effects, have also led to
72 its illegal use both to increase the yield of muscle mass in livestock and as a
73 performance-enhancing substance in sports. The former use has been specifically
74 prohibited by national authorities around the world [5]. The latter use is explicitly
75 forbidden in sports “at all times” (i.e., both in- and out-of-competition) by the World
76 Anti-Doping Agency (WADA). In particular, clenbuterol is included in group S1
77 (anabolic agents), sub-group S1.2 (other, not androgenic steroid agents) of its “List of
78 Prohibited Substances and Methods” [6]. These two illicit uses are strictly connected,
79 since professional athletes have been proven to have received adverse analytical

80 findings (AAF, i.e., positive results to an anti-doping test) for involuntarily consuming
81 meat or other foodstuffs contaminated with CBT [7].

82 Thus, new analytical means for CBT determination in urine could be useful for several
83 purposes: discriminating between voluntary CBT intake for doping purposes and
84 involuntary intake due to meat contamination; and assessment of CBT elimination
85 during clinical studies of neuroprotection. In particular, the use of CBT enantiomers has
86 recently emerged as a possible means of pinpointing meat contamination. The CBT
87 molecule is chiral, since it contains a single asymmetric carbon, thus it can exist as a
88 pair of enantiomers. The differential biological activity of CBT enantiomers has not
89 received much scientific attention in recent years, however it seems the *R*-(-)-CBT
90 enantiomer is responsible for most of the observed pharmacological effects (both at β_2
91 and at β_1 receptors), while the *S*-(+)-CBT enantiomer has low biological activity [8].
92 Neuroprotective effects seem to be strictly enantioselective as well, since they likely
93 depend on β -receptor agonism [9]. Nonetheless, CBT is currently found in formulations,
94 and thus administered, as a racemate. Humans taking racemic CBT seem to eliminate
95 the drug still in racemic form through urine [10]. On the contrary, non-racemic mixtures
96 are found in the meat of several animal species, including cattle, after racemic CBT
97 administration; the enantiomeric excess seems to be maintained in the urine of humans
98 eating it [7, 11]. This enantiomeric excess, however, is highly variable and does not
99 seem to depend on easily predictable parameters or conditions [12].

100 This fact highlights the importance of carrying out the enantioselective analysis of CBT
101 for anti-doping testing purposes, and possibly also for other purposes, such as
102 therapeutic drug monitoring (TDM) and illicit drug administration testing. Although
103 urine is not usually exploited as the main matrix in TDM, as an adjunctive matrix it can

104 provide useful information, e.g., on the elimination and metabolic patterns of the
105 monitored drug.

106 Several published methods have dealt with the enantioseparation and analysis of
107 clenbuterol in human biological matrices. For example, enantioselective determination
108 of CBT in urine for anti-doping testing has been reported by GC-MS [13], LC-MS
109 [7,12,14-17] or UHPLC-MS [18]. A method for human hair [19], again using LC-MS,
110 is also available.

111 However, none of these rely on microsampling. The dried microsampling approach
112 represents a promising alternative to current procedures involving wet samples: the
113 almost complete water loss increases analyte stability, and as a consequence also
114 broadens the detection time window [20-22]. It produces logistics savings due to the
115 small volume to be transported and to the possibility of storing the specimens at room
116 temperature, with significant implications in terms of overall analysis costs [23,24].

117 Dried microsamples are unlikely to be tampered, stably storable and shippable with no
118 particular precautions [25]. The main drawbacks of microsampling are still relatively
119 low sensitivity, due to the impossibility of pre-concentrating the sample, and limited
120 sample availability, which restricts the number of different assays that can be carried
121 out on a single microsample. Until now just one method has been published, in 2020,
122 regarding the use of dried microsampling (namely dried blood spots – DBS) for the
123 analysis of CBT enantiomers in biological matrices [26]; however, it is not
124 enantioselective and it focuses on detectability and not on quantification.

125 The results of a study on sampling by volumetric absorptive microsampling (VAMS)
126 are reported therein. This technique aims to provide fixed-volume microsamples that are
127 not subject to the haematocrit effect like DBS are. VAMS has been coupled to a

128 miniaturised pretreatment technique, namely by the use of microextraction by stop-and-
129 go extraction (StAGE) tips, that is eminently suitable for the purification of small-
130 volume, dried specimens. Finally, the extracted samples were analysed using an original
131 enantioselective method based on LC-MS/MS. The results obtained suggest that the
132 method could be reliably applied for anti-doping purposes.

133

134

135 **2. Materials and Methods**

136

137 **2.1. Chemicals and standard solutions**

138 CBT hydrochloride ($\geq 98\%$ purity), CBT-D₉ hydrochloride ($\geq 98\%$ purity, $\geq 97\%$
139 isotopic purity) used as the internal standard (IS) and acetonitrile, methanol, formic acid
140 (FA), sodium carbonate – all reagents for mass spectrometry – in addition to phosphoric
141 acid, sodium hydroxide and all the solvents used for sample preparation (all analytical
142 grade) were purchased from Sigma Aldrich (Darmstadt, Germany). *S*-(+)-CBT and
143 *R*-(-)-CBT, pure enantiomers (purity $\geq 98\%$, enantiomeric excess $> 99\%$) were
144 purchased from Toronto Research Chemicals (Toronto, ON, Canada). Ultrapure water
145 (18.2 M Ω cm) was obtained by means of a Milli-Q apparatus from Millipore (Milford,
146 MA, USA). The analyte and IS stock solutions (1 mg/mL) were prepared by dissolving
147 suitable amounts of pure powders in methanol. The corresponding standard solutions
148 were prepared daily by dilution with a water/acetonitrile mixture (50:50) containing
149 0.25% FA. All solutions were stored protected from light in amber glass vials certified
150 for mass spectrometry from Waters Corporation (Milford, MA, USA).

151

152 **2.2. LC-MS/MS instrumentation and conditions**

153 LC-MS/MS analysis was performed on a Waters Alliance e2695 chromatographic
154 system with autosampler coupled to a Waters 2998 photodiode array detector and a
155 Waters Micromass Quattro Micro triple-quadrupole mass spectrometer equipped with
156 an electrospray ion source (ESI). Data processing was performed using Waters
157 MassLynx 4.1 software. Separations were obtained on a Phenomenex (Darmstadt,
158 Germany) Lux Cellulose-3 column (150 × 3.0 mm I.D., 3 µm), maintained at room
159 temperature and equipped with a Lux Cellulose-3 guard column. The mobile phase was
160 a mixture of 0.5% formic acid in water brought to pH 7.2 with 1 M carbonate solution
161 and acetonitrile (80:20), flowing at a constant rate of 0.1 mL/min. The injection volume
162 was 10 µL.

163 Multiple reaction monitoring (MRM) transitions were used, acquiring in positive
164 ionisation mode (ESI+) and exploiting two different exclusive transitions for both CBT
165 and the IS: the most abundant one for quantitative purposes, the second one for identity
166 confirmation. The optimised parameters were as follows: ion source voltage, 3 kV; cone
167 voltage, 13 V, ion source temperature, 120 °C; desolvation temperature, 300°C;
168 desolvation gas flow, 600 L/h; -1.8 kV; extractor potential, 3 V; collision exit potential,
169 1 V; scan duration: 0.3 s. (nitrogen as the desolvation gas, argon as the collision gas).
170 The precursor ions and the product ions, with dwell time, cone voltage and collision
171 energy, were optimised and are shown in Table 1.

172

173 **2.3. Compliance with Ethical Standards**

174 Urine samples, used as blank matrices, were obtained from drug-free healthy
175 volunteers. Real urine samples were from patients receiving CBT as part of their

176 standard treatment and had already been collected for general needs related to the
177 therapy. All subjects provided informed consent prior to their participation.

178

179 **2.4. Urine microsampling: volumetric absorptive microsampling (VAMS)**

180 Urine aliquots of 100 μ L were spiked with 5 μ L of analyte standard and/or IS
181 mixtures at known concentrations. Mitra[®] VAMS microsamplers (20 μ L) were provided
182 by Neoteryx (Torrance, CA, USA). A VAMS micro sampler includes a polypropylene
183 handle (about 4 cm long) topped with a small tip (about 2-mm diameter) of a
184 proprietary polymeric porous material. The surface of the sample mixture was touched
185 with a VAMS micro sampler for 5 s, dried at room temperature (RT) for 1 h and stored
186 at RT, in the dark for 2 months at most, in a dedicated clamshell in order to avoid
187 contact with any surface. Clamshell was stored in a sealed polyethylene bag containing
188 desiccant. The micro sampler tip was detached from the handle and subjected to
189 ultrasounds for 20 min in 1 mL of MeOH. The resulting solution was brought to dryness
190 under a nitrogen stream, re-dissolved with 100 μ L of 5:95 mixture of 0.1% FA in
191 acetonitrile and 0.1% FA in water and subjected to StAGE pretreatment.

192

193 **2.5. Microsample pretreatment: stop-and-go extraction (StAGE) tips**

194 The procedure to obtain home-made StAGE tips was carried out by using Kartell
195 Labware (Noviglio, Italy) automatic pipette tips and Supelco Analytical (Darmstadt,
196 Germany) Empore[™] polymeric C8 disks. 3 layers of circular disk fragments (3-mm
197 diameter) were punched out by means of a puncher, then assembled inside the pipette
198 tip. Each obtained StAGE tip was placed into a microcentrifuge tube through the lid and
199 centrifuged using specific temperature, speed and time after each solvent application. At

200 first, the sorbent was activated with 100 μ L of methanol and then equilibrated with 100
201 μ L of ultrapure water ($3000 \times g$, 5 min, 4 $^{\circ}$ C). The sample loading was represented by a
202 100- μ L aliquot of urine extract spiked with 5 μ L of analyte standard and/or IS (it dealt
203 with the extraction solution obtained after VAMS procedure). After the sample loading
204 ($3000 \times g$, 10 min, 4 $^{\circ}$ C), the cartridge was washed with 100 μ L water ($3000 \times g$, 5 min,
205 4 $^{\circ}$ C) and 100 μ L of 50:50 mixture of 0.1% FA in acetonitrile and 0.1% FA in water
206 ($3000 \times g$, 5 min, 4 $^{\circ}$ C). The analytes were eluted using 2 x 300 μ L ACN ($3000 \times g$, 10
207 min, 4 $^{\circ}$ C).

208 The eluate was brought to dryness under a nitrogen stream, re-dissolved with 100 μ L of
209 5:95 mixture of 0.1% FA in acetonitrile and 0.1% FA in water and injected into the LC-
210 MS/MS system.

211

212 **2.6. Method validation**

213 The analytical method was validated according to World Anti-Doping Agency's
214 (WADA) guidelines [27]. The tested parameters were linearity (including limits of
215 detection and limit of quantitation), extraction yield, precision, matrix effect, stability,
216 and accuracy.

217 Urine samples from volunteers not undergoing CBT therapy were spiked with racemic
218 CBT standard solutions at seven different concentrations, containing the IS at a constant
219 concentration, subjected to VAMS and StAGE and injected into the LC-MS/MS
220 system. The analysis was carried out in triplicate for each concentration. The obtained
221 analyte / IS enantiomer peak area ratios were plotted against the corresponding
222 concentrations (expressed as ng/mL) and the calibration curves were obtained by means

223 of the least-square method. LOQ and LOD were calculated as the analyte concentrations
224 which give rise to peaks whose areas are 10 and 3 times the baseline noise, respectively.
225 Extraction yields were evaluated by repeatedly subjecting to the previously described
226 procedure urine samples spiked with racemic CBT standard solutions at three different,
227 known concentrations (corresponding to a low, an intermediate and a high value of the
228 linearity range). The obtained analyte enantiomer peak areas were compared with those
229 obtained by injecting standard solutions at the same theoretical concentrations in order
230 to calculate extraction yield values. Precision assays were carried out on the same urine
231 microsamples, which were analysed six times in the same day to obtain *intraday*
232 precision and six times over six different days to obtain *interday* precision, expressed as
233 percentage relative standard deviation (RSD %).

234 IS-corrected matrix effect of urine VAMS was evaluated on blank sample extracts from
235 6 different sources, fortified post-extraction by adding known amounts of the analyte
236 and then analysed by chiral LC-MS/MS. Mean analyte/IS peak area ratios of each
237 extract were compared with analyte/IS peak area ratios from standard CBT solutions
238 and the resulting percentage matrix effect was calculated as: $100 \times (\text{std response} -$
239 $\text{extract response}) / \text{std response}$.

240 To test analyte stability, blank urine samples were spiked with racemic CBT at two
241 concentration levels (high and low concentrations with respect to the calibration curve),
242 sampled by VAMS then stored at RT, protected from light, in sealed polyethylene bags
243 containing desiccant for 3 months. At regular intervals (1 week), microsamples were
244 pretreated and analysed in triplicate. The measured analyte concentrations were
245 compared to those of the same samples extracted and analysed immediately after
246 biosampling and drying. The stability values thus obtained were also compared to those

247 of conventional urine samples stored at -20°C or -80°C. For autosampler stability of
248 processed VAMS, samples spiked at the same two concentration levels were freshly
249 pretreated in triplicate and stored in the autosampler at RT for 24 hours before re-
250 analysis, while for VAMS bench-top stability, spiked VAMS samples were stored for 3
251 days at room temperature without using neither polyethylene bags nor desiccant.
252 Samples were considered stable when % bias from the nominal concentrations was
253 within $\pm 15\%$.
254 Recovery assays were carried out in order to evaluate method accuracy: standard
255 solutions containing known amounts of racemic CBT (corresponding to a low, an
256 intermediate and a high value of the calibration curves) and a fixed amount of IS were
257 added to urine microsamples which had been already analysed. The obtained spiked
258 samples were then analysed, and the recovery of spiked CBT enantiomers was
259 calculated.

260

261

262 **3. Results and Discussion**

263

264 **3.1. Enantioselective HPLC analysis with UV and MS detection**

265 Efficient chromatographic methods enabling the enantioseparation of CBT both in
266 biological samples and pharmaceutical preparations have been already developed by
267 other authors with different types of chiral stationary phases (CSPs) [12,14-17, 28-30].
268 In this framework, the highly versatile cellulose and amylose tris(phenylcarbamates)
269 and tris(benzoates) CSPs [31-33] did allow the successful separation of CBT
270 enantiomers in biological matrices both under normal-phase (NP) [29] and RP
271 conditions [30], with the latter application being developed with a mobile phase system

272 not compatible with MS detection. Availing in our “laboratory toolbox” of a number of
273 different polysaccharide-based CSPs, these materials have been selected in the present
274 study.

275 In order to identify the best experimental conditions for the enantioseparation of CBT,
276 five different polysaccharide-based chiral stationary phases (CSPs) of identical
277 dimensions were preliminarily evaluated with the same RP eluent system [50 mM
278 ammonium bicarbonate / acetonitrile, 80/20 (V/V)] (see section 2.2. *LC-MS/MS*
279 *instrumentation and conditions*) and UV detection exploiting a RP-HPLC-PDA system
280 ($\lambda = 244$ nm).

281 In the present study, RP conditions were preferred over NP ones, since the former are
282 more indicated for applications with MS detection systems [31] due to the easier
283 ionisation of the sample components. More to the point, aqueous mobile phases are
284 useful to cope with solubility limitations often found in NP experiments, especially
285 when involving the analysis of polar compounds in complex biological matrices.
286 Furthermore, a series of preliminary analysis performed under polar-organic (PO)
287 conditions (data not shown) did not allow satisfactory results.

288 The screened CSPs are characterized by the following chiral selectors: cellulose tris(4-
289 methylbenzoate) (CSP 1), cellulose tris(3,5-dimethylphenylcarbamate) (CSP 2),
290 cellulose tris(3-chloro-4-methylphenylcarbamate) (CSP 3), cellulose tris(4-chloro-3-
291 methylphenylcarbamate) (CSP 4), and amylose tris(5-chloro-2-methylphenylcarbamate)
292 (CSP 5). These CSPs were chosen with the aim of evaluating the impact of the
293 following variables on the overall chromatographic performance: (i) polymer winding
294 (linkage of glucopyranose units, CSPs 1-4 vs CSP 5), since the amylose- and cellulose-
295 based chiral selectors feature a different winding (α -1,4- and β -1,4-linkage of

296 glucopyranose units, respectively) which affects the morphology of the polymer and
297 hence access to the stereoselective binding sites; (ii) type and position of the
298 substituents in the phenyl ring of the carbamate moiety (CSP 2 vs CSP 3 vs CSP 4),
299 since the stereorecognition ability of phenylcarbamate cellulose-type chiral selectors is
300 very sensitive to inductive effects originating from the nature and position of the
301 substituents on this aromatic region; (iii) type of functionalization on the original sugar
302 unit hydroxyls, since the carbamate and ester residues induce different polymer
303 morphologies and binding opportunities.

304 The comparative evaluation allowed us to identify CSP 1 as the best enantioselective
305 material for the purpose of the study: $\alpha = 1.73$; $R_S = 2.1$, within reasonable run times (<
306 7 min) (Table S1). This result is in strict accordance with the work by Velasco-Bejarano
307 and co-workers [28] where CSP 1 was very profitably used for the CBT
308 enantioseparation in pharmaceutical preparations and black-market products.

309 After the preliminary optimization with an RP-HPLC-PDA system, the method was
310 transferred to an LC-MS/MS apparatus, where the analysis was performed exploiting
311 CSP 1 and the same mobile phase (a mixture of 0.5% formic acid in water brought to
312 pH 7.2 with 1 M carbonate solution and acetonitrile, 80:20). Under these conditions, the
313 separation outline was similar to that provided by the HPLC-PDA apparatus, without
314 appreciable suppression of positive analyte ionisation.

315 Optimised mass spectrometry conditions were studied by syringe infusion of analyte
316 solutions directly into the ESI+ source, thus obtaining MS and MS/MS spectra.

317 Quantification was based on the most prominent MRM transition, while a second
318 transition was monitored for qualitative purposes. Table 1 reports all MRM data for
319 CBT and the IS.

320 The LC-MS/MS chromatogram of a racemic CBT mixture at the concentration of 25
321 ng/mL for CBT (IS concentration: 50 ng/mL) is shown in Figure 2. The enantiomer
322 elution order (EEO) with CSP 1 was established by injecting standard solutions of the
323 two enantiomers. The least retained enantiomer was *R*-(-)-CBT, followed by *S*-(+)-
324 CBT.

325 As far as the enantio-recognition mechanism is concerned, with NP eluents, the analyte
326 enantiomers are able to engage stereoselective interactions with the polar carbamate or
327 ester residues (*via* H-bonding and dipole-dipole interaction) [32]. Additionally, π - π
328 stacking interactions between the phenyl groups on the chiral selector and an aromatic
329 region of the solute can make a contribution to the overall chiral recognition event.

330 The importance of interactions involving aromatic regions of both the analyte and chiral
331 selector structure is especially evident when analyses are run in the RP mode. In RP
332 applications like the one described in the present paper, these and other types of
333 hydrophobic interactions, *viz.* those involving the carbon backbone of the sugar
334 moieties, can influence both non-stereoselective and stereoselective retention.

335 Nevertheless, it has been largely demonstrated [31] that a balance between hydrophobic
336 and hydrophilic interactions governs the chromatographic process with water-ACN
337 mobile phases, with the former playing a primary role for analyte retention. In the
338 present study, the importance of H-bond interactions could contribute to explain the
339 stronger retention of CBT enantiomers on CSP 3 and CSP 4 over CSP 2
340 [32].

341

342 **3.2. Urine Microsampling**

343 Initially, microsampling by dried urine spots (10 and 20 μ L) on cellulose cards was
344 tested. However, it was soon evident that clenbuterol is strongly retained by the
345 cellulose matrix of the cards, thus low extraction yield (< 51%) and unsatisfactory
346 analytical sensitivity (LOQ > 2 ng/mL) were obtained with this kind of sampling (see
347 Table S2), irrespective of the extraction solvent or procedure applied.

348 On the contrary, preliminary assays confirmed that VAMS provides reproducible
349 sampling volume (20 μ L, according to the chosen tip size) and promising extraction
350 yields. For this reason, VAMS devices were used for all subsequent collection assays.

351

352 **3.3. Miniaturised urine VAMS pretreatment: Stop-And-Go Extraction (StAGE)**

353 The VAMS technique has been herein applied for the first time for the sampling of
354 miniaturised urine samples followed by the enantioselective determination of
355 clenbuterol. On the other hand, StAGE has been developed and successfully applied for
356 the first time for clenbuterol purification from a biological matrix and in general for the
357 first time from urine-derived samples, allowing a reproducible and high-throughput
358 processing.

359 After urine collection and drying, VAMS tips were extracted with 1 mL of methanol
360 and the resulting clear solution was brought to dryness, re-dissolved in mobile phase
361 and subjected to pretreatment by StAGE tips.

362 StAGE is based on the same principles as SPE. In this case, miniaturisation is achieved
363 by packing the sorbent (in the form of SPE disk fragments cut out with a common card
364 puncher) into a common automatic pipette tip. This arrangement is extremely flexible,
365 since it allows the operator to stack two or more different sorbents, or several layers of

366 the same sorbent, into a single tip, thus obtaining a huge array of combinations and a
367 wide choice of selectivity possibilities for the procedure.
368 In this case, three or six layers were used for the preliminary assays, in the following
369 ordered combinations: 1×C8; 3×C8; 6×C8; 1×C18; 3×C18; 6×C18; 1×SDVB;
370 3×SDVB; 6×SDVB; 3×C8+3×SDVB; 3×C8+3×C18; 3×C18+3×SDVB;
371 3×C8+3×SDVB (alternate layers). Generally speaking, homogenous sorbent layers gave
372 better results than mixed ones, and three-layer assemblies provided higher extraction
373 yields than the corresponding six-layer ones (see Table 2). In particular, homogenous
374 three- and six-layer C8 assemblies provided the best results; of these, the three-layer C8
375 assembly was chosen both for its easier preparation and for its (marginally) better
376 performance.

377

378 **3.4. Method validation**

379 *Linearity:* for the setup of calibration curves, urine was spiked with racemic standard
380 solutions of the analyte (seven concentrations) and the IS at a constant concentration of
381 50 ng/mL. Method sensitivity on urine collected by VAMS and treated by StAGE for
382 both enantiomers was 0.3 and 0.1 ng/mL in terms of LOQ and LOD, respectively;
383 method linearity on the same matrix was assessed between 0.3 and 200 ng/mL. Good
384 linearity was obtained for both enantiomers ($r^2 \geq 0.9995$). For the *R*-(-)-CBT
385 enantiomer, standard error (SE) of the slope was 0.01; SE of the intercept was 0.60; and
386 SE for the *y* estimate was 0.92. For the *S*-(+)-CBT enantiomer, SE of the slope was
387 0.01; SE of the intercept was 0.66; and SE for the *y* estimate was 1.02 (95% Confidence
388 Interval). Despite the very small microsample volume, which prevents sample pre-
389 concentration, suitable sensitivity has been achieved, although it is still lower than that

390 of comparable applications using millilitre-volume samples. The LC-MS/MS
391 chromatogram of a urine VAMS sample spiked with a racemic CBT mixture at the
392 LOQ level is shown in Figure 3.

393 *Extraction yield, precision:* blank urine was spiked at three analyte levels. As can be
394 surmised from Table 3, extraction yield was satisfactory, in the 87-90% range, for both
395 analyte enantiomers and IS. Precision was good, producing RSD values lower than
396 4.3%.

397 *Selectivity:* blank urine samples (from healthy volunteers not undergoing CBT treatment
398 and not consuming meat) were microsampled, pretreated and analysed. No interfering
399 peak was detected in any of the samples. The LC-MS/MS chromatogram of a blank
400 urine VAMS sample is shown in Figure 4.

401 *Matrix effect:* Both VAMS sampling and StAGE pretreatment contributed to producing
402 remarkably clean microsamples. Urine VAMS spiked at three analyte levels after
403 pretreatment were analysed by LC-MS/MS to test for possible ionisation
404 suppression/enhancement. Matrix effect was always lower than 15% (see Table 2); thus,
405 this parameter was within the satisfactory range.

406 *Stability:* dried micromatrices are often more stable than “traditional” macromatrices,
407 even when the former ones are stored at RT. In this case, analyte stability in VAMS
408 spiked with CBT (1 and 50 ng/mL) and stored at RT, protected from light, in sealed
409 polyethylene bags containing desiccant was compared to that in urine samples under
410 freezing (-20°C) and deep-freezing (-80°C) conditions. After 1 month, VAMS stored
411 at RT consistently provided higher mean extraction yields ($> 98.3\%$) than urine samples
412 stored at -80°C ($> 91.8\%$). After 3 months, mean stability was $> 96.2\%$ for urine
413 VAMS and $> 88.7\%$ for fluid urine samples at -80°C . The higher stability of VAMS

414 can be attributed, at least in part, to the water loss which slows or stops most chemical
415 and enzymatic reactions. The two enantiomers had no significant stability differences,
416 so they seem to be configurationally stable in the working conditions. Moreover, both
417 CBT enantiomers proved to be stable (> 98.1%) after 3 days at room temperature
418 without using neither polyethylene bags nor desiccant (bench-top stability), as well as
419 after pretreatment and storage at RT for 24 hours (autosampler stability, > 96.0%).

420

421 **3.5 Analysis of real samples and accuracy**

422 The developed methodology was applied to the analysis of urine VAMS from patients
423 undergoing therapy with racemic CBT.

424 Table 4 reports the quantitative data obtained from the analysis of real samples from
425 patients. Concentration differences between the two enantiomers were small and not
426 statistically significant. This confirms what is currently known from previous studies on
427 the non-enantioselective metabolism of CBT in humans [12]. Analyte identification was
428 carried out on the basis of retention time and two MRM transitions.

429 Good accuracy was reached, with recovery values always higher than 91%.

430 As can be seen from Table 4, urine CBT levels were in the 0.8-2.5 ng/mL range.

431 Although no specific urine concentration ranges are currently available for (current or
432 prospective) therapeutic applications, these levels are reasonably expected for the use
433 reported. Urine levels in post-mortem specimens after overdose of heroin mixed with
434 CBT were in the 9-13 ng/mL range [34]. These concentrations are generally higher, by
435 about one order of magnitude, than those expected in the urine of athletes who use the
436 drug illicitly. Nevertheless, the performance of the LC-MS/MS analytical method, as
437 reported above, is suitable for anti-doping testing applications.

438 In conclusion, an innovative analytical approach, based on microsampling by VAMS
439 followed by miniaturised pretreatment by StAGE and LC-MS/MS, was developed and
440 validated for the determination of CBT enantiomers in dried urine microsamples. It is
441 now proposed for possible future applications in the field of anti-doping testing and
442 clinical neuroprotection studies. VAMS application to urine in anti-doping, in
443 particular, is at its beginnings: in fact, urine VAMS had been until now applied only by
444 us [24,35] and another research group [36] in recent clinical applications. The use of
445 dried urine VAMS samples provide significant advantages over traditional anti-doping
446 sampling, with increased sample resilience to ambient temperature and more practical
447 storage and transportation procedures.

448 The VAMS technique still suffers from the known drawbacks of all microsampling
449 techniques, such as lower sensitivity, due to the impossibility of pre-concentrating the
450 sample, and limited sample availability, which restricts the number of different assays
451 that can be carried out on a single microsample. However, this latter limitation can be
452 partially offset by the use of multiple VAMS for each sampling session.

453 In any case, the resulting VAMS-StAGE-LC-MS/MS workflow produced satisfactory
454 validation results. In particular, enantioselective VAMS-StAGE-LC-MS/MS seems to
455 be a suitable, innovative prospect for possible future applications of microsampling to
456 the anti-doping and clinical testing of CBT enantiomers, with satisfactory analytical
457 performance.

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459

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465 effective doping control” (call year: 2014).

466

467

468 **Conflict of Interest**

469 All authors declare that they have no conflict of interest.

470 **Figure captions**

471

472 **Figure 1.** Chemical structures of (+)-*S*-Clenbuterol (a) and (–)-*R*-Clenbuterol (b)

473

474 **Figure 2.** LC-MS/MS chromatogram of a racemic CBT mixture at the concentration of
475 25 ng/mL for CBT (IS concentration: 50 ng/mL).

476

477 **Figure 3.** LC-MS/MS chromatogram of a urine VAMS sample spiked with a racemic
478 CBT mixture at the LOQ level (0.3 ng/mL).

479

480 **Figure 4.** LC-MS/MS chromatogram of a blank urine VAMS sample.

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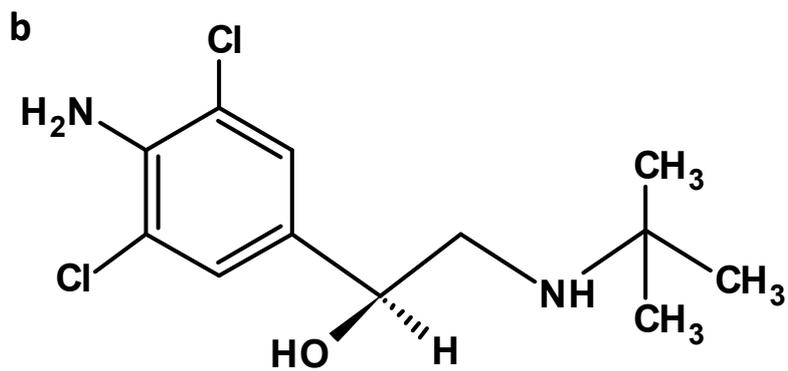
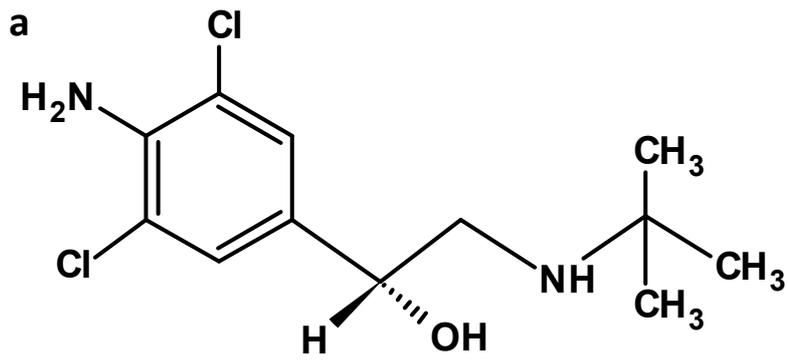


Figure 1
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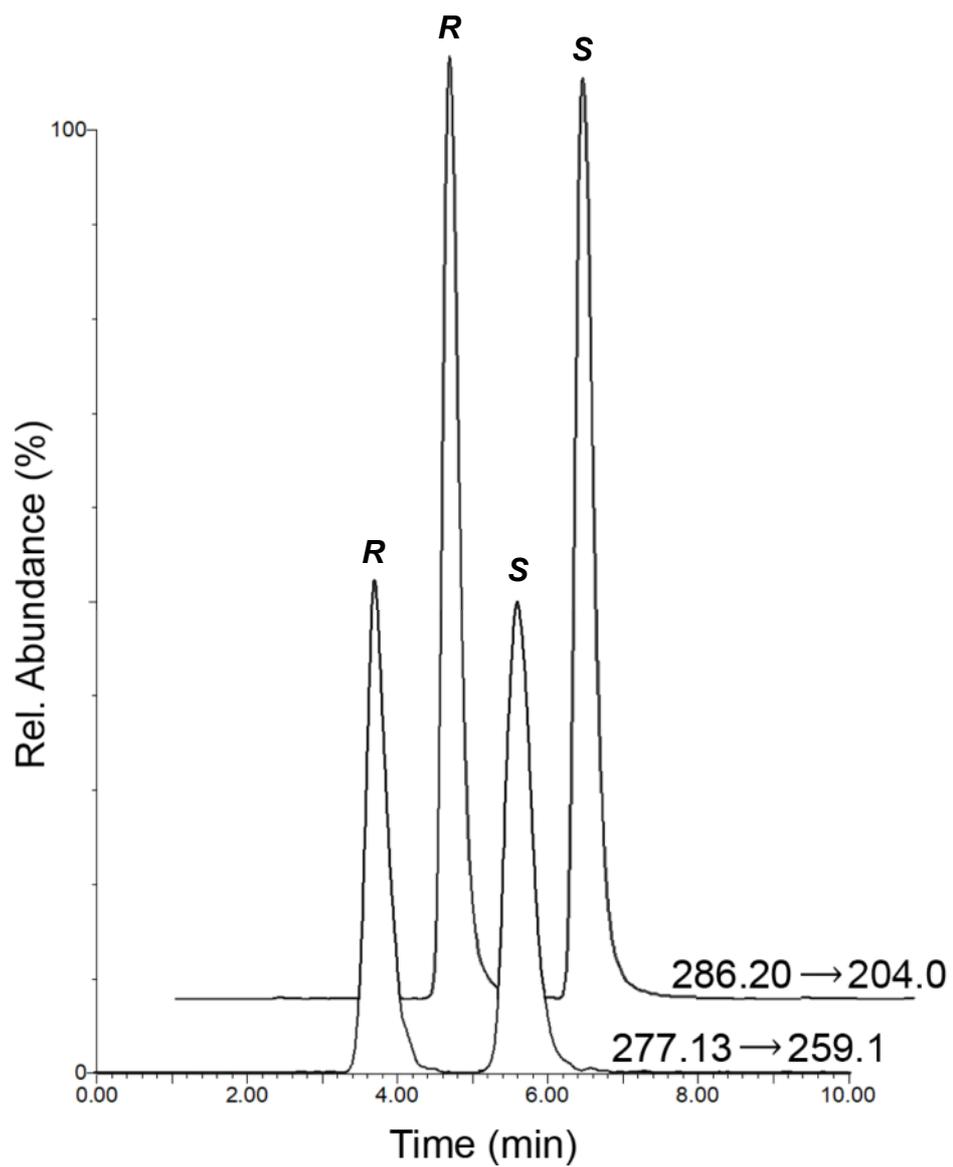


Figure 2
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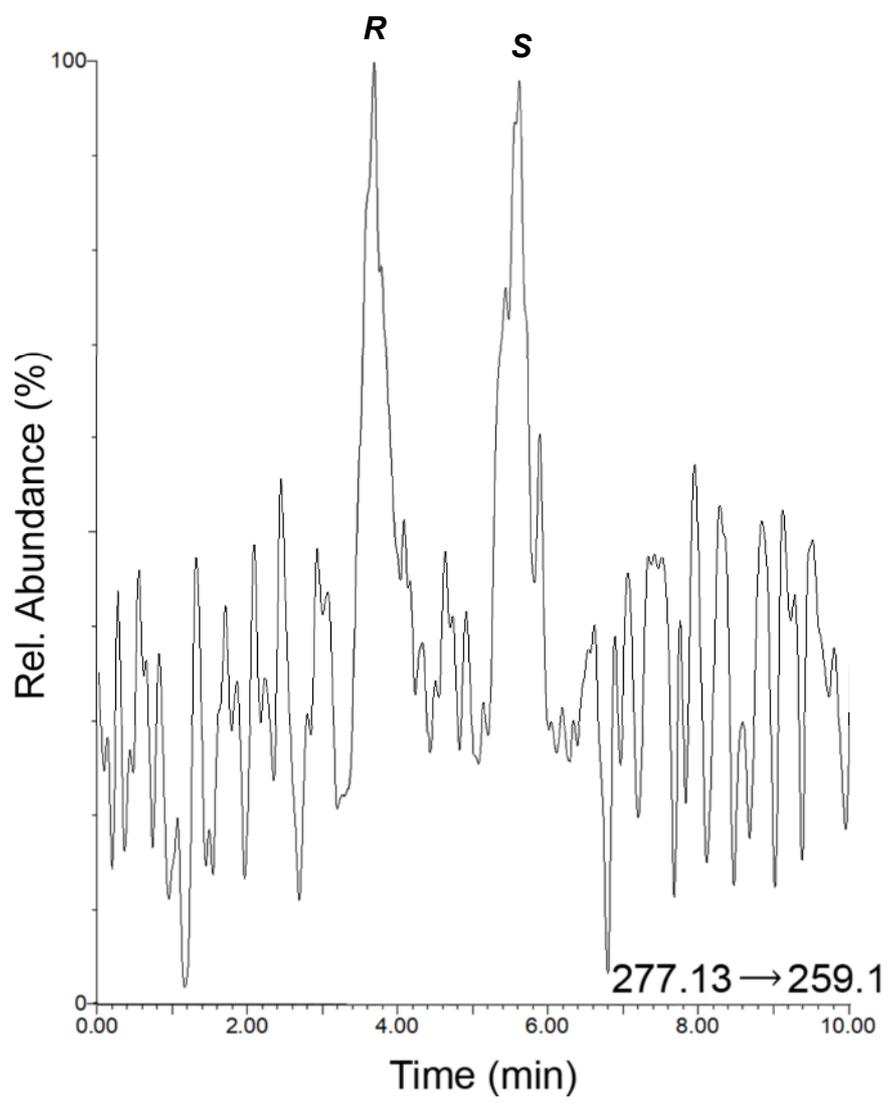


Figure 3
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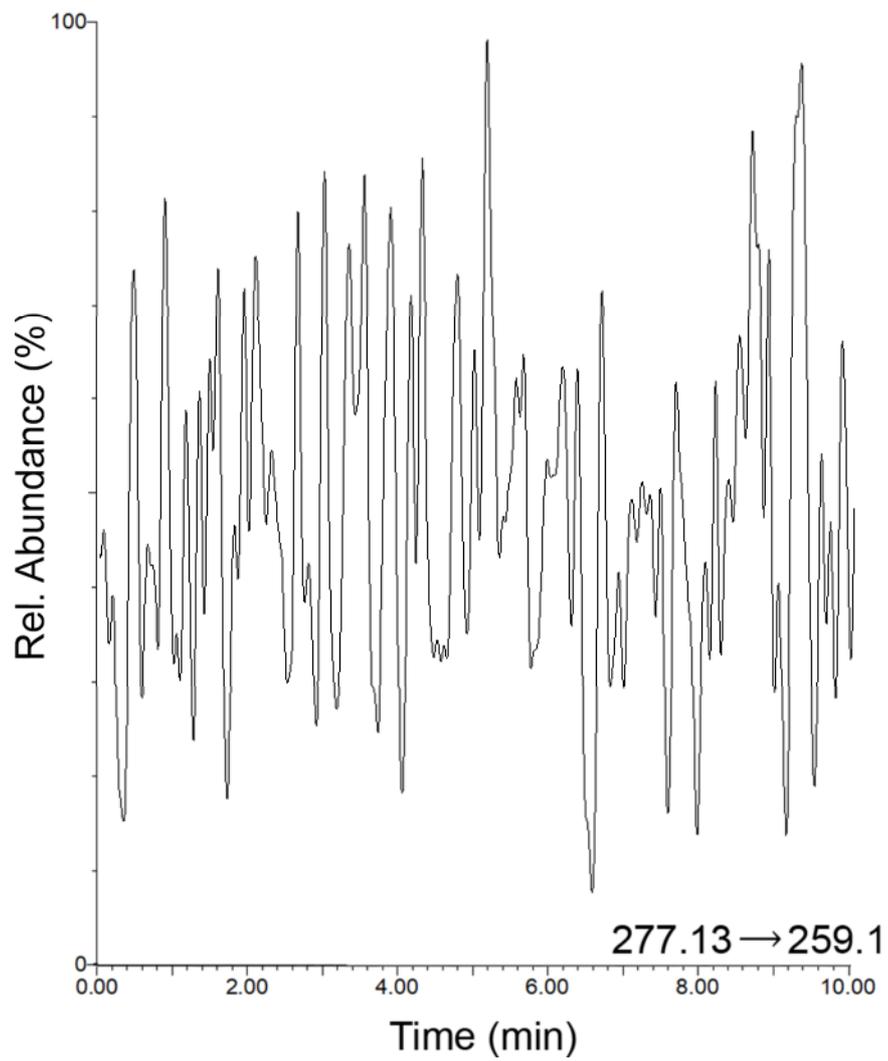


Figure 4
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Table 1. Optimised MRM parameters.

Compound	Molecular weight (g/mol)	Monoisotopic mass (g/mol)	Parent ion [M+H]⁺ (m/z)	Daughter ions (m/z)	Collision energy (eV)	Dwell time (ms)
CBT	277.19	276.08	277.13	259.1	12	300
				203.1 ^a	18	300
IS	286.24	285.14	286.20	204.0	18	300
				268.1 ^a	13	300

^a confirmatory product ion.

Table 2. StAGE tips sorbent optimisation.

Sorbent type	Number of sorbent layers	Extraction yield (% \pm SD) ^{a,b}	Matrix effect (% \pm SD) ^{a,b}
C8	1	70 \pm 3	19 \pm 1
	3	87 \pm 2	14 \pm 1
	6	75 \pm 3	28 \pm 1
C18	1	69 \pm 4	30 \pm 1
	3	77 \pm 4	20 \pm 2
	6	71 \pm 4	27 \pm 3
SDVB	1	64 \pm 4	34 \pm 3
	3	67 \pm 3	31 \pm 2
	6	69 \pm 3	39 \pm 3
C8 + SDVB	3 + 3	67 \pm 5	26 \pm 3
C8 + C18	3 + 3	69 \pm 5	37 \pm 3
C18 + SDVB	3 + 3	51 \pm 3	47 \pm 4
C8 + SDVB (alternate layers)	3 + 3	69 \pm 3	37 \pm 3

^a $n = 6$.^b Mean of the two enantiomers.

Table 3. Linearity, extraction yield and precision on blank spiked urine VAMS samples.

Compound	Analytical system	Linearity range (ng/mL) ^a	Racemate concentration (ng/mL)	R-(-)-Clenbuterol				S-(+)-Clenbuterol			
				<i>r</i> ²	Mean extraction yield (%) ^a	Intraday precision (RSD%) ^a	Interday precision (RSD%) ^a	<i>r</i> ²	Mean extraction yield (%) ^a	Intraday precision (RSD%) ^a	Interday precision (RSD%) ^a
CBT	LC-MS/MS	0.3-200.0	0.3	0.9997	87	4.0	4.2	87	4.1	4.2	
			20	0.9997	86	3.1	3.8	0.9996	87	3.0	3.8
			100		88	2.8	3.6		89	2.9	3.5
IS	LC-MS/MS	/	50	/	90	3.0	3.3	90	3.1	3.4	

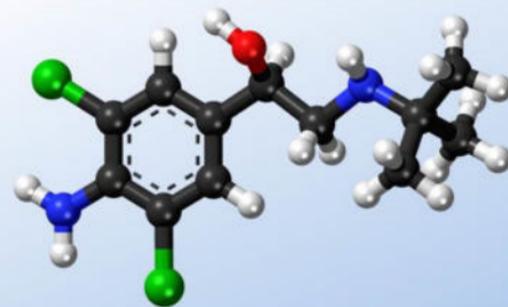
^a *n* = 6.



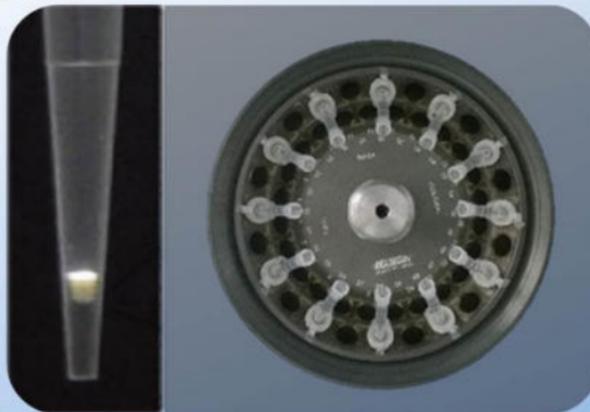
10 μ L
urine
microsampling



volumetric
absorptive
microsampling
VAMS



clenbuterol

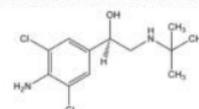
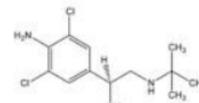


stop-and-go
extraction
STAGE

liquid
chromatography
-
tandem mass
spectrometry

LC-MS/MS

CSP-based
enantioselective
separation



Enantioseparation
↓
ADMET studies
Anti-doping