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Sensitive liquid chromatographic/tandem mass spectrometric method for the determination of beclomethasone dipropionate and its metabolites in equine plasma and urine

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Beclomethasone dipropionate (BDP) is a potent pro-drug to beclomethasone (BOH) and is used in the treatment of chronic and acute respiratory disorders in the horse. The therapeutic dose of BDP (325 μg per horse) by inhalation results in very low plasma and urinary concentrations of BDP and its metabolites that pose a challenge to detection and confirmation by equine forensic laboratories. To solve this problem, a method involving the use of a liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) was developed for the detection, confirmation and quantification of the analytes in equine samples. Ammonium formate or acetate buffer added to LC mobile phase favored the formation of $[M + H]^+$ ions from BDP and its metabolites, whereas formic acid led to the formation of sodium and potassium adduct ions ($[M + Na]^+$, $[M + K]^+$) together with $[M + H]^+$ ions. Acetonitrile, on the other hand, favored the formation of abundant solvent adduct ions $[M + H + CH_3CN]^+$ with the analytes under electrospray ionization (ESI) and atmospheric pressure chemical ionization conditions. In contrast, methanol formed much less solvent adduct ions than acetonitrile. The solvent adduct ions were thermally stable and could not be completely desolvated under the experimental conditions, but they were very fragile to collision-induced dissociation (CID). Interestingly, these solvent adduct ions were observed on a triple-quadrupole mass spectrometry but not on an ion trap instrument where helium used as a damping gas in the ion trap might cause the solvent adduct ions desolvated by collision. By CID studies on the $[M + H]^+$ ions of BDP and its metabolites, their fragmentation paths were proposed. In equine plasma at ambient temperature over 2 h, BDP and B21P were hydrolyzed in part to B17P and BOH, respectively, but B17P was not hydrolyzed. Sodium fluoride added to equine plasma inhibited the hydrolysis of BDP and B21P. The matrix effect in ESI was evaluated in equine plasma and urine samples. The method involved the extraction of BDP and its metabolites from equine plasma and urine samples by methyl *tert*-butyl ether, resolution on a C_8 column with a mobile phase gradient consisting of methanol and ammonium formate (2 mmol l^{-1}, pH 3.4) and multiple reaction monitoring for the analytes on a triple-quadrupole mass spectrometer. The detection limit was 13 pg ml^{-1} for BDP and B17P, 25 pg ml^{-1} for BOH and 50 pg ml^{-1} for B21P in plasma and 25 pg ml^{-1} for BOH in urine. The method was successfully applied to the analysis of equine plasma and urine samples for the analytes following administration of BDP to horses by inhalation. B17P, the major and active metabolite of BDP, was detected and quantified in equine plasma up to 4 h post-administration by inhalation of a very low therapeutic dose (325 μg per horse) of BDP.

KEYWORDS: beclomethasone; corticosteroid; electrospray; tandem mass spectrometry; horse

INTRODUCTION

Beclomethasone dipropionate (BDP) is a pro-drug to beclomethasone (BOH). BOH is one of the large family of synthetic corticosteroids and is similar in structure to dexamethasone. Synthetic corticosteroids are used in the treatment of many disease conditions because of their wide-scale anti-inflammatory properties.[1] Corticosteroids are a restricted classes of substances by the International Olympic Committee (IOC).[2] Methods for the detection and quantification of synthetic corticosteroids by gas chromatography/mass spectrometry (GC/MS) have been reported.[3–6] Liquid chromatographic/mass spectrometric (LC/MS) methods have also been reported for the detection and quantification of

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corticosteroids in biological samples,[1,7] human hairs,[2] milk replacer[8] and human plasma.[9] In a few publications, LC/MS methods for quantification of BDP and its metabolites in human plasma were briefly mentioned,[10–13] but details of the methods were not provided.

BDP is formulated as an inhalation aerosol for management of asthma in humans. Aerosolized BDP administered to horses with recurrent chronic obstructive pulmonary disease (COPD) attenuated the neutrophilic pulmonary inflammation and prevented alteration in lymphocyte subpopulations in horses with heaves. BDP also improved pulmonary function and clinical signs of airway obstruction in the horses.[14–16] On the other hand, since BDP is administered in low doses by inhalation, it is likely that this drug will become a 'race day medication' unless strict regulatory policies and enforcements are in place. The majority of Racing Jurisdictions in North America prohibit race day administration of medication to horses. However, the challenge faced by the regulators is the inability of the equine forensic laboratories to detect very low concentrations of BDP and/or its metabolites in plasma or urine of racehorses. This problem is posed by the inadequate sensitivity by traditional methods used in screening and confirmation of test samples. To address this concern, BDP and its metabolites must be monitored in plasma and/or urine samples from racehorses in competition, and for this purpose, a sensitive method capable of detection and confirmation of the analytes in the equine samples is desired.

BDP is metabolized to beclomethasone-17-propionate (B17P) in human lungs[10] and the homogenates,[17] and to B21P and minor concentration of beclomethasone (BOH) in human plasma, *in vivo*.[10] Following incubation of BDP in human plasma at 37 °C, B17P, beclomethasone-21-propionate (B21P) and BOH were formed.[18] The extensive metabolism of BDP and a therapeutic dose (325 μg) by inhalation in the equine requires a very sensitive method for detection, confirmation and quantification of the equally low concentrations of B17P, B21P and BOH.

A sensitive LC/MS/MS method for the detection, confirmation and quantification of BDP, B17P, B21P and BOH (Figure 1) in equine plasma and urine is described in this paper.

EXPERIMENTAL

Chemicals and reagents

BDP and BOH were purchased from Sigma (St. Louis, MO, USA) and B17P, B21P and desoximetasone, used as an internal standard (IS), from Steraloids (Newport, RI, USA). Ammonium formate (Certified), ammonium acetate (HPLC grade), sodium acetate (Certified ACS), acetic acid (glacial, Certified ACS Plus), sodium fluoride (Certified ACS) and HPLC-grade methanol and water were purchased from Fisher Scientific (Pittsburgh, PA, USA). Formic acid was purchased from EM Science (Gibbstown, NJ, USA). Water and methanol used were of HPLC grade unless otherwise described.

Ammonium formate buffer comprising 1.0 mol l^{-1} ammonium formate and 1.0 mol l^{-1} formic acid was prepared from the dry chemical powder and concentrated formic acid. Ammonium formate (6.3 g) was dissolved in 80 ml of water in a beaker and transferred in to a 100 ml volumetric flask. After adding 4.3 ml of formic acid, it was brought to a final volume of 100 ml with water. The pH of the formate buffer was 3.4. Ammonium acetate buffer (pH 4.6) comprising 1.0 mol l^{-1} ammonium acetate and 1.0 mol l^{-1} acetic acid, and sodium acetate buffer (pH 4.3) consisting of 0.5 mol l^{-1} sodium acetate and 1.0 mol l^{-1} acetic acid were similarly prepared. Another ammonium acetate solution (1 mol l^{-1}) of pH 6.7 was prepared.

Preparation of stock and working standard solutions

Each stock standard solution (1.0 mg ml^{-1}) of BDP, B21P, B17P, BOH, and IS was prepared by dissolving the dry chemical powder in HPLC-grade methanol and stored at 4 °C. Working standard solutions of 100, 10 and 1.0 ng ml^{-1} were prepared from the stock standard solutions by consecutive one-tenth dilution with methanol and stored at 4 °C. The stock and working standard solutions were discarded after 2 months.

Drug administration and sample collection

Female horses ranging in age from 4 to 10 years with an average weight of 550 ± 49 kg were used. The University of Pennsylvania Institutional Animal Care and Use Committee approved the study protocol. Doses of BDP were administered to horses by inhalation using an Equine Aerosol Delivery Device System (3M Corporation, St. Paul, MN, and Boehringer Ingelheim Vetmedica, St. Joseph, MO, USA). Blood and urine samples were collected before drug administration and at various intervals post-drug administration. Blood samples were collected via a venous catheter placed in the jugular vein. Tubes containing potassium oxalate as an anticoagulant and sodium fluoride (10 mg ml^{-1}) as an inhibitor of plasma esterases were used and centrifuged (2500–3000 rpm or 776–1318 *g*) at 4 °C for 15 min to obtain plasma. The plasma samples were transferred into separate tubes, frozen and stored at −20 °C until analysis was performed.

Urine samples were collected via an indwelling catheter placed in the bladder and attached to a drainage bag prior to and post-drug administration. Urine samples were divided into 50 ml aliquots and stored at −20 °C. Each aliquot was used once to avoid any effects of freeze–thaw cycles on the concentration of BDP and its metabolites.

Preparation of analyte standards in equine plasma and extraction of plasma samples

Control equine plasma samples used in this study were previously confirmed to be free of BDP and its metabolites by the same LC/MS method as described. To the control plasma, sodium fluoride powder was added to a final concentration of 10 mg ml^{-1} to inhibit hydrolysis of BDP by plasma esterases. An aliquot of 2.0 ml of plasma was transferred into different disposable glass tubes (16 × 125 mm). To each plasma sample in a tube BDP, B17P, B21P and BOH (25–5000 pg in 25–100 μl of methanol) and IS (2500 pg in 25 μl of methanol) were added. Each sample was briefly mixed by vortexing. The

analyte standards spiked into plasma were used either as calibrators or quality control (QC) samples, and the spiked plasma samples were freshly prepared immediately before use.

BDP and its metabolites were recovered from plasma by liquid–liquid extraction (LLE). To each plasma sample was added methyl *tert*-butyl ether (MTBE) (4 ml) and mixed by rotorack (Thermolyne, Dubuque, IA, USA) for 4 min and then centrifuged at 2500–3000 rpm (839–1409 *g*) for 5 min. The organic layer (top) was transferred into a clean glass tube (16 × 100 mm) using a Pasteur pipette. The sample was re-extracted with an additional 4 ml of MTBE as described above. Both extracts were pooled and dried at 40 °C (Dri-Block DB·3, Techne, Duxford, Cambridge, UK) under a steady stream of nitrogen. The dried extracts were reconstituted in100 µl of LC sample solvent (mobile phase) and analyzed by LC/MS on the same day as the extraction was performed. Plasma samples collected from BDP administration were similarly prepared except that BDP, B17P, B21P and BOH were not added.

Preparation of analyte standards in equine urine and extraction of analyte from urine samples

Negative equine urine samples used in this study were previously confirmed to be free of BDP and its metabolites as described above. An aliquot of 1.0 ml of negative urine was pipetted into different disposable glass tubes (16 × 125 mm) and BDP, B17P, B21P, BOH and IS were added to each sample tube as described above for plasma samples. The spiked urine samples were freshly prepared immediately before use.

To determine the effect of pH on the quantification of the analytes in urine, urine samples pH-adjusted and -unadjusted were extracted by LLE. For adjustment of sample pH, 0.1 ml of sodium acetate buffer was added to each urine sample (1 ml). Each urine sample, pH-adjusted or -unadjusted, was extracted twice with MTBE (3 ml) and the extracts were pooled and dried as described above for plasma samples. Urine samples collected from BDP administration to horses were similarly prepared except that BDP, B17P, B21P and BOH were not added.

To evaluate the effect of enzyme hydrolysis on the release of BDP metabolites from conjugates, urine samples were hydrolyzed with β-glucuronidase. To 1.0 ml of negative urine in a disposable glass tube, BDP, B17P, B21P and BOH (50–5000 pg in 25–100 µl of methanol), 0.20 ml of sodium acetate buffer and 0.50 ml of β-glucuronidase (*Patella vulgata*, 5500 units ml^{-1}) were added and mixed by vortexing. The mixture was incubated at 65 °C for 3 h and cooled to room temperature prior to adding IS (5000 pg in 50 µl of methanol). The hydrolyzed urine samples were extracted twice with 4 ml of MTBE each as described above for plasma samples.

Evaluation of the stability of BDP, B17P, B21P and IS in equine plasma

Each of BDP, B17P and B21P (5 ng each) was spiked into 2 ml of negative equine plasma with or without sodium fluoride (NaF), then IS (2.5 ng) was added to each plasma sample. A set of plasma samples with and without NaF was extracted immediately after the addition of the analyte and IS, and they were designated as '0 h' samples. Another set of plasma samples with and without NaF were allowed to incubate at ambient temperature (25 °C) for 2 h and then extracted, and this set of samples was designated '2 h' samples. The peak areas averaged from duplicate samples were compared for the relevant '0 h' and '2 h' samples and thus the percentage changes of BDP, B17P, B21P and IS concentrations were calculated.

Evaluation of 'matrix effect'

BDP, B17P, BOH (1 ng each in 100 µl of methanol) and IS (2.5 ng in 25 µl of methanol) were added to the dried extracts of 2 ml of negative equine plasma, dried and dissolved in 100 µl of the LC sample solvent. The same concentration each of BDP, B17P, BOH (in 100 µl of methanol) and IS standards (in 25 µl of methanol) were dried and dissolved in the same volume of the LC sample solvent. An aliquot of 20 µl was injected into the LC/MS system and analyzed by the positive electrospray ionization liquid chromatographic/multiple reaction monitoring (ESI (+) LC/MRM) method. The chromatographic peak areas averaged from three sample duplicates for the standards in sample solvent and for the standards added to plasma extracts and reconstituted as described above were used for the estimation of ion suppression or enhancement. The same procedure was used to evaluate equine urine samples for matrix effect. BOH was quantified by the ESI (−) LC/MRM method. The chromatographic peak areas that were averaged from six sample duplicates were used for estimating ion suppression or enhancement.

Instrumentation and operating parameters

Analysis of sample extracts was performed using an LC/MS system (Thermo-Finnigan, San Jose, CA, USA) consisting of a Surveyor MS pump with an on-line degasser, a Surveyor autosampler and a TSQ Quantum triple-quadrupole mass spectrometer equipped with an ESI probe. Another LC/MS system (Thermo-Finnigan) consisting of a Surveyor MS pump, a Surveyor autosampler and an LCQ Deca XP Plus ion trap mass spectrometer was used in the early phase of the method development.

LC separation was performed on an Ace C_8 column (2.1 × 50 mm, 5 µm) (Mac-Mod Analytical, Chadds Ford, PA, USA) with an Ace C_8 guard column (2.1 × 12.5 mm) (Mac-Mod Analytical) that was at ambient temperature. An LC mobile phase gradient in composition and flow-rate, as shown in Table 1, was used for the resolution of BDP and its metabolites. It should be noted that sample injections were performed at a low flow-rate (0.2 ml min^{-1}) so that high responses of analytes could be achieved, and that separations were performed at a high flow-rate (0.4 ml min^{-1}) for the purpose of shortening the run time while achieving good resolution of analytes by starting the LC gradient at a low percentage of organic solvent. The LC eluent was split using a zero dead-volume T-type PEEK connector, and only one quarter (100 µl min^{-1}) of the LC eluent was allowed to enter the mass spectrometer via an atmospheric pressure ionization (API) interface. The splitting ratio could be modified by adjusting the relative length and/or inner diameter of the PEEK tubing leading to API to that of

the tubing voiding to waste. PEEK tubing of 0.005 in inner diameter was used between the injector valve and the LC column and between the LC column and the ESI probe. The splitting lowered the detection limits of the analytes.

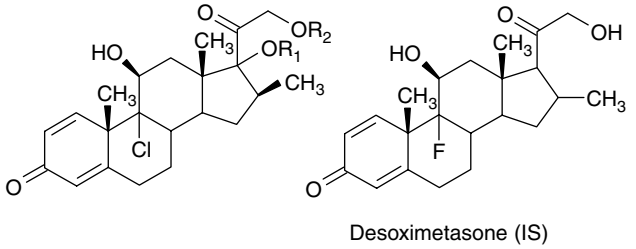
Each dried extract was dissolved in 100 µl of sample solvent (40% methanol in aqueous ammonium formate buffer, 2 mmol l^{-1}, pH 3.4) and an aliquot of 20 µl was injected on to the LC column.

The TSQ Quantum mass spectrometer was equipped with an ESI source and operated in either positive or negative ion mode. The ESI source spray was set orthogonal to the ion transfer capillary tube that guided ion beams into the mass spectrometer. The mass spectrometer was calibrated with a solution of polytyrosine-1,3,6 according to the manufacturer. MS and MS/MS were performed by infusion of the analyte standards in 50% methanol in ammonium formate buffer (2 mmol l^{-1}, pH 3.4) at 5 µl min^{-1}, under the ESI source conditions pre-established for syringe infusion flow-rates. The ESI source parameters were then tuned to accommodate LC flow-rates by syringe infusion of each analyte into LC flow into the ESI source. The ESI source parameters tuned for maximum abundance of $[M+H]^+$ ions of each analyte at the LC flow-rate are shown in Table 2. For quantification, the mass spectrometer was set to the data acquisition mode of MRM and the acquisition parameters are presented in Table 2. The acquisition parameters common to all analytes were as follows: scan width (m/z), 0.80; scan time, 0.5 s; peak width (FWHM), 0.7 for both Q1 and Q3; and collision gas pressure, 1.5 mTorr (1 Torr = 133 pa). Data acquisition and analysis were accomplished with Xcaliber software v.1.3 (Thermo-Finnigan).

Table 1. Gradients of LC mobile phase and flow-rate

LC run time (min)	Formate buffer[a] (%)	Methanol (%)	Flow-rate (µl min^{-1})
0	60	40	200
0.4	60	40	200
0.5	60	40	400
6.0	20	80	400
7.0	10	90	400
7.1	60	40	400
8.8	60	40	400
8.9	60	40	200
9.0	60	40	200

[a] Ammonium formate, 2 mmol l^{-1}, pH 3.4.



Desoximetasone (IS)

Name	Molecular formula	Molecular weight (mono-isotopic)	R1	R2
BOH	$C_{22}H_{29}ClO_5$	408.2	H	H
B17P	$C_{25}H_{33}ClO_6$	464.2	Propionate	H
B21P	$C_{25}H_{33}ClO_6$	464.2	H	Propionate
BDP	$C_{28}H_{37}ClO_7$	520.2	Propionate	Propionate

Figure 1. Structures of BDP and its metabolites and desoximetasone (IS).

Table 2. Parameters for ESI source in positive and negative ion modes and MRM acquisition of BDP and its metabolites[a]

	BOH	IS	B21P	B17P	BDP	BOH	IS
Ionization mode	+	+	+	+	+	−	−
Spray voltage (V)	3800	3800	4300	4300	4200	3000	3000
Sheath gas pressure (psi)	33	33	41	41	37	49	49
Auxiliary valve flow (arbitrary units)	25	25	25	25	25	0	0
Capillary temperature (°C)	275	275	275	275	275	300	300
Tube lens offset (V)	143	143	143	143	143	−105	−105
LC run time (min)	0–5.20	0–5.20	5.21–6.20	5.21–6.20	6.21–8.0	0–6.0	0–6.0
Ion transition (m/z) for quantification (collision energy, V)	409.3 → 279.2 (24)	377.3 → 339.3 (18)	465.3 → 237.1 (28)	465.3 → 337.2 (18)	521.4 → 301.2 (26)	453.1 → 377.3 (18)	421.1 → 355.2 (24)
Ion transitions (m/z) for MRM confirmation (collision energy, V)	409.3 → 355.2 (18) 409.3 → 391.3 (18)			465.3 → 337.2 (18) 465.3 → 373.2 (18)	521.4 → 337.2 (26) 521.4 → 429.2 (18)		

[a] The ESI source conditions including spray voltage, sheath gas pressure, auxiliary valve flow and tube lens offset were tuned for BOH, B17P and BDP, respectively, at an LC flow-rate of 100 µl min^{-1}.

RESULTS

Positive ion MS and MS/MS

The ESI (+) mass spectra of BDP and its metabolites were compared regarding the formation of $[M + H]^+$ ions from methanol–water (50:50, v/v) containing different buffers. In the sample solvent containing 0.1% formic acid, BDP and its metabolites formed $[M + H]^+$, $[M + Na]^+$ and $[M + K]^+$ ions, as indicated by the mass spectra (Fig. 2). In Fig. 2 (a), the $[M + Na]^+$ at m/z 543 was the most abundant ion while $[M + H]^+$ at m/z 521 and $[M + K]^+$ at m/z 559 were weak. The same observation was true for B21P adduct ions (Figure 2(c)). In the mass spectra of B17P and BOH (Figure 2(b) and (d)), the $[M + H]^+$, $[M + Na]^+$ and $[M + K]^+$ ions had comparable abundances. We recognize that the formation of $[M + Na]^+$ and $[M + K]^+$ adduct ions is undesirable for the quantification of very low concentrations of analytes and only the formation of $[M + H]^+$ ion is most desirable. However, when 0.1% formic acid was substituted by ammonium formate (2 mmol l^{-1}, pH 3.4) in methanol–water (50:50, v/v) sample solvent, the $[M + H]^+$ ions became predominant in the mass spectra, but the sodium and potassium adduct ions were less visible (Fig. 2(e), (f), (g) and (h)). Similar results were obtained from the sample solvent containing ammonium acetate (2 mmol l^{-1}, pH 4.6 or 6.7). Based on the selective formation of $[M + H]^+$ ions, ammonium formate buffer was chosen as an additive to the LC mobile phase.

Under LC/MS conditions, BDP and its metabolites formed undesirable positive adduct ions with acetonitrile from the LC mobile phase. As shown in Fig. 3(a) and (c), the mass spectra of B17P showed a strong solvent adduct ion $[M + H + CH_3CN]^+$ (m/z 506.5) formed with acetonitrile in atmospheric pressure chemical ionization (APCI) and ESI sources, respectively. On the other hand, methanol formed less abundant solvent adduct ions $[M + H + CH_3OH]^+$ (m/z 497.5) with B17P in APCI and ESI sources than acetonitrile (Fig. 3(b) and (d)). Acetonitrile also formed, with BOH, a strong adduct ion $[M + H + CH_3CN]^+$ (m/z 450.5) in APCI and ESI sources (Fig. 3(e) and (f)), but methanol formed less solvent adduct ion $[M + H + CH_3OH]^+$ (m/z 441.5). Interestingly, the solvent adduct ions were not observed under syringe infusion conditions where the flow-rate (5 µl min^{-1}) was much lower than that of LC. The observations described above may indicate that the solvent adduct ions of B17P and BOH were not thermally unstable since they were observed under APCI conditions with a vaporizer temperature at 400 °C but could not be desolvated by the 'ion transfer capillary' (a desolvation device) at 275 °C under ESI conditions. Based on these observations, methanol was chosen over acetonitrile as the organic solvent for LC mobile phase.

The collision-induced dissociation (CID) of the $[M + H]^+$ ions of BDP, B17P, B21P, BOH and IS was performed

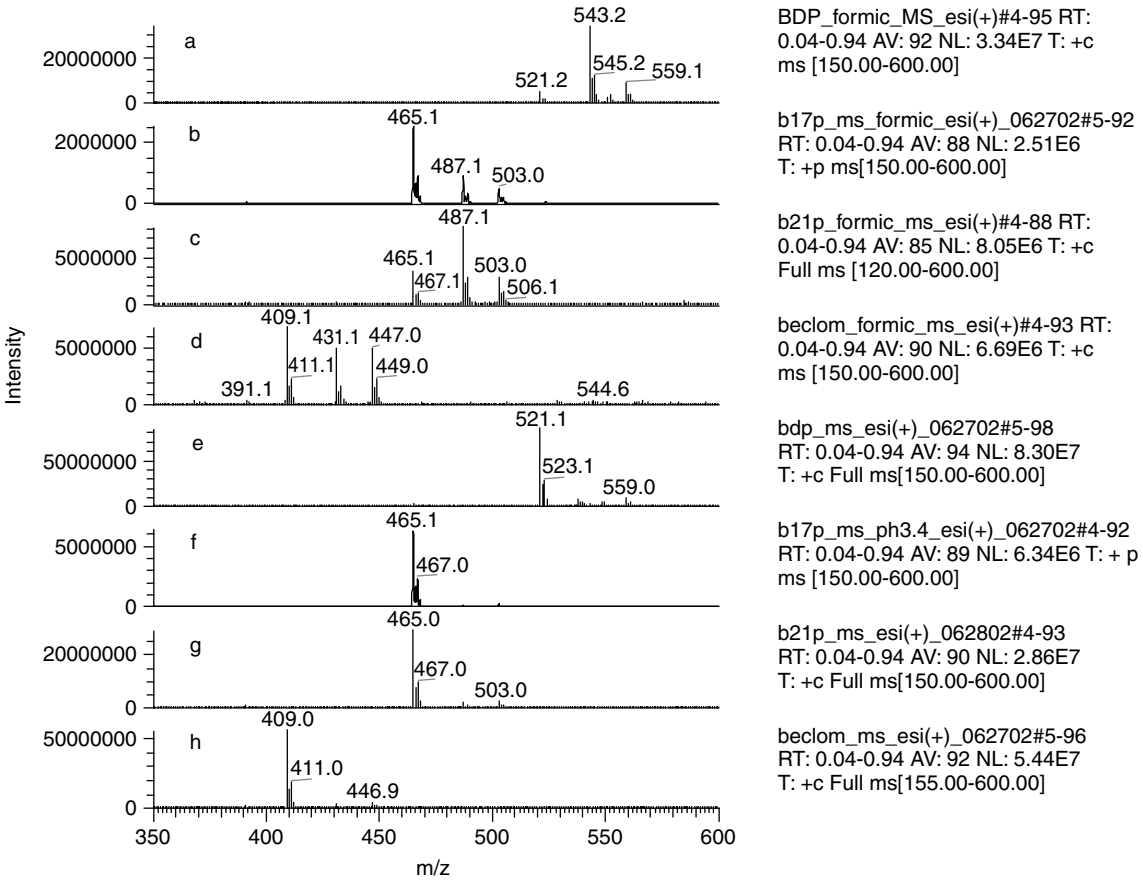


Figure 2. ESI (+) mass spectra of BDP (a, e), B17P (b, f), B21P (c, g) and BOH (d, h) acquired on LCQ Deca XP Plus ion trap mass spectrometer by infusion at 5 µl min^{-1} of 20 µg ml^{-1} of each analyte in methanol–water (50 : 50, v/v) containing 0.1% formic acid (a, b, c and d) and 2 mmol l^{-1} ammonium formate (e, f, g and h).

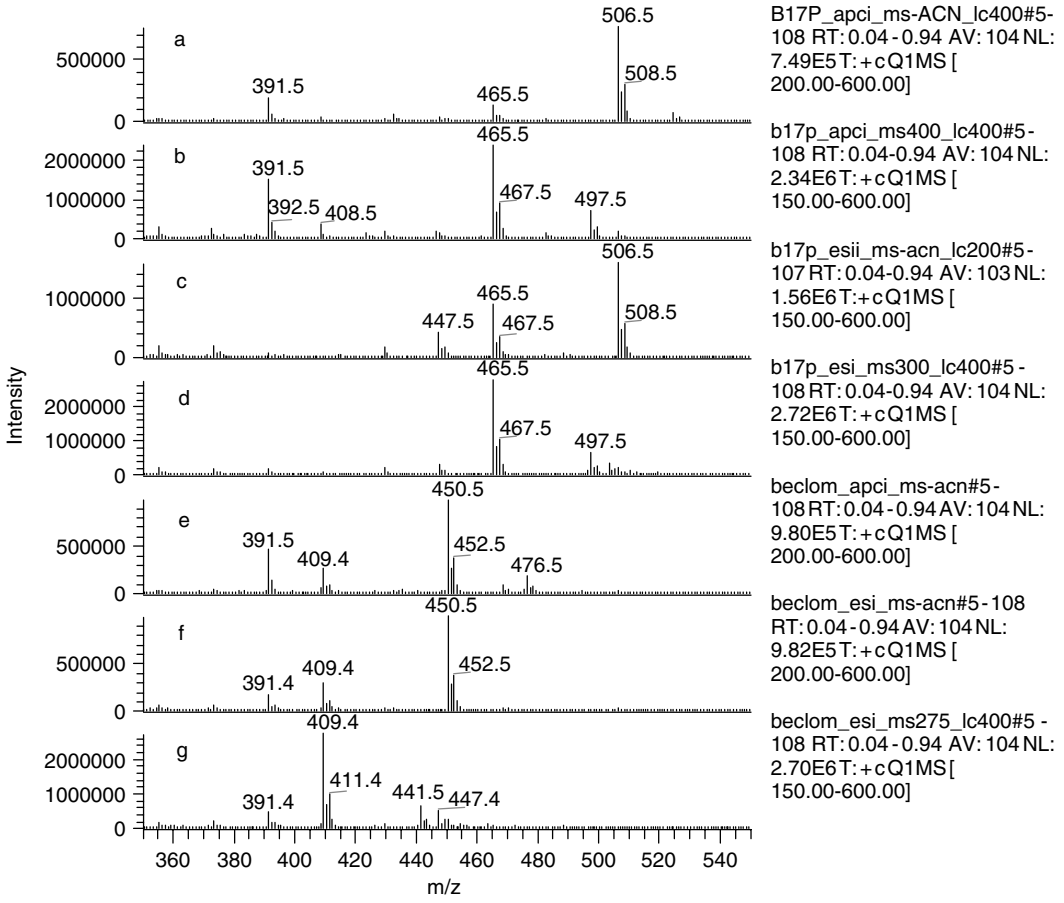


Figure 3. Mass spectra of B17P and BOH showing the solvent adduct ions for B17P at *m/z* 506.5 for $[M + H + CH_3CN]^+$ in (a) and (c) and *m/z* 497.5 for $[M + H + CH_3OH]^+$ in (b) and (d) together with $[M + H]^+$ ion at *m/z* 465.5 and the solvent adduct ions for BOH at *m/z* 450.5 for $[M + H + CH_3CN]^+$ in (e) and (f) and *m/z* 441.5 for $[M + H + CH_3OH]^+$ in (g) together with $[M + H]^+$ ion at *m/z* 409.4. The mass spectra were acquired on the TSQ Quantum triple quadrupole mass spectrometer: (a) acquired with APCI (+) source by infusion of 20 μg ml^{-1} of B17P at 5 μl min^{-1} into an LC flow of 400 μl min^{-1} of acetonitrile–water (50 : 50, v/v) containing 2 mmol l^{-1} ammonium formate (pH 3.4), capillary temperature 200 °C, vaporizer temperature 400 °C; (b) acquired under the same conditions as in (a) except for methanol in place of acetonitrile; (c) acquired with ESI (+) source by infusion of 20 μg ml^{-1} of B17P at 5 μl min^{-1} into an LC flow of 400 μl min^{-1} of acetonitrile–water (50 : 50, v/v) containing 2 mmol l^{-1} of ammonium formate (pH 3.4), capillary temperature 275 °C; (d) acquired under the same conditions as in (c) except for methanol in place of acetonitrile; (e) acquired under the same conditions as in (a) except for BOH in place of B17P; (f) acquired under the same condition as in (c) except for BOH in place of B17P; and (g) acquired under the same conditions as in (d) except for BOH in place of B17P.

for the purpose of selecting abundant product ions for MRM detection. Selection of abundant product ions and optimization for their yield against collision energy were automatically conducted by the instrument control software. At low collision energy (18 V), the $[M + H]^+$ ions of BDP, B17P, B21P and BOH all fragmented to $[M + H - H_2O]^+$ ions at *m/z* 503.3, 447.3 and 391.3, respectively, and they were dominant in the spectra (Fig. 4(a), (c) and (e)). As the collision energy was increased, the $[M + H]^+$ ions fragmented to multiple product ions (Fig. 4(b), (d) and (f)). CID fragmentation of the $[M + H]^+$ ions involved loss of multiple water molecules and a hydrochloride molecule, and in some fragmentation steps it was difficult to differentiate the consecutive loss of two water molecules (36 Da) from the loss of a hydrochloride molecule (also 36 Da). However, BDP and its metabolites contain a chlorine atom in their molecules that has two isotopes with comparable abundances, and the loss of an isotopic hydrochloride molecule ($H^{37}Cl$, 38 Da) could be differentiated from the consecutive loss of two water molecules (36 Da). From the tandem mass spectra of the isotopic $[M + H]^+$ ions containing a ^{37}Cl atom (Fig. 5), the fragmentation paths of BDP and its metabolites were proposed (Fig. 6). The fragmentation paths were confirmed by MS/MS/MS of the product ions from BDP and its metabolites performed on the LCQ-XP Plus ion trap instrument that produced further fragmentation of the product ions. The common product ion at *m/z* 279 from fragmentation of the $[M + H]^+$ ions of BOH, B17P and BDP and that at *m/z* 237 from BOH and B21P (Fig 4 and 5) were not included in the proposed fragmentation paths, and they were formed by rearrangement.[1]

Negative ion MS and MS/MS

In addition to the positive $[M + H]^+$ ions formed in the ESI (+) source, BDP and its metabolites formed, in the ESI (−) source, adduct negative ions with formate from the LC

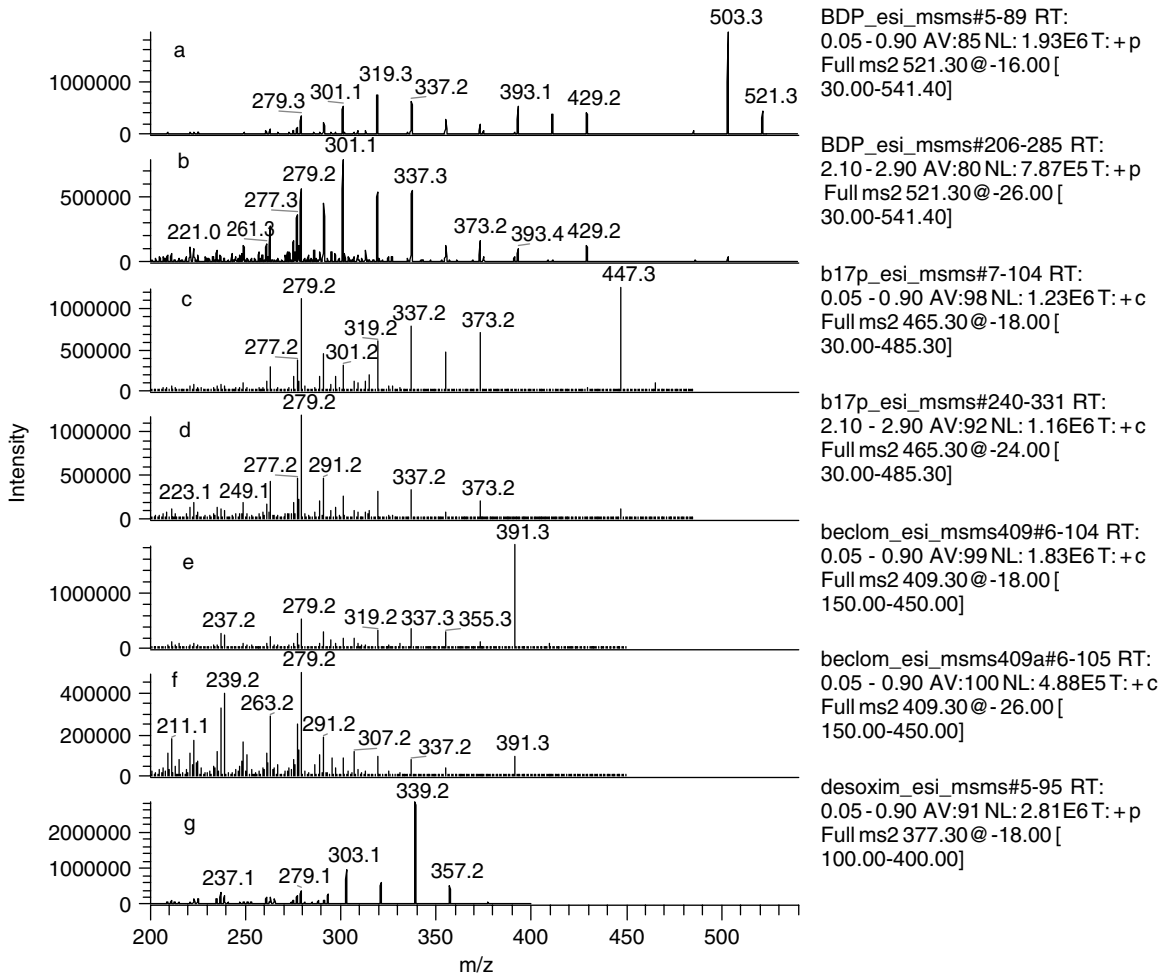


Figure 4. ESI (+) tandem mass spectra of BDP at collision energies (CE) of (a) 16 and (b) 26 V, B17P at CE of (c) 18 and (d) 24 V, BOH at CE of (e) 18 and (f) 26 V and (g) IS at CE of 18 V. These mass spectra were acquired on the TSQ Quantum triple-quadrupole mass spectrometer with infusion of 1.0 μg ml^{-1} of each analyte in methanol–water (50 : 50, v/v) containing 2 mmol l^{-1} ammonium formate (pH 3.4) at 5 μl min^{-1}.

mobile phase. $[M + formate]^-$ ion was the only abundant ion observed in each mass spectrum of BDP, B17P and BOH (Fig. 7(a), (b) and (c)), and there was no apparent formation of $[M - H]^-$ ion or other adduct or fragment ions. Under CID conditions, the $[M + formate]^-$ ions of BDP and B17P underwent fragmentations to produce $[M - H]^-$ ions as the predominant ions (Fig. 7(d) and (e)). However, fragmentation of the $[M + formate]^-$ ion of BOH produced an abundant ion at m/z 377 and a minor $[M - H]^-$ ion at m/z 407 (Fig. 7(f)). It was interesting that there were differences between the tandem mass spectra of $[M + formate]^-$ of BOH acquired on an ion trap instrument (Fig. 7(f)) and on a triple-quadrupole instrument (Fig. 7(g)). In Fig. 7 (g), the product ions at m/z 407, 377, 341 and 297 correspond to $[M - H]^-$, $[M - H - CH_2O]^-$, $[M - H - CH_2O\text{-}HCl]^-$ and $[M - H - CH_2O - HCl - CO_2]^-$, respectively. The product ion at m/z 377 ($[M - H - CH_2O]^-$) may have resulted from the loss of formaldehyde (CH_2O) involving cleavage of the bond between C_{20} and C_{21} in BOH molecule. These fragmentation patterns were in accordance with those of other corticosteroid compounds reported in the literature that the $[M + CH_3COO]^-$ ions of corticosteroid compounds with a 17α-hydroxyl group fragmented to $[M - H - CH_2O]^-$ whereas those of the compounds without a 17α-hydroxyl group did not.[7] In short, only the $[M + formate]^-$ ion of BOH fragmented to product ions (other than $[M - H]^-$ ion) that could be used for MRM quantification.

LC/MRM for quantification and confirmation

The resolution of BDP and its metabolites from each other and from endogenous components in sample matrices was achieved on a reversed-phase C_8 column with gradient elution. The MRM detection was initially performed on the ion transition $[M + H]^+$ to $[M + H - H_2O]^+$ for each analyte, and the MRM on these ion transitions was expected to produce high detection sensitivity because of the high yield (or abundance) of the $[M + H - H_2O]^+$ product ions as indicated in fig. 4(a), (c) and (e). However, when the extracts of plasma samples were analyzed with the MRM on the ion transitions $[M + H]^+$ to $[M + H - H_2O]^+$, the background noise was high and the detection limit for BDP, B17P and B21P was not appreciably low, which implies low specificity of MRM on the ion transitions. This observation is explained by the fact that loss of a water (H_2O) molecule is common among $[M + H]^+$ ions that contain hydroxyl groups. Three other abundant product ions of the $[M + H]^+$

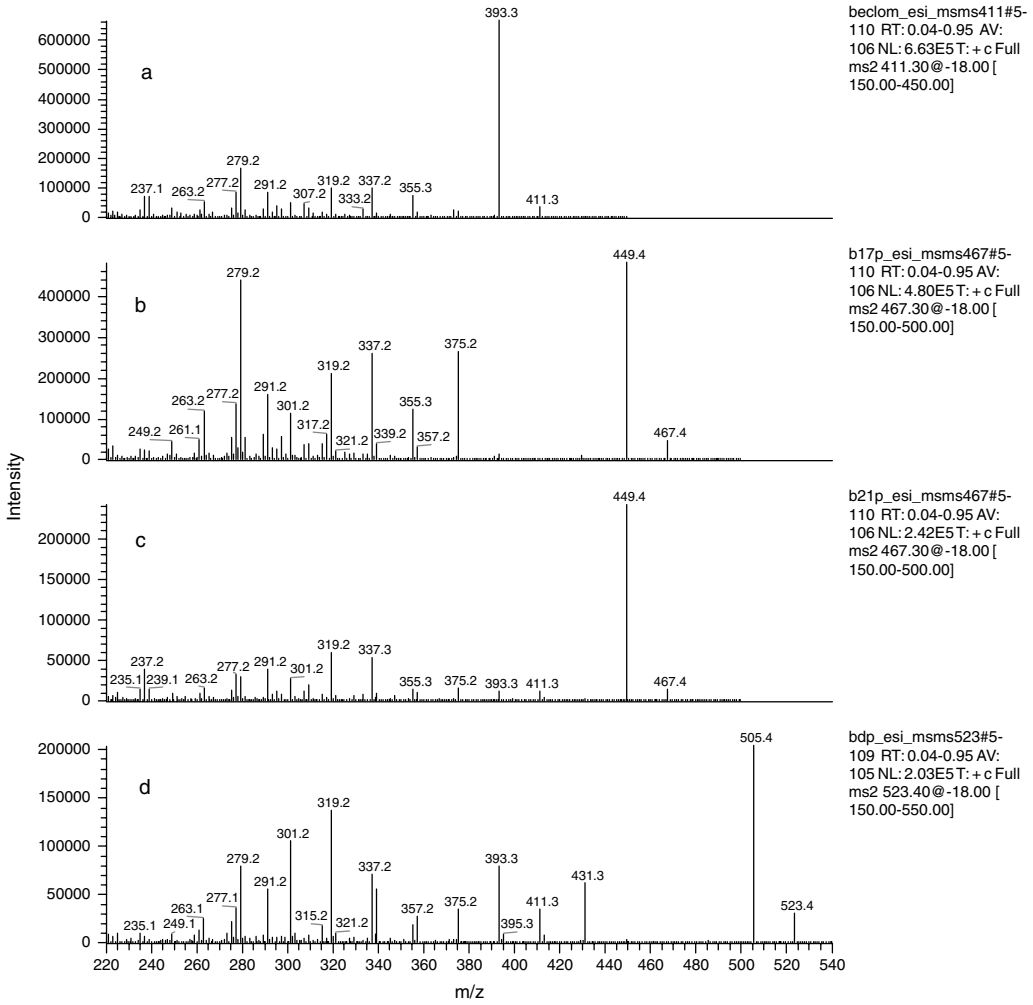


Figure 5. ESI (+) tandem mass spectra of the isotopic $[M + H]^+$ ions containing a ^{37}Cl atom of (a) BOH, (b) B17P, (c) B21P and (d) BDP at collision energies of 18 V acquired under the same conditions as in Fig. 4.

ion of each analyte were chosen and evaluated for MRM quantification and confirmation (Table 2). Quantification was performed by internal standard calibration. For confirmation by triple-quadrupole instruments, it is generally acceptable that chromatographic peaks detected by MRM on three ion transitions at the same retention time are sufficient to confirm the presence of a compound in a test sample. As shown in Fig. 8, the lowest confirmable concentration was 25 pg ml^{-1} for B17P and BDP in plasma.

Preliminary results showed that the ESI (+) LC-MRM method described above was not sufficiently sensitive for the detection of BOH in urine samples. Based on the ESI (−) MS and MS/MS of BOH described earlier, ion transitions m/z 453 → 407, 453 → 377 and 453 → 341 were evaluated for ESI (−) MRM of BOH in urine. The ion transition m/z 453 → 377 was the only one that resulted in a chromatographic peak at a retention time of 4.46 min with low background noise and high signal-to-noise ratio. Hence this ion transition was used for quantification of BOH in equine urine.

Extraction

BDP and its metabolites are neutral molecules and are extractable from acidic, neutral and basic solutions. In preliminary experiments, diethyl ether, MTBE, dichloromethane and 1-chlorobutane were compared for extraction of BDP and its metabolites from equine plasma samples without pH adjustment. MTBE extraction resulted in the highest extraction recovery and cleaner extracts than with other solvents. The extraction efficiency by MTBE from equine plasma is shown in Table 3, and was >60% for all the analytes.

For ESI (+) MRM of BDP and B17P, urine samples were adjusted with acetate buffer to pH ≈ 5 before MTBE extraction so that the extraction of undesirable endogenous basic compounds in urine was avoided. The extraction efficiency was >89% for both analytes (Table 4).

For ESI (−) MRM of BOH from urine, MTBE extraction of urine samples without pH adjustment resulted in cleaner extracts and therefore a lower detection limit than extraction of urine samples pre-adjusted to pH 5 with acetate buffer. The extraction efficiency of BOH was >94% (Table 4).

Stability of BDP and its metabolites

BDP contains two ester groups in its molecule and is a good candidate for hydrolysis to monoester B17P and B21P by plasma esterases, and B17P and B21P might be further hydrolyzed by plasma esterases to BOH. Therefore, it was necessary to evaluate the stability or decomposition of BDP, B17P and B21P in equine plasma at ambient temperature

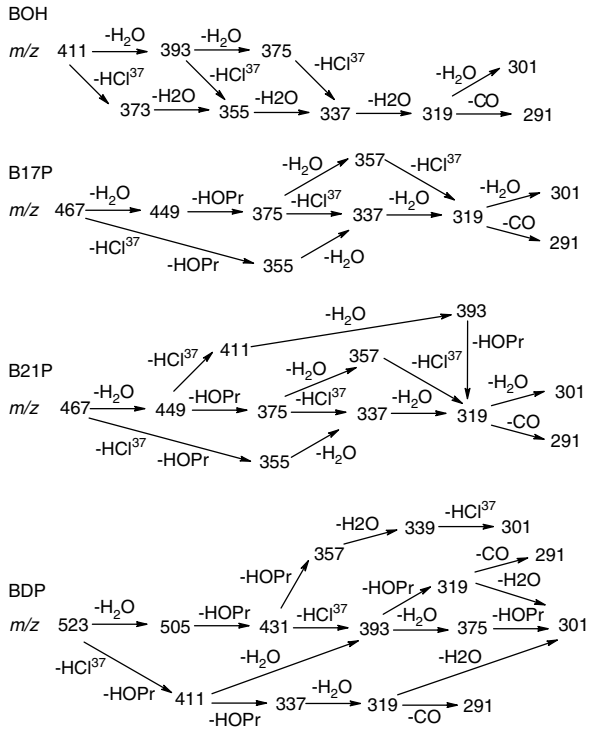


Figure 6. The fragmentation paths proposed for the isotopic $[M + H]^+$ ions of BOH, B17P, B21P and BDP containing a ^{37}Cl atom. HOPr represents propionic acid.

(25 °C) over a period of 2 h. The 2 h time period was chosen because that was the maximum time from start to completion of plasma sample preparations on any given day of sample analysis. The results are shown in Table 5. The concentration of BDP decreased by 36% over 2 h and that of B21P by 86%, but the concentration of B17P did not change over the 2 h period. The results showed that BDP and especially B21P were not stable but B17P was stable in equine plasma at ambient temperature during the time period (2 h). The hydrolysis product of BDP in equine plasma was B17P under the experimental conditions, and that of B21P was BOH. To prevent the hydrolysis of BDP and B21P, NaF was added to equine plasma to inhibit plasma esterases since it has been reported to prevent the hydrolysis of procaine in equine plasma.[19,20] With the addition of NaF (10 mg ml^{-1}), the concentrations of BDP and B21P in equine plasma decreased by <6% over 2 h (Table 5), and the IS (similar to BOH in structure) concentration also remained unchanged during the same time period. These results suggested that NaF inhibited the hydrolysis of BDP and B21P in equine plasma due to inhibition of plasma esterases. Therefore, NaF (10 mg ml^{-1}) was added to negative plasma used in preparing calibrators or QC samples and to the tubes for collecting plasma samples from horses following the administration of BDP.

The stability of BDP, B17P, B21P and BOH in stock standard solutions at 4 °C over 2 months was evaluated

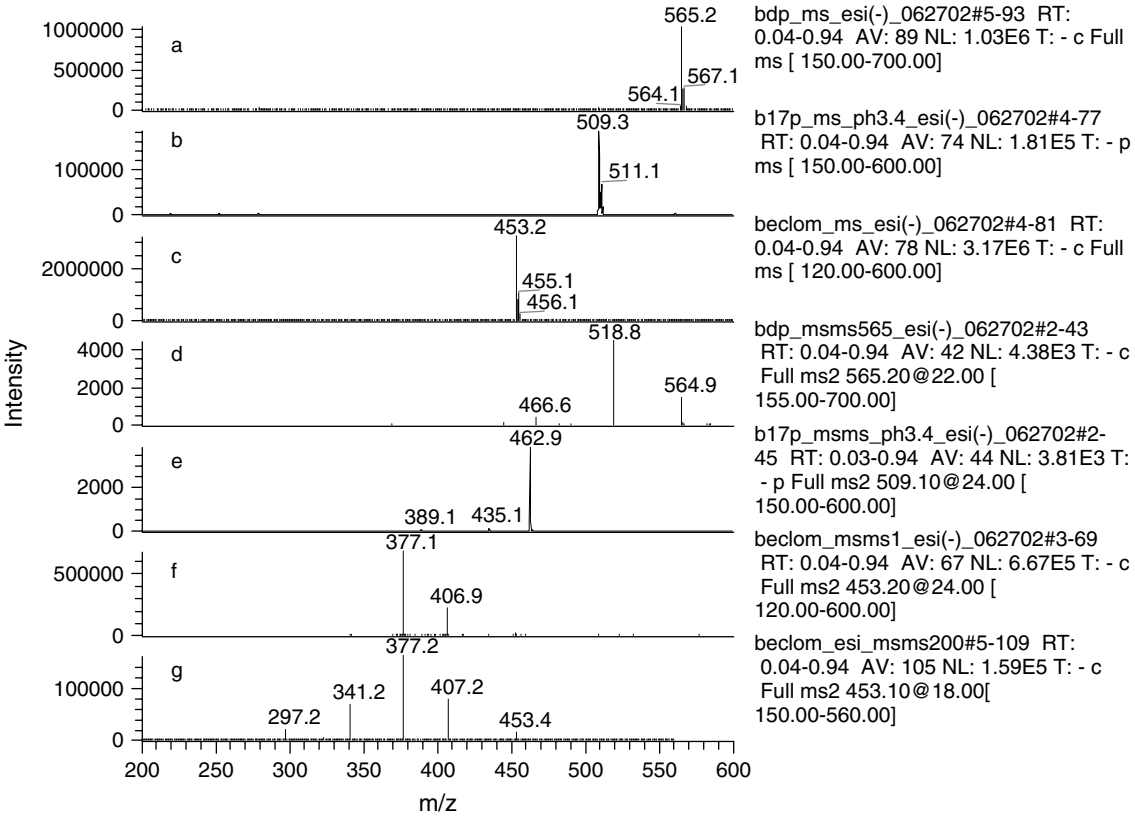


Figure 7. ESI (−) mass spectra of (a) BDP, (b) B17P and (c) BOH and tandem mass spectra of (d) BDP at 22% relative collision energy (RCE), (e) B17P at 24% RCE and (f) BOH at 24% RCE, acquired on the LCQ Deca XP Plus ion trap mass spectrometer with infusion at 5 μl min^{-1} of 20 μg ml^{-1} of each analyte in methanol–water (50 : 50, v/v) containing 2 mmol l^{-1} ammonium formate (pH 3.4). ESI (−) tandem mass spectrum of (g) BOH at collision energy of 18 V acquired on the TSQ Quantum triple-quadrupole mass spectrometer by infusion of BOH (20 μg ml^{-1} at 5 μl min^{-1}) into an LC flow of 200 μl min^{-1}.

Table 3. Extraction recovery, accuracy[a] and precision (RSD)[b] for quantification of BOH, B17P and BDP in plasma ($n = 6$)

	Added (pg/ml)	Extraction efficiency [c] (± SD, %)	Intra-day Detected (± SD, pg ml^{-1})	Intra-day Accuracy (%)	Intra-day RSD (%)	Inter-day Detected (± SD, ng ml^{-1})	Inter-day Accuracy (%)	Inter-day RSD (%)
BOH	50	84 ± 7.3	40 ± 3.8	80	9.4	41 ± 10	82	24
	250	104 ± 4.6	269 ± 12	108	4.5	251 ± 17	100	6.9
	1250	96 ± 3.7	1259 ± 49	101	3.9	1188 ± 43	95	3.6
B17P	50	60 ± 18	39 ± 5.7	78	14	46 ± 6.9	92	15
	250	93 ± 5.0	240 ± 12	96	5.1	260 ± 21	104	8.2
	1250	98 ± 4.5	1218 ± 55	97	4.5	1193 ± 71	95	5.9
BDP	50	60 ± 12	58 ± 7.3	116	13	49 ± 9.1	98	18
	250	78 ± 5.1	255 ± 15	102	6.0	276 ± 32	110	12
	1250	83 ± 3.9	1267 ± 58	101	4.5	1160 ± 152	93	13

[a] Accuracy = quantified/added × 100.

[b] RSD = standard deviation of the quantified/mean of the concentration quantified × 100.

[c] Extraction efficiency = peak area of the extracted/peak area of the standard spiked into the extract of negative plasma × 100.

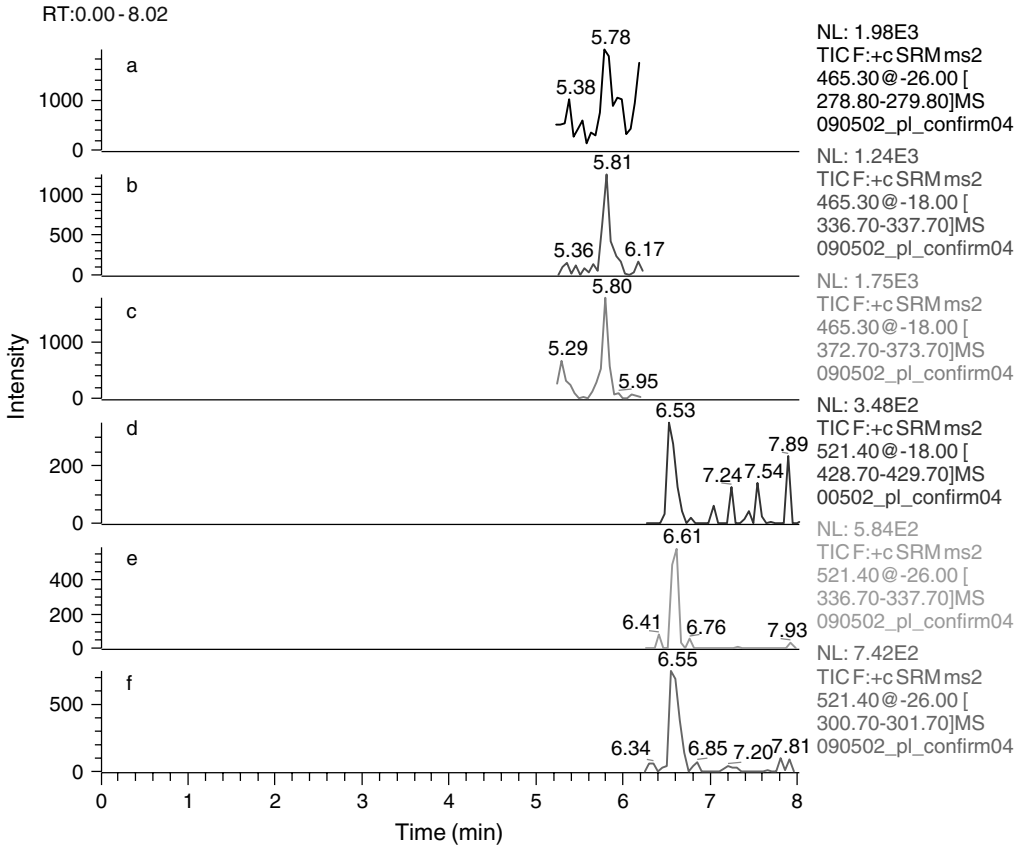


Figure 8. ESI (+) LC/MRM chromatograms showing confirmation of B17P and BDP at 25 pg ml^{-1} in equine plasma. Graphs (a), (b) and (c) show the three ion transitions *m/z* 465 → 279, 465 → 337 and 465 → 373 for confirmation of B17P and (d), (e) and (f) show the three ion transitions *m/z* 521 → 429, 521 → 337 and 521 → 301 for confirmation of BDP. For details on the collision energy for each ion transition, see Table 2.

using the LC/MS method developed in this study. At the concentration of 1.00 mg ml^{-1} in methanol, <4% of BDP was converted to B17P, and <2% of B21P was converted to BOH. At a concentration of 100 ng ml^{-1} in methanol, <3% of BDP was converted to B17P and <2% of B21P was converted to BOH. Conversion of BDP to B21P or B17P to BOH was not observed under the experimental conditions of this study.

The stability of the extracted BDP, B17P, B21P, BOH and IS in the LC sample solvent at ambient temperature over 24 h was also evaluated. They were stable under the experimental conditions.

Matrix effects — ion suppression or enhancement

In API, endogenous components extracted from a sample matrix might suppress or enhance ionization of analytes

Table 4. Extraction recovery, accuracy[a] and precision (RSD)[b] for quantification of BOH, B17P and BDP in urine ($n = 6$)

	Added (pg ml^{-1})	Extraction efficiency[c] (± SD, %)	Intra-day			Inter-day		
			Detected (± SD, pg/ml)	Accuracy (%)	RSD (%)	Detected (± SD, ng/ml)	Accuracy (%)	RSD (%)
BOH[d]	50	99 ± 8.8	43.4 ± 4.4	86	10	49.4 ± 5.9	98	12
	250	94 ± 5.0	255 ± 12.6	102	4.9	238 ± 16.1	95	6.8
B17P[e]	100	113 ± 33	112 ± 37	112	33			
	500	101 ± 6.6	541 ± 25	108	4.6	505 ± 37	101	7.3
	2500	107 ± 4.2	3013 ± 89	121	3.0	2522 ± 68	101	2.7
BDP[e]	100	92 ± 11	87 ± 10	87	12			
	500	104 ± 3.6	526 ± 16	105	3.1	492 ± 37	98	7.4
	2500	104 ± 4.0	2756 ± 86	110	3.1	2356 ± 135	94	5.7

[a] Accuracy = quantified/added × 100.

[b] RSD = standard deviation of the quantified/mean of the concentration quantified × 100.

[c] Extraction efficiency = peak area of the extracted/peak area of the standard spiked into the extract of negative urine × 100.

[d] BOH spiked into urine was quantified by ESI (−) LC/MRM.

[e] B17P and BDP spiked into urine were quantified by ESI (+) LC/MRM.

Table 5. Percentage decrease in concentrations of BDP, B17P, B21P and IS in equine plasma with or without NaF (10 mg ml^{-1}) at ambient temperature within a 2 h period

	Plasma alone	Plasma + NaF (10 mg ml^{-1})	Hydrolysis product
BDP	36	5	B17P
B17P[a]	0	1.4	
B21P	86	5.8	BOH
IS	0	0	

[a] The presence or absence of NaF did not affect the concentration of B17P in plasma.

Table 6. Ion suppression (%) showing 'matrix effect' for ESI (+) and (−)-LC/MRM of BDP, B17P, B21P, BOH and IS

	BOH	IS	B21P	B17P	BDP
Plasma[a]	0.0	3.0	−5.8	15.1	21.5
Urine[b]	16	7.0	—	—	—

[a] For plasma samples, the ion suppression was evaluated at concentrations of 1000 pg per 2 ml for BDP, B17P, B21P and BOH and 2500 pg per 2 ml for IS, and it was calculated from the peak areas averaged from three duplicates of the standards and those added to plasma extracts. The analytes were quantified by ESI (+) LC-MRM.

[b] For urine samples, the ion suppression was evaluated at the concentration of 100 pg ml^{-1} for BOH and 5000 pg ml^{-1} for IS, and it was calculated from the peak areas averaged from six duplicates of the standards and those added to urine extracts. BOH and IS were quantified by ESI (−) LC/MRM.

recovered from that matrix and, thus, affect the signal intensities of the analytes. Matrix effect may occur in plasma, urine and other biological samples during analysis. We examined the matrix effect on the analytes of interest. As a measurement of the matrix effect, ion suppression could be calculated by comparing the chromatographic peak areas of each of BDP, B17P, B21P and BOH standards with those of the standards added to the extracts of 2 ml of negative plasma, according to the following equation:

$$\text{Ion suppression}(\%) = [1 - (A_{\text{extr}}/A_{\text{st}})] \times 100$$

where A_{st} is the peak area of a certain quantity of a drug standard and A_{extr} is the peak area of the same quantity of the drug standard added to the extract of a plasma or urine sample. As shown in Table 6, the ion suppression or enhancement by plasma or urine was <22% for all the analytes, which means that the matrix effect was not severe under the experimental conditions. It should be pointed out that ion enhancement effect by plasma was observed for B21P.

The matrix effect might affect the sensitivity of an analytical method and its reproducibility. One way to eliminate or minimize the matrix effect is to obtain clean sample extracts that contain less endogenous impurities. Another way is to achieve better LC resolution of the analytes from the impurities so that they do not interfere with ionization of the target analytes. We paid meticulous attention to this problem by using very clean extracts by MTBE. The LC separation time was increased from 7 to 9 min in an effort to minimize the matrix effect. No further effort was exercised to eliminate it, since the ion suppression was <22% and the precision of the method was acceptable (Tables 3 and 4).

Validation of the method

Based on the experiments and observations described above, the LC/MRM method for detection and quantification of BDP and its metabolites in equine plasma involved extraction of the analytes from plasma samples by MTBE, separation on a C_8 column, ESI in the positive ion mode and MRM on the ion transitions for each analyte shown in Table 2. Figure 9 shows the chromatographic peaks of BOH, B21P, B17P and BDP spiked into negative equine plasma and extracted prior

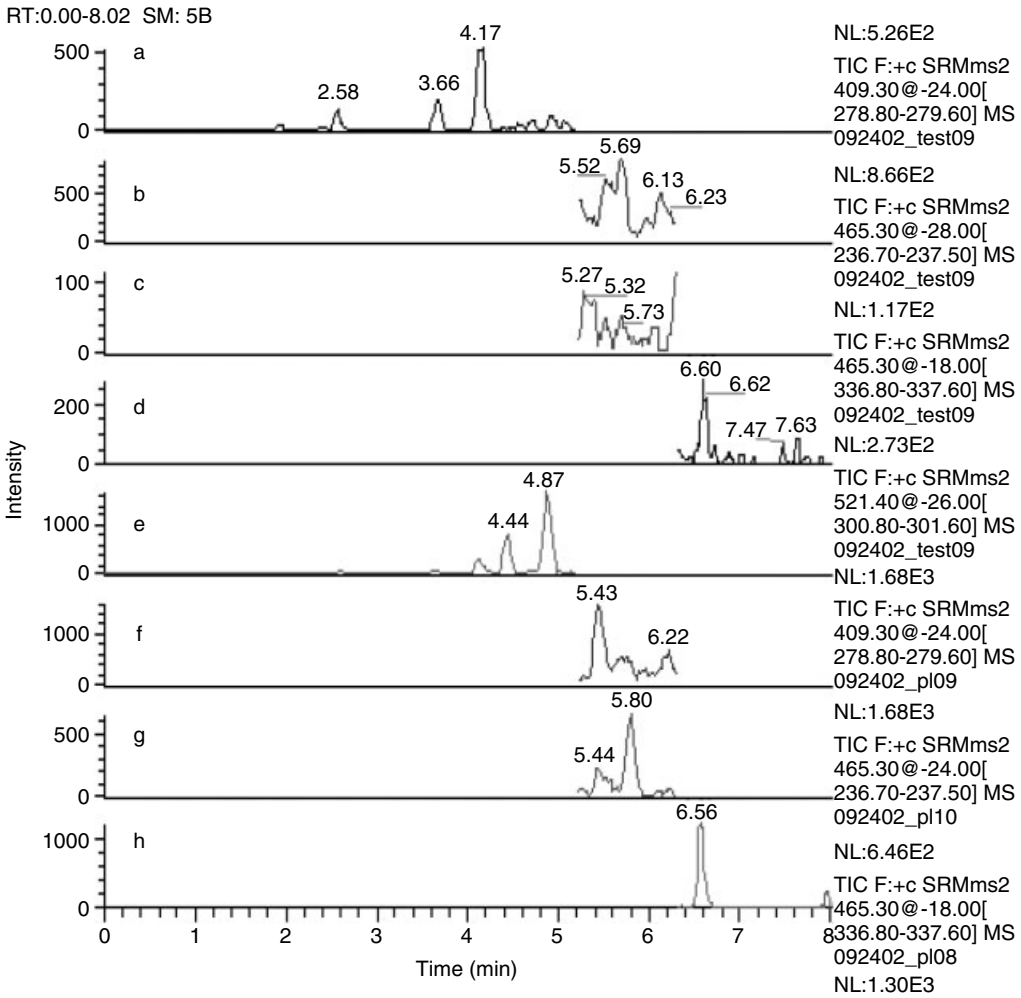


Figure 9. ESI (+) LC/MRM chromatograms for negative and spiked equine plasma samples (2 ml) showing the chromatographic peaks of BOH (50 pg ml^{-1} spiked), B21P (100 pg ml^{-1} spiked), B17P and BDP (25 pg ml^{-1} each spiked) at retention times of 4.44, 5.43, 5.80 and 6.56 min, respectively, in (e), (f), (g) and (h). Graphs (a), (b), (c) and (d) show the absence of BOH, B21P, B17P and BDP from the negative plasma.

to analysis. All the analytes were chromatographically well separated. The method was also applied to the detection and quantification of BOH in equine urine samples, using ESI (−) MRM instead of ESI (+) MRM. Figure 10(a) and (b) show the ESI (−) MRM chromatograms of negative equine urine and that spiked with BOH, respectively.

The method was evaluated with regard to the limit of detection, limit of confirmation, quantification range, precision and accuracy. The limit of detection was 13 pg ml^{-1} for BDP and B17P, 25 pg ml^{-1} for BOH, and 50 pg ml^{-1} for B21P in plasma, and 25 pg ml^{-1} for BOH and 50 pg ml^{-1} for B17P and BDP in urine. The quantification range was 25–2500 pg ml^{-1} for BDP, B17P and BOH and 125–2500 pg ml^{-1} for B21P in plasma, 25–1000 pg ml^{-1} for BOH and 100–2500 pg ml^{-1} for B17P and BDP in urine, with correlation coefficients of the calibration curves >0.99. The limit of confirmation was 25 pg ml^{-1} for BDP and B17P and 50 pg ml^{-1} for BOH in plasma. The accuracy and intra-day and inter-day precision are shown in Tables 3 and 4 for plasma and urine samples, respectively. The quantification accuracy was within the range 78–116% for all the analytes in plasma and urine, which was satisfactory for quantification at very low concentrations. The intra-day relative standard deviation (RSD) was <16% for all the analytes and the inter-day RSD was <24%.

The 'carryover' (or memory effect) is a concern in the quantification of very low concentrations of analytes. We therefore evaluated the method for carryover. No carryover effect was observed for any of the analytes immediately after analysis of the highest concentrations in the quantification ranges.

Analysis of equine plasma and urine samples after administration of BDP to horses by inhalation

The method described was applied to the analysis of equine plasma and urine samples obtained following the administration of BDP by inhalation to research horses. At a therapeutic dose of BDP (325 μg) administered once, only B17P, the major metabolite of BDP, was detected and quantified in plasma (Fig. 11). The detection and quantification results for BDP, B17P, and BOH in equine plasma samples obtained from four horses post-administration of BDP are summarized in Table 7. B17P was detected and quantified in

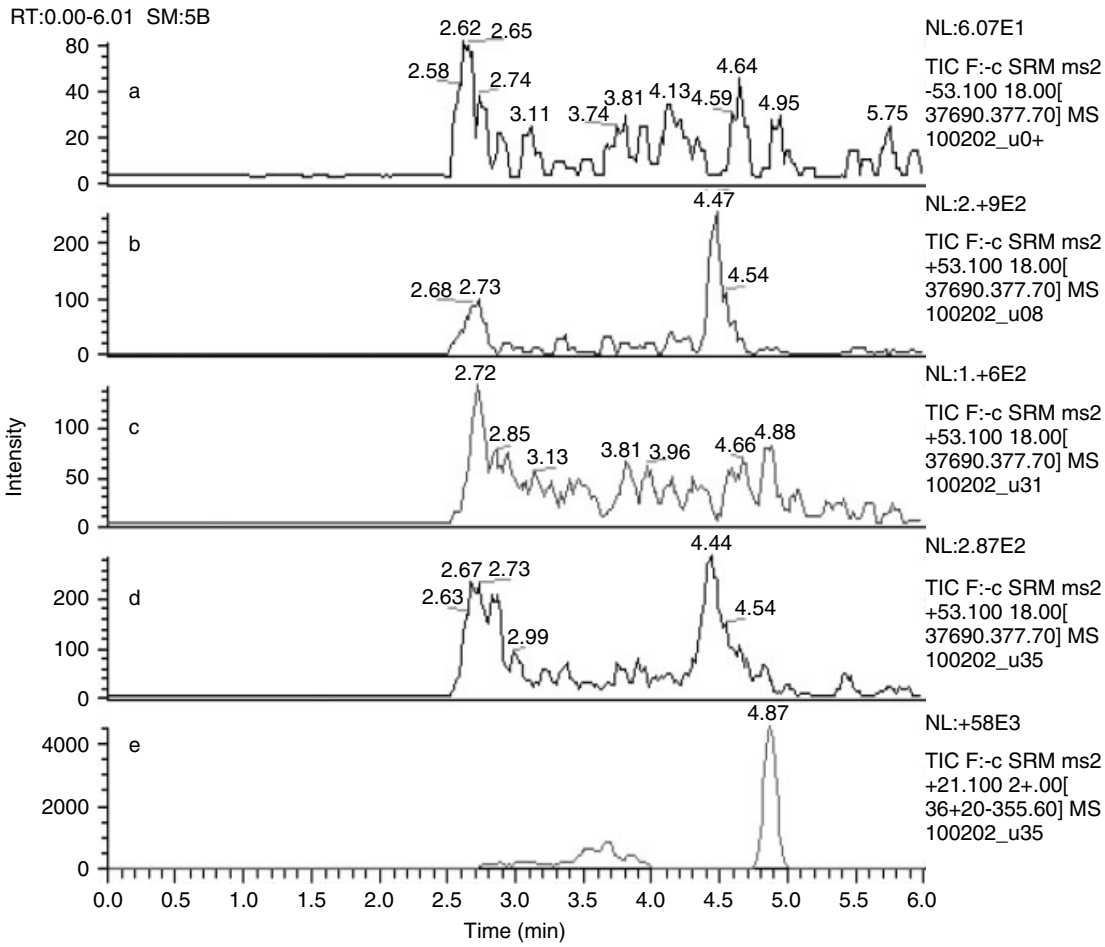


Figure 10. ESI (−) LC/MRM chromatograms for (a) negative urine, (b) negative urine spiked with 25 pg of BOH and the urine samples (1 ml) collected at (c) 0 h and (d) 2 h after the last dose of a 3-day administration of BDP (therapeutic dose of 325 μg, twice a day). Graph (e) shows the chromatographic peak of IS spiked into the urine sample collected 2 h post-administration. Ion transitions monitored were *m/z* 463 → 377 for (a)–(d) and *m/z* 421 → 355 for (e). The chromatographic peak at a retention time of ~4.4 min indicates the presence of BOH.

plasma up to 4 h post-administration of a single therapeutic dose of BDP by inhalation, up to 6 h after the last dose of the 3-day administration (therapeutic dose, twice per day for 3 days) and up to 10 h post-administration of a single high dose (3 × the therapeutic dose of 325 μg per horse per dose). BDP and BOH were detected in some plasma samples collected at early hours post-administration of the high dose, but their concentrations were low. B21P was not detected in plasma at any time post-administration of BDP.

In urine samples obtained from horses post-administration of BDP at a therapeutic dose or high dose (three times the therapeutic dose) by inhalation, B17P and BDP were not detected by ESI (+) LC-MRM. The absence of B17P and BDP from urine is reasonable if one considers the hydrophobic properties of B17P and BDP and the fact that they may be metabolized to unidentified/unknown metabolites in the horse. The metabolites of BDP in urine have not yet been identified and reported for horses or other species. BOH was expected to be a possible metabolite of BDP in urine. Indeed, a very low concentration of BOH was detected and quantified, by the ESI (−) LC-MRM method, in the urine sample collected 2 h after the last dose of the 3-day administration of BDP (Fig. 10(c) and (d)).

Drugs are sometimes excreted in conjugated form of their metabolites in urine, and the metabolites could be released from conjugates by enzyme hydrolysis. In this study, urine samples that were either enzyme-hydrolyzed or unhydrolyzed were compared for detection and quantification of BOH. BOH was detected and quantified in the unhydrolyzed equine urine up to 10 h post-administration of the high dose (three times the therapeutic dose) of BDP (Table 7). However, enzyme hydrolysis resulted in much higher background noise and a higher detection limit (250 pg ml^{-1}) for urine samples. As a result of this drawback, BOH at very low concentrations that was detected in the unhydrolyzed urine samples could not be detected after enzyme hydrolysis.

BOH is similar to flumethasone in structure. It has been reported that the concentration of free flumethasone in calf urine after administration of flumethasone was only 20–35% lower than the total concentration of its free and conjugate forms,[21] suggesting that flumethasone exists mainly in the free unconjugated form in calf urine. This information might be helpful when one considers the case of BOH as a metabolite of BDP in equine urine.

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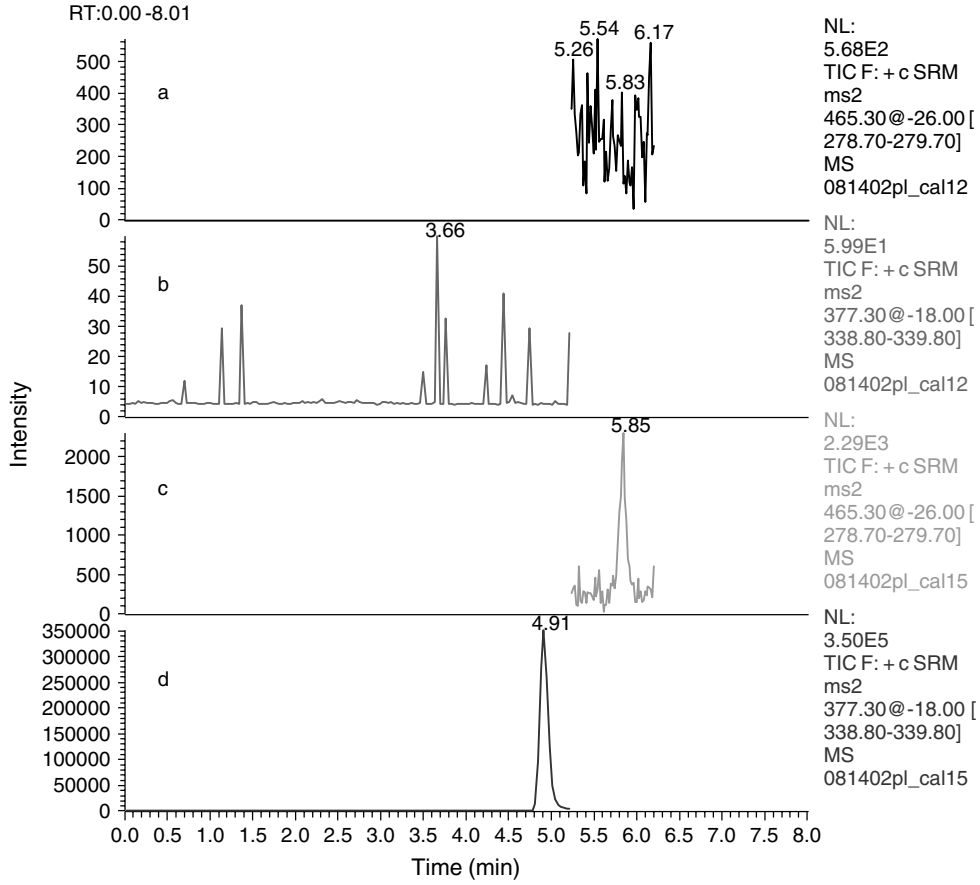

Figure 11. ESI (+) LC/MRM chromatograms for negative and administration equine plasma samples. Graphs (a) and (b) show the absence of B17P and IS from plasma collected at 0 h post-administration of BDP, while (c) indicates the detection of B17P in a plasma sample collected 4 h post-administration of a therapeutic dose (325 μg per horse) of BDP by inhalation and (d) shows the peak of IS spiked into the plasma sample.

Table 7. Quantification results for BOH, B17P and BDP in equine plasma and urine collected at different times post-administration of BDP by inhalation

			Plasma			Urine
Horse No.	Dose (μg)	Time (h)	BOH (pg ml^{-1})	B17P (pg ml^{-1})	BDP (pg ml^{-1})	BOH (pg ml^{-1})
257	325	0	ND[a]	ND	ND	ND
	325	2	ND	15[b]	ND	ND
	325	4	ND	21[b]	ND	ND
	325	6	ND	Detected	ND	ND
	325	24	ND	ND	ND	ND
282	3 × 325	0	ND	ND	ND	ND
	3 × 325	2	ND	229	33	178
	3 × 325	4	26	148	21[b]	139
	3 × 325	6	19[b]	96	13[b]	104
	3 × 325	24	ND	Detected	ND	28
185	3 × 325	0	ND	ND	ND	ND
	3 × 325	1	30	173	16 [b]	91
	3 × 325	2	17[b]	104	Detected	72
	3 × 325	3	ND	72	ND	51
	3 × 325	4	ND	52	ND	56
	3 × 325	6	ND	42	ND	61
	3 × 325	8	ND	41	ND	67
	3 × 325	10	ND	33	ND	81
	3 × 325	24	ND	ND	ND	ND

Table 7. cont

Horse No.	Dose (μg)	Time (h)	Plasma			Urine
			BOH (pg ml^{-1})	B17P (pg ml^{-1})	BDP (pg ml^{-1})	BOH (pg ml^{-1})
152	325	0	ND	ND	ND	ND
	325	D3 2[c]	ND	68	ND	49
	325	D3 4	ND	27	ND	ND
	325	D3 6	ND	23	ND	ND
	325	D3 24	ND	ND	ND	ND

[a] ND: not detected.

[b] Estimated results.

[c] Sampling time after the last dose of BDP administration (twice a day) of BDP on the third day.

DISCUSSION

In addition to the $[M + H]^+$ ions, sodium and potassium adduct ions ($[M + Na]^+$, $[M+K]^+$) were formed from BDP and its metabolites in the sample solvent containing 0.1% formic acid. A similar result has been reported by other investigators that $[M + Na]^+$ and $[M + K]^+$ adduct ions were formed from other drugs in a sample solvent containing 0.01% formic acid.[22] The adduct ions are generally undesirable in quantification as they decrease the abundance of $[M + H]^+$ ions used for quantification, even though they might be useful for the identification and confirmation of drugs that are able to form such specific adduct ions. The replacement of formic acid with ammonium formate or acetate buffer would eliminate the formation of the sodium and potassium adduct ions and enhance the formation of $[M + H]^+$ ions, which is beneficial for quantification.

BDP and its metabolites formed solvent adduct ions $[M + H + CH_3CN]^+$ and $[M + H + CH_3OH]^+$ in APCI (+) and ESI (+) sources. These solvent adduct ions are fragile under CID conditions: they were fragmented to $[M + H]^+$ ions when they collided with CID gas (argon at 1.5 mTorr, the normal pressure for CID) even at a very low collision energy (2 V). These solvent adduct ions were observed on the triple-quadrupole instrument, but not on the ion trap instrument. This observation may be explained on the basis of possible effects of helium gas, that is used as a 'damping gas' in an ion trap instrument, on the stability of the solvent adduct ions formed in an API source. The helium gas as a 'damping gas' may collide with the solvent adduct ions and render them susceptible to fragmentation, and it may even have desolvation effects. The results indicate that the triple-quadrupole instrument is soft enough to allow the detection of adduct ions of weak non-covalent combinations, whereas the ion trap instrument is not soft enough for the detection of weakly combined adduct ions. This difference between triple-quadrupole and ion trap instruments may have implications in choosing the right mass analyzer to study loose complexes between molecules such as proteins and substrates.

In the ESI (+) mode, fragmentation of the $[M + H]^+$ ions of BDP and its metabolites under CID conditions produced multiple product ions, which is useful for confirmation but unfavorable for quantification. On the other hand, fragmentation of the $[M + \text{formate}]^-$ ions of BDP and its metabolites under CID conditions resulted in the formation of less product ions, which favors quantification but not confirmation.

The results from the analysis of plasma samples after BDP administration indicated that B17P was the major analyte that was detected. Hence it is evident that B17P is the target analyte to detect the use of BDP in racehorses.

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