Received: 15 August 2018 | Revised: 24 November 2018 | Accepted: 24 November 2018

DOI: 10.1002/dta.2553

RESEARCH ARTICLE

WILEY

Phenylbutazone blood and urine concentrations, pharmacokinetics, and effects on biomarkers of inflammation in horses following intravenous and oral administration of clinical doses

Heather K. Knych[1,2] | Rick M. Arthur[3] | Dan S. McKemie[1] | Kelsey Seminoff[1] | Briana Hamamoto-Hardman[1] | Philip H. Kass[4]

[1] K.L. Maddy Equine Analytical Chemistry Laboratory, School of Veterinary Medicine, University of California, Davis, CA, USA

[2] Department of Veterinary Molecular Biosciences, School of Veterinary Medicine, University of California, Davis, CA, USA

[3] School of Veterinary Medicine, University of California, Davis, CA, USA

[4] Department of Population Health and Reproduction, School of Veterinary Medicine, University of California, Davis, CA, USA

Correspondence
Heather Knych, K.L. Maddy Equine Analytical Chemistry Laboratory, University of California, Davis, School of Veterinary Medicine, 620 West Health Science Drive, Davis, CA 95616, USA.
Email: hkknych@ucdavis.edu

Funding information
California Horse Racing Board; Racing Medication and Testing Consortium

Abstract

Phenylbutazone (PBZ) is a potent mon-steroidal anti-inflammatory drug used commonly in performance horses. The objectives of the current study were to describe blood and urine concentrations and the pharmacokinetics of PBZ and its metabolites following intravenous (IV) and oral administration and to describe the duration of pharmacodynamic effect. To that end, 17 horses received an IV administration and 18 horses an oral administration of 2 g of PBZ. Blood and urine samples were collected prior to and for up to 96 hours post drug administration. Whole blood samples were collected at various time points and challenged with lipopolysaccharide or calcium ionophore to induce ex vivo synthesis of eicosanoids. Concentrations of PBZ and eicosanoids were measured using liquid chromatography–tandem mass spectrometry (LC–MS/MS) and non-compartmental pharmacokinetic analysis performed on concentration data from IV and oral administration. Serum concentrations of PBZ and its metabolites were below the limit of quantitation at 96 hours post administration. The volume of distribution at steady state, systemic clearance, and terminal half-life was 0.194 ± 0.019 L/kg, 23.9 ± 4.48 mL/h/kg, and 10.9 ± 5.32 hours, respectively. The terminal half-life following oral administration was 13.4 ± 3.01 (paste) and 15.1 ± 3.96 hours (tablets). Stimulation of PBZ treated whole blood with lipopolysaccharide and calcium ionophore resulted in an inhibition of TXB_2, PGE_2, LTB_4 and 15-HETE production for a prolonged period of time post drug administration. The results of this study suggest that PBZ has a prolonged anti-inflammatory following IV or oral administration of 2 g to horses.

KEYWORDS

eicosanoids, horse, inflammation, pharmacokinetics, phenylbutazone

1 | INTRODUCTION

Phenylbutazone (PBZ) is arguably one of the more commonly used non-steroidal anti-inflammatory drugs (NSAIDs) in equine medicine. It is a potent anti-inflammatory and as such is frequently used in the treatment of training- and performance-related injuries. The use of NSAIDs, such as PBZ, is highly regulated in performance horse disciplines. The potential for these drugs to mask injuries from detection by trainers and riders or to interfere with detection during pre-competition fitness and lameness examinations has been an ongoing

concern. In many North American racing jurisdictions, under the Model Rules adopted by the Association of Racing Commissioners International (ARCI), the administration of PBZ is not permitted within 24 hours of racing and cannot exceed a regulatory threshold of 2 μg/mL in serum or plasma. Whenever possible, United States racing prefers to regulate therapeutic substances in blood (serum or plasma). The International Federation of Horseracing Authorities (IFHA) does not authorize thresholds for therapeutic substances but does recommend a screening limit of 100 ng/mL for PBZ in both blood (plasma) and urine.[1,2] The European Horseracing Scientific Liaison Committee advises a 168-hour (6-day) detection time for PBZ,[3] the same detection time as the Fédération Équestre Internationale (FEI).[4] While its use is prevalent, and it has been well studied in the horse,[5-11] there is a limited number of comprehensive published studies describing blood and urine concentrations and the pharmacokinetics of this drug and its metabolites in a large population of young horses that are suitable for the establishment of regulatory recommendations.

In the United States, the ARCI regulatory recommendation includes a withdrawal period of 24 hours for PBZ following intravenous (IV) administration of 2 g. The decision to choose a 24-hour withdrawal period is historic and not based on the duration of pharmacologic effect. Racehorses in the USA are examined on race day for fitness to compete by regulatory veterinarians. An equine pre-race examination is highly dependent on the identification of signs of inflammation, which NSAIDs inhibit. As the examination may occur as many as 14 hours prior to post time, concerns have been raised with respect to the regulatory examining veterinarian's ability to effectively conduct an examination if PBZ is administered at 24 hours prior to racing. This concern necessitates further study of the duration of the pharmacodynamic effect of PBZ. Recently, US racing has discussed extending the prohibition of NSAID administration to no less than 48 hours prior to racing. Therefore, in addition to describing the pharmacokinetics of PBZ, this study also sought to describe pharmacodynamic effects.

In vitro and ex vivo models are commonly used to describe the effect of NSAIDs through assessment of relative inhibition of cyclooxygenase (COX) and lipoxygenase (LOX) enzymes and ultimately production of pro-inflammatory mediators that play a crucial role in perpetuating the inflammatory process.[12] The advantage to using an ex vivo model over an in vitro model is that it is directly relevant to the whole animal and considers drug behavior in vivo. In this model, incubation of whole blood with calcium ionophore (CI), aids in the calcium-dependent translocation of cytoplasmic PLA2 to cellular membranes. Subsequent release of free arachidonic acid provides precursors for production of eicosanoids including prostaglandins and thromboxane through the action of COX enzymes.[13] Similarly, CI stimulates translocation of 5-lipooxygenase (LO) and 15-LO to cellular membranes leading primarily to the production of hydroxyeicosatetraenoic acids (HETEs). Another well-characterized stimulus of the arachidonic acid pathway is lipopolysaccharide (LPS). Incubation of LPS with whole blood stimulates COX enzymes leading to the production of Thromboxane B2 (TXB2) and Prostaglandin E2 (PGE2).[14]

Whether permitted at an established blood or urine concentration or considered a prohibited substance, published concentration data is imperative to the proper use of this drug and in order to prevent inadvertent positive regulatory findings. To that end, the goal of the current study was (a) to describe blood and urine concentrations of phenylbutazone and its metabolites following IV and oral administration, (b) to describe the pharmacokinetics of phenylbutazone following IV and oral administration, and (c) to describe the pharmacodynamic effects of phenylbutazone using an established ex vivo model.

2 | MATERIALS AND METHODS

2.1 | Animals

A total of 35 healthy, exercised Thoroughbred research horses (age: 3–6 years; weight: 501 ± 36.8 kg) were studied. Prior to and throughout the course of the study (with the exception of the day of drug administration), horses were exercised five days a week, following standard protocols established by our laboratory.[15] Before beginning the study, horses were determined healthy and free of disease by physical examination, complete blood count, and a serum biochemistry panel that included aspartate aminotransferase, creatinine phosphokinase, alkaline phosphatase, total bilirubin, sorbitol dehydrogenase, blood urea nitrogen, and creatinine. Blood analyses were performed by the Clinical Pathology Laboratory of the William R. Pritchard Veterinary Medical Teaching Hospital of the University of California, Davis, using their standard protocols. Horses did not receive any other medications for at least four weeks prior to commencement of this study. This study was approved by the Institutional Animal Care and Use Committee of the University of California, Davis.

2.2 | Study design, instrumentation, and drug administration

Seventeen horses received a 2g IV dose (Phenylbutazone Injection 20%, VetOne, Boise, ID, USA) and 18 horses received a 2 g oral dose of PBZ. All horses in the oral dose groups were fasted for 10–12 hours prior (after finishing evening meal) until 2 hours post PBZ administration. In the oral administration group, 9 horses received a paste formulation (Vetribute®, VetOne, Boise, ID, USA) and 9 horses received tablets (EQUI-PHAR®, Vedco, St Joseph, MO, USA). Tablets were crushed and suspended in 20–30 mL of a mixture of water and Karo syrup and administered using a dosing syringe directly into the oral cavity.

For IV administration, a 14-gauge catheter was aseptically placed in both external jugular veins immediately prior to drug administration. One catheter was used for IV drug administration while the contralateral catheter was used for sample collection. The dosing catheter was removed following drug administration. Horses receiving an oral administration of PBZ had a single catheter placed in one external jugular vein for sample collection.

2.3 | Sample collection for determination of PBZ concentrations

Blood samples were collected at time 0 (immediately prior to drug administration) and at 5, 10 (IV), 15, 30, and 45 minutes and at 1, 2, 3, 4, 6, 8, 12, 18, 24, 30, 36, 48, 72, and 96 hours post drug administration. Prior to drawing each sample of blood for analysis of drug concentrations, 10 mL of blood was aspirated from the catheter and T-port extension set and discarded. The catheter was flushed with 10 mL of a dilute heparinized saline solution (10 IU/mL) following each sampling. For both routes of administration, catheters used for sample collection were removed following collection of the 24-hour sample and the remaining samples were collected by direct venipuncture. Blood samples were collected into serum tubes and placed at room temperature for 30–60 minutes prior to centrifugation at 3000 x g for 10 minutes. Serum was immediately transferred into storage cryovials and stored at 20°C until analyzed.

Urine samples were collected at time 0 (immediately prior to drug administration) and at approximately (within 10 minutes of the time point) 24, 48, 72, hours and 96 hours post drug administration by free catch. Urine samples were stored at −20° C until analysis.

2.4 | Sample collection for determination of eicosanoid concentrations

Twelve horses (6 intravenous, 3 paste, and 3 tablets) had samples collected for ex vivo determination of eicosanoid concentrations. Horses were weighed immediately prior to drug administration. Concentrations of inflammatory biomarkers (5(s)-HETE, leukotriene B_4 (LTB_4), 15(s)-HETE, TXB_2, and PGE_2) were determined in blood samples collected at time 0 (immediately prior to drug administration) and 2, 4, 6, 8, 24, 30, 48, 72, and 96 hours following PBZ administration. For determination of TXB2 concentrations, a single sample was collected into non-heparinized blood tubes and incubated at 37°C for 1 hour. Samples were subsequently centrifuged, and serum harvested and stored at −20°C until analyzed. A whole blood sample was also collected into an ethylenediaminetetraacetic acid (EDTA) containing blood tube and separated into three aliquots (5 mL each). One aliquot was stimulated with CI; (dissolved in 2% methanol, final concentration of 10uM), incubated for 2 hours at 37°C and plasma harvested by centrifugation. The second aliquot was stimulated with LPS; (dissolved in water, final concentration of 50ug/mL), incubated for 24 hours at 37°C and plasma harvested by centrifugation. The third aliquot was stimulated with methanol (ME; 2% as vehicle), incubated for 2 hours at 37°C and plasma harvested by centrifugation. All plasma samples were stored at −20°C until analyzed by liquid chromatography–tandem mass spectrometry (LC–MS/MS) for determination of eicosanoid concentrations.

2.5 | Determination of PBZ concentrations

2.5.1 | Chemicals and reagents

The analytical reference standard for PBZ was obtained from US Pharmacopeia (Rockville, MD, USA); oxyphenbutazone (OPB) and γ-hydroxy phenylbutazone (GHPB) were obtained from Toronto Research Chemicals (Toronto, ON, Canada). The internal standard d10-PBZ was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA) and d9-OPB was obtained from Toronto Research Chemicals (Toronto, ON, Canada). Stock solutions were prepared at 1 mg/mL in methanol, except for PBZ which was prepared in acetonitrile (ACN). ACN and water were purchased from Burdick and Jackson (Muskegon, MI, USA). Methanol, hexane, ethyl acetate, glacial acetic acid, and buffer reagents were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Formic Acid, 98%, was purchased from Sigma Aldrich (St Louis, MO, USA). The solvents were HPLC grade or better.

2.5.2 | Serum samples

For analysis, PBZ, OPB, and GHPB were combined into one working solution, in duplicate, by dilution of the 1 mg/mL stock solutions with methanol to concentrations of 0.0001, 0.001, 0.01, and 0.1 μg/μL. Serum calibrators were prepared by dilution of the working standard solutions with drug-free equine serum to concentrations ranging from 0.005 to 40 μg/mL. Calibration curves and negative control samples were prepared fresh for each quantitative assay. In addition, quality control samples [drug free equine serum fortified with analyte at three concentrations within the standard curve high, medium and low (3X LOQ of the assay)] were included with each sample set as an additional check of accuracy.

Prior to analysis, 500 μL of serum was diluted with 500 μL of ACN:1 M acetic acid (9:1, v:v) containing 200 ng/mL of d10-PBZ and d9-OPB internal standard, to precipitate proteins. The samples were vortexed for 2 minutes to mix, refrigerated for 20 minutes, vortexed for an additional 1.5 minutes, centrifuged at 3830 g for 10 minutes at 4°C and 20 μL of the supernatant injected into the LC–MS/MS system.

The concentration of PBZ, OPB, and GHPB was measured in serum by LC–MS/MS using positive heated electrospray ionization [HESI(+)]. Quantitative analysis of serum was performed on a TSQ Vantage triple quadrupole mass spectrometer (Thermo Scientific, San Jose, CA, USA) coupled with a turbulent flow chromatography system (TFC TLX2 Thermo Scientific, San Jose, CA, USA) having LC-10ADvp liquid chromatography systems (Shimadzu, Kyoto, Japan) and operated in laminar flow mode. The spray voltage was 3500 V, the vaporizer temperature was 316°C, and the sheath and auxiliary gas were 40 and 30 respectively (arbitrary units). Product masses and collision energies were optimized by infusing the standards into the mass spectrometer. Chromatography employed an ACE 3 C18 10 cm x 2.1 mm 3 μm column (Mac-Mod Analytical, Chadds Ford, PA, USA) and a linear gradient of ACN in water, both with 0.2% formic acid, at a flow rate of 0.40 mL/min. The initial ACN concentration was held at 1% for 0.33 minutes, ramped to 60% over 4.17 minutes, ramped to 99% over 1 minute, held at that concentration for 0.33 minutes, before re-equilibrating for 3.83 minutes.

Detection and quantification was conducted using selective reaction monitoring (SRM) of initial precursor ion for PBZ [mass to charge ratio (m/z) 309.1], OPB [(m/z) 325.1], GHPB [(m/z) 324.9], and the internal standards d10-PBZ [(m/z) 319.1] and d9-OPB [(m/z) 334.1].

The response for the product ions for PBZ (m/z 65.1, 211.1), OPB (m/z 148.0, 204.1), GHPB (m/z 211.1, 253.1), and the internal standards d10-PBZ (m/z 82.1, 221.1) and d9-OPB (m/z 149.0, 213.2) were plotted and peaks at the proper retention time integrated using Quanbrowser software (Thermo Scientific, San Jose, CA, USA). Quanbrowser software was used to generate calibration curves and quantitate analytes in all samples by linear regression analysis. A weighting factor of 1/X was used for all calibration curves.

2.5.3 | Urine sample analysis

Working solutions were the same as those described for serum analysis. Urine calibrators were prepared by dilution of the working standard solutions with drug free equine urine to concentrations ranging from 0.025 to 60 μg/mL. Calibration curves, negative control samples, and quality control samples (high, medium, and low (3X LOQ of the assay)), were prepared fresh for each quantitative assay.

Prior to analysis, 1 mL of urine was diluted with 0.1 mL of water containing 2000 ng/mL of d10-PBZ and d9-OPB internal standard and 0.4 mL of β-glucuronidase enzyme, (Sigma Aldrich, St Louis, MO, USA) at 10 000 Units/mL in pH 5, 1.6 M acetate buffer. The pH of the samples was adjusted to 5 ± 0.5 with 2 N NaOH or 2 N HCl, as necessary, and heated in a sonicating water bath at 65°C for 2 hours with 99 minutes of sonication. After cooling to room temperature, the pH was adjusted to 6 ± 0.5 with 1.5 mL of pH 6.5, 0.6 M phosphate buffer, and 2 N NaOH or 2 N HCl, as necessary. Samples were mixed gently and centrifuged at 3310 g for 5 minutes at 4°C. The samples were subjected to solid-phase extraction (SPE) using CleanScreen Extraction Columns DAU (130 mg/3 mL; United Chemical Technologies, Bristol, PA, USA). In brief, the columns were conditioned with 2 mL of methanol, and then 3 mL of 0.1 M phosphate buffer at pH 6 before the samples were loaded onto the column. The samples were passed through the column using a CEREX system 48 Processor with positive pressure SPE manifold (SPE Ware, Baldwin Park, CA, USA). A minimum of 2 minutes was allowed for samples to pass through the column. The columns were rinsed consecutively with 3 mL of water and 2 mL of 1 M acetic acid prior to elution with 3 mL (1:1, v:v) hexane:ethyl acetate. Samples were dried under nitrogen in a Zymark TurboVap (McKinley Scientific, Sparta, NJ, USA) at 45°C, reconstituted in 150 μL of 5% ACN in water with 0.2% formic acid and 30 μL injected into the LC–MS/MS system. Detection and quantification were the same as described above for serum analysis.

2.6 | Determination of eicosanoid concentrations

2.6.1 | Chemicals and reagents

The analytical reference standards for PGE_2, TXB_2, 15(S)-HETE, 5(S)-HETE, Leukotriene-B_4 (LTB_4), and internal standards (IS) d4-PGE_2, d4-TXB_2, d8–15(S)-HETE, d8–5(S)-HETE, and d4-LTB_4 were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Ethanol was purchased from Sigma Aldrich (St Louis, MO, USA). Water and ACN were purchased from Burdick and Jackson (Muskegon, MI, USA). Methanol, MTBE, and methylene chloride were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Formic Acid, 97%, was purchased from Alfa Aesar (Ward Hill, MA), USA. The solvents were HPLC grade or better.

2.6.2 | Plasma sample analysis

Reference standard working solutions were prepared by dilution of the 1 mg/mL or 0.1 mg/mL stock solutions with methanol to concentrations of 0.001, 0.01 0.1, and 1 ng/μL. Prior to spiking the plasma calibrators and quality control samples, endogenous eicosanoids were removed from equine plasma by charcoal stripping as described previously.[16] Calibration curves (0.05 to 110 ng/mL) were prepared fresh for each quantitative assay. In addition, quality control samples (at three concentrations within the standard curve) were included with each sample set as an additional check of accuracy

Prior to analysis, 0.5 mL sample was mixed with 100 μL of the 7 mix internal standard solution in 25% methanol in water (d4-PGE_2:0.0025 ng/μL, d4-LTB_4 was at 0.005 ng/μL and the remaining IS were at 0.0075 ng/μL). The samples were extracted with 2 mL MTBE:methylene chloride (60:40, v:v), vortexed for 2 minutes and then rotated for 5 minutes at 40 revolutions per minute, before the samples were centrifuged at 1860 g for 2 minutes at 4°C. The organic layer was transferred to a tube and the extraction procedure repeated a second time with 2 mL 100% MTBE. The two extracts were mixed together and dried under nitrogen at room temperature in an evaporator. All samples were re-constituted in 100 μL of water:methanol:ACN (50:40:10, v:v:v) and 20 μL was injected into the LC–MS/MS system.

The concentration of eicosanoids was measured in plasma by LC–MS/MS using negative electrospray ionization [ESI(−)]. Quantitative analysis of plasma was performed on a triple quadrupole mass spectrometer coupled with a turbulent flow chromatography system having LC-10ADvp liquid chromatography systems and operated in laminar flow mode. The spray voltage was 3500 V, the vaporizer temperature was 0°C, and the sheath and auxiliary gas were 40 and 20 respectively (arbitrary units). Product masses and collision energies of each analyte were optimized by infusing the standards into the mass spectrometer. Chromatography employed an ACE 3 C18 10 cm x 2.1 mm, 3 μm column, and a linear gradient of acetonitrile in water with a constant 0.2% formic acid at a flow rate of 0.35 mL/min. The initial ACN concentration was held at 25% for 0.33 minutes, ramped to 80% over 15 minutes, ramped to 90% over 0.17 minutes, before re-equilibrating for 4.0 minutes at initial conditions.

Detection and quantification were conducted using SRM of initial precursor ion and production ion transitions (Table 1). The response for the product ions were plotted and peaks at the proper retention time integrated using Quanbrowser software. Quanbrowser software was used to generate calibration curves and quantitate eicosanoids in all samples by linear regression. A weighting factor of 1/X was used for all calibration curves.

2.7 | Pharmacokinetic calculations

Pharmacokinetic analysis was performed using commercially available software (Phoenix WinNonlin v8.0, Pharsight, Princeton, NJ, USA) and

TABLE 1 Analyte transitions and collision energy (CE) for each eicosanoid

Analyte	Transition	CE (eV)
PGE2	351/271	20
d4-PGE2	355/275	20
TXB2	369/169	20
d4-TXB2	373/173	21
15(s)-HETE	319/257	18
5(s)-HETE	319/257	17
d8–15(s)-HETE	327/308	17
d8–5(s)-HETE	327/309	15
LTB4	335/195	19
d4-LTB4	339/197	19

non-compartmental analysis. The area under the curve (AUC) was calculated using the log-linear trapezoidal rule and extrapolation to infinity using the last measured serum concentration divided by the terminal slope λ_z. Bioavailability was calculated using the formula $F = AUC_{PO}/AUC_{IV}$. The AUC_{IV} and AUC_{PO} used for the bioavailability calculations was determined using the formula above. Pharmacokinetic parameters for both metabolites were calculated using non-compartmental analysis.

2.8 | Statistical analysis

To establish an appropriate regulatory threshold concentration for 48 and 72 hours, a 95/95 confidence interval analysis was performed on PBZ serum concentrations. Statistical analyses were performed using commercially available software (Stata/IC 13.1, College Station, TX, USA) to assess significant differences in eicosanoid concentrations between baseline and each time point following PBZ administration. Data were analyzed using mixed effects analysis of variance, with the horse as a random effect and with time as a fixed effect. Post hoc comparisons were performed using a Bonferroni multiple-comparison adjustment to preserve a nominal significance of 0.05.

TABLE 2 Accuracy and precision values for LC–MS/MS analysis of phenylbutazone (PBZ), oxyphenbutazone (OPB), γ-hydroxy phenylbutazone (GHPB) and various eicosanoids in equine biological matrices. Values represent the average of 6 replicates

A.)

Drug	Matrix	Concentration (μg/mL)	Intra-day Accuracy (% Nominal Concentration)	Intra-day Precision (% Relative SD)	Inter-day Accuracy (% Nominal Concentration)	Inter-Day Precision (% Relative SD)
PBZ	Serum	0.02	114	4.0	97.0	3.0
		0.25	95.0	9.0	102	2.0
		15.0	107	6.0	96.0	3.0
	Urine	0.08	97.0	11.0	NA	NA
		2.0	98.0	4.0	NA	NA
		30.0	100	7.0	NA	NA
OPB	Serum	0.02	96.0	6.0	96.0	5.0
		0.25	97.0	3.0	99.0	3.0
		2.5	101	3.0	102	3.0
	Urine	0.08	113	8.0	NA	NA
		2.0	97.0	6.0	NA	NA
		30.0	96.0	7.0	NA	NA
GHPB	Serum	0.02	98.0	7.0	97.0	6.0
		0.25	96.0	2.0	101	2.0
		2.5	101	2.0	105	3.0
	Urine	75.0	107	10.0	NA	NA
		2000	94.0	6.0	NA	NA
		30,000	88.0	5.0	NA	NA

B.)

Analyte	Concentration (ng/mL)	Intra-day Accuracy (% Nominal Concentration)	Intra-day Precision (% Relative SD)	Inter-day Accuracy (% Nominal Concentration)	Inter-day Precision (% Relative SD)
TXB_2	0.3	96.0	7.0	99.0	6.0
	4.0	107	2.0	106	4.0
	40.0	103	5.0	103	5.0
PGE_2	0.3	100	6.0	105	5.0
	4.0	105	5.0	107	5.0
	40.0	106	4.0	108	5.0
LTB_4	0.3	107	5.0	105	5.0
	4.0	107	5.0	107	4.0
	40.0	105	4.0	105	5.0
15(s)-HETE	0.3	104	6.0	105	6.0
	4.0	106	5.0	105	5.0
	40.0	108	5.0	105	5.0
5(s)-HETE	0.3	99.0	4.0	104	4.0
	4.0	106	5.0	106	5.0
	40.0	105	4.0	103	7.0

NA, not applicable

3 | RESULTS

The responses for PBZ, OPB, GHPB, and all eicosanoids were linear and gave correlation coefficients of 0.99 or better. The intra-day (serum and urine) and inter-day (serum) precision and accuracy of the assay were determined by assaying quality control samples in replicates (n = 6). Accuracy was reported as percent nominal concentration and precision was reported as percent relative standard deviation (Table 2A, B). Accuracy and precision for all matrices were considered acceptable based on the Food and Drug Administration's guidelines for Bioanalytical Method Development. The presence of PBZ and its metabolites in post administration samples was confirmed by matching the retention times as well as relative abundances of ions in the selected reaction monitoring ion chromatogram MS/MS spectra. The limit of quantitation (LOQ) was the lowest calibrator that could be measured with acceptable precision (back calculated concentration not exceeding 20% of the CV) and accuracy (within 20% of the nominal concentration) and the limit of detection (LOD) was established based on the lowest calibrator with a 3:1 signal-to-noise ratio. The technique was optimized to provide an LOQ of 25 ng/mL and an LOD of 0.5 ng/mL for PBZ, OPB, and GHPB in serum. For the urine samples, the LOQ was 25 ng/mL for PBZ and GHPB and 50 ng/mL for OPB; the LOD was 10 ng/mL for PBZ and GHPB and 25 ng/mL for OPB. The LOQ and LOD for all eicosanoids were 0.05 ng/mL approximately 0.025 ng/mL, respectively. A representative chromatograph for all eicosanoids is shown in Figure 1.

PBZ, OPB, and GHPB serum concentrations over time curves are depicted in Figures 2 and 3 for IV and oral administration, respectively and the mean ± SD serum concentrations are listed in Tables 3 and 4. At the final time point sampled (96 hours), PBZ and metabolite concentrations were at or below the LOQ of the assay for both routes of administration. Results of the 95/95 confidence interval analysis indicate a regulatory threshold of 0.30 and 0.10 µg/mL in serum would be appropriate for an IV 2 g dose of PBZ, administered at 48 and 72 hours, respectively. Urinary concentrations of PBZ and its major metabolites were above the LOQ of the assay in all horses at the final time point (96 hours) sampled (Table 5). Pharmacokinetic parameters for PBZ and metabolites following IV and oral administration are summarized in Tables 6 and 7, respectively.

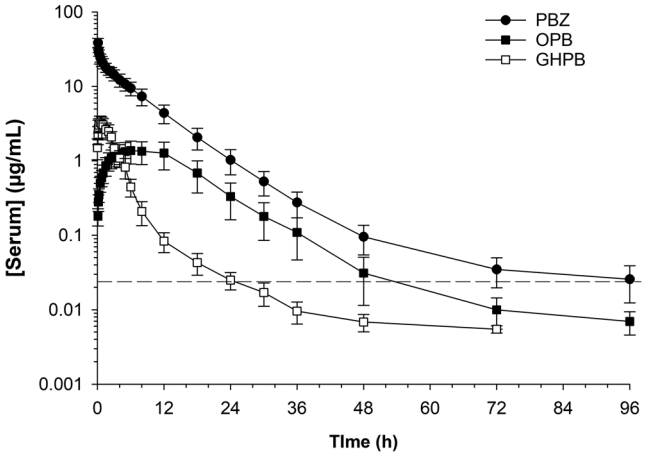

FIGURE 2 Mean ± SD phenylbutazone (PBZ), oxyphenbutazone (OPB) and gamma-hydroxy phenylbutazone (GHPB) serum concentrations over time following intravenous administration of 2 g of phenylbutazone to 17 exercised thoroughbred horses. The dotted line represents the limit of quantitation of the assay

Thromboxane B_2 concentrations in non-heparinized blood were significantly reduced ($p < 0.05$), relative to baseline for 24 hours post PBZ administration (Figure 4). Eicosanoid concentrations following stimulation with LPS and CI are depicted in Figures 5 and 6. Concentrations of TXB_2 (in LPS and CI stimulated whole blood) and PGE_2 (in LPS stimulated whole blood) were significantly reduced ($p < 0.05$), relative to baseline in blood collected from PBZ-treated horses. Concentrations of 15(s)- HETE were significantly greater than baseline for up to 30 hours post administration and significantly reduced thereafter in LPS treated blood and significantly reduced compared to baseline at

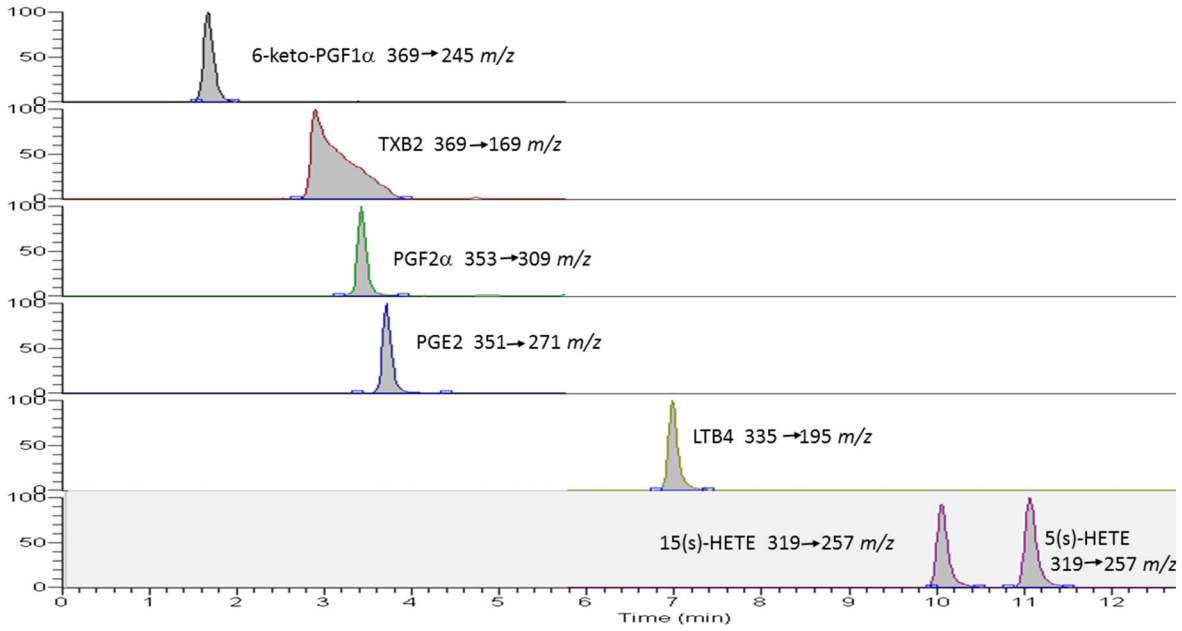

FIGURE 1 Chromatograms of eicosanoids at a concentration of 10 ng/mL [Colour figure can be viewed at wileyonlinelibrary.com]

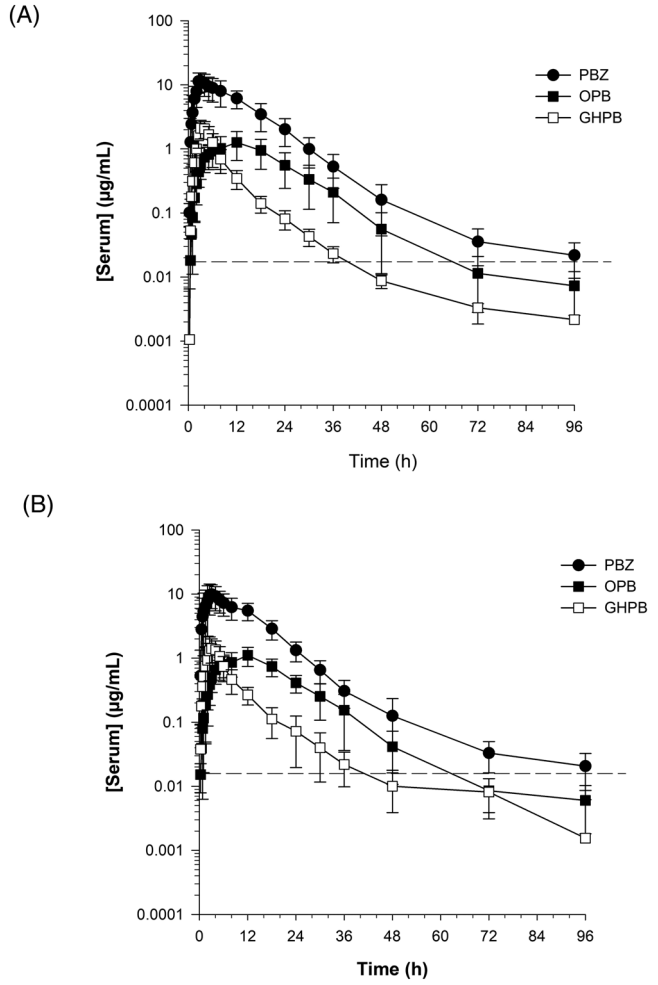

FIGURE 3 Mean ± SD serum concentrations of phenylbutazone (PBZ), oxyphenbutazone (OPB) and gamma-hydroxy phenylbutazone (GHPB) over time following oral administration of 2 g of A, phenylbutazone paste or B, tablets to 18 exercised thoroughbred horses. The dotted line represents the limit of quantitation of the assay

all time points up to 72 hours post PBZ treatment in CI stimulated while blood. Concentrations of 5(s)-HETE were significantly reduced, relative to baseline, from 48–96 hours following stimulation with LPS.

All eicosanoids, with the exception of TXB_2 were either non-detectable or below the LOQ in methanol stimulated whole blood.

4 | DISCUSSION

The primary goal of the current study was to describe serum concentrations and the pharmacokinetics of PBZ following administration to horses with the ultimate goal of providing information to veterinarians and regulatory authorities regarding the disposition of this drug. A secondary goal was to describe the duration of pharmacologic effect using an ex vivo model to predict the effects of in vivo administration of PBZ on eicosanoid production.

The Vd_{ss} reported in the current study (0.203 ± 0.021 L/kg) is in close agreement with that reported previously (0.165 ± 0.014 L/kg)[8] following intravenous administration of a 4.4 mg/kg dose. The systemic clearance reported in the current study was 24.0 ± 4.6 mL/h/kg, which is within the range reported previously (16–26 mL/h/kg).[6-8] In the current study, following IV administration of 2 g (~4 mg/kg), the terminal half-life was 10.9 ± 5.32 hours (harmonic mean ± SD). This is longer than that reported previously (4.0–6.0 hours) following administration of equivalent doses.[17,18] The most likely explanation for the discrepancy is the more sensitive analytical assay utilized in the current study as well as the prolonged duration of sample collection relative to previous studies, thus allowing for quantitation of PBZ for a longer period of time. It is important to note when comparing systemic clearance and the elimination half-life across studies as well as when establishing withdrawal times for higher doses, that PBZ elimination is reportedly dose dependent in the horse with increased doses prolonging the elimination time.[19,20]

In the current study, following oral administration to fasted horses (for 10–12 hours prior and 2 hours post PBZ administration), maximal PBZ concentrations were achieved at 4.72 ± 4.14 (mean ± SD) and 2.78 ± 0.57 for tablets and paste, respectively. Similarly, previous studies report maximal concentrations between 4 and 6 hours when food is withheld for 3–4 hours prior to and post PBZ administration. Previous studies reported bioavailability between 69 and 91%,[6,8,10,21,22] which is in agreement with the current study (87.0% and 74.1% for the paste and oral formulations, respectively).

The current ARCI recommended serum threshold for horseracing in the USA is 2 µg/mL, with a corresponding withdrawal time of 24 hours following IV administration of PBZ up to doses of 4 mg/kg.

TABLE 3 Mean ± SD serum concentrations of phenylbutazone (PBZ), oxyphenbutazone (OPB) and γ-hydroxy phenylbutazone (GHPB) following a single intravenous administration of 2 g of phenylbutazone to 17 exercised thoroughbred horses

	PBZ	OPB	GHPB
Time (h)	Concentration (µg/mL)		
0	ND	ND	ND
0.08	38.6 ± 5.55	0.18 ± 0.05	1.50 ± 0.47
0.16	31.0 ± 2.61	0.28 ± 0.07	2.28 ± 0.62
0.25	28.3 ± 2.81	0.35 ± 0.07	2.72 ± 0.81
0.5	24.2 ± 2.98	0.50 ± 0.15	3.03 ± 0.66
0.75	21.9 ± 3.35	0.69 ± 0.19	3.26 ± 0.74
1.0	20.4 ± 3.20	0.86 ± 0.24	3.15 ± 0.72
1.5	18.0 ± 2.49	1.01 ± 0.28	3.06 ± 0.64
2.0	16.4 ± 2.18	1.09 ± 0.31	2.63 ± 0.61
2.5	15.6 ± 2.41	1.18 ± 0.34	2.49 ± 0.56
3.0	14.3 ± 2.39	1.28 ± 0.38	2.09 ± 0.37
4.0	12.2 ± 2.44	1.33 ± 0.43	1.47 ± 0.28
5.0	10.8 ± 2.09	1.38 ± 0.47	1.09 ± 0.28
6.0	9.48 ± 2.00	1.35 ± 0.46	0.82 ± 0.25
8.0	7.36 ± 1.86	1.27 ± 0.52	0.45 ± 0.12
12.0	4.40 ± 1.23	0.69 ± 0.32	0.21 ± 0.07
18.0	2.07 ± 0.67	0.33 ± 0.17	0.08 ± 0.02
24.0	1.03 ± 0.38	0.18 ± 0.09	0.04 ± 0.01
30.0	0.53 ± 0.19	0.11 ± 0.06	0.30 ± 0.01
36.0	0.28 ± 0.10	0.03 ± 0.02	<LOQ
48.0	0.10 ± 0.04	<LOQ	<LOQ
72.0	0.03 ± 0.02	<LOQ	<LOQ
96.0	0.03 ± 0.01	<LOQ	<LOQ

TABLE 4 Mean ± SD serum concentrations of phenylbutazone (PBZ), oxyphenbutazone (OPB) and γ-hydroxy phenylbutazone (GHPB) following a single oral administration of 2 g of phenylbutazone paste or capsules to 18 exercised Thoroughbred horses

	Paste (n = 9)			Tablets (n = 9)		
	PBZ	OPB	GHPB	PBZ	OPB	GHPB
Time (h)	Concentration (μg/mL)					
0	ND	ND	ND	ND	ND	ND
0.25	0.101 ± 0.04	<LOQ	0.001 ± 0.01	0.532 ± 0.80	0.02 ± 0.01	0.04 ± 0.05
0.5	1.28 ± 1.71	0.02 ± 0.02	0.05 ± 0.11	2.82 ± 3.07	0.04 ± 0.04	0.18 ± 0.31
0.75	2.44 ± 2.96	0.05 ± 0.05	0.18 ± 0.34	4.44 ± 4.21	0.08 ± 0.07	0.36 ± 0.235
1.0	3.69 ± 3.12	0.09 ± 0.07	0.31 ± 0.47	5.43 ± 4.91	0.12 ± 0.10	0.52 ± 0.58
1.5	6.06 ± 3.42	0.17 ± 0.10	0.61 ± 0.54	6.53 ± 5.03	0.19 ± 0.14	0.75 ± 0.70
2.0	7.95 ± 3.50	0.29 ± 0.15	1.00 ± 0.56	7.87 ± 5.66	0.27 ± 0.18	0.93 ± 0.77
2.5	11.4 ± 3.97	0.44 ± 0.19	1.61 ± 0.58	9.54 ± 4.84	0.39 ± 0.20	1.30 ± 0.85
3.0	11.5 ± 3.78	0.56 ± 0.22	2.11 ± 0.68	9.91 ± 4.00	0.50 ± 0.21	1.41 ± 0.58
4.0	10.6 ± 4.00	0.74 ± 0.31	2.07 ± 0.61	9.26 ± 3.80	0.66 ± 0.27	1.35 ± 0.50
5.0	9.42 ± 3.73	0.82 ± 0.35	1.68 ± 0.56	8.06 ± 3.02	0.74 ± 0.29	1.07 ± 0.44
6.0	8.99 ± 3.61	0.95 ± 0.43	1.26 ± 0.48	7.28 ± 2.76	0.81 ± 0.31	0.74 ± 0.31
8.0	8.02 ± 3.52	1.02 ± 0.54	0.70 ± 0.28	6.24 ± 2.35	0.86 ± 0.35	0.47 ± 0.19
12.0	6.14 ± 1.91	1.26 ± 0.60	0.35 ± 0.11	5.51 ± 1.66	1.11 ± 0.37	0.27 ± 0.08
18.0	3.48 ± 1.62	0.95 ± 0.46	0.14 ± 0.04	2.88 ± 0.96	0.74 ± 0.23	0.11 ± 0.06
24.0	2.03 ± 0.94	0.56 ± 0.31	0.08 ± 0.03	1.33 ± 0.45	0.41 ± 0.13	0.07 ± 0.05
30.0	0.99 ± 0.49	0.34 ± 0.22	0.04 ± 0.01	0.65 ± 0.26	0.25 ± 0.15	0.04 ± 0.03
36.0	0.53 ± 0.29	0.21 ± 0.14	<LOQ	0.31 ± 0.14	0.15 ± 0.12	<LOQ
48.0	0.16 ± 0.12	0.06 ± 0.04	<LOQ	0.13 ± 0.11	0.04 ± 0.03	<LOQ
72.0	0.04 ± 0.02	<LOQ	<LOQ	0.03 ± 0.02	<LOQ	<LOQ
96.0	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

TABLE 5 Mean ± SD urine concentrations of phenylbutazone (PBZ), oxyphenbutazone (OPB) and γ-hydroxy phenylbutazone (GHPB) following a single administration of 2 g of intravenous or oral phenylbutazone to exercised thoroughbred horses

		PBZ	OPB	GHPB
Formulation	Time (h)	(μg/mL)		
Injectable	0	ND	ND	ND
	24	5.72 ± 2.72	28.2 ± 17.1	7.68 ± 4.29
	48	0.43 ± 0.28	1.70 ± 1.06	0.65 ± 0.51
	72	0.09 ± 0.04	0.23 ± 0.14	0.15 ± 0.08
	96	0.05 ± 0.03	0.12 ± 0.06	0.09 ± 0.05
Paste	0	ND	ND	ND
	24	3.87 ± 2.48	22.1 ± 6.76	7.56 ± 2.92
	48	0.33 ± 0.14	2.30 ± 1.18	0.69 ± 0.19
	72	0.08 ± 0.02	0.32 ± 0.16	0.17 ± 0.08
	96	0.04 ± 0.01	0.08 ± 0.04	0.08 ± 0.03
Tablets	0	ND	ND	ND
	24	4.41 ± 1.09	17.2 ± 10.1	5.65 ± 6.99
	48	0.37 ± 0.37	1.91 ± 1.73	0.42 ± 0.35
	72	0.06 ± 0.02	0.18 ± 0.07	0.09 ± 0.05
	96	0.03 ± 0.01	0.04 ± 0.01	0.05 ± 0.02

In the current study, serum concentrations were below this recommended threshold at 24 hours post IV administration of a total dose of 2 g (~4 mg/kg). Using the 95/95 confidence interval approach, which is commonly utilized in the USA to establish regulatory threshold recommendations for therapeutic substances, the 0.10 μg/mL screening limit in plasma used by the IFHA and the FEI would allow an IV 2 g dose of PBZ at 72 hours, but there would be at a substantial risk of exceeding the urine screening limit utilized by these two organizations. In the current study, following oral administration of 2 g of PBZ, five horses that received the paste formulation and one horse that received tablets exceeded the 2 μg/mL threshold concentration. Based on these results, it is prudent to observe an extended withdrawal time when using oral doses of 4 mg/kg.

In spite of low serum PBZ concentrations, some veterinarians and horseracing regulatory officials have raised concerns over their ability to accurately evaluate a horse with respect to lameness prior to competition if they have been administered anti-inflammatory drugs within 24 hours of post time. In support of this a previously reported study suggests that although PBZ blood concentrations are relatively low, the anti-inflammatory effects of this compound still exists 24 hours post administration.[23] Other investigators have hypothesized that the prolonged duration of action of this drug is due to penetration and sequestration of PBZ and/or the presence of the active metabolite OPB in inflammatory exudate.[24] In the current study, determination of the extent of inhibition of pro-inflammatory biomarkers was used in an attempt to develop a more objective approach to ascertain the pharmacodynamic effects of PBZ.

Following PBZ administration, production of TXA2 was significantly inhibited, as evidenced by decreased production of its stable, inactive metabolite TXB2. Prolonged inhibition (up to 24 hours for IV and oral tablet formulations and 48 hours for oral paste administration) of TXB2 production in non-heparinized blood in the presence of PBZ indicates that this compound is an effective inhibitor of COX1 enzymes. This finding is in agreement with a previous report in horses whereby TXB2 concentrations were significantly decreased (up to 50%), relative

TABLE 6 Serum pharmacokinetic parameters (mean ± SD) for (A) phenylbutazone and (B) oxyphenbutazone (OPB) and γ-hydroxy phenylbutazone (GHPB) following a single intravenous 2 g administration to 17 horses. All data was generated using non-compartmental analysis

A	
Parameter	**Phenylbutazone**
Vd_{ss} (L/kg)	0.194 ± 0.019
Cl (mL/h/kg)	23.9 ± 4.48
$Lambda_z$ (1/h)	0.064 ± 0.026
HL $Lambda_z$ (h)*	10.9 ± 5.32
AUC_{last} (μg*h/mL)	173 ± 34.5
AUC_{inf} (μg*h/mL)	173 ± 34.6

B		
Parameter	**OPB**	**GHPB**
C_{max} (μg/mL)	1.41 ± 0.49	3.28 ± 0.94
T_{max} (h)	6.94 ± 2.70	1.13 ± 0.79
$Lambda_z$ (1/h)	0.075 ± 0.017	0.061 ± 0.029
HL $Lambda_z$ (h)*	9.86 ± 3.23	15.3 ± 11.2
AUC_{last} (μg*h/mL)	26.5 ± 10.2	16.0 ± 3.10
AUC_{inf} (μg*h/mL)	26.7 ± 10.3	16.2 ± 3.14

*,harmonic mean; V_{ss}, volume of distribution at steady state; Cl, total systemic clearance' C_{max}, maximum serum concentration; T_{max}, time of maximum serum concentration; $lambda_z$, slope of terminal portion of plasma concentration curve; HL $lambda_z$, terminal half-life; AUC_{last}, area under the serum concentration curve until the last measured concentration; $AUC_{inf,}$ area under the serum concentration curve extrapolated to infinity.

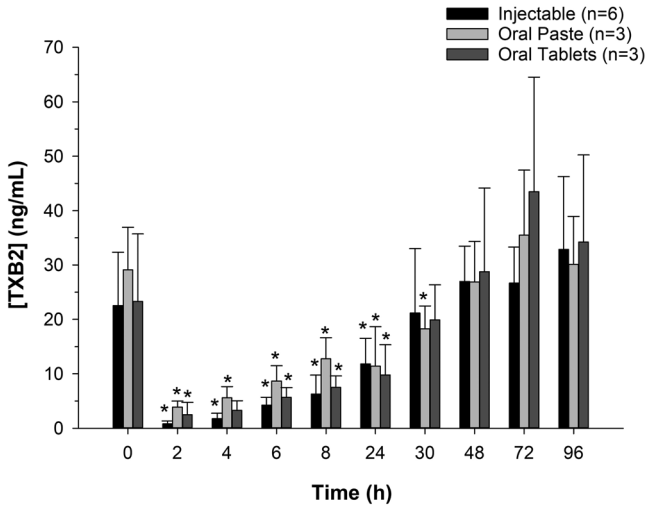

FIGURE 4 Thromboxane B2 concentrations over time in non-heparinized blood following intravenous or oral (paste or tablets) administration of 2 g of phenylbutazone to exercised thoroughbred horses. * indicates concentrations that are significantly different from baseline

to baseline for up to 24 hours, not returning to baseline concentrations until 48 hours post PBZ administration.[9] Sustained inhibition of TXB2 production in CI stimulated whole blood previously treated with PBZ was also noted in the current study. This further supports the prolonged COX 1 inhibitory actions of PBZ as CI stimulation leads to activation and release of TXA2 from platelets.[13] Similar to the effects noted in non-heparinized blood and CI stimulated whole blood, TXB2 production was also significantly inhibited for a prolonged period of time in PBZ treated whole blood following stimulation with LPS. Incubation with LPS stimulates COX2 activity and therefore continued inhibition of TXB2 in these samples suggests a prolonged COX2 inhibitory effect (up to 48 hours) along with inhibition of COX1.

TABLE 7 Serum pharmacokinetic parameters (mean ± SD) for (A) phenylbutazone and (B) oxyphenbutazone (OPB) and γ-hydroxy phenylbutazone (GHPB) following a single oral 2 g administration to 18 horses. All data was generated using non-compartmental analysis

A		
	Phenylbutazone	
Parameter	**Paste (n = 9)**	**Tablets (n = 9)**
C_{max} (μg/mL)	12.2 ± 4.0	10.5 ± 4.25
T_{max} (h)	2.78 ± 0.57	4.72 ± 4.14
$Lambda_z$ (1/h)	0.052 ± 0.009	0.046 ± 0.011
HL $Lambda_z$ (h)*	13.4 ± 3.01	15.1 ± 3.96
AUC_{last} (μg*h/mL)	150.7 ± 44.4	128.2 ± 34.1
AUC_{inf} (μg*h/mL)	151.2 ± 44.4	128.7 ± 34.1
F (%)	87.0	74.1

B				
	Paste		**Tablets**	
Parameter	**OPB**	**GHPB**	**OPB**	**GHPB**
C_{max} (μg/mL)	1.35 ± 0.57	1.92 ± 0.75	1.14 ± 0.32	1.60 ± 0.67
T_{max} (h)	12.0 ± 3.00	3.11 ± 0.55	14.7 ± 6.08	2.94 ± 0.64
$Lambda_z$ (1/h)	0.074 ± 0.017	0.059 ± 0.027	0.067 ± 0.020	0.079 ± 0.024
HL $Lambda_z$ (h)*	9.81 ± 2.20	14.3 ± 7.06	11.3 ± 3.71	9.90 ± 4.34
AUC_{last} (μg*h/mL)	26.9 ± 11.6	13.5 ± 3.51	21.9 ± 3.50	10.7 ± 3.65
AUC_{inf} (μg*h/mL)	27.0 ± 11.7	13.7 ± 3.46	22.0 ± 3.48	10.8 ± 3.72

*harmonic mean; C_{max}, maximum serum concentration; T_{max}, time of maximum serum concentration; $lambda_z$, slope of terminal portion of plasma concentration curve; HL $lambda_z$, terminal half-life; AUC_{last}, area under the serum concentration curve until the last measured concentration; $AUC_{inf,}$ area under the serum concentration curve extrapolated to infinity; F, bioavailability.

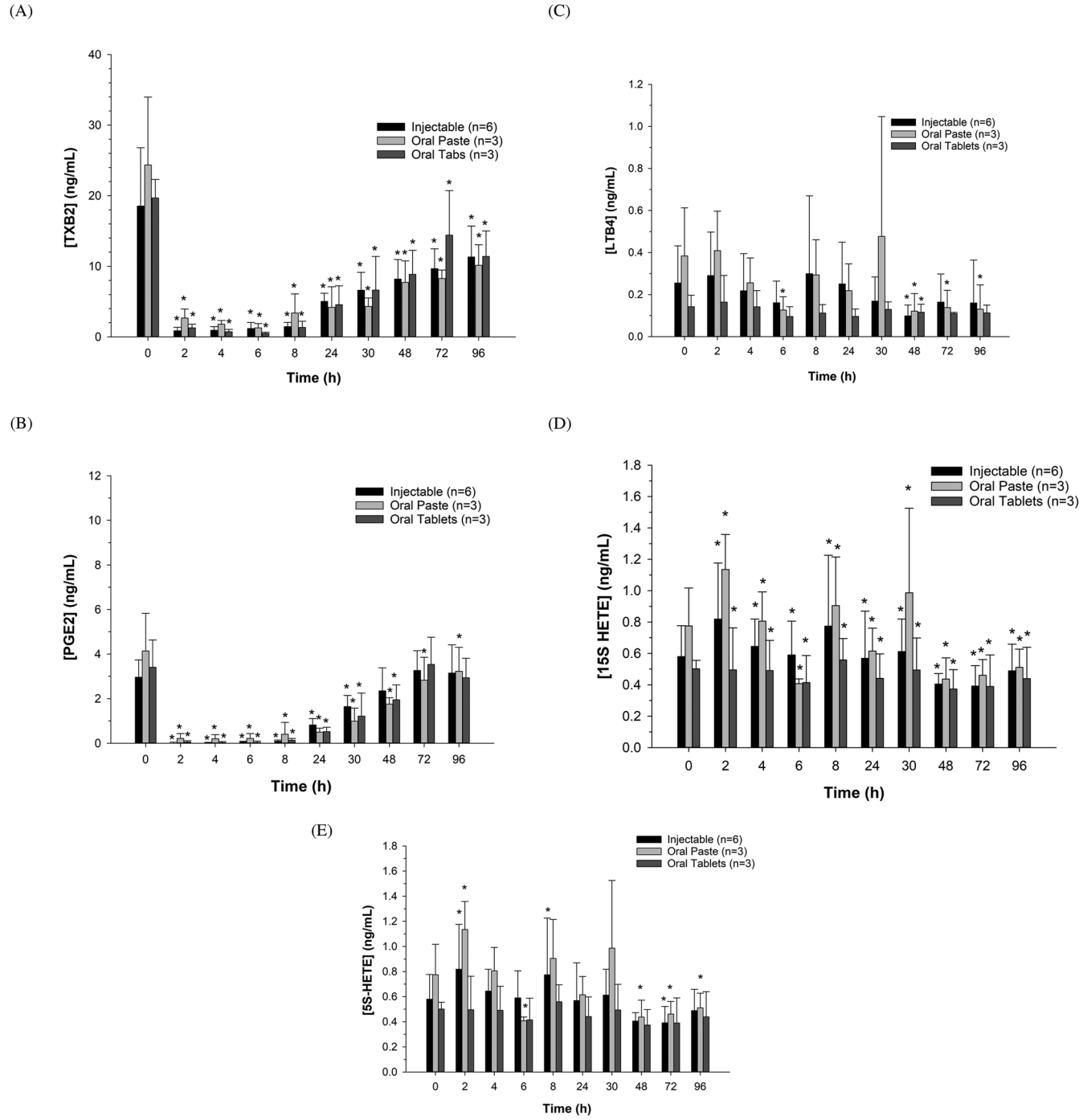

FIGURE 5 Effect of intravenous or oral (paste or tablets) administration of 2 g of phenylbutazone on eicosanoid production in lipopolysaccharide treated equine whole blood at various times post drug administration. * indicates concentrations that are significantly different from baseline

PGE2 is another well-established biomarker used to assess the effects of NSAIDs, specifically their ability to inhibit COX2 activity. Lipopolysaccharide is a potent activator of COX2 enzymes and therefore decreased production of eicosanoids resulting from activation of COX2 (such as PGE2) in LPS stimulated whole blood pretreated with an enzyme inhibitor, is a good indicator of the ability of a drug to inhibit the activity of this enzyme. Similar to what was observed for COX1 in the current study and what has been reported in previous studies for COX2,[25] PBZ had a significant and prolonged (30 hours for IV and up to 96 hours for oral) COX2 inhibitory effect.

Concentrations of 15(s)-HETE in CI stimulated whole blood are an indicator of 15-LOX activity and concentrations in LPS stimulated whole blood an indicator of COX activity.[26] In the current study, concentrations of 15(s)-HETE were significantly increased in LPS stimulated blood until the 24-hour time point and significantly decreased, relative to baseline, thereafter (48–96 hours). While it is not possible to ascertain from the current study why 15(s)-HETE production was not inhibited more quickly in LPS stimulated blood, results suggest a delayed onset of COX2 inhibition with respect to effects on 15(s)-HETE. In contrast to the results observed in LPS stimulated whole

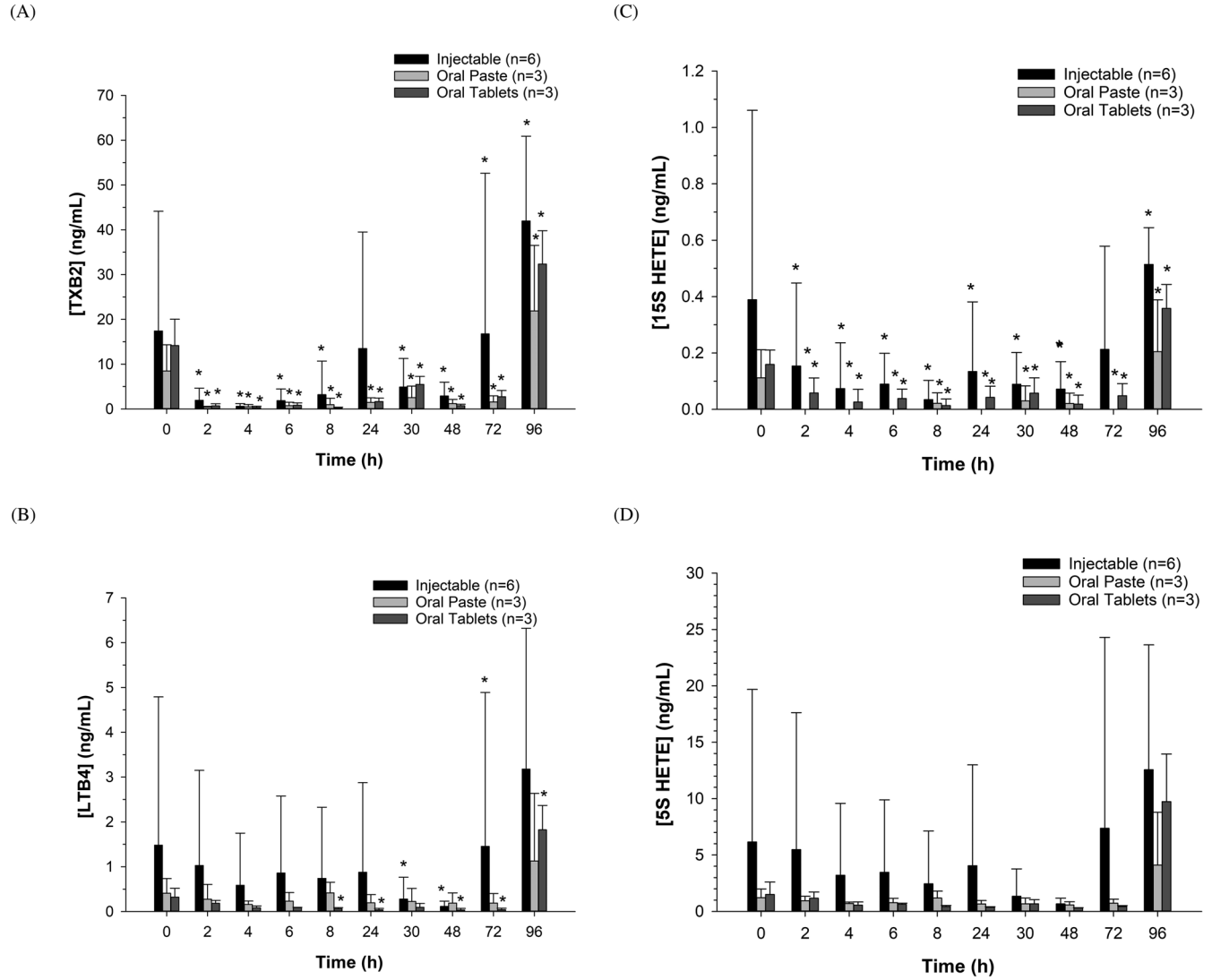

FIGURE 6 Effect of intravenous or oral (paste or tablets) administration of 2 g of phenylbutazone on eicosanoid production in calcium ionophore treated equine whole blood at various times post drug administration. * indicates concentrations that are significantly different from baseline

blood, inhibition of 15(s)-HETE was significant through 48 hours and 72 hours following intravenous and oral administration, respectively, in CI stimulated whole blood. While to the best of the authors' knowledge inhibition of 15-LOX by PBZ has not been reported previously, the significant decrease in 15(s)-HETE relative to baseline, in CI stimulated whole blood suggests that PBZ may have an inhibitory effect on 15-LOX activity.

In the current study, serum and urine concentrations, pharmacokinetics and pharmacodynamics of PBZ following administration of a 2 g total dose (~ 4 mg/kg) both intravenously and orally are reported. While a limited number of horses were studied, results of the current study support the ARCI recommended withdrawal time of 24 hours for PBZ following an intravenous dose of 4 mg/kg. However, quantitation of concentrations exceeding the regulatory threshold at 24 hours in several horses following oral administration suggest a prolonged withdrawal time is necessary following administration of 2 g by this route. Lastly, based on determination of TXB2 concentrations in non-heparinized blood and concentrations of various pro-inflammatory eicosanoids in CI and LPS stimulated whole blood pre-treated with PBZ, the duration of anti-inflammatory affect appears to exceed 24 hours and suggest a longer withdrawal time prior to racing may be warranted.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the Racing Medication and Testing Consortium and the California Horse Racing Board for providing financial support for this study. The authors would like to thank Russell Baden, Kirsten Kanarr, Stacy Steinmetz, and Sandy Yim for their assistance with this project.

ORCID

Heather K. Knych https://orcid.org/0000-0002-8676-4970

REFERENCES

1. IFHA. http://www.ifhaonline.org/default.asp?section=IABRW&area=1.
2. IFHA. http://www.ifhaonline.org/default.asp?section=IABRW&area=6.
3. EHSLC. https://www.ehslc.com/detection-times.

4. FEI. https://inside.fei.org/system/files/FEI Detection Times 2018.pdf.
5. Gerring EL, Lees P, Taylor JB. Pharmacokinetics of phenylbutazone and its metabolites in the horse. *Equine Vet J*. 1981. https://doi.org/10.1111/j.2042-3306.1981.tb03472.x;13(3):152-157.
6. Lees P, Higgins AJ. Clinical pharmacology and therapeutic uses of non-steroidal anti-inflammatory drugs in the horse. *Equine Vet J*. 1985;17(2):83-96. http://www.ncbi.nlm.nih.gov/pubmed/3987667. Accessed August 10, 2018
7. Lees P, Higgins AJ, Mawhinney IC, Reid DS. Absorption of phenylbutazone from a paste formulation administered orally to the horse. *Res Vet Sci*. 1986;41(2):200-206. http://www.ncbi.nlm.nih.gov/pubmed/3775111. Accessed August 10, 2018
8. Maitho TE, Lees P, Taylor JB. Absorption and pharmacokinetics of phenylbutazone in Welsh Mountain ponies. *J Vet Pharmacol Ther*. 1986;9(1):26-39. http://www.ncbi.nlm.nih.gov/pubmed/3701913. Accessed August 10, 2018
9. Lees P, Ewins CP, Taylor JBO, Sedgwick AD. Serum thromboxane in the horse and its inhibition by aspirin, phenylbutazone and flunixin. *Br Vet J*. 1987;143(5):462-476. https://doi.org/10.1016/0007-1935(87)90024-8
10. Smith PBW, Caldwell J, Smith RL, Horner MW, Moss MS. The bioavailability of phenylbutazone in the horse. *Xenobiotica*. 1987. https://doi.org/10.3109/00498258709043950;17(4):435-443.
11. Knych HK, Stanley SD, Seminoff KN, McKemie DS, Kass PH. Pharmacokinetics of methocarbamol and phenylbutazone in exercised thoroughbred horses. *J Vet Pharmacol Ther*. 2016;39(5):469-477. https://doi.org/10.1111/jvp.12298
12. Granström E. The arachidonic acid cascade. The prostaglandins, Thromboxanes and leukotrienes. *Inflammation*. 1984;8 Suppl: S15-S25. http://www.ncbi.nlm.nih.gov/pubmed/6090312. Accessed August 10, 2018
13. Sweeney FJ, Eskra JD, Ernest MJ, Carty TJ. Spectrophotometric monitoring of lipoxygenase and cyclooxygenase pathway activity using Ionophore stimulated whole blood. *Agents Actions*. 1987;21(3/4). https://rd.springer.com/content/pdf/10.1007%2FBF01966526.pdf. Accessed August 10, 2018
14. Young JM, Panah S, Satchawatcharaphong C, Cheung PS. Human whole blood assays for inhibition of prostaglandin G/H synthases-1 and -2 using A23187 and lipopolysaccharide stimulation of thromboxane B2 production. *Inflamm Res*. 1996;45(5):246-253. https://doi.org/10.1007/BF02259611
15. Knych HK, Arthur RM, Mitchell MM, et al. Pharmacokinetics and selected pharmacodynamics of cobalt following a single intravenous administration to horses. *Drug Test Anal*. 2015;7(7):619-625. https://doi.org/10.1002/dta.1737
16. Thakare R, Chhonker YS, Gautam N, et al. Simultaneous LC-MS/MS analysis of eicosanoids and related metabolites in human serum, sputum and BALF. *Biomed Chromatogr*. 2018;32(3). https://doi.org/10.1002/bmc.4102
17. Toutain PL, Autefage A, Legrand C, Alvinerie M. Plasma concentrations and therapeutic efficacy of phenylbutazone and flunixin meglumine in the horse: pharmacokinetic/pharmacodynamic modelling. *J Vet Pharmacol Ther*. 1994;17(6):459-469. http://www.ncbi.nlm.nih.gov/pubmed/7707492. Accessed October 27, 2018
18. Lees P, Toutain P-L. Pharmacokinetics, pharmacodynamics, metabolism, toxicology and residues of phenylbutazone in humans and horses. *Vet J*. 2013;196(3):294-303. https://doi.org/10.1016/j.tvjl.2013.04.019
19. Piperno E, Ellis DJ, Getty SM, Brody TM. Plasma and urine levels of phenylbutazone in the horse. *J Am Vet Med Assoc*. 1968;153(2):195-198. http://www.ncbi.nlm.nih.gov/pubmed/5655055. Accessed August 10, 2018
20. Lees P, Landoni MF, Giraudel J, Touitain PL. Pharmacodynamics and pharmacokinetics of nonsteroidal anti-inflammatory drugs in species of veterinary interest. *J Vet Pharmacol Ther*. 2004;27(6):479-490. https://doi.org/10.1111/j.1365-2885.2004.00617.x
21. Chay S, Woods WE, Nugent TE, et al. Population distributions of phenylbutazone and oxyphenbutazone after oral and i.V dosing in horses. *J Vet Pharmacol Ther*. 1984;7(4):265-276. http://www.ncbi.nlm.nih.gov/pubmed/6512917. Accessed August 10, 2018
22. Lees P, Higgins AJ. Effects of a phenylbutazone paste in ponies: model of acute nonimmune inflammation. *Am J Vet Res*. 1986;47(11):2359-2363. http://www.ncbi.nlm.nih.gov/pubmed/3466561. Accessed August 10, 2018
23. Dunn PS. A clinician's views on the use and misuse of phenylbutazone. *Equine Vet J*. 1972;4(2):63-65. http://www.ncbi.nlm.nih.gov/pubmed/4677296. Accessed August 11, 2018
24. Lees P, Taylor JB, Higgins AJ, Sharma SC. Phenylbutazone and oxyphenbutazone distribution into tissue fluids in the horse. *J Vet Pharmacol Ther*. 1986;9(2):204-212. http://www.ncbi.nlm.nih.gov/pubmed/3723663. Accessed October 26, 2018
25. Cuniberti B, Odore R, Barbero R, et al. In vitro and ex vivo pharmacodynamics of selected non-steroidal anti-inflammatory drugs in equine whole blood. *Vet J*. 2012;191(3):327-333. https://doi.org/10.1016/j.tvjl.2011.03.016
26. Mangal D, Uboh CE, Soma LR, Liu Y. Inhibitory effect of triamcinolone acetonide on synthesis of inflammatory mediators in the equine. *Eur J Pharmacol*. 2014;736:1-9. https://doi.org/10.1016/j.ejphar.2014.04.013

How to cite this article: Knych HK, Arthur RM, McKemie DS, Seminoff K, Hamamoto-Hardman B, Kass PH. Phenylbutazone blood and urine concentrations, pharmacokinetics, and effects on biomarkers of inflammation in horses following intravenous and oral administration of clinical doses. *Drug Test Anal*. 2019;11:792–803. https://doi.org/10.1002/dta.2553