

1 Postprint of: Waraksa E., Owczarek K., Kubica P., Kłodzińska E., Ozimek M., Wrzesień R.,
2 Bobrowska-Korczak B., Namieśnik J., Determination of bromhexine and its metabolites in
3 equine serum samples by liquid chromatography – Tandem mass spectrometry: Applicability
4 to the elimination study after single oral dose, Journal of Chromatography B, Vol. 1151 (2020),
5 122197, DOI: [10.1016/j.jchromb.2020.122197](https://doi.org/10.1016/j.jchromb.2020.122197)

6 © 2020. This manuscript version is made available under the CC-BY-NC-ND 4.0
7 license <http://creativecommons.org/licenses/by-nc-nd/4.0/>

8 **Determination of bromhexine and its metabolites in equine serum samples by liquid**
9 **chromatography – tandem mass spectrometry: Applicability to the elimination study**
10 **after single oral dose**

11 Emilia Waraksa^{a,b*}, Katarzyna Owczarek^a, Paweł Kubica^a, Ewa Kłodzińska^b, Mariusz Ozimek^b,
12 Robert Wrzesień^c, Barbara Bobrowska-Korczak^d, Jacek Namieśnik^{‡ a}

13 **Affiliations**

14 ^a Gdańsk University of Technology, Faculty of Chemistry, Department of Analytical
15 Chemistry, G. Narutowicza 11/12 Street, 80-233 Gdańsk, Poland

16 ^b Institute of Sport - National Research Institute, Department of Analytical Chemistry and
17 Instrumental Analysis, Trylogii 2/16 Street, 01-982 Warsaw, Poland

18 ^c Medical University of Warsaw, Central Laboratory of Experimental Animal, Banacha 1 B
19 Street, 02-097 Warsaw, Poland

20 ^d Medical University of Warsaw, Department of Bromatology, Banacha 1 B Street, 02-097
21 Warsaw, Poland

22 * Correspondence to: Emilia Waraksa, e-mail: emilia.waraksa@insp.waw.pl

‡ This article is dedicated to the memory of Prof. Jacek Namieśnik (1949 – 2019).

23 **Keywords:** bromhexine, metabolites of bromhexine, liquid chromatography – tandem mass
24 spectrometry, elimination study, doping, equestrian sports

25 **Highlights**

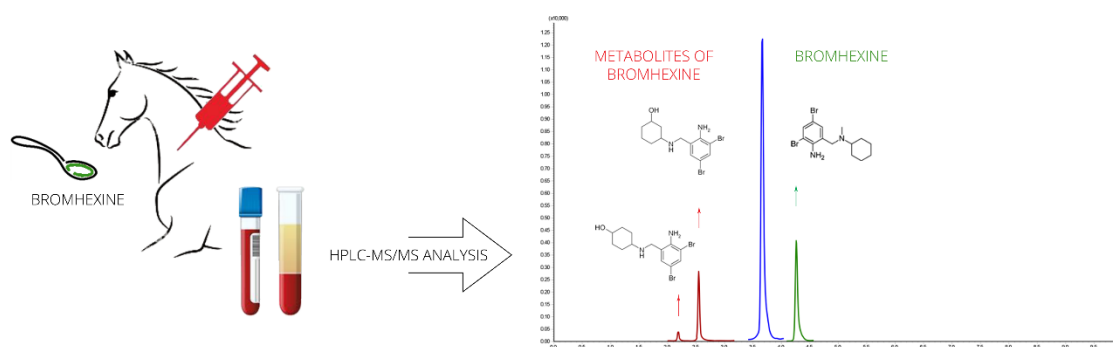
- 26 • Time courses of BH and its metabolites concentrations determined for the first time in
27 equine serum.
- 28 • Fully validated LC-MS/MS method for determination of BH and its metabolites in
29 equine serum samples.
- 30 • Simple sample preparation utilizing acetonitrile protein precipitation.

31 **Abstract**

32 Bromhexine (BH), expectorant used in the treatment of respiratory disorders associated
33 with viscid or excessive mucus, is not permitted for use in the competing horse by many
34 authorities in horseracing and Olympic disciplines. Metabolic studies are of the great
35 importance in anti-doping field because they allow for updating the selection of the most
36 appropriate markers for prohibited substances, such as metabolites present at higher
37 concentration levels and/or lasted for a longer period of time in biological samples than a parent
38 drug. This study describes LC-MS/MS-based method for simultaneous determination of BH
39 and its metabolites, including 4-(2-amino-3,5-dibromobenzylamino)cyclohexanol (4-HDMB),
40 3-(2-amino-3,5-dibromobenzylamino)cyclohexanol (3-HDMB), in equine serum samples. The
41 2-(2-amino-3,5-dibromobenzylamino)cyclohexanol (2-HDMB) was monitored as well. The
42 assay was validated in terms of linearity ($R^2 > 0.9951$), intra- and inter-assay accuracy (91.6 –
43 109.1%) and precision ($CV < 9.6\%$) as well as recovery (94.8 – 105.65%). The LODs were
44 0.0052, 0.0053, 0.0056 and 0.0043 ng/mL for BH, 2-HDMB, 3-HDMB and 4-HDMB,
45 respectively. The developed method was applied to determine the time courses of BH and its
46 metabolites concentrations in equine serum collected for 95.25 h following a single oral

‡ This article is dedicated to the memory of Prof. Jacek Namieśnik (1949 – 2019).

47 administration of BH to two healthy mares (in dose of 0.8 mg/kg). The parent drug was found
48 at higher concentration levels than 3-HDMB (major metabolite) and 4-HDMB (minor
49 metabolite), however, both BH metabolites lasted for a longer period of time in equine serum
50 than the parent drug. Thus, both metabolites of BH can be considered as BH abuse markers.



51
52 **1. Introduction**
53 The issue of affecting efficiency of horses using performance-enhancing and
54 performance-impairing substances or methods is of the most importance in equestrian sports.
55 Although, there is no single organization regulating anti-doping framework, the individual
56 authorities provide rules and regulations to ensure the integrity of the sport as well as the welfare
57 of horses [1]. Among them, the International Federation of Horseracing Authorities (IFHA)
58 implemented a guide for horseracing activities – the International Agreement on Breeding,
59 Racing and Wagering (IABRW) [2], and the authority for Olympic disciplines, Fédération
60 Equestre Internationale (FEI), published the Equine Prohibited Substances List (EPSL) [3] that
61 categorize prohibited substances.

62 Bromhexine (BH), known as 2-amino-3,5-dibromo-N-cyclohexyl-N-
63 methylbenzylamine, is an expectorant used in the treatment of respiratory disorders associated

‡ This article is dedicated to the memory of Prof. Jacek Namieśnik (1949 – 2019).

64 with viscid or excessive mucus. BH acts on respiratory system and it is listed in the EPLS as a
65 controlled substance, so its usage is prohibited by both IFHA and FEI regulations. In mammals,
66 BH was found to be extensively converted to several metabolites, including 4-(2-amino-3,5-
67 dibromobenzylamino)cyclohexanol (4-HDMB, ambroxol) and 3-(2-amino-3,5-
68 dibromobenzylamino)cyclohexanol (3-HDMB) [4-9]. In general, the presence of doping
69 substance metabolite(s) in biological samples provide(s) additional confirmation of drug
70 identification and information on its misuse, even when the primary compound could not be
71 detected. This is especially important for highly metabolized doping agents, which are present
72 in biological samples at higher concentrations and/or lasted for a longer period of time than a
73 parent drug. Moreover, doping substances can be converted to active metabolites, such as BH
74 into 4-HDMB, which also causes an action on the respiratory tract and it is listed in the EPLS
75 as a controlled compound. The most recent reports on pharmacokinetics [8], excretion and
76 metabolic patterns of BH [9] in horses is dated more than 20 years ago. Several metabolites
77 have been identified in equine urine samples following BH and its active metabolite 4-HDMB
78 administration [9]. Nevertheless, metabolites of BH in equine plasma, serum neither whole
79 blood following drug administration have not been investigated yet.

80 Among different methods for determination of BH and/or its metabolites in biological
81 samples [6-7, 10-13] and pharmaceutical formulations [14-22] one can distinguish UV-Vis
82 spectrophotometry [14-16], thin layer chromatography (TLC) [17], capillary electrophoresis
83 (CE) [10, 18], high performance liquid chromatography – ultraviolet detection (HPLC-UV)
84 [12-13, 19-21], liquid chromatography – mass spectrometry (LC-MS) [22] and liquid
85 chromatography – tandem mass spectrometry (LC-MS/MS) [6-7]. Although methods using
86 HPLC-UV are still widely applied in quantification of BH and 4-HDMB in pharmaceutical
87 formulations, LC-MS and LC-MS/MS-based assays are probably the most popular choice for
88 their determination in biological samples. Methods using LC-MS and LC-MS/MS provide fast

‡ This article is dedicated to the memory of Prof. Jacek Namieśnik (1949 – 2019).

89 analysis, often omitting time-consuming and complicated sample preparation, including liquid-
90 liquid extraction (LLE) [12-13] and solid-phase extraction (SPE) [11], while guaranteeing
91 significantly better sensitivity compared to HPLC-UV-based procedures. The recent literature
92 reports [6-7] indicated LC-MS/MS-procedures as a powerful tool for determination of BH and
93 its metabolites in biological samples of mammals.

94 The purpose of this study was to develop and validate a novel, rapid and sensitive
95 LC-MS/MS method for simultaneous determination of BH and its metabolites in equine serum
96 samples. The previously described study was focused on a method based on SPE and
97 RRLLC-MS/MS for determination of these compounds in human plasma samples [7]. To the
98 best of our knowledge, it is the first study describing elimination process of BH in horses. The
99 sample preparation procedure was limited to protein precipitation (PPT) using acetonitrile and
100 centrifugation of samples. The method proposed in this study allowed for simultaneous
101 determination of BH and its metabolites in a 10-min-long analytical run. The proposed assay
102 seems to be suitable for routine doping control analysis due to effortlessness of the sample
103 preparation procedure, obtained values of LOQs at pg/mL (13 – 17 pg/mL; in comparison – the
104 previously described method [7] achieved LOQs within 50 -150 pg/mL) and high recovery
105 (94.8 – 105.65%; in comparison – in the previously described method [7] recovery was in the
106 range of 57.0 – 70.9%). The developed and fully validated method has been successfully
107 applied to analysis of real samples collected for 95.25h after a single oral administration of BH
108 to two healthy mares. As a result, concentration-time curves of 3-HDMB and 4-HDMB in
109 equine serum were determined for the first time along with BH. The study indicated both BH
110 metabolites as appropriate BH abuse markers since they last for a longer period of time in
111 equine serum than the parent drug

‡ This article is dedicated to the memory of Prof. Jacek Namieśnik (1949 – 2019).

112

113 **2. Materials and methods**

114 **2.1 Standards and reagents**

115 *Racemic*-4-(2-amino-3,5-dibromobenzylamino)cyclohexanol (4-HDMB), *racemic*-3-
116 (2-amino-3,5-dibromobenzylamino)cyclohexanol (3-HDMB), *racemic*-2-(2-amino-3,5-
117 dibromobenzylamino)cyclohexanol (2-HDMB) were purchased from ChiroBlock GmbH
118 (Bitterfeld Wolfen, Germany). Diphenhydramine (used as ISTD) was obtained from Sigma-
119 Aldrich Chemical Co. (St. Louis, MO, USA). All of the standards have a minimum purity of
120 95%.

121 Water (LC-MS grade), methanol (MeOH; LC-MS grade), and acetonitrile (ACN;
122 LC-MS grade) were obtained from Honeywell Burdick & Jackson Company (NJ, USA).
123 Hydrochloric acid (35–38%; pure p.a.) was purchased from POCH (Gliwice, Poland).

124 **2.2 LC-MS/MS conditions**

125 The analysis of equine serum samples were performed on a LC-MS/MS system (LCMS-
126 8060, Shimadzu, Japan) equipped with an electrospray ionisation source (ESI) working in a
127 positive mode of multiple reaction monitoring (MRM). The parameters of ion source were as
128 follows: nebulizing gas flow of 3 L/min; heating gas flow of 10 L/min; interface temperature
129 of 300°C; desolvation line temperature of 250°C; heat block temperature of 400°C; and drying
130 gas flow of 10 L/min. Conditions of ion transitions were optimized for all analytes using
131 LabSolutions v.5.85 Software. Detailed information on MS/MS transitions, specific parameters
132 and structures of compounds are given in Table 1.

133 Chromatographic separation was carried out using UPLC Nexera X2 system (Shimadzu,
134 Japan) consisting of degasser DGU-20A5R, controller CBM-20A, binary pump LC-30AD,

‡ This article is dedicated to the memory of Prof. Jacek Namieśnik (1949 – 2019).

135 autosampler SIL-30AC and column oven CTO-20AC. Chromatographic separation was
136 achieved using Kinetex core-shell C₁₈ column (15cm × 2.1mm, 2.6µm) with C₁₈ guard column
137 (0.5cm x 2.1mm, 2.6µm) from Phenomenex (Torrance, USA). The column temperature was
138 kept at 30°C. The flow rate of the mobile phase was set at 1 mL/min; and the injection volume
139 was set at 5 µL. For the mobile phase, following solvents were used: A) water + 0.1% formic
140 acid, and B) acetonitrile + 0.1% formic acid. The analytes were eluted with following gradient
141 program: 0 – 0.50 min 10% B, 0.50 – 5.50 min 10 – 35% B, 5.50 – 7.00 min 35 – 95% B. The
142 column was stabilized after each analysis for 3 min. The total time of the chromatographic run
143 was 10 min.

144 **2.3 Drug administration, samples collection and storage**

145 Two healthy mares received a single oral dose of bromhexine (400 mg; 0.8 mg/kg). The
146 blood samples were collected before and after 1,3, 5, 8, 11.5, 15.5, 19.5, 23.5, 29.25, 35, 41,
147 48, 53, 59.5, 72, 83 and 95.25 h of drug administration. All samples were stored at –20°C before
148 the analysis. The study was approved by the Local Ethical Committee for Animal
149 Experimentation at the Faculty of Biology, University of Warsaw, Poland (Decision no.
150 565/2018).

151 **2.4 Preparation of stock, working and quality control solutions**

152 A stock solutions of each analyte were prepared at concentration of 1 mg/mL by
153 dissolving of accurately weighted reference substances in water. Working solutions were
154 prepared as a mixture of analytes at concentrations of 100, 10, 1, 0.1, 0.01 µg/mL for each
155 compound. ISTD stock solution was prepared at concentration of 1 mg/mL by dissolving
156 accurately weighted reference substance in water. ISTD working solutions were prepared at
157 concentrations of 1 and 0.02 µg/mL by diluting the ISTD stock solution with water.

‡ This article is dedicated to the memory of Prof. Jacek Namieśnik (1949 – 2019).

158 Because the method covers a wide concentration range (0.025 – 500 ng/mL), two
159 separate calibration curves were prepared (0.025 – 1 and 1 – 500 ng/mL). For this purpose,
160 calibration solutions were prepared in triplicate at concentrations of 0.025, 0.05, 0.15, 0.25, 0.5,
161 1, 5, 25, 50, 100, 200 and 500 ng/mL for all analytes by fortifying 200 μ L of blank equine
162 serum with appropriate volumes of the working solutions. ISTD concentration was kept at
163 1 ng/mL in calibration samples in the range of 0.025 – 1 ng/mL, and at 50 ng/mL in calibration
164 solutions within 1 – 500 ng/mL.

165 Quality control (QC) samples at six concentration levels (0.05, 0.25, 1, 5, 50 and
166 200 ng/mL) were prepared. ISTD concentration was at 1 ng/mL for QC samples at
167 concentrations of 0.05, 0.25 and 1 ng/mL, and at 50 ng/mL for QC samples at concentrations
168 of 5, 50 and 200 ng/mL.

169 Both calibration solutions and QC samples were treated according to the sample
170 preparation procedure (matrix-matched calibration curve) and analyzed by LC-MS/MS system.

171 **2.5 Sample preparation**

172 A 200 μ L of equine serum, 10 μ L of the ISTD working solution at 0.02 μ g/mL, 200 μ L
173 of acetonitrile were added to a Eppendorf vial and refrigerated (3°C) for 1 h. After
174 centrifugation for 5 min at 13000 rpm, supernatant was analyzed by LC-MS/MS system. When
175 analyte(s) was (were) determined at concentration(s) above 1 ng/mL, a 200 μ L of equine serum
176 was spiked with 10 μ L of the ISTD working solution at 1 μ g/mL and reanalyzed as described
177 above.

178 **2.6 Method validation**

179 The method was validated in terms of matrix effects (ME), linearity, limit of detection
180 (LOD) and quantification (LOQ) values, accuracy, precision, recovery (RE) and carry over

‡ This article is dedicated to the memory of Prof. Jacek Namieśnik (1949 – 2019).

181 effects. All validation experiments were designed according to the principles for bioanalytical
182 method validation [23-26].

183 **3. Results and discussion**

184 **3.1 Optimization of sample preparation procedure**

185 In this work, sample pretreatment strategy was focused on proving high selectivity and
186 sensitivity using a fast and simple procedure with a small sample volume (200 μ L) and low
187 consumption of organic solvents (200 μ L of acetonitrile). A one-step sample preparation
188 method using protein precipitation (PPT) through the use of acetonitrile was proven to get
189 sufficient clean samples usable for LC-MS/MS analysis. Proteins can be irreversibly adsorbed
190 onto the chromatographic support, which causes the deterioration of separation efficiency,
191 peaks symmetry and a rapid column clogging. Therefore, protein-rich matrices, such as serum
192 samples containing large amounts of albumin and immunoglobulins, require protein removal
193 before analysis. For this purpose, PPT was chosen due to the simplicity of the technique. Two
194 organic precipitation agents, ACN and MeOH, were tested. Data on the recovery rates of all the
195 analytes when ACN and MeOH was used are presented in Supplementary Table 1. Finally,
196 ACN was chosen on the basis of significantly higher recoveries for all analytes compared to
197 using MeOH.

198 **3.2 Optimization of LC-MS/MS parameters**

199 The LC-MS/MS conditions were optimized to ensure selectivity and sensitivity of the
200 method. Preliminary studies, involving the investigation of two chromatographic columns for
201 the analytes separation were performed. Structural (geometric isomers) and physicochemical
202 similarities of the target compounds make them difficult to separate, so high resolution is
203 desirable. Because of that reason, columns packed with core-shell particles were tested, i.e.
204 Kinetex core-shell C₁₈ column (15 cm \times 2.1 mm, 2.6 μ m) with guard column

‡ This article is dedicated to the memory of Prof. Jacek Namieśnik (1949 – 2019).

205 (0.5 cm x 2.1 mm, 2.6 μ m) from Phenomenex (Torrance, USA) and Ascentis Express®
206 C₁₈ column (15 cm x 2.1 mm, 2.7 μ m) with guard column (0.5 cm x 2.1 mm, 2.7 μ m). The use
207 of the Kinetex column allowed to obtain higher instrumental responses (mainly for BH), more
208 symmetrical and narrower peaks, increased separation efficiency and significantly shorter
209 analysis time compared to using Ascentis Express® column. Therefore, Kinetex column was
210 chosen for future studies. Appropriate chromatograms obtained in the preliminary studies are
211 presented in Supplementary Fig. 1.

212 Formic acid and ammonium formate were tested as the mobile phase modifiers.
213 Compared to the use of ammonium formate, formic acid additive provided better resolution and
214 higher response for all analytes. Better peak shapes, including improved symmetry factor and
215 minimized tailing, were obtained when formic acid was used compared to ammonium formate.
216 The gradient profile, column temperature, flow rate and injection volume were adjusted as well.

217 MRM transitions were chosen for each analyte on the basis of signal and characteristic
218 fragment ions.

219 **3.3 Method validation**

220 The developed procedure meets specified performance requirements and it is acceptable
221 for its intended use. The summary of method validation results was shown in Table 2 and
222 Table 3.

223 Two linear calibration curves covering the low concentration range within
224 0.025 – 1 ng/mL and the high concentration range of 1 – 500 ng/mL were used for BH,
225 2-HDMB, 3-HDMB and 4-HDMB. Linear calibration equations were obtained for calibration
226 curves constructed by plotting analyte-to-ISTD peak area ratios versus corresponding
227 concentrations. Calibration curves covered a broad concentration range, thus weighting factor
228 1/x was applied to each one for increasing the accuracy at the lowest concentration levels.

‡ This article is dedicated to the memory of Prof. Jacek Namieśnik (1949 – 2019).

229 Obtained coefficients of determination for the calibration curves were greater than 0.9951 for
230 all target analytes.

231 LODs and LOQs were established on the basis of six-point matrix-matched calibration
232 curves in the concentration range of 0.025 – 1 ng/mL for all analytes, using following equations:
233 $LOD = (3.3S_b)/a$ and $LOQ = (10S_b)/a$ where: S_b – standard deviation of intercept of the
234 calibration curve, a – slope of the calibration curve. The proposed method was found to be
235 sensitive (LOD was 0.0052 ng/mL for the parent compound and within 0.0043 – 0.0056 ng/mL
236 for metabolites of BH).

237 Matrix effects (ME) were calculated by comparing the slopes of calibration curves,
238 according to the following formula: $ME=(a_m/a_r - 1)\times 100\%$, where a_m and a_r are the slopes of
239 the curves prepared in blank equine serum and solvent, respectively. Negative values of ME
240 indicate signal suppression, while positive values signify enhancement of the signal. Matrix
241 effects at values of $-20\% < ME < 20\%$ were considered as soft and insignificant; $20 < ME < 50$ and
242 $-50 < ME < -20$ as medium, and $ME > \pm 50$ as large. The obtained results indicated signal
243 suppression for all analytes, medium for BH (in both the low and the high concentration ranges),
244 soft (in the high concentration range) and medium (in the low concentration range) for
245 2-HDMB, 3-HDMB and 4-HDMB. For medium and/or large ME, the application of methods
246 for reducing the influence of the matrix are required. Therefore, the matrix-match calibration
247 was used for all investigated compounds.

248 The intra- and inter-assay accuracy and precision were assessed. For this purpose,
249 QC samples ($n=6$) were analyzed by LC-MS/MS system on the same day and over three
250 consecutive days, respectively. Intra- and inter-day accuracy was calculated according to the
251 following formula: $A=(C_m - C_n)/C_n \times 100\%$, where C_m is the mean measured concentration and
252 C_n is the nominal concentration. Intra- and inter-assay precision was assessed as the coefficients

‡ This article is dedicated to the memory of Prof. Jacek Namieśnik (1949 – 2019).

253 of variation (CVs). Both intra and inter-day accuracy was in the range of 91.6 – 109.1%.
254 CV was found to be less than 9.6%.

255 The recovery was assessed by comparing the analyte-to-ISTD peak area ratios obtained
256 for pre- and post-extracted fortified blank equine serum samples ($n=6$) at QC samples levels.
257 The results indicated that recovery was within 94.8 – 105.65%.

258 Carry-over effect was verified by injecting blank samples after the upper limit of
259 quantification in all analytical runs. Carry-over effect was found to be insignificant on the basis
260 of analyte-to-ISTD peak area ratios (below 0.1%) for blank samples.

261 Stability of the analytes were investigated at QC samples levels by analyzing samples
262 stored at room temperature (RT) for 24 h, samples stored at 4°C for 24 h, and samples three
263 times frozen (at -20°C) and then defrosted. The accuracy was within 94.8 – 109.8% for samples
264 stored at RT for 24 h, 91.6 – 109.7% for samples stored at 4°C for 24 h, and 95.4 – 109.7% for
265 samples after three freeze/thaw cycles. The CV was found to be less than 9.9% for all examined
266 samples.

267 **3.4 Analysis of real samples**

268 The developed and validated method was applied to the quantification of BH and its
269 metabolites in real samples. For this purpose, equine serum samples ($n=3$) were treated
270 according to the sample preparation procedure (described above) and analyzed by LC-MS/MS
271 system. BH and its two metabolites, 3-HDMB (major) and 4-HDMB (minor) were detected and
272 determined in serum samples collected following a single oral administration (in dose of
273 0.8 mg/kg) to two mares. The 2-HDMB was monitored as probable metabolite of BH. However,
274 it was not detected in any of the real samples. Exemplary LC-MS/MS chromatograms obtained
275 for the calibration solution at $c=1$ ng/mL for all target analytes fortified with ISTD at $c=1$ ng/mL
276 and a real serum sample collected from horse H1 in 1 h post-administration fortified with ISTD

‡ This article is dedicated to the memory of Prof. Jacek Namieśnik (1949 – 2019).

277 at $c=1$ ng/mL are presented in Fig. 3. The parent drug was found at higher concentration levels
278 than its metabolites with the maximum concentration (C_{max}) within 18.2 – 23.7 ng/mL found in
279 1 h post-administration (t_{max}). The C_{max} values obtained for 3-HDMB (10.26 – 12.72 ng/mL)
280 and 4-HDMB (0.279 – 2.4 ng/mL) were found in 5 – 15.5 h after BH administration. More
281 detailed data on maximum concentrations obtained for BH and its metabolites are presented in
282 Table 4. Although the parent drug was found at higher concentrations than 3-HDMB and
283 4-HDMB, both BH metabolites lasted for a longer period of time in equine serum. The parent
284 drug was detectable at up to 29.25 – 35 h post-administration, while 3-HDMB at up to 59.5 h
285 in serum samples collected from both horses, and 4-HDMB at up to 35 – 53 h. BH, 3-HDMB,
286 and 4-HDMB serum concentrations over time curves are shown in Fig. 2. Serum concentrations
287 of BH and its metabolites (4-HDMB and 3-HDMB) swing up and down wildly. Each of the
288 examined compound (BH, 4-HDMB and 3-HDMB) serum concentration has a peak around
289 15-20 h. These phenomena are closely related to the process of drug absorption, which is linked
290 to the route of administration of the drug (BH was orally administered with feed to horses H1
291 and H2 for 30 and 40 min, respectively), race, age, sex, diet, physical effort as well as inter-
292 and intra-individual variability of the drug elimination. In this case the explanation of
293 metabolites and parent compound concentration increase may be mainly connected to the drug
294 administration process, dissolution profile of the drug itself and the dissolution from the horses'
295 digestion system.

296 **Conclusions**

297 The presented LC-MS/MS-based method allows for a quick, simultaneous
298 determination of BH and its metabolites, including 3-HDMB (major) and 4-HDMB (minor), as
299 well as 2-HDMB. The fast and simple sample preparation procedure was based on only two
300 operations: protein precipitation through the use of acetonitrile and centrifugation of samples.
301 The assay was fully validated achieving low limits of detection (0.0057 – 0.0078 ng/mL), high

‡ This article is dedicated to the memory of Prof. Jacek Namieśnik (1949 – 2019).

302 recoveries (94.8 – 105.65%) and good repeatability (CV<9.6%) for all analytes. Thus, the
303 proposed method seems to be suitable for routine doping control analysis.

304 The assay was successfully applied to the analysis of real samples collected after a single
305 oral administration of BH to two healthy mares. Time courses of BH and its metabolites
306 (3-HDMB and 4-HDMB) concentrations in equine serum were determined for the first time.
307 The 2-HDMB, which was considered as a probable BH metabolite, was not found in the real
308 samples. The parent drug was found at higher concentration levels than 3-HDMB (major
309 metabolite) and 4-HDMB (minor metabolite), however, both BH metabolites can be considered
310 as appropriate BH abuse markers because they lasted for a longer period of time in equine
311 serum. Nevertheless, the presented data were obtained from the pilot study involving only two
312 horses in the experiment. Therefore, more extensive study may be needed in the future.

313 **Acknowledgements** This work was supported by the Polish National Science Center [grant
314 number 2016/21/N/ST4/02342].

315 **Conflict of interest statement** The authors declare that they have no conflict of interest.

316 5. References

317 [1] A.G. Fragkaki, N. Kioukia-Fougia, P. Kioussi, M. Kioussi, M. Tsiyou, Challenges in
318 detecting substances for equine anti-doping, *Drug Test Anal.* 9 (2017) 1291-1303.

319 [2] <https://www.ifhaonline.org/resources/2018Agreement.pdf> (Accessed 14 March 2019).

320 [3] https://inside.fei.org/sites/default/files/2019%20Prohibited%20Substances%20List_0.pdf
321 (Accessed 29 March 2019).

322 [4] E. Schraven, F.W. Koss, J. Keck, G. Beisenherz, Excretion, isolation and identification of
323 the metabolites of Bisolvon®, *Eur. J. Pharmacol.* 1 (1967) 445-451.

‡ This article is dedicated to the memory of Prof. Jacek Namieśnik (1949 – 2019).

- 324 [5] Z. Kopitar, R. Jauch, R. Hankwitz, H. Pelzer, Species difference in metabolism and
325 excretion of bromhexine in mice, rats, rabbits, dogs and man, *J. Pharmacol.* 21 (1973) 6-10.
- 326 [6] L.A. Meijer, J.C.M. Verstegen, S. Bull, J. Fink-Gremmels, Metabolism of bromhexine in
327 pig hepatocyte cultures, *J. Vet. Pharmacol. Ther.* 27 (2004) 219-225.
- 328 [7] J. Liu, X. Chen, Y. Hu, G. Cheng, D. Zhong, Quantification of the major metabolites of
329 bromhexine in human plasma using RRLC-MS/MS and its application to pharmacokinetics, *J*
330 *Pharm Biomed Anal.* 51 (2010) 1134-1141.
- 331 [8] P. de Backer, L.M. Vandecasteele-Thienpont, J.A. Jonckheere, F.M. Belpaire, M.
332 Debackere, A.P. de Leenheer, Bioavailability of bromhexine in the horse, *Zentralbl*
333 *Veterinarmed A.* 27 (1980) 740-745.
- 334 [9] C.E. Uboh, J.A. Rudy, L.R. Soma, M. Fennell, L. May, R. Sams, F.A. Railing, J.
335 Shellenberger, M. Kahler, Characterization of bromhexine and ambroxol in equine urine: effect
336 of furosemide on identification and confirmation, *J Pharm Biomed Anal.* 9 (1991) 33-39.
- 337 [10] T. Pereze-Ruiz, C. Martineze-Lozano, A. Sanz, E. Bravo, Determination of bromhexine
338 and ambroxol in pharmaceutical dosage forms, urine and blood serum, *J. Chromatogr. B* 692
339 (1997) 199-205.
- 340 [11] M. Javanbakht, M. H. Namjumanesh, B. Akbari-adergani, Molecularly imprinted solid-
341 phase extraction for the selective determination of bromhexine in human serum and urine with
342 high performance liquid chromatography, *Talanta* 80 (2009) 133-138.
- 343 [12] M.H. Botterblom, T.J. Janssen, P.J. Guelen, T.B. Vree, Rapid and sensitive determination
344 of ambroxol in human plasma and urine by high-performance liquid chromatography, *J*
345 *Chromatogr.* 421 (1987) 211-215.

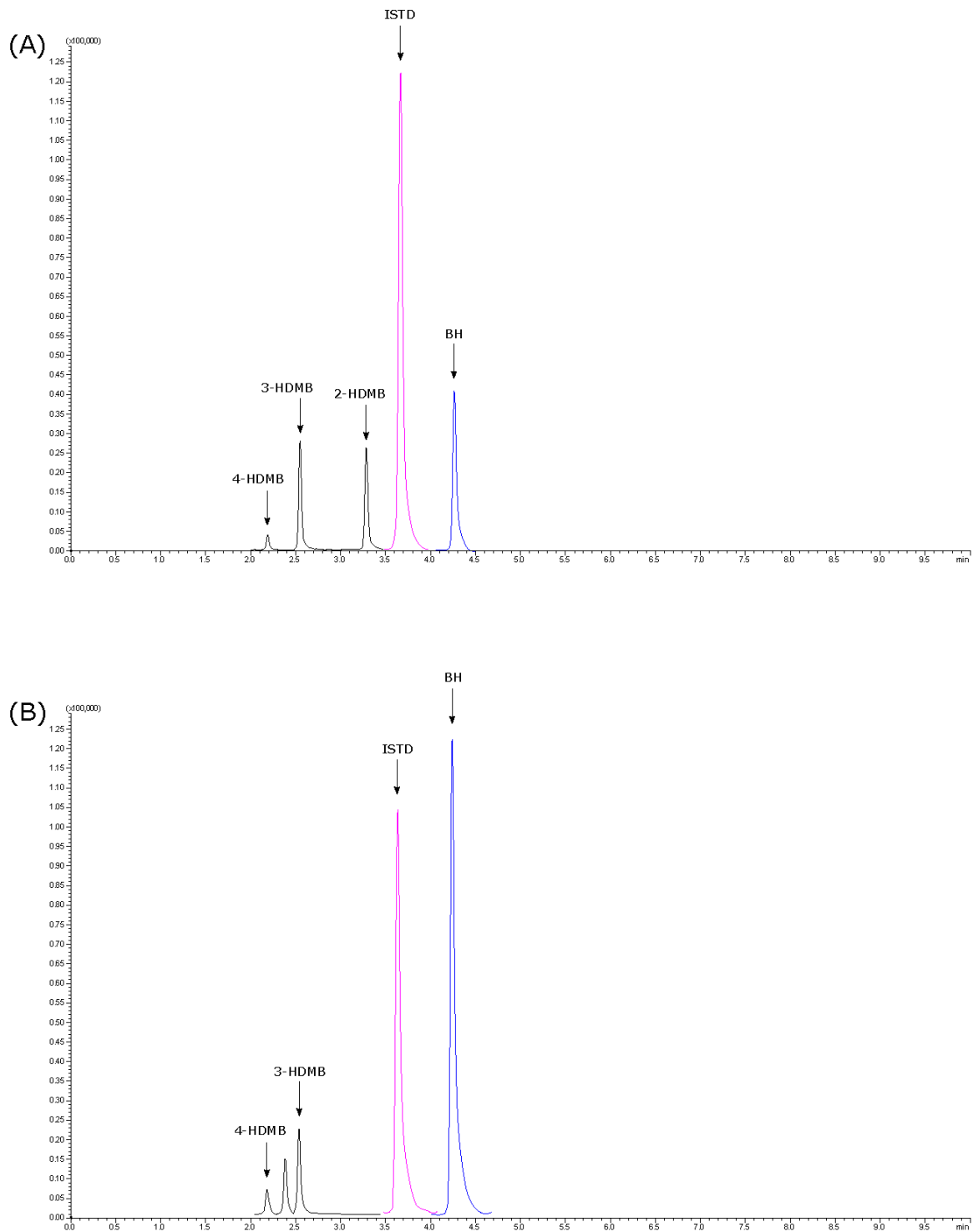
‡ This article is dedicated to the memory of Prof. Jacek Namieśnik (1949 – 2019).

- 346 [13] M. Nobilis, J. Pastera, D. Svoboda, J. Květina, K. Macek, High-performance liquid
347 chromatographic determination of ambroxol in human plasma, *J Chromatogr.* 23 (1992)
348 251-255.
- 349 [14] T. Perez-Ruiz, C. Martinez-Lozano, A. Sanz, S. Mondejar, Flow-injection extraction-
350 spectrophotometric determination of bromhexine with orange IV, *J. Pharm Biomed Anal.* 13
351 (1995) 1101-1106.
- 352 [15] A.C.B. Dias, J.L.M. Santos, J.L.F.C. Lima, E.A.G. Zagatto, Multi-pumping flow system
353 for spectrophotometric determination of bromhexine, *Anal. Chim. Acta* 499 (2003) 107-113.
- 354 [16] V. Tantishaiyakul, C. Poeaknapo, P. Sribun, K. Sirisuppanon, Simultaneous determination
355 of dextromethorphan HBr and bromhexine HCl in tablets by first-derivative spectrophotometry,
356 *J. Pharm Biomed Anal.* 17 (1998) 237-243.
- 357 [17] E. Sumarlik, G. Indrayanto, TLC densitometric determination of bromhexine hydrochloride
358 in pharmaceuticals, and its validation, *J. Liq. Chrom. & Relat. Technol.* 27 (2004) 2047-2056.
- 359 [18] D. C. Oliva, K. T. Vélez, A. L. R. Vázquez, Simultaneous Determination of Bromhexine
360 and Amoxicillin in Pharmaceutical Formulations by Capillary Electrophoresis, *J. Mex. Chem.*
361 *Soc.* 55 (2011) 79-83.
- 362 [19] P. N. S. Pai, G. K. Rao, M. S. Murthy, A. Agarwal, S. Puranik, Simultaneous Determination
363 of Salbutamol Sulphate and Bromhexine Hydrochloride in Tablets by Reverse Phase Liquid
364 Chromatography, *Indian J Pharm Sci.* 71 (2009) 53–55.
- 365 [20] A. Porel, Sanjukta Haty, A. Kundu, Stability-indicating HPLC Method for Simultaneous
366 Determination of Terbutaline Sulphate, Bromhexine Hydrochloride and Guaifenesin, *Indian J*
367 *Pharm Sci.* 73 (2011) 46–56.

‡ This article is dedicated to the memory of Prof. Jacek Namieśnik (1949 – 2019).

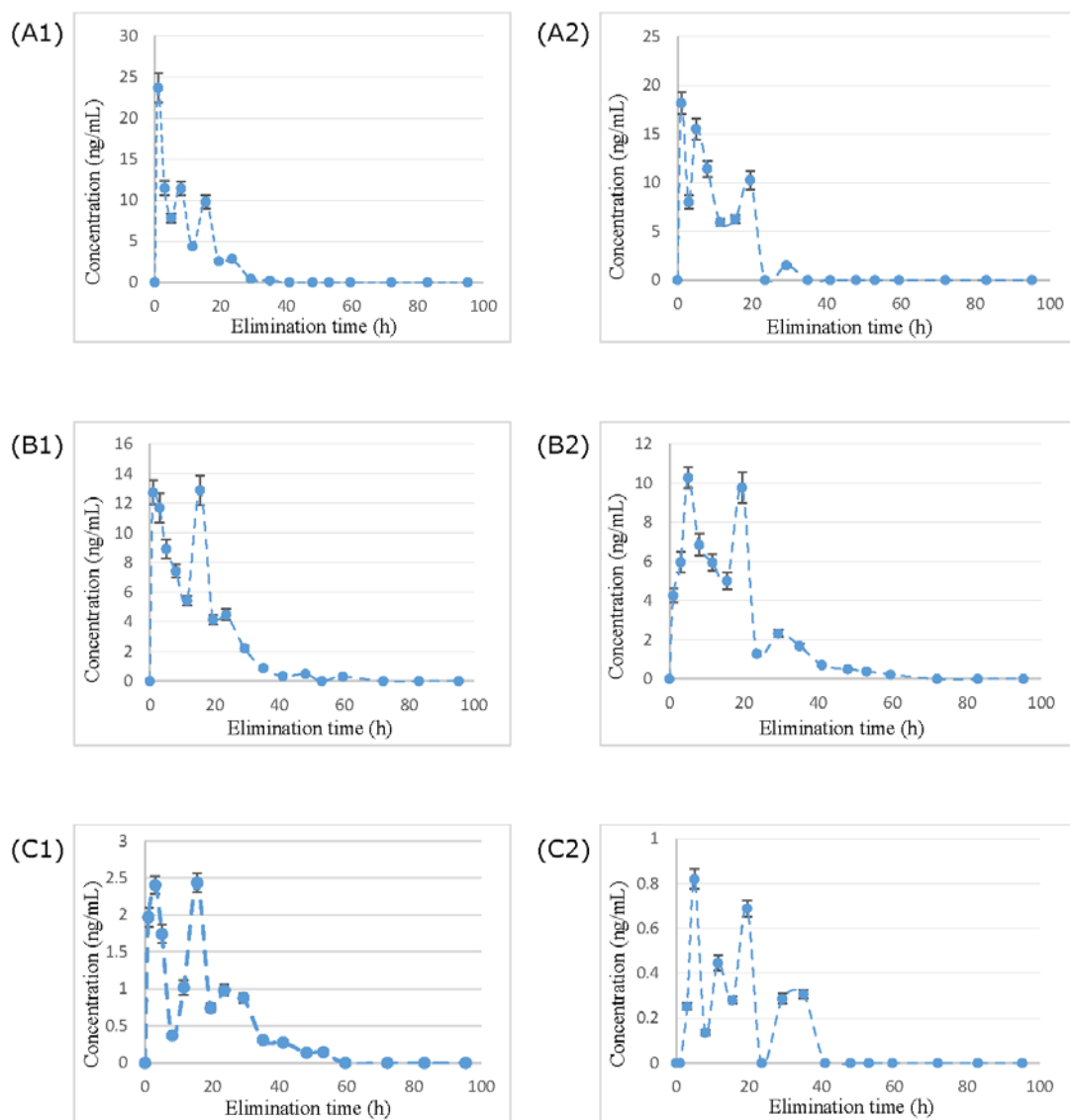
- 368 [21] P. M. Njaria, K. O. Abuga, F. N. Kamau, H. K. Chepkwony, A Versatile HPLC Method
369 for the Simultaneous Determination of Bromhexine, Guaifenesin, Ambroxol,
370 Salbutamol/Terbutaline, Pseudoephedrine, Triprolidine, and Chlorpheniramine Maleate in
371 Cough–Cold Syrups, *Chromatographia* 79 (2016) 1507-1514.
- 372 [22] W. Zhang, F. Feng, Y. Wang, Bioequivalence study of bromhexine by liquid
373 chromatography–electrospray ionization-mass spectrometry after oral administration of
374 bromhexine hydrochloride tablets, *J. Pharm Biomed Anal.* 48 (2008) 1206-1210.
- 375 [23] <https://www.fda.gov/downloads/drugs/guidances/ucm073384.pdf> (Accessed 12 April
376 2019).
- 377 [24] <https://www.fda.gov/media/70858/download> (Accessed 12 April 2019).
- 378 [25] https://pacificbiolabs.com/wp-content/uploads/2017/12/Q2_R1__Guideline-4.pdf
379 (Accessed 12 April 2019).
- 380 [26] [https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-bioanalytical-](https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-bioanalytical-method-validation_en.pdf)
381 [method-validation_en.pdf](https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-bioanalytical-method-validation_en.pdf) (Accessed 20 February 2019).

‡ This article is dedicated to the memory of Prof. Jacek Namieśnik (1949 – 2019).



383

384 **Fig. 1** LC-MS/MS chromatograms obtained for: (A) calibration solution at $c=1$ ng/mL for all385 target analytes fortified with ISTD at $c=1$ ng/mL; (B) real serum sample collected from horse386 H1 in 1 h post-administration fortified with ISTD at $c=1$ ng/mL.



387

388 **Fig. 2** Time curves of BH and its metabolites concentrations in equine serum: (A1) BH serum
 389 concentrations over time curve for horse H1, (A2) BH serum concentrations over time curve
 390 for horse H2, (B1) 3-HDMB serum concentrations over time curve for horse H1, (B2) 3-HDMB
 391 serum concentrations over time curve for horse H2, (C1) 4-HDMB serum concentrations over
 392 time curve for horse H1, (C2) 4-HDMB serum concentrations over time curve for horse H2.

393

394

395 **List of tables**

396 **Table 1.** Optimized MS/MS conditions for positive mode MRM analysis for target analytes.

397 **Table 2.** Data obtained from the equations of calibration curves, including information on
398 linearity, matrix effects, limit of detection and quantification.

399 **Table 3.** Intra- and inter-day accuracy and precision, recovery \pm SD, stability.

400 **Table 4.** Maximum concentrations of BH and its metabolites in equine serum obtained after a
401 single oral administration of BH to two horses.

402 **Supplementary material**

403 Supplementary Table 1. Recovery rates obtained for all the analytes when ACN and MeOH
404 was used as the solvent for the deproteinization.

405 Supplementary Figure 1. The chromatograms obtained in preliminary studies using: (A)
406 Ascentis Express® C18 column (15 cm \times 2.1 mm, 2.7 μ m) with guard column (0.5 cm \times 2.1
407 mm, 2.7 μ m), (B) Kinetex core-shell C18 column (15 cm \times 2.1 mm, 2.6 μ m) with guard column
408 (0.5 cm \times 2.1 mm, 2.6 μ m) from Phenomenex (Torrance, USA).

‡ This article is dedicated to the memory of Prof. Jacek Namieśnik (1949 – 2019).

Table 1 Optimized MS/MS conditions for positive mode MRM analysis for target analytes.

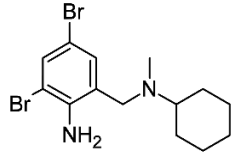
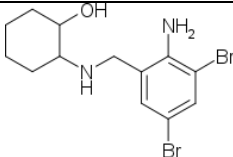
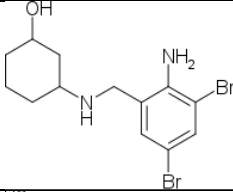
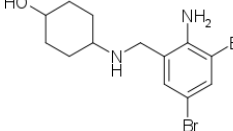
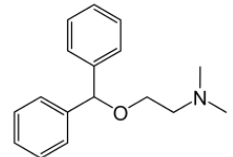
Compound	Molecular formula	Chemical structure	Precursor ion ➤ quantifier ion ➤ qualifier ion M+H [m/z]	Collision energy [V]	Dwell time [msec]	RT [min]
BH	C ₁₄ H ₂₀ Br ₂ N ₂		376.9 ➤ 114.25 ➤ 263.9	➤ -17 ➤ -29	97	4.23
2-HDMB	C ₁₃ H ₁₈ Br ₂ N ₂ O		378.9 ➤ 116.2 ➤ 263.9	➤ -20 ➤ -17		3.26
3-HDMB	C ₁₃ H ₁₈ Br ₂ N ₂ O		378.9 ➤ 116.2 ➤ 263.9	➤ -20 ➤ -17		2.47
4-HDMB	C ₁₃ H ₁₈ Br ₂ N ₂ O		378.9 ➤ 263.9 ➤ 116.2	➤ -20 ➤ -17		2.12
Diphenhydramine (ISTD)	C ₁₇ H ₂₁ NO		256.1 ➤ 167.2 ➤ 152.1	➤ -14 ➤ -37		3.69



Table 2. Data obtained from the equations of calibration curves, including information on linearity, matrix effects, limit of detection and quantification.

Compound	Calibration range (ng/mL)	Calibration curve equation	S _a	S _b	Linearity (R ²)	LOD/LOQ (ng/mL)	ME (%)
BH	0.025 – 1	y=0.128x + 0.0298	0.0012	0.0002	0.9971	0.0052/0.016	-21.6
	1 – 500	y=0.0043x + 0.0001	0.000049	0.0013	0.9995		-34.6
2-HDMB	0.025 – 1	y=0.0927x + 0.0027	0.00089	0.00015	0.9951	0.0053/0.016	-33.6
	1 – 500	y=0.0023x + 0.0008	0.000011	0.00030	0.9997		-10.4
3-HDMB	0.025 – 1	y=0.0509x - 0.0001	0.00053	0.000087	0.9983	0.0056/0.017	-41.4
	1 – 500	y=0.0016x + 0.0033	0.0000098	0.00026	0.9994		-1.2
4-HDMB	0.025 – 1	y=0.1313x - 0.0009	0.0010	0.00017	0.9978	0.0043/0.013	-32.9
	1 – 500	y=0.0037x +0.012	0.000027	0.00071	0.9993		-3.4

S_a: standard deviations of slope; S_b: standard deviations of constant terms; R²: coefficient of determination; LOD: limit of detection; LOQ: limit of quantification.



Table 3. Intra- and inter-day accuracy and precision, recovery \pm SD, stability.

Compound	Calibration range (ng/mL)	Concentration of quality control sample (ng/mL)	Intra-day accuracy (precision) (%) ($n=6$)	Inter-day accuracy (precision) (%) ($n=18$)	RE (%) \pm SD ($n=6$)	Stability: accuracy (precision) (%)		
						RT ($n=6$)	4°C ($n=6$)	Three freeze/thaw cycles ($n=6$)
BH	0.025 – 1	C ₁ =0.05	93.5 – 106.3 (2.4 – 9.4)	99.7 (7.4)	94.8 \pm 7.5	99.3 (4.8)	96.3 (3.1)	102.7 (9.9)
		C ₂ =0.25	98.1 – 108.8 (0.20 – 7.3)	102.4 (6.5)	104.21 \pm 0.87	98.1 (0.41)	100.8 (1.9)	106.6 (0.86)
		C ₃ =1	104.0 – 104.7 (1.6 – 8.1)	105.8 (4.4)	105.65 \pm 0.14	105.6 (0.14)	103.3 (2.3)	108.1 (1.7)
	1 – 500	C ₁ =5	93.3 – 102.6 (2.8 – 5.8)	98.0 (5.9)	98.3 \pm 1.5	103.9 (6.0)	108.9 (0.43)	96.2 (6.4)
		C ₂ =50	91.6 – 94.8 (1.5 – 2.8)	93.4 (2.4)	97.6 \pm 1.1	104.6 (7.6)	103.6 (0.66)	95.4 (5.4)
		C ₃ =200	98.7 – 102.1 (0.12 – 3.0)	100.6 (2.1)	94.8 \pm 1.0	100.2 (1.7)	101.7 (0.80)	100.3 (0.92)
2-HDMB	0.025 – 1	C ₁ =0.05	96.0 – 100.9 (1.4 – 3.8)	97.9 (3.3)	102.9 \pm 7.2	107.7 (4.2)	91.6 (5.6)	97.1 (1.8)
		C ₂ =0.25	98.2 – 109.1 (0.96 – 5.0)	102.2 (5.9)	100.3 \pm 2.8	101.1 (2.9)	95.6 (0.36)	98.0 (1.5)



		C ₃ =1	104.8 – 105.4 (2.3 – 3.9)	105.2 (2.6)	97.9 ± 9.3	107.6 (2.0)	107.4 (3.9)	108.4 (1.1)
	1 – 500	C ₁ =5	92.7 – 96.7 (1.4 – 3.4)	94.8 (2.8)	98.3 ± 1.1	100.3 (0.72)	109.7 (0.78)	107.2 (5.9)
		C ₂ =50	98.4 – 101.9 (1.4 – 3.1)	100.4 (2.3)	100.1 ± 4.2	109.8 (3.9)	106.1 (7.7)	100.2 (5.5)
		C ₃ =200	99.1 – 101.8 (0.21 – 1.4)	100.8 (1.5)	101.0 ± 3.8	99.3 (2.2)	97.2 (1.9)	99.7 (7.2)
3-HDMB		0.025 – 1	C ₁ =0.05	97.5 – 100.5 (1.4 – 9.6)	98.8 (4.8)	99.3 ± 4.2	103.9 (1.8)	101.0 (2.8)
	C ₂ =0.25		94.4 – 102.1 (5.6 – 8.2)	98.9 (6.4)	100.6 ± 5.2	106.6 (4.6)	103.2 (3.5)	99.2 (5.5)
	C ₃ =1		95.4 – 100.8 (1.4 – 4.1)	97.9 (3.3)	101.0 ± 3.8	94.8 (1.6)	107.8 (7.2)	108.0 (1.6)
	1 – 500	C ₁ =5	99.0 – 101.5 (0.27 – 2.9)	100.1 (1.9)	97.5 ± 2.5	108.2 (1.6)	108.9 (7.1)	107.8 (1.4)
		C ₂ =50	102.3 – 105.7 (1.6 – 2.2)	104.3 (2.1)	96.5 ± 2.2	104.9 (1.4)	104.9 (3.0)	105.8 (0.70)
		C ₃ =200	102.6 – 104.5 (0.44 – 1.2)	103.8 (1.2)	101.3 ± 4.8	107.2 (1.4)	103.2 (1.4)	102.5 (0.46)

4-HDMB	0.025 – 1	C ₁ =0.05	92.6 – 94.9 (0.41 – 0.60)	93.8 (1.2)	94.5 ± 3.5	98.0 (3.7)	91.8 (1.2)	102.6 (0.97)
		C ₂ =0.25	97.2 – 103.3 (1.1 – 3.4)	99.8 (3.6)	100.3 ± 5.5	103.6 (3.2)	101.1 (2.2)	96.7 (2.2)
		C ₃ =1	107.7 – 108.6 (1.8 – 5.2)	108.2 (2.8)	101.1 ± 2.9	109.5 (1.5)	105.1 (2.5)	107.7 (0.19)
	1 – 500	C ₁ =5	99.0 – 107.3 (0.14 – 3.2)	104.2 (4.7)	103.9 ± 5.4	104.2 (7.7)	101.0 (2.6)	109.7 (2.2)
		C ₂ =50	101.8 – 105.3 (1.7 – 2.1)	103.5 (3.3)	102.2 ± 2.2	108.6 (6.2)	99.1 (2.6)	108.1 (1.3)
		C ₃ =200	105.5 – 106.9 (0.18 – 1.5)	106.4 (1.0)	100.6 ± 2.2	106.3 (1.6)	106.0 (2.1)	105.8 (0.55)

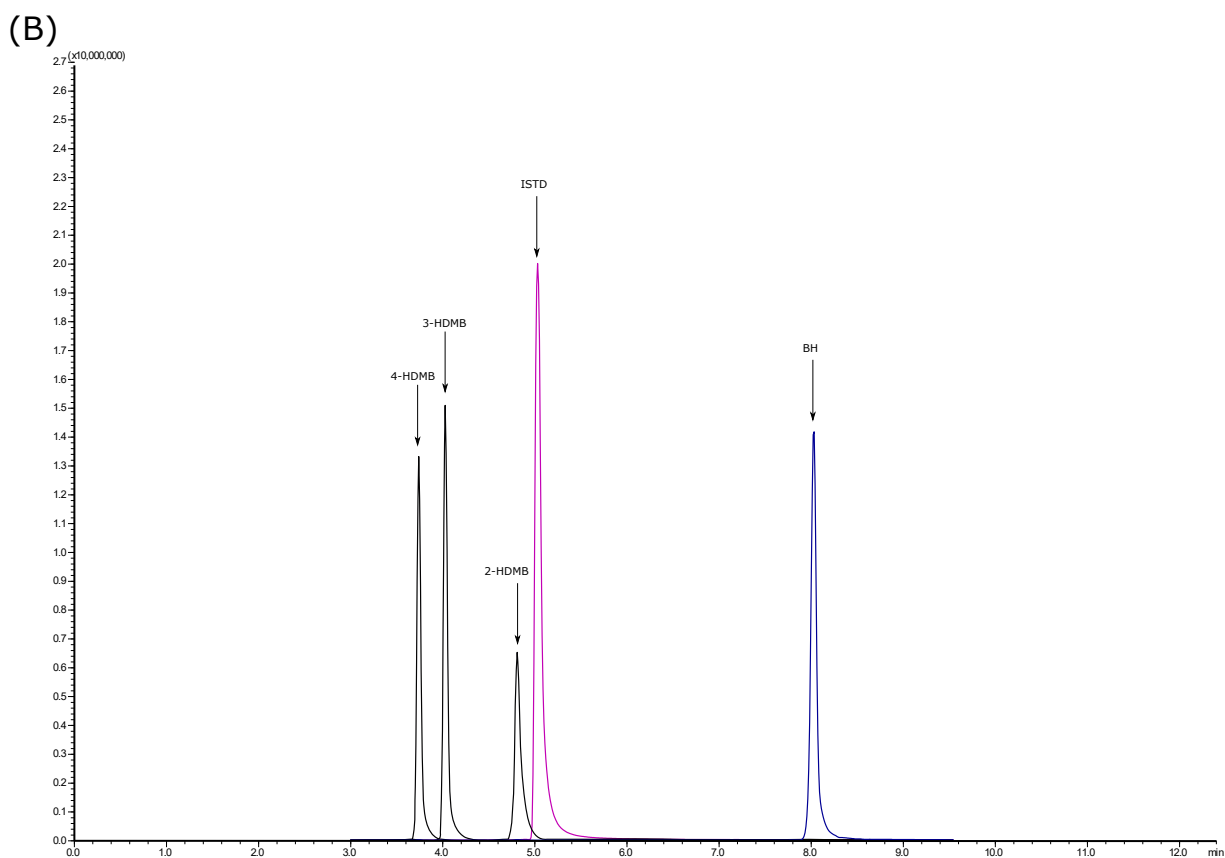
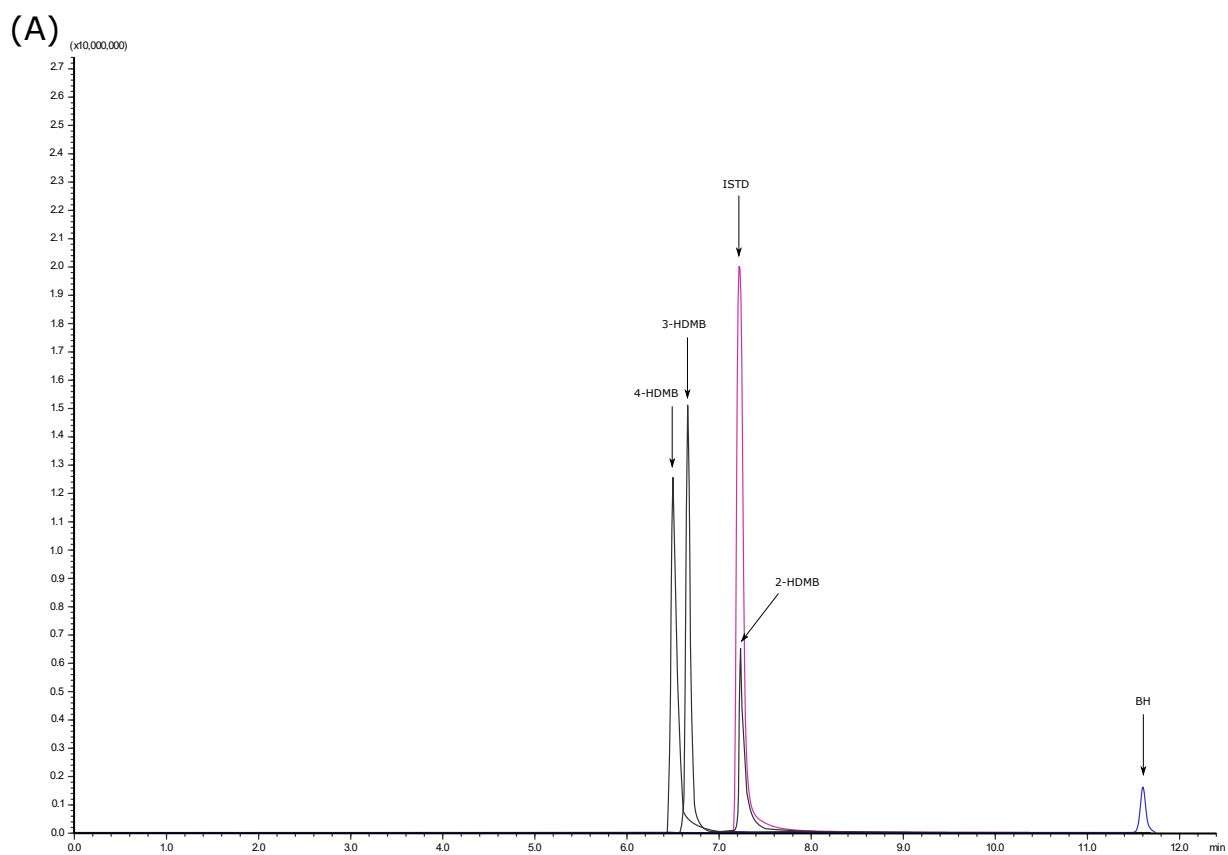
n: number of measurements; RE: recovery; CV: coefficient of variation; RT: room temperature.

Table 4. Maximum concentrations of BH and its metabolites in equine serum obtained after a single oral administration of BH to two horses.

Horse name		H1	H2
BH dose (mg/kg)		0.8	
BH	C_{\max} (ng/mL \pm SD; $n=3$)	23.7 ± 1.8	18.2 ± 1.1
	t_{\max} (h)	1	1
3-HDMB	C_{\max} (ng/mL \pm SD; $n=3$)	12.72 ± 0.82	10.26 ± 0.54
	t_{\max} (h)	15.5	5
4-HDMB	C_{\max} (ng/mL \pm SD; $n=3$)	2.40 ± 0.12	0.279 ± 0.016
	t_{\max} (h)	15.5	5



Supplementary Figure 1. The chromatograms obtained in preliminary studies using: (A) 406 Ascentis Express® C18 column (15 cm × 2.1 mm, 2.7 μm) with guard column (0.5 cm × 2.1mm, 2.7 μm), (B) Kinetex core-shell C18 column (15 cm × 2.1 mm, 2.6 μm) with guard column (0.5 cm × 2.1 mm, 2.6 μm) from Phenomenex (Torrance, USA).



Supplementary Table 1. Recovery obtained for all the analytes when ACN and MeOH was used as the solvent for the deproteinization.

Compound	Concentration of QC (ng/mL)	RE (%) \pm SD ($n=3$)	
		MeOH	ACN
BH	C ₁ =0.25	99.1 \pm 4.6	73.7 \pm 5.7
	C ₂ =50	97.7 \pm 4.1	73.0 \pm 2.8
2-HDMB	C ₁ =0.25	97.8 \pm 1.5	74.2 \pm 1.6
	C ₂ =50	97.7 \pm 1.7	73.3 \pm 2.1
3-HDMB	C ₁ =0.25	97.6 \pm 5.0	77.9 \pm 5.8
	C ₂ =50	98.2 \pm 3.9	77.9 \pm 5.0
4-HDMB	C ₁ =0.25	109.3 \pm 2.3	70.0 \pm 2.2
	C ₂ =50	108.4 \pm 2.8	73.9 \pm 1.6

n : number of measurements; RE: recovery.

