VETERINARY DRUG RESIDUE

The Analysis of Phenylbutazone and Its Active Metabolite, Oxyphenbutazone, in Equine Tissues (Muscle, Kidney, and Liver), Urine, and Serum by LC-MS/MS

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This study reports the use of two validated LC with tandem MS (MS/MS) methods to study the residue depletion profile of phenylbutazone (PBZ) and its metabolite oxyphenbutazone (OXPBZ) from equine serum, urine, and muscle, kidney, and liver tissues. One LC-MS/MS method, with an LOQ of 1.0 ng/mL for PBZ and 2.0 ng/mL for OXPBZ, was used for the analysis of the two drugs in the biological fluids (equine urine and serum); the other LC-MS/MS method, with an LOQ of 0.5 ng/g for PBZ and OXPBZ, was used for the analysis of the drugs in the equine tissue samples. PBZ was administered intravenously to two horses dosed with 8.8 mg/kg PBZ once daily for 4 days and sacrificed humanely at a slaughter plant 7 days after the last drug administration. Urine, serum, and kidney, liver, and muscle tissues were collected from the two horses and shipped on ice to the laboratory and stored at −20°C until analysis. The concentrations of PBZ and OXPBZ residues in the biological fluid and tissue samples collected at slaughter were measured with the two validated LC-MS/MS methods using deuterated internal standards. The results demonstrate that the validated methods are fit for studying the depletion kinetics of PBZ residues in equine tissues and biological fluids.

Phenylbutazone (PBZ; 4-butyl-1,2-diphenyl-3,5-pyrazolidinedione) is a nonsteroidal anti-inflammatory drug (NSAID) commonly used in treating musculoskeletal disorders such as rheumatoid arthritis and arthritic diseases and for pain relief in non-food-producing animals, including horses not destined for the food chain. It is approved for use in horses in Canada and the United States, but product labeling carries the warning "not for use in horses intended for human food." Because PBZ is very effective, inexpensive, and generally safe in horses, it continues to be extensively used in many countries. The use of PBZ to treat any horse that may end up for slaughter is an "extra-label" drug use (ELDU). Under those conditions, it is the responsibility of the owner and the prescribing veterinarian to ensure that unsafe residues are not present in treated animals at the time of slaughter. In Canada, veterinarians are allowed to prescribe drugs for use in a manner not expressly specified on the product labeling. ELDU is common practice because there are numerous situations in which approved product labeling does not cover the specific medical needs of an animal. The European Medicines Agency (EMA) evaluated PBZ in 1997 for the purpose of establishing maximum residue limits (MRLs) in food of animal origin. The data available at that time did not allow the EMA to establish an MRL, and as a result, the European Union (EU) banned the use of PBZ in food-producing animals (1). Globally, the use of PBZ in horses has been very controversial (2), and the detection of undeclared horse meat in food products in the EU in 2013 caused much concern that people were being exposed to "unsafe" residues of PBZ (3).

In 2012, a search of the published literature revealed that although there were several methods (4–9) available for the study of the pharmacokinetics of PBZ in biological fluids (including serum, plasma, and urine) of horses for up to 21 days, none of the methods had been applied to study the depletion of PBZ from equine tissues. The few tissue methods that were published before (10–13) and subsequent to that literature search (14, 15) while the study was underway were primarily for regulatory monitoring purposes and none had been used for the depletion study of PBZ residues in the horse. Asea et al. (10) had published a validated LC–UV method with an LOQ of 5 ng/g for PBZ in equine, porcine, and bovine tissues using diclofenac as an internal standard in 2004, but the method did not include the metabolite oxyphenbutazone (OXPBZ). In 2010, Jedziniak et al. (11) published a method using LC with tandem MS (MS/MS) for the quantitative analysis of 10 NSAIDs, including PBZ and the metabolite OXPBZ in bovine muscle tissues, with PBZ-D_{10} as the internal standard. The method, with LOQs of 2.8 and 2.1 µg/kg for PBZ and OXPBZ, respectively, was also demonstrated to be applicable to the analysis of the 10 NSAIDs

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in swine, horse, and chicken muscle tissues. In 2012, Gentili et al. (12) published two multiresidue LC-MS/MS methods based on a versatile extraction procedure for isolating NSAIDs from bovine milk and muscle tissues that included PBZ, but not the metabolite OXPBZ. The authors calculated a decision limit (CCβ) of 1.84 µg/kg for PBZ with this method. In that same year, Olejnik et al. (13) reported that animal tissue matrix (bovine, swine, equine, and chicken) had significant effects on the performance of the method for official residue control of 10 NSAIDs, including PBZ and OXPBZ, and should be taken into consideration in the analysis of these drugs.

Because there appeared to be a huge outcry against the slaughter of horses, we hypothesized that if a sensitive enough method could be developed to enable us to perhaps determine whether the residues in edible horse tissues could be correlated with residues in biological fluids, then such a correlation factor (or factors) could be used to provide a scientifically sound basis for testing biological fluids as a means to support regulatory enforcement without having to sacrifice the horse of interest at slaughter. In 2012, it was considered that the LC/UV method our group had developed in 2004 (10) would not be sensitive enough to be used for the depletion study we were contemplating conducting. Therefore, we modified the previously validated LC/UV method to include OXPBZ and made changes to the procedure to significantly improve the detection sensitivity by changing the UV detection to MS/MS and by replacing diclofenac with a more suitable stable isotope internal standard for quantification. The modified method was validated and implemented into the routine diagnostic program for the Canadian National Chemical Residue Monitoring Program (NCRMP) for drug residues. It was this validated LC-MS/MS method that was used for the analysis of PBZ and OXPBZ residues in equine tissue samples generated from a PBZ depletion pilot study. The other LC-MS/MS method used for the analysis of the biological fluid samples (serum and urine) was one that had been validated at Maxxam Analytics, a contract laboratory.

One of the requirements for method validation is to use incurred residue material whenever possible (these were not available to us at the time of validation) to verify the characteristic performance parameters of the method determined with fortified material. In our study, we used biological fluid and tissue samples (collected from horses) that contained physiologically generated incurred residues to evaluate the suitability of the two validated LC-MS/MS methods for the analysis of PBZ and OXPBZ residues before the methods were applied to study the depletion of PBZ in equine tissues and biological fluids. This paper reports the results obtained when the two sensitive validated LC-MS/MS methods were used for the analysis of PBZ and OXPBZ residues obtained from serum, urine, and kidney, liver, and muscle tissues of two horses experimentally administered with PBZ and slaughtered 7 days after the last drug administration.

Experimental

Equipment

(a) *Centrifuge and tubes*.—Centrifuge capable of 2850 × *g*, with 50 and 15 mL tube carriers with associated centrifuge tubes: 50 mL disposable polypropylene (Falcon, VWR International, Edmonton, Canada) and 15 mL glass, conical bottom with snap caps (Cat. No. 21020-764; Kimble, VWR International).

(b) *Syringe filters*.—0.2 µm, 13 mm, PVDF (Cat. No. 28143-942; Acrodisc, VWR International).

(c) *Homogenizer*.—Polytron Model PT 3100 D with PT-DA 3020/2T aggregate (Brinkmann Instruments, Ltd, Rexdale, ON, Canada), or equivalent.

(d) *LC-MS/MS System 1*.—Shimadzu UFLC XR-CTO-20AC series binary LC delivery system interfaced to an AB Sciex API 4000 QTRAP mass spectrometer. (*Note*: This system was used at Maxxam Analytics for the biological fluid sample analysis.)

(e) *LC column*.—Waters Acquity Ultra-Performance LC (UPLC) Halo-C_{18} column, 2.7 µm, 2.1 × 50 mm (Part No. 186002350; Waters Corp., Mississauga, ON, Canada).

(f) *LC-MS/MS System 2*.—Waters Acquity UPLC system with a Waters Micromass Quattro Premier MS/MS detector equipped with an electrospray ionization (ESI) source and MassLynx 4.1 software (Waters Corp.), or equivalent. (*Note*: This system was used at the Centre for Veterinary Drug Residues for all the tissue sample analysis.)

(g) *LC column*.—Poroshell 120 EC-C_{18} column, 2.1 × 50 mm, 2.7 µm (Agilent Technologies, Mississauga, ON, Canada).

(h) *Liquid dispensers adjustable to 1–10 and 5–50 mL*.—Brinkmann Instruments (Mississauga, ON, Canada), or equivalent.

(i) *Nitrogen evaporator with heating bath*.—Organomation Associates, Inc. (Berlin, MA), or equivalent.

(j) *Pipettors adjustable to 10–100 and 100–1000 µL, plus tips*.—Eppendorf, or equivalent.

(k) *pH Meter*.—Orion STAR 211A, or equivalent.

(l) *Shaker*.—Two-speed, flat-bed (Eberbach Model 6010; VWR International).

(m) *SPE cartridge*.—Waters Oasis MCX 3 cc/60 mg SPE column (Waters Corp.).

(n) *Vacuum manifold for SPE*.—Supelco (Oakville, ON, Canada), or equivalent.

(o) *Vortex mixer*.—One-speed (Thermolyne Maxi Mix, VWR International).

(p) *Nitrogen*.—Compressed, minimum 99.995% (Praxair, Saskatoon, SK, Canada).

(q) *Whatman filter vial, 0.2 µm PTFE syringe filter*.—Cole-Parmer (Toronto, Canada).

(r) *Argon for collision gas reaction*.—Compressed, minimum 99.99% (Praxair).

(s) *Ultrasonic bath*.—Part No. 8891 (Cole-Parmer).

Chemicals and Reagents

(a) *PBZ and DL-dithiothreitol (DL-DTT; 99%)*.—Sigma-Aldrich Canada (Oakville, ON, Canada).

(b) *OXPBZ*.—Toronto Research Chemicals (North York, ON, Canada)

(c) *PBZ-D_{10} (99%)*.—C/D/N Isotopes, Inc. (Pointe-Claire, QC, Canada)

(d) *PBZ-D_9 (99%)*.—C/D/N Isotopes, Inc.

(e) *Acetic acid (glacial), ascorbic acid, phosphoric acid, ammonium hydroxide (reagent grade)*.—Fisher Scientific (Montreal, QC, Canada).

(f) *PBZ formulation*.—Vétoquinol N.-A., Inc. (Georges, Lavaltrie, QC, Canada).

(g) *Acetonitrile (HPLC grade), methanol (HPLC grade distilled in glass), methylene chloride (distilled in glass), hexane*

(distilled in glass), and ethyl acetate (distilled in glass).—Caledon Laboratories, Ltd (Georgetown, ON, Canada).

(h) *Formic acid (approximately 98%) for MS and ammonium formate*.—EM Science (Gibbstown, NJ).

(i) *Methyl tertiary butyl ether and diethyl ether*.—Anachemia Canada Co. (Montreal, QC, Canada).

Stock and Standard Solutions

(a) *Mobile phase for the LC-MS/MS analysis of urine and serum*.—(*1*) *Mobile phase A*.—5 mM Ammonium formate, pH 3.9.

(*2*) *Mobile phase B*.—Acetonitrile.

(b) *Mobile phase for the LC-MS/MS analysis of tissues*.—(*1*) *Mobile phase A*.—Methanol.

(*2*) *Mobile phase B*.—5 mM Ammonium formate, pH 3.9.

Reagents and Solution Preparation

(a) *Water*.—All water used in the method was purified by reverse osmosis, followed by deionization, adsorption, and filtration.

(b) *Stabilization solution (Solution A)*.—Prepared by dissolving 250 mg DL-DTT in 1000 mL ethyl acetate. Prepare monthly as needed.

(c) *Extraction solution (Solution B)*.—Prepared by mixing 420 mL Solution A and 60 mL methanol. This solution should be prepared fresh daily as required.

(d) *SPE conditioning solution (Solution C)*.—Prepared by mixing 2 mL methanol, 2 mL ammonium hydroxide, 140 mL methylene chloride, and 140 mL Solution A. This solution should be prepared fresh daily as required.

(e) *Elution solution (Solution D)*.—Prepared by mixing 4 mL glacial acetic acid and 8 mL methanol, diluting this mixture to 200 mL with methylene chloride, and then adding 200 mL diethyl ether. This solution can be prepared fresh daily as required.

(f) *5 mM Ammonium formate, pH 3.9*.—Prepared by dissolving 0.134 g ammonium formate and 0.132 g formic acid in approximately 500 mL water, diluting to 1000 mL with water, and then mixing. This solution can be stored at 4°C for 2 weeks.

(g) *Reconstitution solution*.—Prepared by mixing 53 mL 5 mM ammonium formate (pH 3.9) with 35 mL methanol and 12 mL acetonitrile. This solution can be stored at 4°C and used within 2 weeks.

(h) *1 mg/mL Ascorbic acid solution*.—Prepared by dissolving 10 mg ascorbic acid in 10 mL water.

(i) *1 M Phosphoric acid solution*.—Prepared by measuring approximately 68 mL phosphoric acid and slowly adding to 1 L water, with gentle stirring.

Stock Standard Solutions

(a) *PBZ and OXPBZ standard stock solutions (50 µg/mL)*.—A 0.0050 g portion of the analytical standards PBZ and OXPBZ and the PBZ-D_9 and PBZ-D_{10} internal standards was weighed into separate 100 mL glass volumetric flasks. The standards were dissolved in 80 mL methanol to which 5 mL Solution A was added. The solutions were mixed thoroughly and diluted to volume with methanol. Standard stock solutions in 1 mL aliquots can be transferred into amber-colored cryogenic storage vials and stored for 1 year in a temperature-monitored freezer set at −20°C.

(b) *2.0 µg/mL Mixed working standard solution*.—A 400.0 µL aliquot of each of the standard stock solutions of PBZ and OXPBZ was transferred into a 10 mL glass volumetric flask and diluted to volume with methanol. This solution should be prepared weekly and stored at 4°C in amber glass vials.

(c) *0.2 µg/mL Mixed working standard solution*.—A 40.0 µL aliquot of each of the standard stock solutions of PBZ and OXPBZ was transferred into a 10 mL glass volumetric flask and diluted to volume with methanol. This solution should be prepared weekly and stored at 4°C in amber glass vials.

(d) *0.02 µg/mL Mixed working standard solution*.—A 1000.0 µL aliquot of the 0.2 µg/mL mixed working standard solution of PBZ and OXPBZ was transferred into a 10 mL glass volumetric flask and diluted to volume with methanol. This solution should be prepared weekly and stored at 4°C in amber glass vials.

(e) *Internal standard working solution (2.0 µg/mL)*.—A 1000 µL aliquot of the 50 µg/mL PBZ-D_{10} and PBZ-D_9 stock standard solution was transferred into a 25 mL glass volumetric flask and diluted to volume with methanol. This solution can be prepared annually and stored in a temperature monitored fridge set at 4°C in an amber glass vial.

(f) *Mixed chemical standard for system suitability test*.—A 100 µL aliquot of the 0.2 µg/mL mixed working standard solution and a 10 µL aliquot of the 2.0 µg/mL PBZ-D_{10} (for tissue analysis) or PBZ-D_9 (for biological fluid analysis) internal standard working solution were pipetted into a disposable tube. To this was added a 100 µL aliquot of the reconstitution solution and mixed. This solution was filtered into an autosampler vial and used to evaluate the readiness and suitability of the LC-MS/MS system before injecting any samples on the system.

Animal Dosing Experiments, Animal Care, and Sample Collection for Incurred Residues Analysis

Because the residue depletion profile of PBZ from horse tissues beyond 24 h was unknown, a pilot study was first conducted in July and August of 2012 to determine the sampling time points before conducting a full depletion study. For the pilot study, eight normal, healthy, adult horses of light horse breed (four mares and four geldings) from Bouvry Exports in Fort Macleod, AB, Canada, were assembled and kept together in an outdoor pen and had free-choice access to hay and water. The horses were 3–11 years old, with a mean weight of 400 kg. They had been at the Bouvry equine feedlot for at least 4 months before the study. The study was approved by the University of Saskatchewan Animal Research Ethics Board and complied with the Canadian Council on Animal Care guidelines for humane animal use. Pretreatment blood samples were collected from each horse. The original plan was to dose the horses with oral PBZ twice daily for 4 days but, because half of the horses were feral and could not be easily handled without risk of injury to both humans and horses, the eight horses were instead each intravenously dosed with 8.8 mg/kg PBZ once daily for 4 days and sacrificed humanely at the plant 7, 14, 21, and 28 days (two horses per time point) after the last PBZ dose administration. At postmortem, muscle (gluteal and diaphragm), kidney, liver, blood, and urine samples were collected and analyzed using

the two validated LC-MS/MS methods with deuterated internal standards. Whole blood samples were centrifuged at 2600 × *g* for 10 min to separate the serum. The results of the pilot study and the complete depletion study are forthcoming.

Here, we report on the sample set collected 7 days after the last drug treatment, which was provided to us to evaluate the method for its ability to analyze physiologically incurred PBZ and OXPBZ residues before the methods were used for the depletion study. The urine, serum, and kidney, liver, and muscle tissues collected from the two horses were shipped on ice to the laboratory and stored at −20°C and analyzed within 3 months after sample collection.

Sample Preparation and Extraction

(a) *Urine and serum extraction procedure.*—All the serum and urine sample analyses were conducted by Maxxam Analytics in British Columbia under contract. Serum and urine samples were extracted and analyzed for PBZ and OXPBZ residues using an LC-MS/MS analytical method validated at Maxxam Analytics and operated under ISO/IEC 17025:2005 accreditation. Maxxam Analytics also performs all of the blood and urine testing of race horses for the Canadian Pari-Mutuel Agency and of show horses for Equine Canada.

Appropriate volumes of the mixed working standard solutions (2.0, 0.2, and 0.02 µg/mL) were added to 1 mL serum or 2 mL urine to prepare calibration standards with concentrations between 1.0 and 150 ng/mL for PBZ and between 2.0 and 300 ng/mL for OXPBZ. QC samples were prepared at 3.0 ng/mL for PBZ and 6.0 ng/mL for OXPBZ for urine and serum analyses and were included in each day's sample set in order to monitor the precision and accuracy of the method within each run on each day. Ten microliters of the 50 µg/mL PBZ-D_9 solution was added as internal standard to 1.0 mL serum or 2.0 mL urine. Fifty microliters of a 1 mg/mL freshly prepared ascorbic acid solution was added to stabilize PBZ and OXPBZ and prevent oxidation. (*Note*: It is absolutely necessary to add ascorbic acid to prevent oxidation of PBZ into other, less stable forms). To adjust the pH to 4, 150 or 400 µL 1 M phosphoric acid solution was added to 1.0 mL serum or 2.0 mL urine, respectively. Four milliliters of methyl tertiary butyl ether was added, and the samples were autoshaken for 10 min and centrifuged at 2600 × *g* for 5 min. The organic layer was transferred into culture tubes and evaporated to dryness under a gentle steady stream of nitrogen. The dried extracts were dissolved in 100 µL reconstitution solution and analyzed using the API 4000 QTRAP LC-MS/MS instrument.

(b) *Instrumentation for the analysis of urine and serum samples.*—A Shimadzu UFLC XR-CTO-20AC series binary LC delivery system interfaced to an AB Sciex API 4000 QTRAP mass spectrometer (System 1) was used for the separation and analysis of the serum and urine samples. Analytical separation was achieved on a 2.1 × 50 mm (2.7 µm) Halo-C_{18} column held at 35°C using a binary mobile phase consisting of 5 mM ammonium formate (A) and acetonitrile (B). Chromatographic separation of the components in the extract was achieved at a mobile phase flow rate of 500 µL/min and with the following gradient conditions: Mobile phase A was set at 90% for 0.3 min, linearly reduced to 20% at 2 min and to 10% at 2.1 min, and held at 10% until 2.9 min. It was then linearly increased to initial conditions (90% A) at 3 min and held until the end of the run cycle at 4.0 min. A 1 µL aliquot of the urine or serum extract was injected into the LC-MS/MS system with a run time of 4 min per injection. The mass spectrometer was operated under positive ESI conditions with an ion-spray voltage of 400 V, an entrance potential of 10 V, a source temperature of 650°C, and ion-source gas 1 and 2 pressures on at 60 psi. The detailed MS/MS conditions with the interface heater on are shown in Table 1. The triple quadrupole mass spectrometer was operated in selected-reaction monitoring mode (SRM) detecting three product ion transitions for PBZ and OXPBZ and one product ion transition for the deuterated internal standard. The acquisition and processing of acquired data were controlled by Analyst® software, version 1.6.

(c) *Tissue sample extraction procedure.*—All tissue samples were analyzed for PBZ and its active metabolite, OXPBZ, at the Canadian Food Inspection Agency Centre for Veterinary Drug Residues (CVDR) laboratory in Saskatoon, an internationally accredited laboratory operating under ISO/IEC 17025:2005, using a modified method previously published by one of the authors (10). The CVDR is responsible for developing and validating methods of analysis for veterinary drug residues in food of animal origin using those validated methods to support Canada's regulatory control of the use of veterinary drugs in domestically and imported foods of animal origin.

In brief, tissue samples were prepared by using a modified version of the previously validated LC–UV method for PBZ (which used diclofenac as internal standard), i.e., OXPBZ was included and the use of diclofenac was replaced with a PBZ-D_{10} stable isotope for MS/MS detection. Seven fortified-matrix calibration standards were prepared as follows: 2.00 ± 0.02 g portions of blank tissue samples were weighed into seven individual disposable 50 mL centrifuge tubes. Two of the seven negative control tissues were fortified with 50 and 100 µL aliquots of the 0.02 µg/mL mixed working standard solution, the third tissue sample was fortified with a 100 µL aliquot of the 0.2 µg/mL mixed working standard solution, and the fourth and fifth tissue samples were fortified with 25 and 50 µL aliquots of the 2.0 µg/mL mixed working standard solution to provide matrix standards of 0.5, 1, 10, 25, and 50 ng/g tissue equivalency, respectively. The sixth negative control tissue was fortified with a 100 µL aliquot of the 0.2 µg/mL mixed working standard solution to serve as the 10 ng/g QC sample. The seventh sample was not fortified and served as the negative control sample.

Next, 2.00 ± 0.02 g portions of the test tissue samples were weighed into individual disposable 50 mL centrifuge tubes. To each tube was added 10 µL aliquots of the 2 µg/mL PBZ-D_{10} internal standard working solution, which was mixed and allowed to stand for 15 min.

Note: The following steps before the reconstitution step just prior to SPE cleanup are critical and should be conducted without interruption.

Eight milliliters of Solution B were added to each centrifuge tube, and the contents were homogenized with a Polytron homogenizer. The samples were vortex-mixed for 5 min (on a multitube vortex mixer), after which they were shaken for 2 min on a mechanical shaker and then centrifuged at 5°C for 5 min at 2850 × *g*. The supernatant was poured into a clean polypropylene centrifuge tube. The extraction process

Table 1. Interface, UPLC, and MS/MS parameters for the quantitative and qualitative operation of the two LC-MS/MS systems

Compound	Precursor ion, *m/z*	Product ion, *m/z*	Retention time, s	Dwell time, ms	Collision energy, eV	Desolvation potential, V	Collision cell exit potential
Interface and MS/MS parameters for System 1[a]							
		160.3	2.49	50	31	94	10
PBZ	309.2	106.0	2.49	50	43	94	10
		188.0	2.49	50	25	94	10
		160.2	2.16	50	27	89	10
OXPBZ	325.2	176.2	2.16	50	29	89	10
		204.0	2.16	50	23	89	10
PBZ-D_9	318.4	169.1	2.47	50	33	106	10
UPLC and MS/MS parameters for System 2[b]							
Compound	Precursor ion, *m/z*	Product ion, *m/z*	Retention time, s	Dwell time, ms	Cone voltage, V		Collision voltage, V
		188.2	2.24	20	32		15
PBZ	309.2	120.0	2.24	20	32		20
		160.1	2.24	20	32		20
		204.2	1.84	50	32		15
OXPBZ	325.2	148.0	1.84	50	32		30
		120.0	1.84	50	32		20
PBZ-D_{10}	319.2	165.1	2.23	25	32		25

[a] System 1: Shimadzu UFLC XR-CTO-20AC series binary LC delivery system interfaced to an AB Sciex API 4000 QTRAP mass spectrometer.

[b] System 2: Waters Acquity UPLC system with a Waters Micromass Quattro Premier MS/MS detector equipped with an ESI source and MassLynx 4.1 software.

was repeated twice more. The combined supernatants were centrifuged at 5°C for 10 min at 2850 × *g* at 5°C. The supernatant was poured into a clean centrifuge tube and evaporated under nitrogen at 55°C to near dryness. The residue was reconstituted with 5.0 mL Solution C and vortex-mixed for 2 min. A Waters Oasis MCX 3 cc/60 mg SPE column was loaded onto the vacuum manifold and conditioned with 5 mL Solution C. Glass wool was added onto the SPE. The reconstituted sample extract was loaded onto the cartridge. The cartridge was washed with 1 mL Solution C, after which the glass wool was removed. The cartridge was dried for 5–10 min under vacuum (−15 to −20 mm Hg), washed with 3 mL hexane, and dried for 10–15 min under vacuum (−15 to −20 mm Hg). PBZ and OXPBZ were eluted with 4 mL Solution D into a 5 mL glass tube. All the test samples and calibration samples were evaporated to dryness under nitrogen at 55°C. The residue was reconstituted in 400 µL methanol, vortex-mixed for 10 s, after which 600 µL water was added and vortex-mixed for 10 s. The reconstituted samples were sonicated for 10–15 min and vortex-mixed. The samples were transferred to a microcentrifuge vial and centrifuged at 1700 × *g* for 10 min, and a 500 µL portion of the sample was transferred to a Whatman filter vial, 0.2 µm PTFE (taking precautions to avoid disturbing the tissue pellet), and filtered into an autosampler vial for gradient LC-MS/MS analysis. (*Note*: It is absolutely imperative to use Solutions A, B, C, and D in these procedures to prevent the oxidation of PBZ into other, less stable forms under typical laboratory conditions).

(d) *Instrumentation for the analysis of muscle, kidney, and liver tissue samples.*—A Waters UPLC Acquity System interfaced to a Waters Quattro Premier tandem mass spectrometer (System 2) operated under positive ESI was used for the separation and analysis of the tissue samples. Analytical separation of the tissue extracts was achieved on a Poroshell 120 EC-C_{18} 2.1 × 50 mm, 2.7 µm column held at 50°C, with a mobile phase flow at 0.5 mL/min and a binary mobile phase of methanol (A) and 5 mM ammonium formate, pH 3.9 (B), under the following gradient flow conditions: The composition of the mobile phase was 25% A at time 0 min and was linearly increased to 90% at 2.5 min and then to 95% at 3.75 min. It was decreased linearly to the initial conditions (25% A) at 3.9 min and held at that composition until the end of the run cycle at 5 min. The capillary voltage on the mass spectrometer was held at 2.5 kV, the cone voltage at 32 V, and the extractor and the radio frequency (RF) lens at 3.00 and 0.1 V, respectively. The source temperature was held at 120°C and the desolvation temperature at 450°C. The cone gas and desolvation gas (nitrogen) flows were held at 50 and 1100 L/h, respectively. The quadrupoles were operated at unit mass resolution, and the collision gas (argon) was set at 0.25 mL/min. The multiplier was held at 700 V. Ion energies 1 and 2 were set at 1.5 and 3 V, respectively. A 5 µL volume of tissue extract was injected into the LC-MS/MS system for analysis. The triple quadrupole mass spectrometer was operated in SRM mode detecting at least three product ion transitions for PBZ and OXPBZ, as listed in Table 1 with the associated collision energies. The acquisition and processing of data were controlled by Waters MassLynx software, version 4.1.

Before each day's run, a wet prime of the injector was performed, the syringe was purged, and the needle wash of the LC system was primed. The sample cone was cleaned before the mass spectrometer was turned on. The LC system was allowed

to equilibrate for 15 min. Using the system suitability standard and a QC sample from a previous run, four injections or more were made into the LC-MS/MS system to condition the column and the sample cone. The MS detector response ratio (peak area) of each analyte to that of the internal standard measured at the specified retention time from the SRM chromatograms was plotted against the corresponding concentration of the analyte.

Validation

The two LC-MS/MS methods were validated to assess whether they were selective, accurate, precise, and capable of producing reliable, repeatable results that can stand the test of scrutiny (fit-for-purpose).

Selectivity of the Methods for Serum, Urine, and Tissues

The selectivity of the methods was determined by analyzing six different blank serum, urine and tissue samples from six different horses with no known history of PBZ administration.

Experiments to Measure the Recovery, Precision, and Accuracy of the Methods for PBZ and OXPBZ Residues in Serum, Urine, and Tissues

Recovery samples were prepared by fortifying drug-free serum and blank urine samples, in triplicate, at 3.0, 20, and 120 ng/mL levels for PBZ and at 6.0, 40, and 240 ng/mL levels for OXPBZ.

To characterize the accuracy and precision of the method for the quantification of PBZ and OXPBZ in both serum and urine, QC samples were prepared by the QC manager at the same three concentrations used for the recovery studies in replicates of three at each concentration. These samples were coded, randomized, and provided blind to the analyst, who analyzed them over a period of 3 days using PBZ-D_9 as an internal standard. These fortified samples were extracted and analyzed using the LC-MS/MS method described for System 1. The results of these analyses are shown in Table 2.

For tissue samples, the analyst who was to analyze the samples in the tissue depletion study conducted a series of familiarization experiments to demonstrate that she could repeat the validated method and that the results of her analysis

Table 2. Accuracy and precision data generated for the validation of urine and serum analysis method

Day of analysis	Concentration added, ng/mL	Concentration found in urine, ng/mL			Concentration added, ng/mL	Concentration found in serum, ng/mL		
		Mean ± SD	RSD, %	Accuracy, %[a]		Mean ± SD	RSD, %	Accuracy, %
				PBZ				
Day 1 (*n* = 3)	3.0	3.1 ± 0.1	3	+3	3.0	2.9 ± 0.2	7	−3
Day 2 (*n* = 3)	3.0	3.5 ± 0.2	5	+17	3.0	3.2 ± 0.1	3	+7
Day 3 (*n* = 3)	3.0	2.8 ± 0.1	4	−7	3.0	3.1 ± 0.1	3	+3
Interday (*n* = 9)	3.0	3.1 ± 0.4	11	+3	3.0	3.1 ± 0.2	5	+3
Day 1 (*n* = 3)	20.0	20.6 ± 0.5	2	+3	20.0	19.5 ± 0.5	3	−3
Day 2 (*n* = 3)	20.0	19.1 ± 1.1	6	−5	20.0	20.0 ± 0.5	3	0
Day 3 (*n* = 3)	20.0	19.3 ± 0.9	5	−4	20.0	17.7 ± 1.2	7	−12
Interday (*n* = 9)	20.0	19.1 ± 1.2	6	−5	20.0	19.1 ± 1.2	6	−5
Day 1 (*n* = 3)	120	123.9 ± 3.3	3	+3	40.0	40.7 ± 1.3	3	+2
Day 2 (*n* = 3)	120	110.6 ± 3.3	3	−8	40.0	41.2 ± 1.1	3	+3
Day 3 (*n* = 3)	120	116.9 ± 6.7	6	−3	40.0	39.5 ± 0.9	2	−1
Interday (*n* = 9)	120	116.9 ± 6.7	6	−3	40.0	40.5 ± 0.9	2	+1
				OXPBZ				
Day 1 (*n* = 3)	6.0	5.0 ± 0.1	2	−17	6.0	6.2 ± 0.4	7	+3
Day 2 (*n* = 3)	6.0	6.4 ± 0.4	6	+7	6.0	5.7 ± 0.3	5	−5
Day 3 (*n* = 3)	6.0	6.6 ± 0.4	6	+10	6.0	6.5 ± 0.2	3	+8
Interday (*n* = 9)	6.0	5.8 ± 0.7	12	−3	6.0	6.1 ± 0.4	7	+2
Day 1 (*n* = 3)	40.0	38.0 ± 2.5	7	−5	40.0	42.8 ± 2.7	6	+7
Day 2 (*n* = 3)	40.0	43.7 ± 1.1	3	+9	40.0	39.9 ± 1.2	3	−0.3
Day 3 (*n* = 3)	40.0	45.3 ± 3.3	7	+13	40.0	38.0 ± 1.8	5	−5
Interday (*n* = 9)	40.0	42.3 ± 3.8	9	+6	40.0	40.2 ± 2.4	6	+1
Day 1 (*n* = 3)	240	223.9 ± 18.1	8	−7	80.0	86.3 ± 6.5	8	+8
Day 2 (*n* = 3)	240	258.7 ± 5.2	2	+8	80.0	83.9 ± 3.7	4	+5
Day 3 (*n* = 3)	240	204.0 ± 27.0	13	−15	80.0	79.3 ± 2.3	3	−1
Interday (*n* = 9)	240	228.9 ± 27.7	12	−5	80.0	83.2 ± 3.6	4	+4

[a] Accuracy = [Mean experimentally determined concentration – theoretical (fortified) concentration] × 100 ÷ theoretical (fortified) concentration.

would be considered fit-for-purpose. These familiarization experiments included preparing PBZ and OXPBZ calibration standards in neat solvent and a fortified-matrix and matrix-matched calibration standard set for muscle, kidney, and liver, and then analyzing the data for linearity, recovery, and repeatability. A second familiarization exercise was conducted by preparing a set of calibration standards and estimating the absolute recoveries and within-day and between-day accuracy and precision of the method at three concentrations (1.0, 5, and 20 ng/g). The results of these experiments are shown in Table 3. The final experiment to certify the analyst's proficiency at using the method was done through the analysis of an additional set of "blind-fortified" samples prepared in duplicate by a senior analyst, coded, randomized, and provided to the trained analyst for analysis using the described procedure. The results of the blind-fortified sample analysis experiment are shown in Table 4.

Linearity of the Calibration Curves for Quantitative Analysis

To evaluate the linearity of the calibration curves used for the quantification of PBZ and OXPBZ in serum and urine, a series of drug-free serum or drug-free urine samples fortified with standard solutions to provide concentrations ranging from 1.0 to 150 ng/mL for PBZ and from 2.0 to 300 ng/mL for OXPBZ was prepared in duplicate and injected at the beginning and at the end of each batch. For tissue samples, the linearity between 0.5 and 100 ng/g was assessed using regression analysis.

Calculations

The coefficient of determination, R^2, of the standard curve was calculated each time for PBZ and OXPBZ. From the calibration curves, the LOQ was determined as the concentration corresponding to the intercept, Y_0, of the fortified-matrix regression line $\pm 10\sigma$, where σ = the SD in the response ratio. Peak area response ratios were calculated for each unknown sample, and the corresponding concentrations were interpolated from the linear calibration curves.

Application of the Method: Animal Dosing Experiments to Generate Incurred Tissue Residues

To provide incurred residues for the evaluation of the performance of the validated analytical method, incurred residue samples were generated as described under *Sample Preparation and Extraction*. The results of the analysis of the incurred samples are shown in Table 5.

Table 3. Accuracy and precision data generated on tissue samples from familiarization exercise

Day of analysis	Concentration added, ng/g	Concentration found in kidney, ng/g			Concentration found in muscle, ng/g			Concentration found in liver, ng/g		
		Mean ± SD	RSD, %	Accuracy, %[a]	Mean ± SD	RSD, %	Accuracy, %	Mean ± SD	RSD, %	Accuracy, %
					PBZ					
Day 1 (*n* = 3)	1.0	0.9 ± 0.1	11	−10	1.2 ± 0.2	16	+20	0.9 ± 0.1	13	−10
Day 2 (*n* = 3)	1.0	1.2 ± 0.2	17	+20	0.9 ± 0.1	11	−10	1. ± 0.2	16	+20
Day 3 (*n* = 3)	1.0	0.9 ± 0.1	11	−10	1.2 ± 0.2	17	+20	1.1 ± 0.2	18	+10
Interday (*n* = 9)	1.0	1.0 ± 0.2	20	0	1.1 ± 0.2	18	+10	1.1 ± 0.2	18	+10
Day 1 (*n* = 3)	5.0	5.5 ± 0.4	7	+10	4. 6 ± 0.5	11	−8	4.8 ± 0.7	15	−4
Day 2 (*n* = 3)	5.0	4.4 ± 0.6	14	−12	4.4 ± 0.8	18	−12	5.5 ± 1.0	18	+10
Day 3 (*n* = 3)	5.0	4.8 ± 0.9	19	−4	5.3 ± 0.9	17	+6	5.3 ± 0.6	11	+6
Interday (*n* = 9)	5.0	4.9 ± 0.6	12	−2	4.8 ± 0.5	10	−4	5.2 ± 0.4	8	+4
Day 1 (*n* = 3)	20.0	20.6 ± 1.3	6	+3	21.2 ± 1.4	7	+6	20.8 ± 2.2	11	+4
Day 2 (*n* = 3)	20.0	21.3 ± 3.3	15	+7	19.2 ± 2.1	11	−4	19.7 ± 1.9	10	−2
Day 3 (*n* = 3)	20.0	18.7 ± 2.1	11	−7	20.5 ± 1.8	9	+3	21.8 ± 2.1	10	+9
Interday (*n* = 9)	20.0	20.2 ± 1.3	6	+1	20.3 ± 1.0	5	+2	20.8 ± 1.1	5	+4
					OXPBZ					
Day 1 (*n* = 3)	1.0	0.8 ± 0.1	13	−20	1.1 ± 0.2	18	+10	1.2 ± 0.2	14	+20
Day 2 (*n* = 3)	1.0	1.2 ± 0.2	17	+20	1.4 ± 0.3	21	+40	1.0 ± 0.2	20	0
Day 3 (*n* = 3)	1.0	1.3 ± 0.2	14	+30	0.9 ± 0.1	13	−10	0.9 ± 0.1	14	−10
Interday (*n* = 9)	1.0	1.1 ± 0.2	18	+10	1.1 ± 0.2	18	+10	1.0 ± 0.1	10	0
Day 1 (*n* = 3)	5.0	4.7 ± 0.5	11	−6	5.3 ± 1.1	20	+6	5.2 ± 0.9	17	+4
Day 2 (*n* = 3)	5.0	4.9 ± 0.7	14	−2	5.2 ± 0.9	16	+4	4.8 ± 1.0	21	−4
Day 3 (*n* = 3)	5.0	5.2 ± 1.0	19	+4	4.7 ± 0.3	6	−6	4.5 ± 0.1	2	−10
Interday (*n* = 9)	5.0	4.9 ± 0.3	6	−2	5.1 ± 0.3	6	+2	4.8 ± 0.4	8	−4
Day 1 (*n* = 3)	20.0	17.6 ± 2.1	12	−12	18.9 ± 1.6	8	−6	20.8 ± 2.3	11	+4
Day 2 (*n* = 3)	20.0	22.2 ± 2.4	11	+11	20.8 ± 2.5	12	+4	17.9 ± 1.8	10	−11
Day 3 (*n* = 3)	20.0	18.6 ± 1.9	10	−7	17.9 ± 1.7	9	−11	19.5 ± 2.0	10	−3
Interday (*n* = 9)	20.0	19.5 ± 2.4	12	−3	19.2 ± 1.5	7	−4	19.4 ± 1.5	7	−4

[a] Accuracy = [Mean concentration found – theoretical (fortified) concentration added] × 100 ÷ theoretical (fortified) concentration added.

Table 4. Concentrations of PBZ and OXPBZ determined by a trained analyst in blind-fortified tissue samples using the validated method

	Muscle			Liver			Kidney		
Sample No.	Concn added, ng/g	Concn found, ng/g	Difference, %	Concn added, ng/g	Concn found, ng/g	Difference, %	Concn added, ng/g	Concn found, ng/g	Difference, %
					PBZ				
1	5.0	4.5	−10	8.0	7.0	−12	5.0	5.8	+16
4	5.0	4.8	−4	70.0	69.0	−1	45.0	43.0	−4
3	16.0	17.0	+6	8.0	7.3	−9	5.0	5.5	+10
5	16.0	15.2	−6	19.0	20.1	+6	17.0	16.0	−6
2	37.0	36.2	−2	19.0	17.5	−8	17.0	17.0	0
6	37.0	36.9	0	70.0	68.2	−3	45.0	41.2	−8
					OXPBZ				
7	9.0	9.2	+2	8.0	7.6	−5	9.0	8.8	−2
10	9.0	8.7	−3	70.0	71.0	+1	9.0	8.6	−4
8	20.0	19.5	−3	19.0	19.2	+1	20.0	19.5	−3
12	20.0	19.6	−2	70.0	68.0	−3	20.0	19.7	−2
9	40.0	38.3	−4	8.0	7.8	−3	45.0	43.0	−4
11	40.0	39.0	−3	19.0	18.6	−2	45.0	46.1	+2

Results and Discussion

The chemical structures of PBZ and OXPBZ are shown in Figure 1. The proton located on the carbon in position four of the pyrazolidine ring of PBZ has an acidic property that can form an enol with the neighboring group to form 4-butyl-5-hydroxy-1,2-diphenyl-1,2-dihydro-3H-pyrazol-3-one. This implies that PBZ can act as a very weak acid. The pKa of PBZ is 4.5. PBZ exists in solution in three forms: as the diketo, the enol, and a mesomeric anionic form (16). The primary state in solution is the diketo form, and conversion between the forms is slow. PBZ is also metabolized in the liver and is oxidized to OXPBZ, γ-hydroxy-PBZ, β-hydroxy-PBZ, γ-keto-PBZ, and γ-dihydroxy-PBZ. It is, therefore, imperative that all methods designed for the analysis of PBZ take precautions to ensure that the analyte being measured is the stable form of PBZ in order to prevent oxidation of PBZ to its metabolites, because solutions of PBZ are known to be unstable. In the methods used for the analysis of PBZ and OXPBZ in this study, we took precautions to ensure that experimental conditions for the extraction and analysis would reduce the potential oxidation of PBZ into its metabolites by using ascorbic acid for urine and serum sample extractions and by the addition of DL-DTT for tissue sample extractions. In this way, we ensured that the solution conditions were favorable to the extraction of the stable form of PBZ. We believe that this is one of the reasons why we were able to sensitively detect and analyze PBZ and OXPBZ in the matrixes of interest. We noticed that there were very few published methods for PBZ in which such precautions had been taken in the development of the methods for analysis of PBZ before our study began (10) or thereafter (11, 13, 17).

Table 5. Concentrations of PBZ and OXPBZ (ng/mL or ng/g) measured in a pair of horses administered PBZ intravenously for 4 days and sacrificed 7 days after the last drug administration

					Muscle[b]	
	Serum[a]	Urine[a]	Kidney[b]	Liver[b]	Diaphragm	Gluteal
Horse No.			PBZ			
6 (Mare)	17.0	240	85.2	62.1	11.2	8.1
10 (Gelding)	3.0	6.0	10.8	37.0	2.2	2.5
			OXPBZ			
6 (Mare)	8.1	1050	68.7	9.0	5.7	8.1
10 (Gelding)	1.5	61.0	7.5	6.1	2.3	4.8

[a] Concentrations of PBZ and OXPBZ measured in nanograms per milliliter.

[b] Concentration of PBZ and OXPBZ measured in nanograms per gram.

The full-scan mass spectrum of PBZ and OXPBZ under positive ESI conditions used in this study is shown in Figure 2. The $[M + H]^{+1}$ protonated molecule is the base peak in each case. These precursor ions at m/z = 309.2 and m/z = 325.2 for PBZ and OXPBZ, respectively, generated under (positive) ESI conditions, were used as the quantification ions, whereas the product ions presented in Table 1 were used for confirmation of the identity of the analytes. The precursor ion at m/z = 318.4 from the internal standard PBZ-D_9 (in serum and urine) and the precursor ion at m/z = 319.2 from the internal standard PBZ-D_{10} (in tissues) were monitored and used for quantitative analysis.

The selectivity of the method was evaluated by analyzing six different urine and serum samples according to the described procedure. There were no interfering peaks at the expected retention times of PBZ and OXPBZ under the analytical conditions. It was concluded that because all the negative samples tested negative, the method is capable of detecting the absence of the two analytes in PBZ/OXPBZ-free samples, i.e., it was selective.

Figure 3 shows overlaid SRM mass chromatograms of an extract of a drug-free urine sample fortified with PBZ and OXPBZ at a concentration of 50 ng/mL each, and an extract

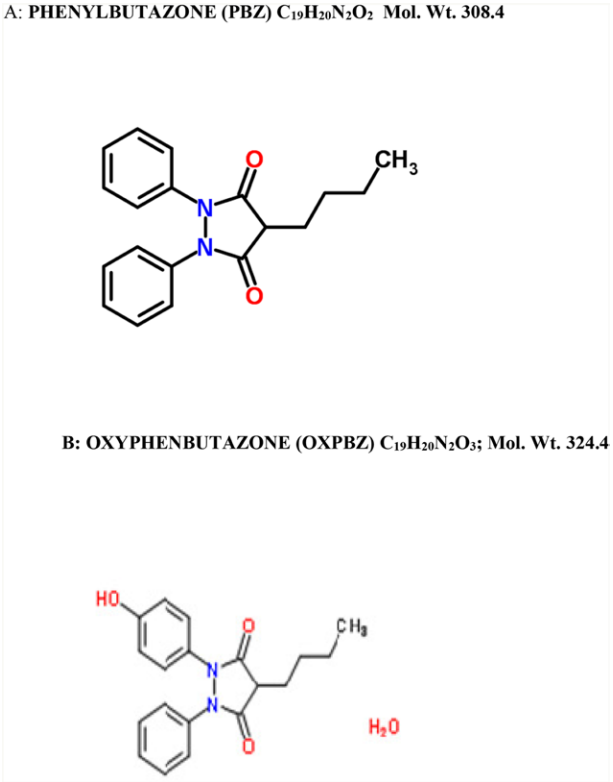

Figure 1. The structural and molecular formulas for PBZ and OXPBZ.

of a drug-free equine urine sample fortified with the internal standard PBZ-D_9. The mass chromatograms show that under (positive) ESI conditions, PBZ was chromatographically resolved from OXPBZ and from other urine endogenous components, whereas the internal standard PBZ-D_9 was mass resolved from PBZ.

A typical overlaid SRM chromatogram for equine serum is shown in Figure 4 for an extract of a drug free serum sample fortified with PBZ and OXPBZ, each at a concentration of 50 ng/mL, and a drug-free serum sample containing the internal standard PBZ-D_9. The mass chromatograms demonstrate that under (positive) ESI conditions, PBZ was chromatographically resolved from OXPBZ and from other serum endogenous components, whereas the internal standard PBZ-D_9 was mass resolved from PBZ.

Figure 5 shows an example of a typical overlaid SRM chromatogram of extracts of a drug-free muscle tissue sample fortified with the internal standard PBZ-D_{10} and drug-free muscle tissue samples containing incurred PBZ and OXPBZ. The mass chromatograms show that under (positive) ESI conditions, PBZ was chromatographically resolved from OXPBZ and from other tissue endogenous components, whereas the internal standard PBZ-D_{10} was mass resolved from PBZ.

Linearity

Analysis of the residuals of the regression data generated for the detector response ratios versus concentration of PBZ and/or OXPBZ in serum and urine demonstrated that the

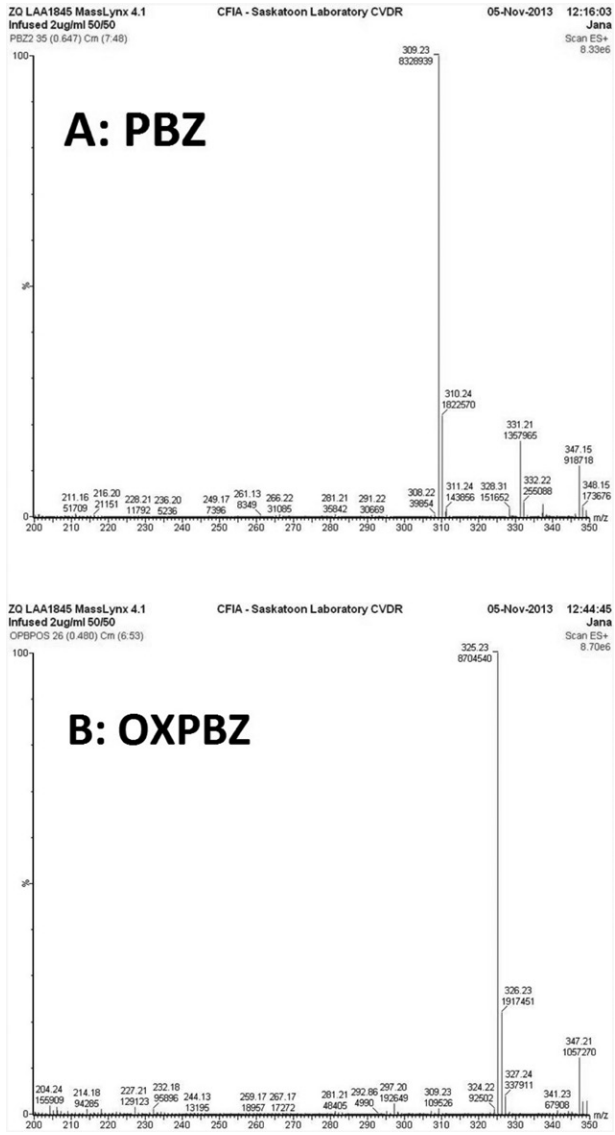

Figure 2. Mass spectra of PBZ and OXPBZ.

data were not homoscedastic, but heteroscedastic. Therefore, a weighted least-squares regression was used to describe the linear regression for serum and urine. For equine urine, the method was determined to be linear over the calibration ranges of 1.0–150 ng/mL for PBZ and 2.0–300 ng/mL for OXPBZ, with a regression coefficient of determination value >0.99. For serum, the method was determined to be linear over the calibration ranges of 1–50 and 2–100 ng/mL for PBZ and OXPBZ, respectively, with a coefficient of determination value >0.99. The LOQs for the LC-MS/MS method for urine and serum were determined to be 1.0 ng/mL for PBZ and 2.0 ng/mL for OXPBZ.

Analysis of the residuals of the regression data for the tissue method showed that the data were homoscedastic; therefore, no weighting factor was applied to the regression data for the quantitative analysis of PBZ and OXPBZ in tissues. The calibration curves for tissues were linear between 0.5 and 100 ng/g, with a coefficient of determination value >0.99. The

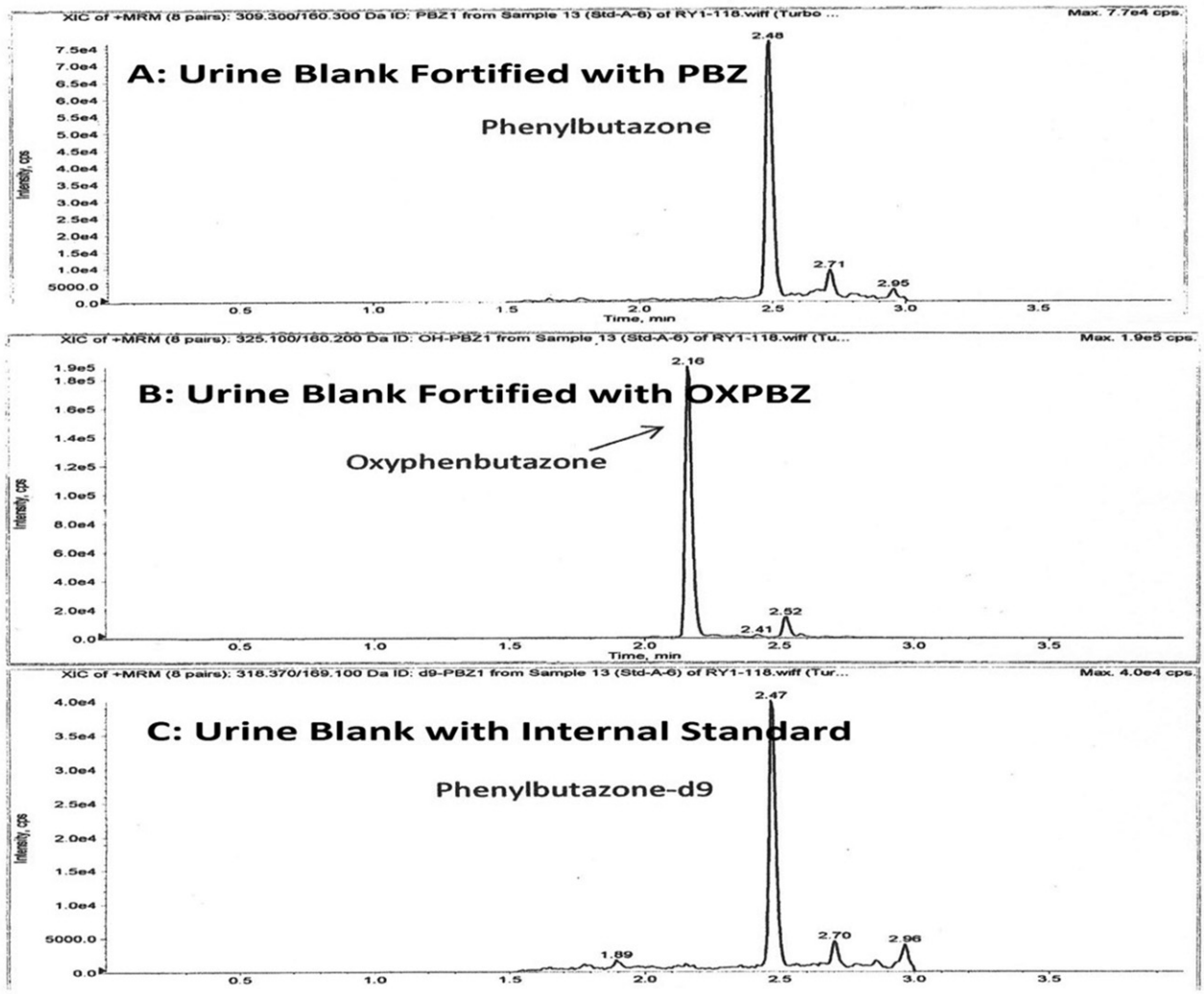

Figure 3. A typical overlaid LC-MS/MS SRM mass chromatogram of an extract obtained from a drug-free urine sample fortified with (A) 50 ng/mL PBZ, (B) 50 ng/mL OXPBZ, or (C) the internal standard PBZ-D_9 at a concentration of 50 µg/mL.

LOQ for the LC-MS/MS method for tissues was determined to be 0.5 ng/g for both PBZ and OXPBZ.

Recovery

The apparent recovery of the method for the extraction of PBZ and OXPBZ residues in urine and serum was evaluated by comparing the results obtained from PBZ/OXPBZ-free serum and urine samples fortified with PBZ and OXPBZ over the calibration range (fortified-matrix samples) with PBZ and OXPBZ concentrations in neat solvent at concentrations over the calibration range. In serum, the apparent recoveries for PBZ were calculated as 75 and 67% at 3.0 and 40 ng/mL, respectively. The apparent recovery for OXPBZ was 83% at 6 ng/mL and 82% at 80 ng/mL. In urine, the apparent recovery for PBZ was 24% at 3 ng/mL and 21% at 120 ng/mL. The apparent recovery for OXPBZ was 23% at 6 ng/mL and 28% at 240 ng/mL. Although the apparent recoveries for PBZ and OXPBZ were low for urine analysis, the high sensitivity, precision, and accuracy of the analytical method enabled the method to qualify for use in the study.

Absolute recoveries of PBZ and OXPBZ in animal tissues were calculated from the slopes of the calibration curves generated for fortified-matrix and matrix-matched curves. The absolute recoveries for PBZ and OXPBZ in muscle, kidney, and liver tissues ranged from 80–95, 83–98, and 79–87%, respectively.

Precision and Accuracy of the Method for Quantitative Analysis in Serum, Urine, and Tissues

The precision and accuracy of the method for serum were evaluated at three concentrations over a 3-day period. Table 2 shows the intraday and interday accuracy and precision characteristic operational parameters of the method for the analysis of PBZ and OXPBZ residues in equine serum and urine. The data show that the method was accurate and precise. There were no replicate results with RSDs >20% for PBZ and/or OXPBZ, and there was no systematic bias observed in the use of the method to quantify serum and urine samples fortified with known concentrations of PBZ and OXPBZ.

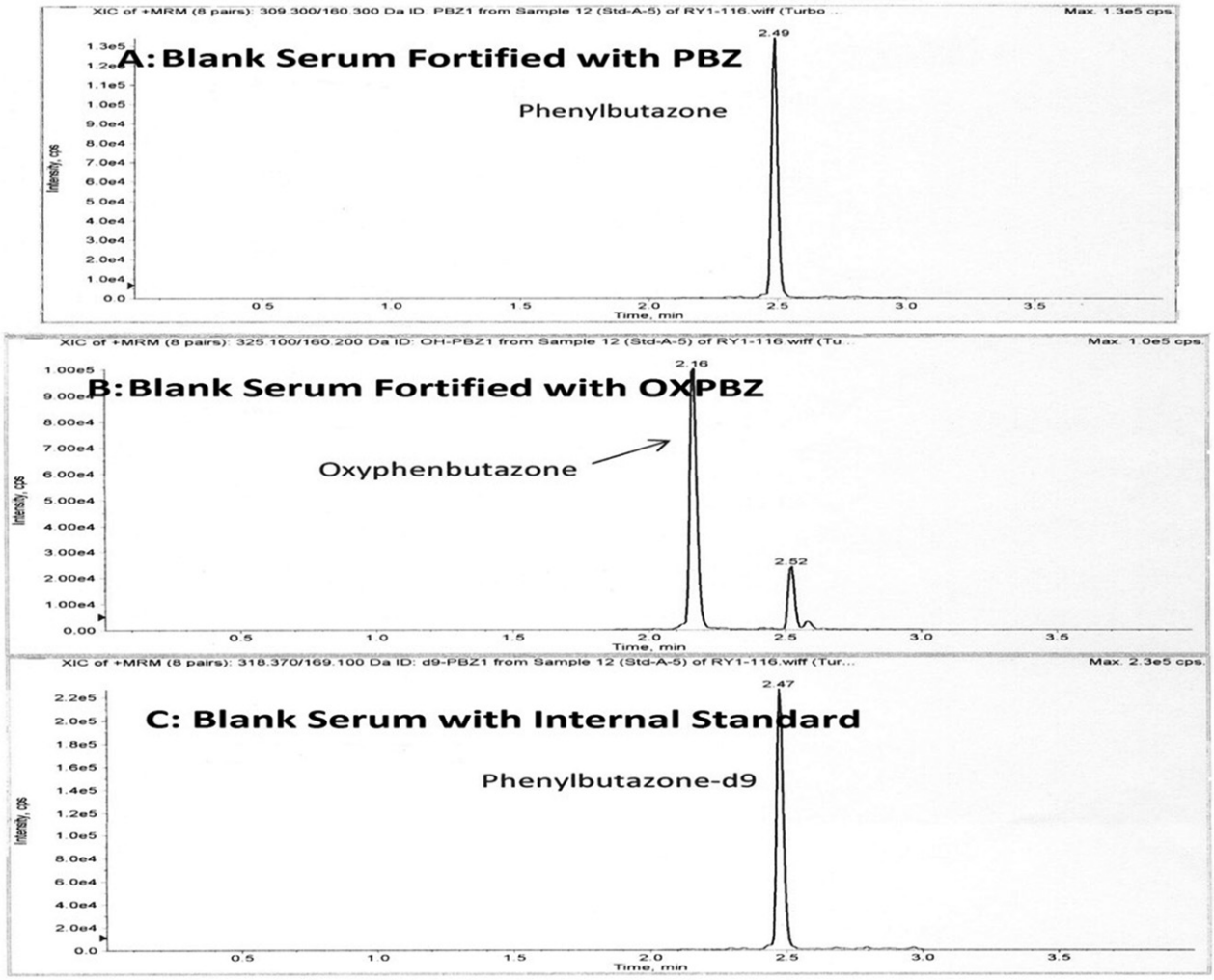

Figure 4. A typical overlaid LC-MS/MS SRM mass chromatogram of an extract obtained from a drug-free serum sample fortified with (A) 50 ng/mL PBZ, (B) 50 ng/mL OXPBZ, or (C) the internal standard PBZ-D_9 at a concentration of 50 µg/mL.

Table 3 shows the intraday and interday accuracy and precision data for the method developed for the quantitative analysis of PBZ and OXPBZ residues in equine muscle, kidney, and liver tissue samples using the familiarization data generated by the analyst. All the replicate results had RSDs ≤20%, and there was no apparent bias observed in the use of the method to quantify tissue samples fortified with PBZ and OXPBZ. Because the tissue method used at the CVDR was already validated for equine, swine, and bovine tissues and was being used in the routine laboratory to support the NCRMP, the abbreviated validation study described was conducted to demonstrate that the analyst who was going to use the method for the analysis of the samples to be generated in the depletion study could reproduce the method and use it for the intended purpose.

In addition, to qualify as a certified analyst able to use the LC-MS/MS method under the laboratory's ISO/IEC 17025:2005 quality system, a set of samples was prepared in duplicate by a senior analyst, coded, randomized, and provided to the trained analyst for analysis using the described procedure. Results of the analysis of blind-fortified samples shown in Table 4 show that all the duplicate results had differences <20% and there was no apparent bias observed in the use of the method to quantify muscle, kidney, and liver tissue samples fortified with PBZ and OXPBZ. This provided an additional assurance that the analyst can use the validated LC-MS/MS method to accurately analyze unknown tissue samples in the absence of certified reference standards.

Application of the Method to Incurred Residues

The analysis results of the tissues and biological fluids taken from the two horses sacrificed 7 days after the last administered drug are shown in Table 5. Seven days after the last drug administration, the concentrations of PBZ and OXPBZ measured in horse 6, the mare, were all higher than the concentrations measured in horse 10, the gelding. Because the objective of the current study was to use these tissue samples to evaluate whether the method would be suitable for the analysis of PBZ and OXPBZ residues in incurred samples and because there were only two horses, we are not able to draw any statistical conclusions from the data. However, it is fair to

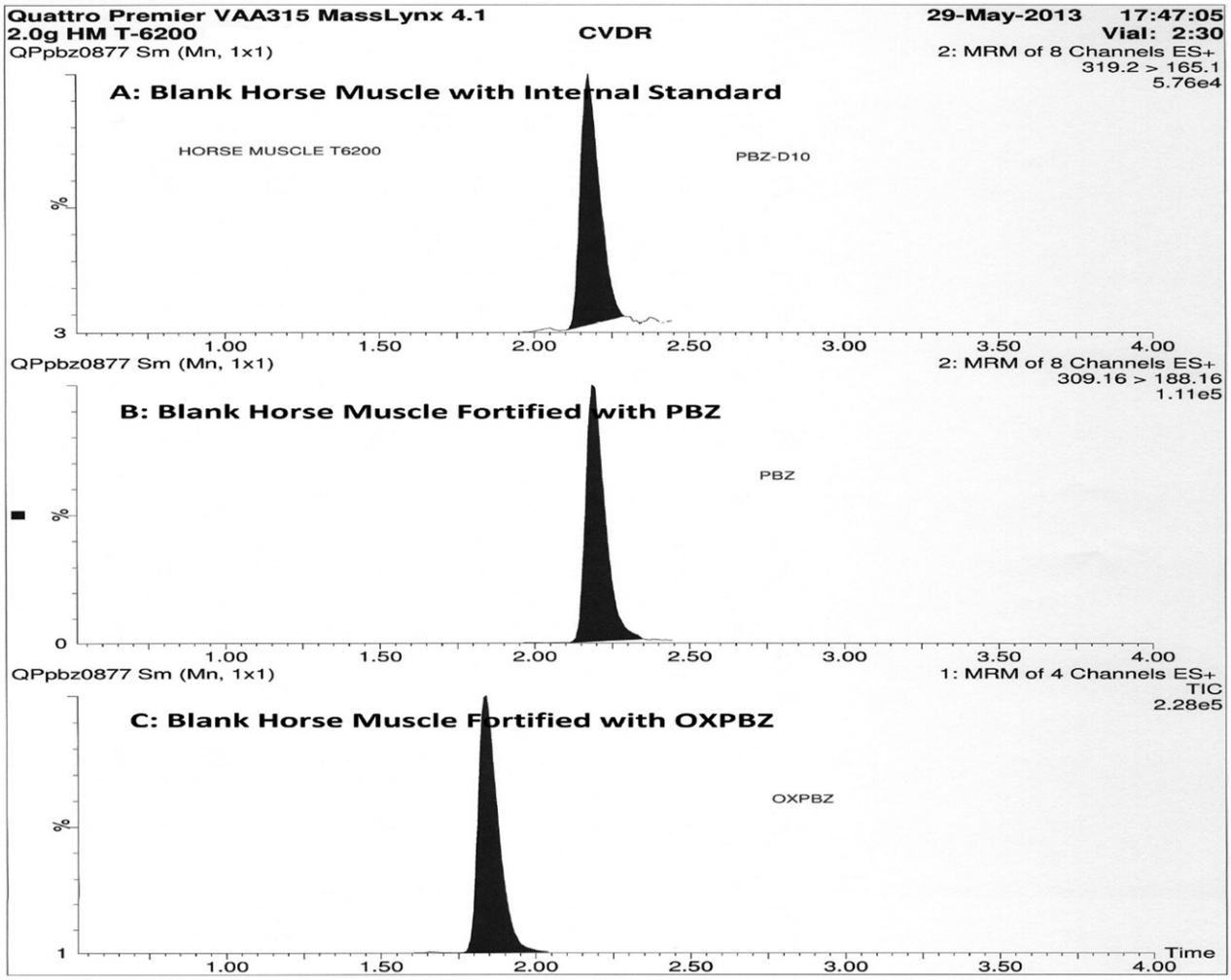

Figure 5. A typical overlaid LC-MS/MS SRM mass chromatogram of an extract obtained from a drug-free horse muscle tissue sample fortified with (A) 50 ng/mL PBZ, (B) 50 ng/mL OXPBZ, or (C) the internal standard PBZ-D_{10} at a concentration of 2 µg/mL.

conclude that the concentrations of PBZ and OXPBZ measured in all the matrixes tested were highest in urine in the mare (240 and 1050 ng/mL, respectively) and in the gelding (6.0 and 61.0 ng/mL, respectively). In urine, the measured concentrations of the metabolite OXPBZ were higher than the corresponding concentrations of PBZ. This appeared to be the only matrix in which the OXPBZ concentrations exceeded those of PBZ.

For drugs like PBZ that are excreted in urine, it was expected that the urine concentrations would exceed the concentrations in serum and tissues (17, 18). Our study confirmed this expectation. The concentrations of PBZ and OXPBZ in urine were also more variable than the concentrations in some of the other matrixes because the urine sample collected at slaughter was from urine accumulated in the individual horse's bladder over a variable period of time. Therefore, the urine sample consisted of an average of drug concentrations over a period of time and was expected to be inconsistent with the corresponding tissue samples in which the PBZ or OXPBZ concentration reflected a single moment in time.

In serum and kidney and liver tissues, the PBZ concentrations measured were slightly higher than the concentrations of OXPBZ on a horse-by-horse basis. In the serum of horse 6 (the mare), the concentration of PBZ was 17.0 ng/mL, almost double the concentration of OXPBZ (8.1 ng/mL); the trend was similar in horse 10 (the gelding), in which the PBZ concentration was 3.0 ng/mL, twice that of OXPBZ (1.5 ng/mL). The concentration of PBZ measured in the gluteal muscle (8.1 ng/g) and the diaphragm muscle (11.2 ng/g) of horse 6 was not significantly different from the PBZ concentration measured in the kidney of horse 10. The OXPBZ concentration in the gluteal muscle (8.1 ng/g) and the diaphragm muscle (5.7 ng/g) of horse 6 was also not significantly different from the concentration of OXPBZ in the kidney of horse 10.

It is of interest to note that 7 days after the last drug administration of PBZ, the two LC-MS/MS methods were still capable of measuring quantifiable concentrations of both PBZ and OXPBZ residues in all the incurred matrixes tested. The low concentrations of both PBZ and OXPBZ residues measured in the incurred samples support our initial assessment that our previous LC/UV method would not have been sensitive enough to have been used to conduct the depletion study we were contemplating, and most of the other methods published after the start of this study may also not have been sensitive enough for the intended purpose. It is worthy to note that the

analytical results from the two LC-MS/MS methods used for the analysis of PBZ and OXPBZ in the 7-day post–drug administration samples showed that whenever there were quantifiable residues of PBZ and/or OXPBZ in the biological fluids, there were also quantifiable concentrations of the drugs in the tissues. However, there were not enough data to enable the derivation of any correlation factor (or factors) between the concentrations in biological fluids and those in the tissues to permit using biological fluid concentrations as a basis for not sending a horse to slaughter.

In the EU, for several prohibited or unauthorized substances such as PBZ, minimum required performance limits (MRPLs) for the analytical methods have been specified. An MRPL is the concentration of a prohibited substance, a metabolite of a prohibited substance, or a marker residue that laboratories shall be able to routinely detect and identify. In 2007, the EU Reference Laboratories for residues of veterinary drugs published a technical guidance document that established a minimum recommended concentration of 5 ng/g for the analytical methods for PBZ residues and its metabolite OXPBZ in matrixes such as muscle, milk, kidney, liver, and plasma. In other words, the detection capability (CCβ) for screening methods, or the decision limit (CCα) for confirmatory methods, must be less than or equal to 5 ng/g. On the basis of the results obtained from the fortified and physiologically incurred biological fluids and equine tissues containing PBZ and OXPBZ, it was concluded that the two LC-MS/MS methods were suitable for the quantitative analysis of PBZ and OXPBZ residues in equine urine, serum, and tissues and were, therefore, applied to the analysis of samples generated from a pilot study in a depletion study of PBZ and its active metabolite, OZPBZ, from the horse.

This is the first report of the use of these two sensitive methods for the analysis of PBZ and OXPBZ residues in biological fluids and tissues of horses after the methods were evaluated for their suitability in analyzing incurred residues of both PBZ and OXPBZ and before they were actually applied for the depletion study. The results of the pilot study and the complete depletion study will be published elsewhere.

Conclusions

Two validated LC-MS/MS methods were evaluated for their suitability for use in a residue depletion study using incurred samples (serum, urine, and kidney, liver, and muscle tissues) generated from one male and one female horse that were experimentally administered with a commercial formulation of PBZ and sacrificed 7 days after the last drug administration. The methods permitted the determination of the concentrations of residues of PBZ and its active metabolite, OXPBZ, in all the matrixes studied. The LC-MS/MS method for horse tissues, with an LOQ of 0.5 ng/g for both PBZ and OXPBZ, and the LC-MS/MS method for biological fluids, with an LOQ of 1.0 ng/g for PBZ and 2.0 ng/g for OXPBZ, were shown to meet the fit-for-purpose criteria for use in conducting a depletion study.

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