Research article

Drug Testing and Analysis

Received: 31 August 2015 Accepted: 7 April 2016 Published online in Wiley Online Library

(www.drugtestinganalysis.com) DOI 10.1002/dta.2020

Analysis of phenylbutazone residues in horse tissues with and without enzyme-hydrolysis by LC-MS/MS

Joe O. Boison,[a]* Trisha Dowling,[b] Ron Johnson[c] and Jana Kinar[a]

Phenylbutazone (PBZ) is permitted to be used for the treatment of musculoskeletal pain and inflammation in race horses but it is not approved for use in horses destined for human consumption. In a recent study initiated in our laboratory to study the disposition of PBZ and its oxyphenbutazone (OXPBZ) metabolite in equine tissues, we compared the effect of an additional enzymatic hydrolysis step with ß-glucuronidase on the results of the analysis for PBZ without enzymatic hydrolysis. Incurred tissue samples obtained from a female horse dosed with PBZ at 8.8 mg/kg for 3 days and sacrificed 6 days following the last administration were used for this study. Liver, kidney, and muscle tissues were collected, extracted, cleaned up on a silica-based solid-phase extraction (SPE) preceded by a weak-anion exchange SPE and analyzed with our in-house validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for PBZ and OXPBZ. Addition of the hydrolysis step resulted in a significant increase in recovery of both PBZ and OXPBZ residues. © 2016 Her Majesty the Queen in Right of Canada. Drug Testing and Analysis © 2016 John Wiley & Sons, Ltd.

Keywords: phenylbutazone; oxyphenbutazone; LC-MS/MS; equine liver; kidney; muscle tissue; enzyme hydrolysis

Introduction

Phenylbutazone (PBZ) has been used for a long time in treating race horses for musculoskeletal pain and inflammation but it is not approved for use in horses destined for human consumption. Canadian horse meat is primarily exported to Switzerland, Japan, France, Belgium, and Kazakhstan.[1–3] North American horses that are sacrificed for human consumption may have received treatment with PBZ during their lifetime. In 1997, the European Medicines Agency (EMA) evaluated PBZ for the purpose of establishing maximum residue limits (MRLs) in food of animal origin. The data available at that time did not allow a conclusion on the amount of PBZ that could be considered safe.[4] As no MRL was established, the European Union (EU) banned the use of PBZ in food-producing animals. Since more and more race horses are finding their way into the food chain, it is imperative that residues of PBZ and its active metabolite, oxyphenbutazone (OXPBZ), be monitored in sacrificed horses to ensure that consumers are not exposed to any unwanted drug residues resulting from PBZ administration. The chemical structures of PBZ, OXPBZ and their deuterated analogues are shown in Figure 1.

A recent study was conducted jointly by the University of Saskatchewan, University of Guelph, and the Canadian Food Inspection Agency to estimate the depletion profile of PBZ residues in horse tissues. After reviewing the results from that study, it was decided to investigate if including an enzymatic hydrolysis step to the sample preparation procedure would have an impact on the recovery and the analysis of PBZ and OXPBZ residues from equine tissue samples. It was well known that PBZ binds strongly to tissue proteins.[5–8] This paper describes the results of a comparative study that was conducted in our laboratory for the analysis of PBZ and OXPBZ with and without hydrolysis. This is the first reported study of the effect of β-glucoronidase hydrolysis on PBZ residues in incurred equine muscle, kidney, and liver tissues.

Materials and method

Incurred equine tissue was generated by dosing a 10-year-old female horse with PBZ at 8.8 mg/kg once a day for 3 days and sacrificed within 6 days. Liver, kidney, and muscle tissue samples obtained from this horse were homogenized prior to extraction.

Acetonitrile, methanol, hexane, and isopropanol were high performance liquid chromatography (HPLC) grade supplied from Caledon. Acetonitrile was supplemented with DL-dithiothreitol (Sigma-Aldrich, Oakville, ON, Canada) at 250 mg/L to help stabilize the phenylbutazone. Ammonium hydroxide and formic acid were purchased from EMD (Millipore, Etobicoke, ON, Canada) and ammonium formate from Fisher.

A pH 4.5 ascorbic/acetate solution was prepared by combining 2.7 g sodium acetate and 0.17 g ascorbic acid with 100 mL with deionized water. Acetic acid was used to pH the solution to 4.5. All the reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada).

An 85 000 units/mL *ß-glucuronidase* – H2 Type enzyme solution from *Helix pomatia* – was purchased from Sigma-Aldrich (Oakville, ON, Canada). Twenty µL of the enzyme solution was added to 4 mL of pH 4.5 solution. Bond Elut Silica 3 cc 500 mg from Agilent

* *Correspondence to: Joe. O. Boison, CFIA Saskatoon Laboratory, 116 Veterinary Road, Saskatoon, SK S7N 2R3, Canada. E-mail: joe.boison@inspection.gc.ca*

a *CFIA Saskatoon Laboratory, 116 Veterinary Road, Saskatoon, SK., Canada S7N 2R3*

b *Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine, Saskatoon, SK, Canada S7N 5B4*

c *Department of Biomedical Sciences, Ontario Veterinary College, University of Guelph, ON, Canada N1G 2W1*

and Oasis WAX 3 cc 60 mg extraction cartridges from Waters were used. Pre-slit Mini-UniPrep 0.2 µm syringeless PTFE filters were purchased from Whatman (VWR Edmonton, AB, Canada).

The mobile phase consisted of methanol (A) and 5 mM Ammonium formate, pH 3.9 (B). Mobile phase B was prepared by adding 100 mL 0.1 M ammonium formate to 10 mL 0.1 % formic acid and making it up to 2 L with water. The gradient conditions used for the analysis are presented in Table 1.

Phenylbutazone (Sigma-Aldrich, Oakville, ON, Canada), phenylbutazone $D_{10,}$ oxyphenbutazone $-D_9$ (CDN Isotopes, Pointe Claire, QUE, Canada), and oxyphenbutazone (Toronto Research Chemical) stock solutions were made at 2 µg/mL in methanol and diluted to working solution concentrations of 0.25 µg/mL.

A Waters Acquity UPLC interfaced to a Waters Micromass Quattro Premier mass spectrometer with a Z-spray interface controlled by MassLynx version 4.1 software operated in the positive electrospray ionization mode was used for the liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. The optimized parameters for the analysis are shown in Table 2.

Collision energies, precursor, and product ions used for the quantitative and confirmatory analysis of PBZ and OXPBZ residues in the samples using PBZ-D_{10} as internal standard are shown in Table 3.

Matrix-fortified (MF) calibration curves were generated by fortifying blank (PBZ-free) kidney, muscle and liver tissue samples with PBZ, OXPBZ, and the internal standard PBZ-D_{10} after they had undergone the overnight incubation with the enzyme. MF standards containing 0.5, 1.0, 2.0, 5.0, 10, 15, 25, 50, and 100 ng/g PBZ and OXPBZ and 20 ng/g PBZ-D_{10} were prepared and used for the quantitative analysis of PBZ and OXPBZ residues in the experimental samples.

OXPBZ $-D_9$ ($C_{19}H_{11}D_9N_2O_3$; MW = 333.43) was obtained as internal standard for the quantification of OXPBZ but, unlike PBZ-D_{10} which showed the same recovery efficiency as PBZ, OXPBZ-D_9 showed recovery efficiencies that were highly variable compared to OXPBZ itself, and so it was decided not to include it in the quantitative analysis procedure for OXPBZ. Quantification for both analytes was based on matrix fortified standard calibration curves. PBZ was quantified using a calibration curve generated from the mass detector response ratio of PBZ to that of PBZ-D_{10} versus concentration of PBZ in the matrix fortified calibration solutions, while OXPBZ was quantified based on a calibration curve generated from the mass detector response of OXPBZ versus concentration of OXPBZ in the matrix fortified calibration solutions.

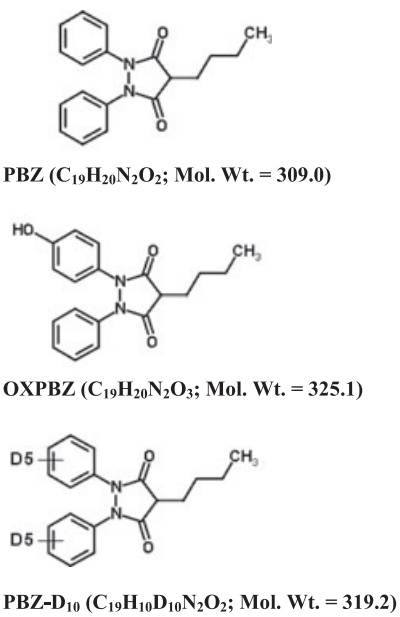

Figure 1. Chemical structures of phenylbutazone, oxyphenbutazone and phenylbutazone-D_{10} (Internal Standard).

Table 1. Gradient conditions used for the LC-MS/MS analysis

Time	%A	%B	Flow mL/min	Curve
0	25	75	0.5	-
3.5	90	10	0.5	6
3.9	95	5	0.5	1
5	95	5	0.5	1
5.5	25	75	0.5	6
6.5	25	75	0.5	6

Table 2. Optimized instrument settings for the MS analysis of PBZ and OXPBZ

Capillary voltage (kV)	0.5	LM1/HM1 resolution	13.6/13.5
RF Lens voltage (V)	0.3	LM2/HM2 resolution	13.0/13.0
Multiplier voltage (V)	700	Ion energy 1	0.3
Extractor voltage (V)	3.0	Ion energy 2	0.8
Cone voltage (V)	25	Column temp (°C)	50
Desolvation gas flow rate (L/h)	1150	Sample temp (°C)	10
Cone gas flow rate (L/h)	50	Desolvation Temp (°C)	450

Table 3. MS/MS operating parameters for the analysis of PBZ and OXPBZ using PBZ-D_{10} as Internal Standard

Analyte	Retention Time (s)	Precursor Ion (CE)	Product Ion 1 (CE)	Product Ion 2 (CE)	Dwell Time (s)	Cone Voltage (V)
PBZ	2.55	309.0	188.2 (15)	120.0 (20)	0.1	32
OXPBZ	2.11	325.1	204.2 (15)	120.0 (20)	0.1	32
PBZ-D_{10}	2.55	319.2	165.1 (25)	125.0 (20)	0.1	32

CE = Collision Energy

Sample preparation

Twenty µL of ß-glucuronidase was pipetted into 4 mL of the pH 4.5 solution and inverted to mix. This was combined with 2.0 g of tissue and incubated overnight in a water bath set at 37 °C. After incubation, the samples were cooled and 8 mL of acetonitrile stabilized with DL-dithiothreitol was added to each sample and vortex-mixed for 10 min. Samples were centrifuged at 5300 rpm for 10 min after which the supernatant was decanted into a fresh tube and the pellet re-suspended in 4 mL acetonitrile. Samples were vortex-mixed for 5 min followed by the same centrifugation step done previously. The supernatant was decanted into first tube. A silica solid-phase extraction (SPE) was conditioned with 3 mL methanol and samples were loaded onto the cartridge and the eluant was collected. The cartridge was washed with 1 mL acetonitrile and also collected with the eluant. The samples were dried at 50 °C until

Table 4. Results of the analysis of incurred equine muscle, kidney and liver tissues with and without β-Glucuronidase enzymatic hydrolysis

Equine Tissue Matrix	Method without Enzymatic Hydrolysis				Method with Enzymatic Hydrolysis			
	[PBZ] (ng/g)		[OXPBZ] (ng/g)		[PBZ] (ng/g)		[OXPBZ] (ng/g)	
	Concn	Mean ± SD	Concn	Mean ± SD	Concn	Mean ± SD Ave*	Concn	Mean ± SD Ave*
Liver	71	74 ± 3	3.3	3 ± 1	97	97 ± 9	16	17 ± 2
	76		2.5		88		15	
	76		3.4		107		19	
Kidney	25	26 ± 1	10	10 ± 0	32	36*	8.2	14*
	27		10		39		19	
	25		10					
Muscle	0.6	0.4 ± 0.2	<1	<1	2.2	2.0*	1.1	1.0*
	0.5		<1		1.8		0.9	
	0.2		<1					

* Average = only 2 samples

approximately 5 mL remained. Samples were then loaded onto a conditioned Oasis WAX SPE. They were washed with 3 mL water, 3 mL of a 20 % acetonitrile/80 % isopropanol solution, followed by 3 mL 1 % ammonium hydroxide/water. Samples were dried under vacuum for 5 min and then eluted with 9 mL of 5% ammonium hydroxide/methanol. They were evaporated to dryness at 55 °C with nitrogen after which it was reconstituted with 200 μL methanol and 300 μL of water. The samples were sonicated for 5 min and filtered through 0.2 μm PTFE filters. Five 5 μL aliquots were injected onto an Agilent Poroshell 2.1 μm, 2.1 × 50 mm column. Data was generated from a Waters Aquity UPLC coupled with a Waters Premier triple quadrupole mass spectrometer.

Results

The results obtained from replicate analysis of incurred muscle, kidney and liver tissues using our regular method without enzyme hydrolysis and the modified procedure that includes an enzymatic hydrolysis step are shown in Table 4.

Discussion

PBZ, a non-steroidal anti-inflammatory drug (NSAID), is rapidly and completely absorbed following oral administration.[9] As is common with many NSAIDs, PBZ is extensively protein bound which results in a number of drug interactions with other acidic drugs such as anticoagulants, sulfonamides, hypoglycemics, other NSAIDs and glucocorticoids. PBZ is metabolized in the liver to para (oxyphenbutazone) and omega-1 metabolites. Since glucuronidation is a major metabolic pathway for PBZ that enables its transformation from a lipophilic substrate into hydrophilic glucuronides to facilitate its elimination through bile and urine, we investigated the effect, if any, β-glucuronidase enzymatic hydrolysis will have on the extractability of PBZ from animal tissue.

In our previously validated method[10] as well as other previously published methods for the analysis of PBZ and its OXPBZ metabolite,[11–13] we made no effort to evaluate whether an enzymatic hydrolysis step would improve the extractability of PBZ and OXPBZ primarily because we didn't have access to naturally incurred equine tissue samples with measurable concentrations of PBZ and OXPBZ that would have enabled us to properly evaluate this additional step.

In a recent pilot study conducted at the University of Saskatchewan to investigate the disposition of PBZ in equine tissues and serum, we learned that there will be measurable concentrations of both PBZ and OXPBZ in the muscle, kidney and liver tissues of a horse administered 8.8 mg/kg PBZ for 3 days and sacrificed within 7 days following the last administration. Therefore, we did that experiment and collected tissue samples from a horse that was sacrificed on the sixth day post dose. The results of the experiment conducted using incurred samples obtained from this animal analyzed with the validated method with a limit of quantification (LOQ) of 0.5 ng/g for both PBZ and OXPBZ are shown in Table 4.

The results show that in the absence of enzymatic hydrolysis, liver tissue obtained from the horse sacrificed 6 days post dose contained the highest concentration of PBZ followed by kidney and muscle. With the additional enzymatic hydrolysis step in the sample preparation procedure, the recovery of PBZ were elevated by about a factor of 1.3 in liver, 1.4 in kidney, and 4.7 times in muscle tissues. The concentration of OXPBZ residues was highest in the kidney followed by liver but it was below the LOQ of the method for muscle using our previously published method without enzymatic hydrolysis.

With enzyme hydrolysis, however, the recovery of OXPBZ in equine liver was elevated by a factor of 5.7, followed by kidney by 1.4. The concentration of OXPBZ which were below the LOQ of the method when the method without enzymatic hydrolysis was used for the analysis were clearly found to be present at quantifiable concentrations when the procedure that included the enzymatic hydrolysis step was used for the sample analysis.

Conclusion

The recovery of PBZ and OXPBZ residues are improved with the addition of a β-glucuronidase enzyme hydrolysis step. It is, therefore, strongly recommended that methods developed for the analysis of PBZ and its OXPBZ metabolite consider the inclusion of this enzyme hydrolysis step.

References

[1] Canadian Meat Council. Horse Meat Production in Canada. Canadian Meat Council, Ottawa, ON, Canada, **2013**.

[2] Alberta Agriculture and Rural Development Information Management. *Agriculture Statistics Yearbook 2012*, 38th Edition, Alberta Agriculture and Rural Development, AB, Canada, **2013**.
[3] Alberta Farm Animal Care. The Alberta Horse Welfare Report. Alberta Equine Welfare Group, High River, AB, Canada, **2008**.
[4] Summary Report CVMP Phenylbutazone 1997. EMEA/MRL/297/97-FINAL. European Agency for Veterinary Medicinal Products, London, UK, **1997**.
[5] P. Lees, P. L. Toutain. Pharmacokinetics, pharmacodynamics, metabolism, toxicology, and residues of phenylbutazone in humans and horses. *Vet. J.* **2013**, *196*, 294.
[6] E. L. Gerring, P. Lees, J. B. Taylor. Pharmacokinetics of phenylbutazone and its metabolites in the horse. *Equine Vet. J.* **1981**, *13*, 152.
[7] P. Lees, J. B. Taylor, T. E. Maitho, J. D. Millar A. J. Higgins. Metabolism, excretion, pharmacokinetics and tissue residues of phenylbutazone in the horse. *Cornell Vet.* **1987**, *77*, 192.
[8] P. G. Dayton, Z. H. Israili, J. M. Perel. Influence of binding on drug metabolism and distribution. *Ann. N.Y. Acad. Sci.* **1973**, *226*, 172.
[9] P.B. Smith, J. Caldwell, R.L. Smith, M. W. Horner, M. S. Moss. The bioavailability of phenylbutazone in the horse. *Xenobiotica* **1987**, *17*, 435.
[10] P. Asea, K. D. Souster, C. D. C. Salisbury, J. O. Boison. Development and Validation of a Method for the Determination of Phenylbutazone Drug Residues in Bovine, Equine, and Porcine Muscle Tissues Using HPLC with UV Detection. *J. Liq. Chromatogr. R. T.* **2004**, *27*, 3013.
[11] L. Bousova, L. Hollosi, E. Ates, M. Godulal. Validated method for the determination of phenylbutazone in horse meat with LC-MS/MS. Thermo Fisher Scientific. Food Safety Response Center, Dreieich, Germany.
[12] J. Blanca, R. Muñoz, P. Muñoz, A. Ángela, R. Díaz, M. Martín de Pozuelo. Analysis of Phenylbutazone residues in horse muscle. National Centre for Food. Spanish Agency for Food Safety and Nutrition (AESAN), Madrid, Spain, **2013**.
[13] S. B. Clark, S. B. Turnipseed, G. J. Nandrea, M. R. Madson. Confirmation of phenylbutazone residues in bovine kidney by liquid chromatography/mass spectrometry. US Food and Drug Administration, Denver Federal Center. *J. AOAC Int.* **2002**, *85*, 1009.