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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**

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‡ 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**

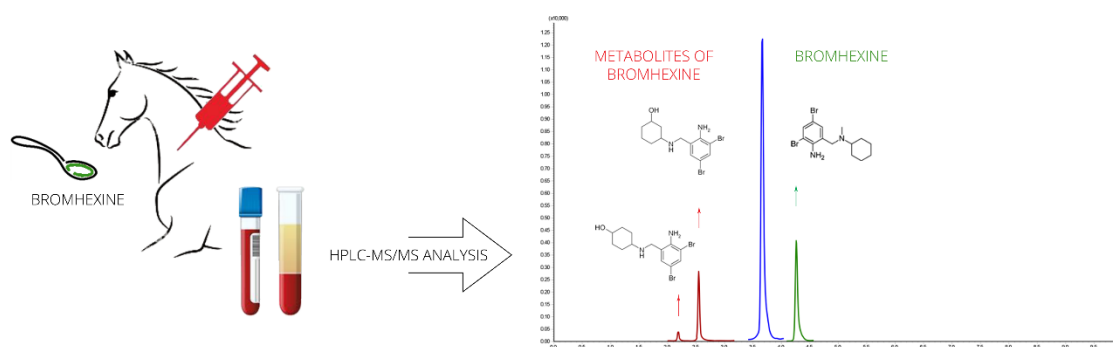
- 26 • Time courses of BH and its metabolites concentrations determined for the first time in  
27 equine serum.
- 28 • Fully validated LC-MS/MS method for determination of BH and its metabolites in  
29 equine serum samples.
- 30 • Simple sample preparation utilizing acetonitrile protein precipitation.

## 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

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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

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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

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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

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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,

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135 autosampler SIL-30AC and column oven CTO-20AC. Chromatographic separation was  
136 achieved using Kinetex core-shell C<sub>18</sub> column (15cm × 2.1mm, 2.6µm) with C<sub>18</sub> 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.

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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  $\mu$ 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  $\mu$ L of equine serum, 10  $\mu$ L of the ISTD working solution at 0.02  $\mu$ g/mL, 200  $\mu$ 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  $\mu$ L of equine serum  
176 was spiked with 10  $\mu$ L of the ISTD working solution at 1  $\mu$ 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

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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  $\mu$ L) and low  
187 consumption of organic solvents (200  $\mu$ 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 C<sub>18</sub> column (15 cm  $\times$  2.1 mm, 2.6  $\mu$ m) with guard column

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205 (0.5 cm x 2.1 mm, 2.6  $\mu$ m) from Phenomenex (Torrance, USA) and Ascentis Express®  
206 C<sub>18</sub> column (15 cm x 2.1 mm, 2.7  $\mu$ m) with guard column (0.5 cm x 2.1 mm, 2.7  $\mu$ 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.

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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

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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 freeze (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

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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

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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.

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

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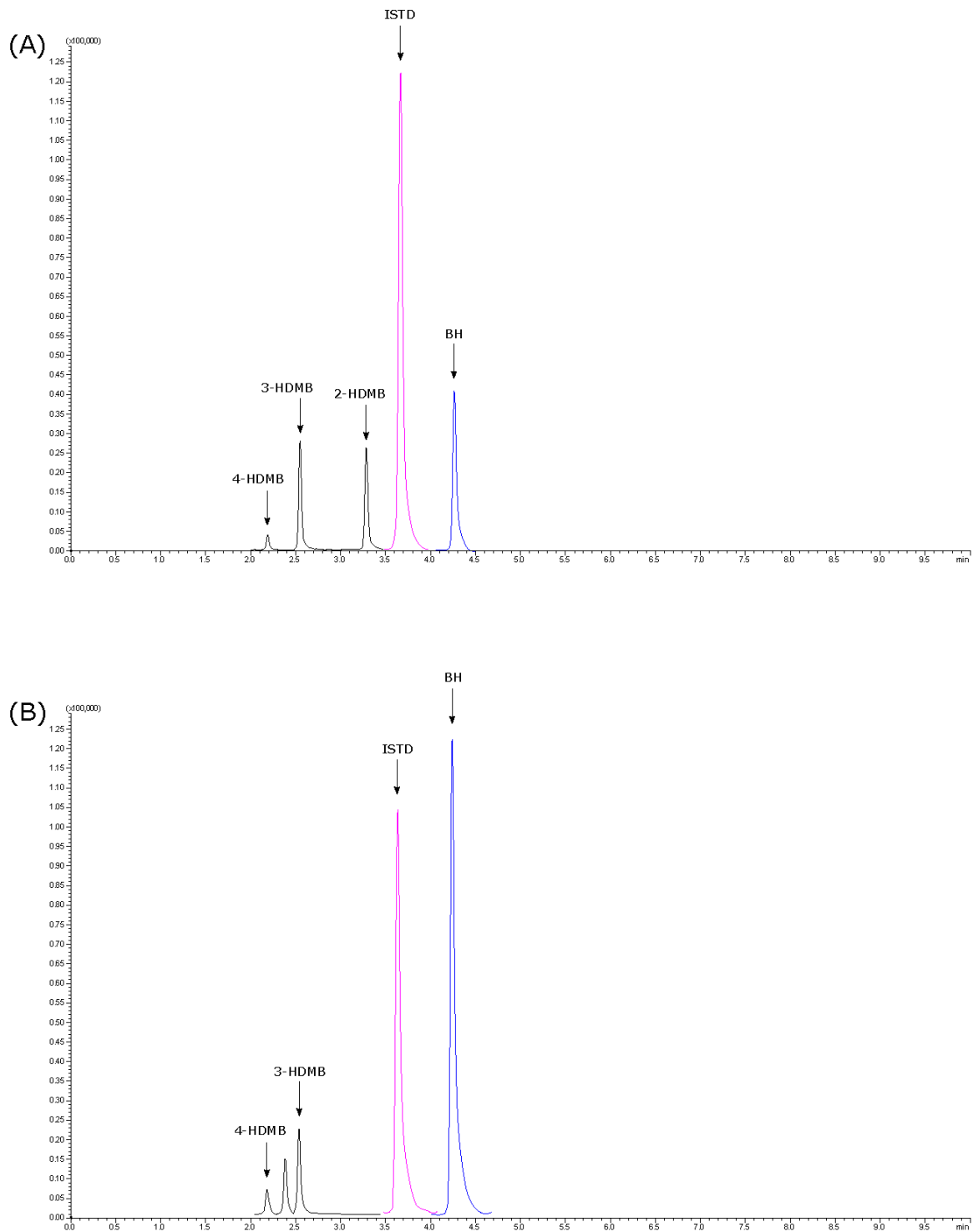
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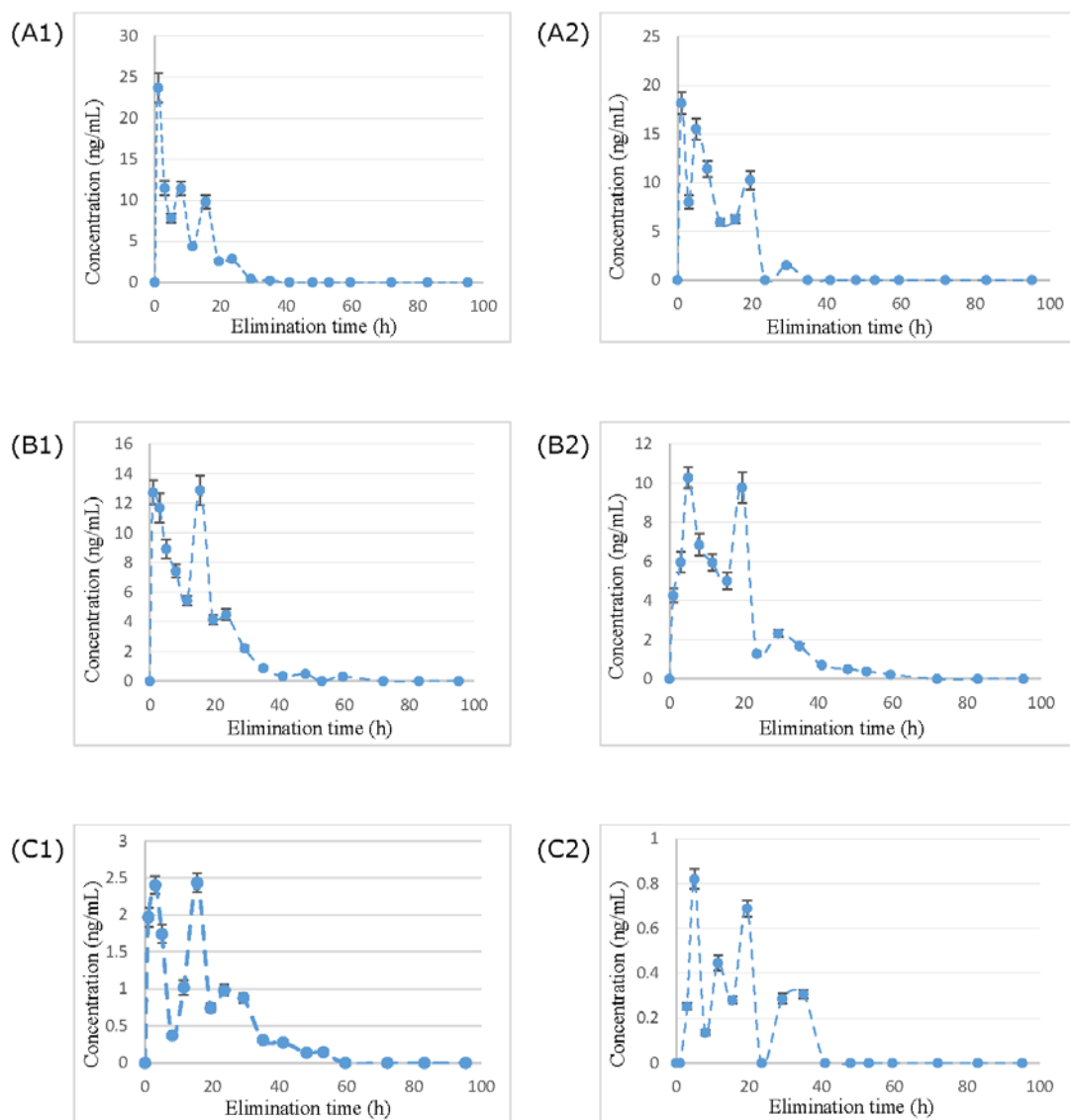
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383

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.

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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  $\mu$ m) with guard column (0.5 cm  $\times$  2.1  
407 mm, 2.7  $\mu$ m), (B) Kinetex core-shell C18 column (15 cm  $\times$  2.1 mm, 2.6  $\mu$ m) with guard column  
408 (0.5 cm  $\times$  2.1 mm, 2.6  $\mu$ m) from Phenomenex (Torrance, USA).

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

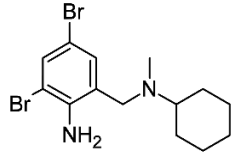
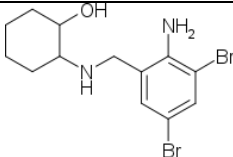
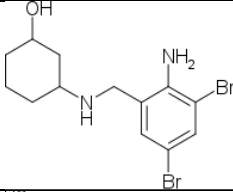
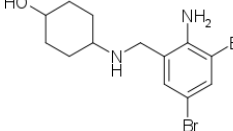
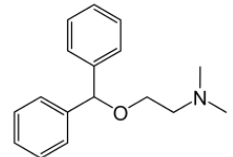
Compound	Molecular formula	Chemical structure	Precursor ion ➤ <b>quantifier ion</b> ➤ <b>qualifier ion</b> M+H [m/z]	Collision energy [V]	Dwell time [msec]	RT [min]
BH	C <sub>14</sub> H <sub>20</sub> Br <sub>2</sub> N <sub>2</sub>		376.9 ➤ <b>114.25</b> ➤ 263.9	➤ <b>-17</b> ➤ -29	97	4.23
2-HDMB	C <sub>13</sub> H <sub>18</sub> Br <sub>2</sub> N <sub>2</sub> O		378.9 ➤ <b>116.2</b> ➤ 263.9	➤ <b>-20</b> ➤ -17		3.26
3-HDMB	C <sub>13</sub> H <sub>18</sub> Br <sub>2</sub> N <sub>2</sub> O		378.9 ➤ <b>116.2</b> ➤ 263.9	➤ <b>-20</b> ➤ -17		2.47
4-HDMB	C <sub>13</sub> H <sub>18</sub> Br <sub>2</sub> N <sub>2</sub> O		378.9 ➤ <b>263.9</b> ➤ 116.2	➤ <b>-20</b> ➤ -17		2.12
Diphenhydramine (ISTD)	C <sub>17</sub> H <sub>21</sub> NO		256.1 ➤ <b>167.2</b> ➤ 152.1	➤ <b>-14</b> ➤ -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 <sub>a</sub>	S <sub>b</sub>	Linearity (R <sup>2</sup> )	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<sub>a</sub>: standard deviations of slope; S<sub>b</sub>: standard deviations of constant terms; R<sup>2</sup>: 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 <sub>1</sub> =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 <sub>2</sub> =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 <sub>3</sub> =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 <sub>1</sub> =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 <sub>2</sub> =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 <sub>3</sub> =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 <sub>1</sub> =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 <sub>2</sub> =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 <sub>3</sub> =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 <sub>1</sub> =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 <sub>2</sub> =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 <sub>3</sub> =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 <sub>1</sub> =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 <sub>2</sub> =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 <sub>3</sub> =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 <sub>1</sub> =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 <sub>2</sub> =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 <sub>3</sub> =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 <sub>1</sub> =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 <sub>2</sub> =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 <sub>3</sub> =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 <sub>1</sub> =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 <sub>2</sub> =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 <sub>3</sub> =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)

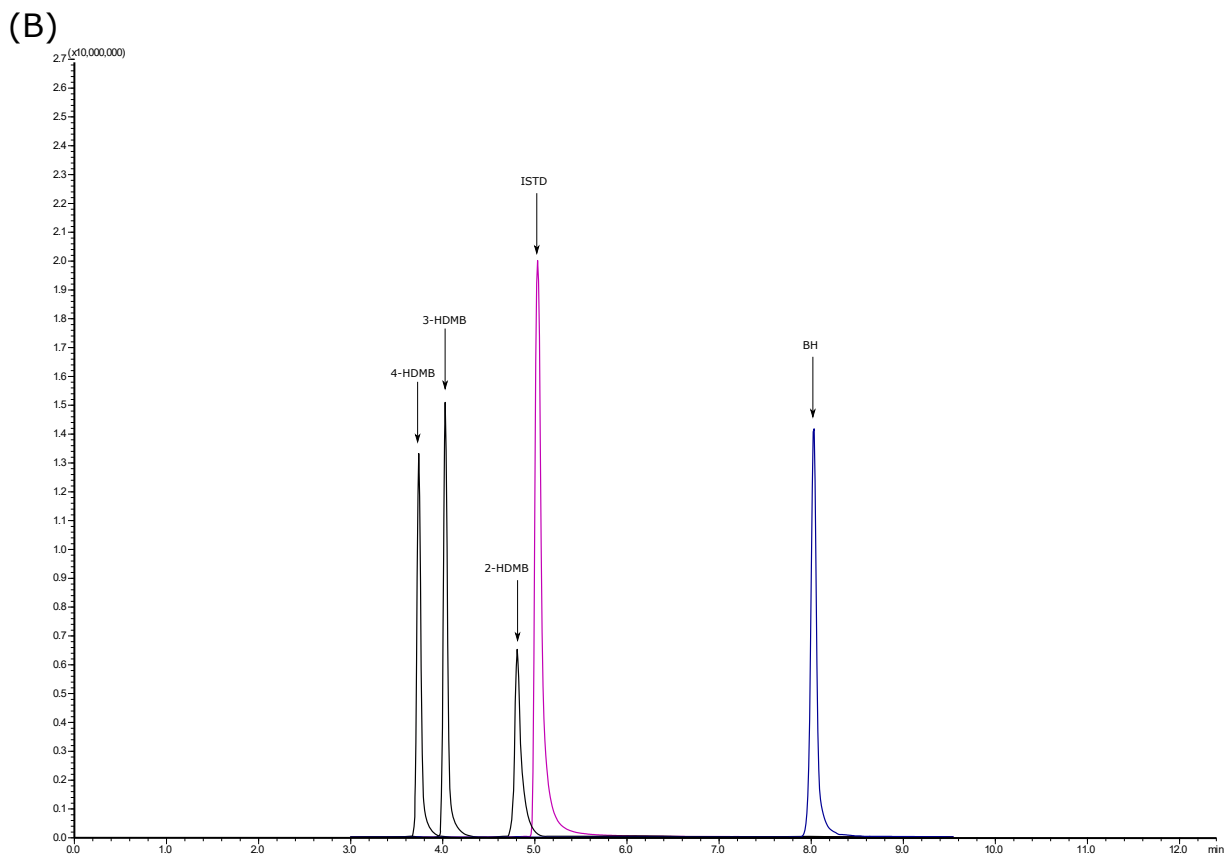
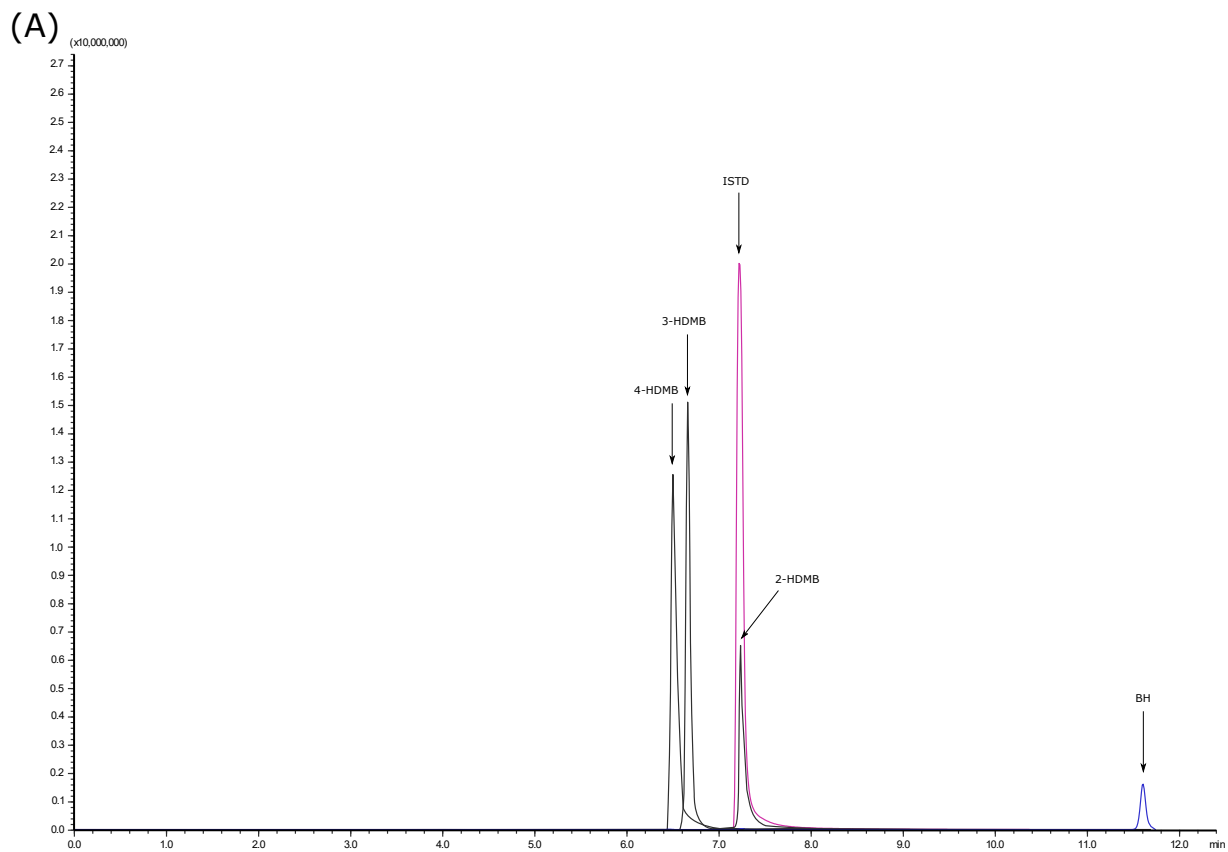
*n*: number of measurements; RE: recovery; CV: coefficient of variation; RT: room temperature.



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; $n=3$ )	$23.7 \pm 1.8$	$18.2 \pm 1.1$
	$t_{\max}$ (h)	1	1
3-HDMB	$C_{\max}$ (ng/mL $\pm$ SD; $n=3$ )	$12.72 \pm 0.82$	$10.26 \pm 0.54$
	$t_{\max}$ (h)	15.5	5
4-HDMB	$C_{\max}$ (ng/mL $\pm$ SD; $n=3$ )	$2.40 \pm 0.12$	$0.279 \pm 0.016$
	$t_{\max}$ (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 <sub>1</sub> =0.25	99.1 $\pm$ 4.6	73.7 $\pm$ 5.7
	C <sub>2</sub> =50	97.7 $\pm$ 4.1	73.0 $\pm$ 2.8
2-HDMB	C <sub>1</sub> =0.25	97.8 $\pm$ 1.5	74.2 $\pm$ 1.6
	C <sub>2</sub> =50	97.7 $\pm$ 1.7	73.3 $\pm$ 2.1
3-HDMB	C <sub>1</sub> =0.25	97.6 $\pm$ 5.0	77.9 $\pm$ 5.8
	C <sub>2</sub> =50	98.2 $\pm$ 3.9	77.9 $\pm$ 5.0
4-HDMB	C <sub>1</sub> =0.25	109.3 $\pm$ 2.3	70.0 $\pm$ 2.2
	C <sub>2</sub> =50	108.4 $\pm$ 2.8	73.9 $\pm$ 1.6

$n$ : number of measurements; RE: recovery.

