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

Emilia Waraksa[a,b*], Katarzyna Owczarek[a], Paweł Kubica[a], Ewa Kłodzińska[b], Mariusz Ozimek[b], Robert Wrzesień[c], Barbara Bobrowska-Korczak[d], Jacek Namieśnik [‡ a]

Affiliations

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

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

[c] Medical University of Warsaw, Central Laboratory of Experimental Animal, Banacha 1 B Street, 02-097 Warsaw, Poland

[d] Medical University of Warsaw, Department of Bromatology, Banacha 1 B Street, 02-097 Warsaw, Poland

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

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Highlights

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

Abstract

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

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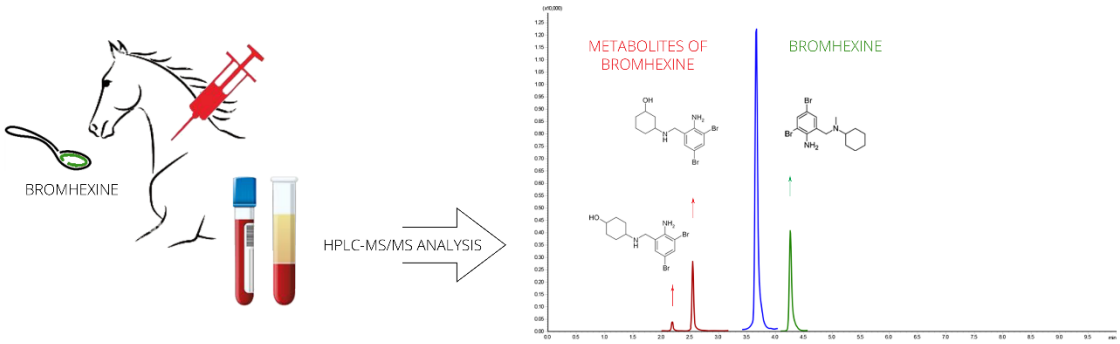
administration of BH to two healthy mares (in dose of 0.8 mg/kg). The parent drug was found at higher concentration levels than 3-HDMB (major metabolite) and 4-HDMB (minor metabolite), however, both BH metabolites lasted for a longer period of time in equine serum than the parent drug. Thus, both metabolites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



1. Introduction

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

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

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

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

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

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

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2. Materials and methods

2.1 Standards and reagents

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

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

2.2 LC-MS/MS conditions

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

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

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autosampler SIL-30AC and column oven CTO-20AC. Chromatographic separation was achieved using Kinetex core-shell C_{18} column (15cm × 2.1mm, 2.6µm) with C_{18} guard column (0.5cm x 2.1mm, 2.6µm) from Phenomenex (Torrance, USA). The column temperature was kept at 30°C. The flow rate of the mobile phase was set at 1 mL/min; and the injection volume was set at 5 µL. For the mobile phase, following solvents were used: A) water + 0.1% formic acid, and B) acetonitrile + 0.1% formic acid. The analytes were eluted with following gradient program: 0 – 0.50 min 10% B, 0.50 – 5.50 min 10 – 35% B, 5.50 – 7.00 min 35 – 95% B. The column was stabilized after each analysis for 3 min. The total time of the chromatographic run was 10 min.

2.3 Drug administration, samples collection and storage

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

2.4 Preparation of stock, working and quality control solutions

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

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

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

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

2.5 Sample preparation

A 200 µL of equine serum, 10 µL of the ISTD working solution at 0.02 µg/mL, 200 µL of acetonitrile were added to a Eppendorf vial and refrigerated (3^{o}C) for 1 h. After centrifugation for 5 min at 13000 rpm, supernatant was analyzed by LC-MS/MS system. When analyte(s) was (were) determined at concentration(s) above 1 ng/mL, a 200 µL of equine serum was spiked with 10 µL of the ISTD working solution at 1 µg/mL and reanalyzed as described above.

2.6 Method validation

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

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

3. Results and discussion

3.1 Optimization of sample preparation procedure

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

3.2 Optimization of LC-MS/MS parameters

The LC-MS/MS conditions were optimized to ensure selectivity and sensitivity of the method. Preliminary studies, involving the investigation of two chromatographic columns for the analytes separation were performed. Structural (geometric isomers) and physicochemical similarities of the target compounds make them difficult to separate, so high resolution is desirable. Because of that reason, columns packed with core-shell particles were tested, i.e. Kinetex core-shell C_{18} column (15 cm × 2.1 mm, 2.6 µm) with guard column

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205 (0.5 cm x 2.1 mm, 2.6 μm) from Phenomenex (Torrance, USA) and Ascentis Express®
206 C_{18} column (15 cm × 2.1 mm, 2.7 μm) with guard column (0.5 cm × 2.1 mm, 2.7 μm). 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 annonium 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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Obtained coefficients of determination for the calibration curves were greater than 0.9951 for all target analytes.

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

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

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

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

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

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

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

3.4 Analysis of real samples

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

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

Conclusions

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

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

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

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

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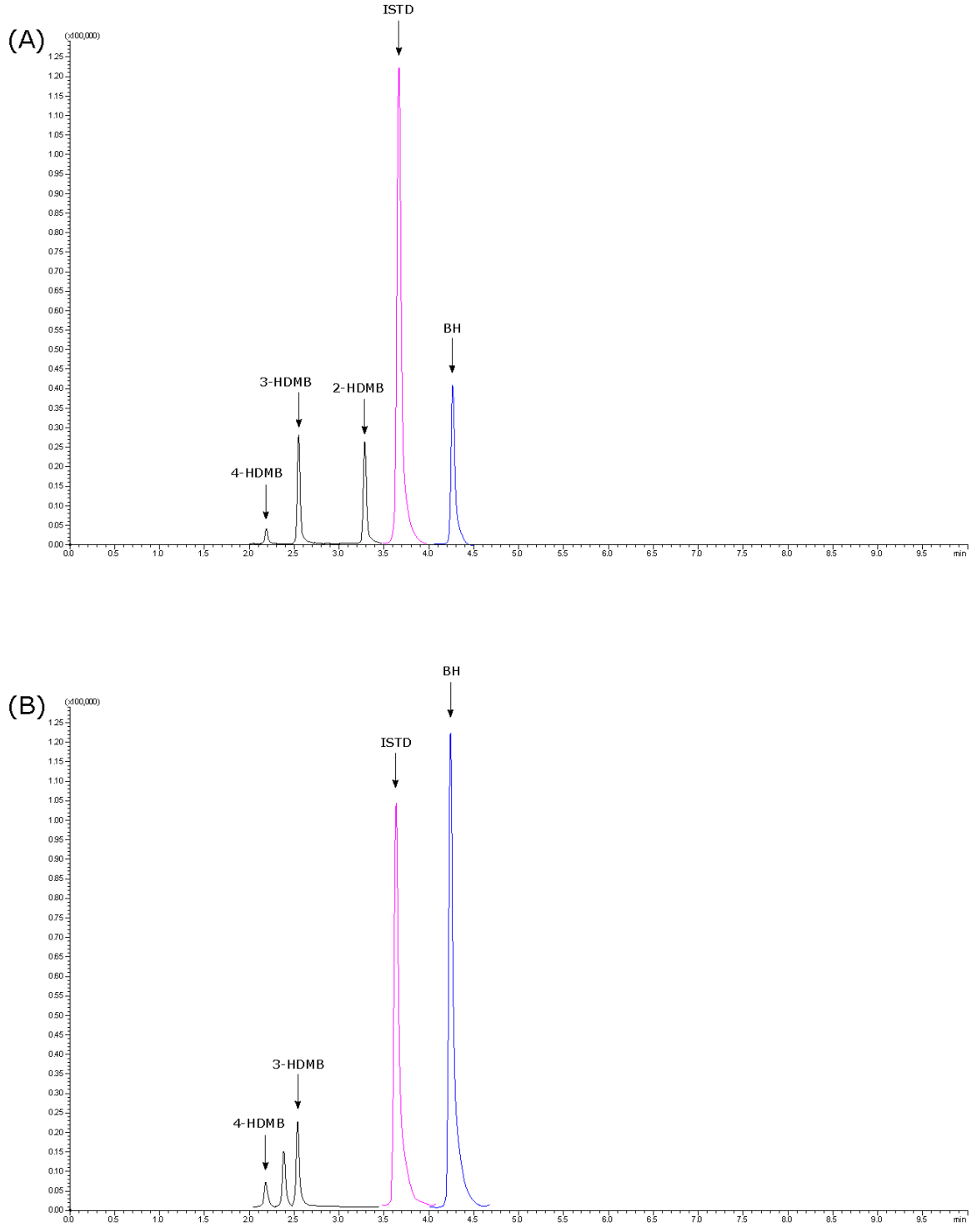
‡ This article is dedicated to the memory of Prof. Jacek Namieśnik (1949 – 2019).

382 **List of figures**



383

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

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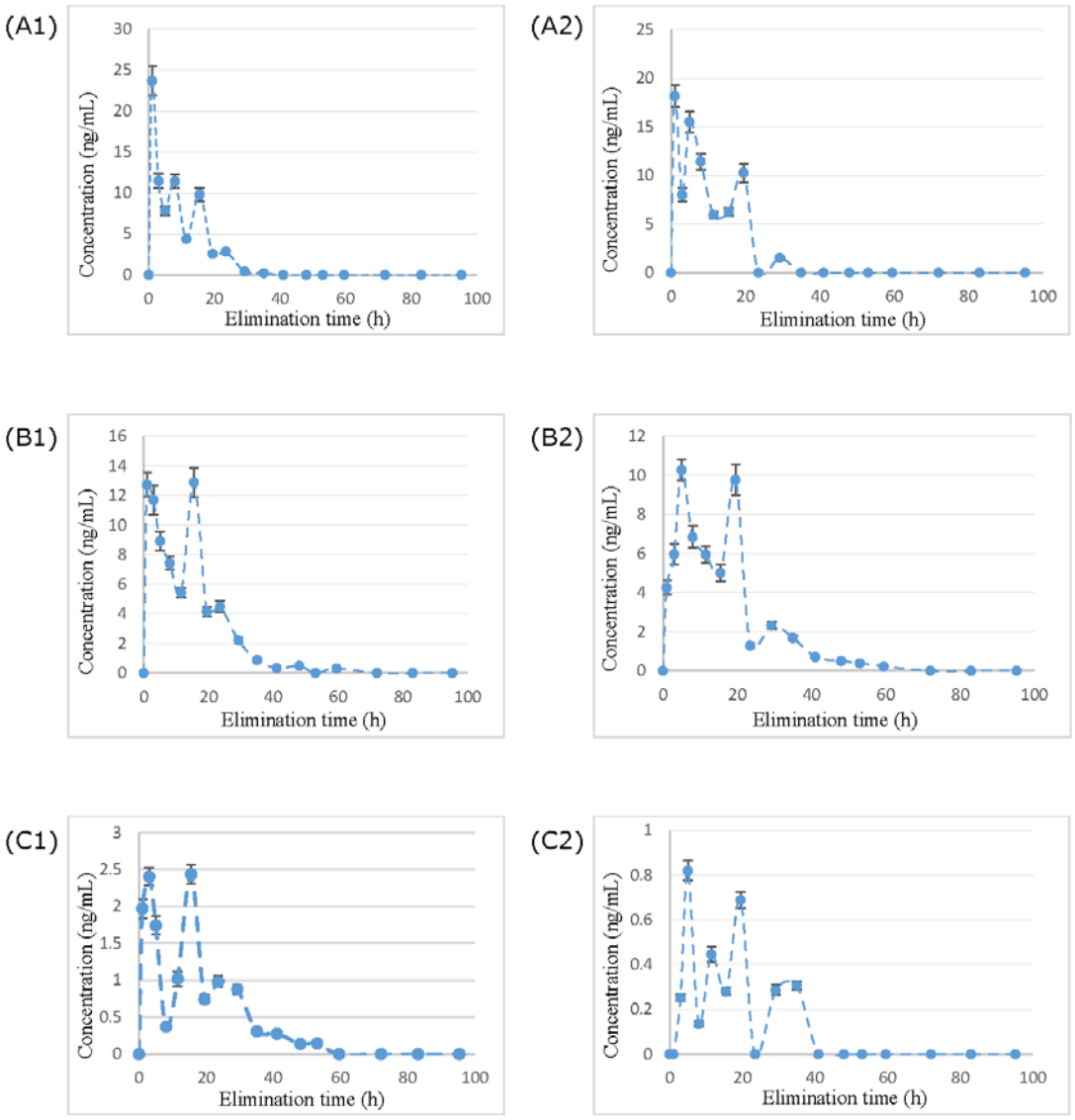

387

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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Supplementary material

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

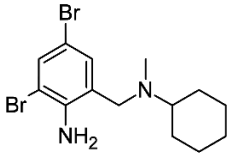
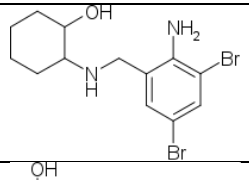
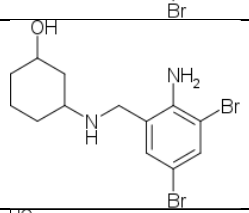
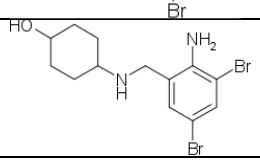
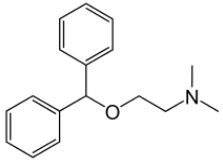
Compound	Molecular formula	Chemical structure	Precursor ion ➢ **quantifier ion** ➢ qualifier ion M+H [m/z]	Collision energy [V]	Dwell time [msec]	RT [min]
BH	$C_{14}H_{20}Br_2N_2$		376.9 ➢ **114.25** ➢ 263.9	➢ **-17** ➢ -29	97	4.23
2-HDMB	$C_{13}H_{18}Br_2N_2O$		378.9 ➢ **116.2** ➢ 263.9	➢ **-20** ➢ -17		3.26
3-HDMB	$C_{13}H_{18}Br_2N_2O$		378.9 ➢ **116.2** ➢ 263.9	➢ **-20** ➢ -17		2.47
4-HDMB	$C_{13}H_{18}Br_2N_2O$		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; R^2: coefficient of determination; LOD: limit of detection; LOQ: limit of quantification.

Table 3. Intra- and inter-day accuracy and precision, recovery ±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 (%) ± 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 ± 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 ± 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 ± 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 ± 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 ± 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 ± 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 ± 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 ± 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)	102.1 (3.6)
		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)

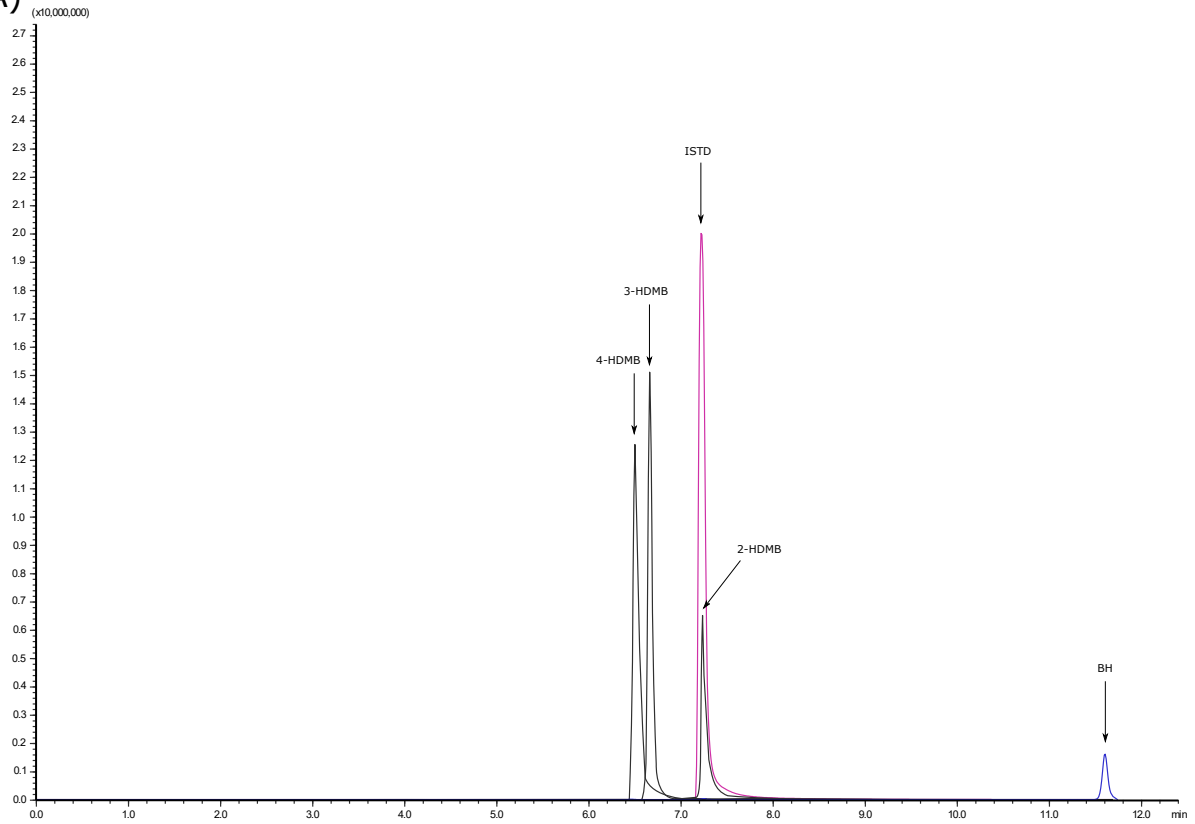
n: number of measurements; RE: recovery; CV: coefficient of variation; RT: room temperature.

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

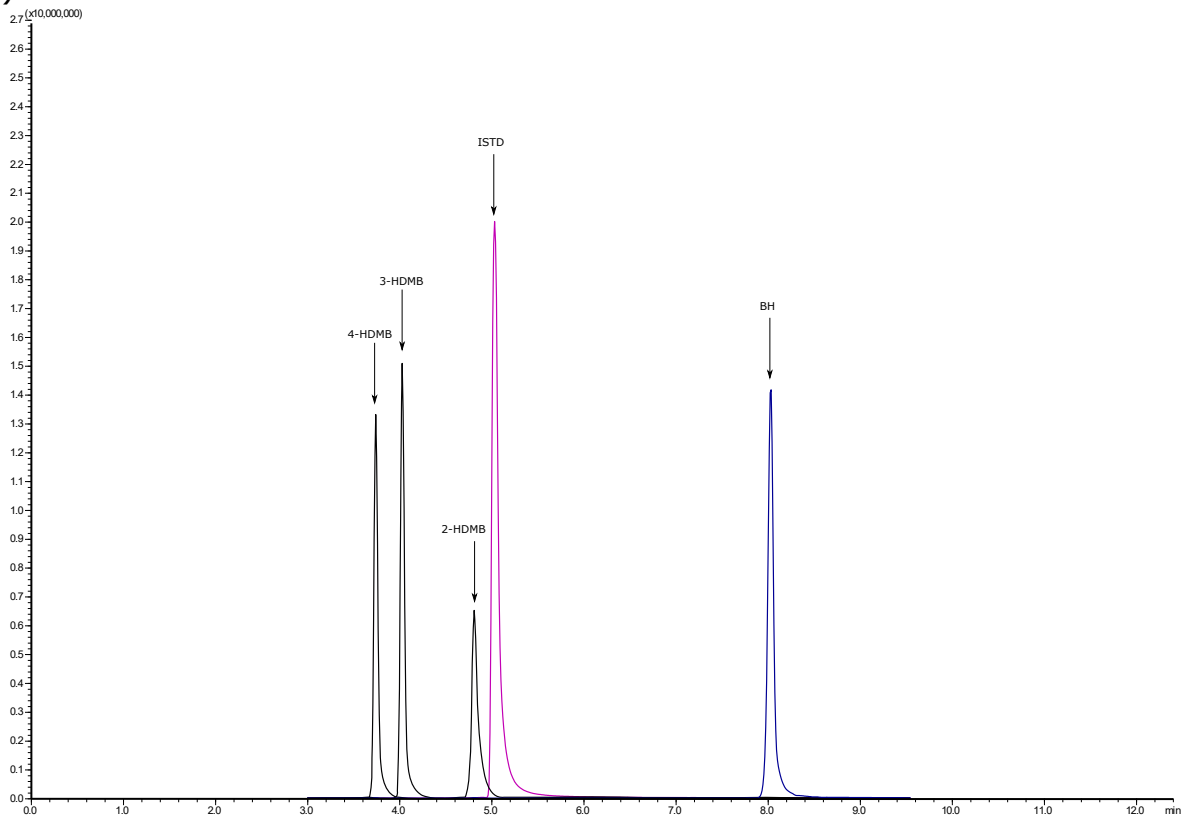
Horse name		H1	H2
BH dose (mg/kg)		0.8	
BH	C_{max} (ng/mL ±SD; *n*=3)	23.7 ± 1.8	18.2 ± 1.1
	t_{max} (h)	1	1
3-HDMB	C_{max} (ng/mL ±SD; *n*=3)	12.72 ± 0.82	10.26 ± 0.54
	t_{max} (h)	15.5	5
4-HDMB	C_{max} (ng/mL ±SD; *n*=3)	2.40 ± 0.12	0.279 ± 0.016
	t_{max} (h)	15.5	5

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 x 2.1 mm, 2.6 µm) from Phenomenex (Torrance, USA).

(A)



(B)



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 (%) ± SD (*n*=3)	
		MeOH	ACN
BH	C_1=0.25	99.1 ± 4.6	73.7 ± 5.7
	C_2=50	97.7 ± 4.1	73.0 ± 2.8
2-HDMB	C_1=0.25	97.8 ± 1.5	74.2 ± 1.6
	C_2=50	97.7 ± 1.7	73.3 ± 2.1
3-HDMB	C_1=0.25	97.6 ± 5.0	77.9 ± 5.8
	C_2=50	98.2 ± 3.9	77.9 ± 5.0
4-HDMB	C_1=0.25	109.3 ± 2.3	70.0 ± 2.2
	C_2=50	108.4 ± 2.8	73.9 ± 1.6

n: number of measurements; RE: recovery.