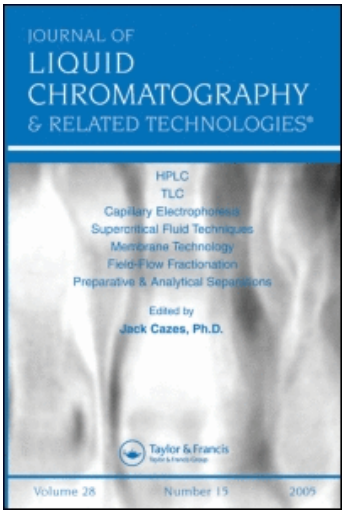

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Development and Validation of a Method for the Determination of Phenylbutazone Drug Residues in Bovine, Equine, and Porcine Muscle Tissues Using HPLC with UV Detection

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ABSTRACT

A sensitive liquid chromatographic method with UV detection has been developed for the analysis of phenylbutazone (PBZ) drug residues in bovine, equine, and porcine muscle tissues. PBZ is extracted from muscle tissues with ethyl acetate/methanol solution containing DL-dithiothreitol. The tissue extract is centrifuged, evaporated to minimum volume under nitrogen, and cleaned up on a conditioned Florisil solid phase extraction cartridge. PBZ is eluted with a 1 : 1 mixture of ether and a solution of

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methylene chloride : methanol : acetic acid (94 : 4 : 2 v/v/v). The eluate is evaporated to dryness with nitrogen, reconstituted in mobile phase, filtered, and analyzed by reversed-phase liquid chromatography with UV detection at 270 nm. The method, with a detection limit of 3 ppb, was used to monitor the prevalence of PBZ drug residues in equine, porcine, and bovine muscle tissues obtained from animals slaughtered in federally inspected Canadian abattoirs.

Key Words: Phenylbutazone; Validation; HPLC; UV detection.

INTRODUCTION

Phenylbutazone (PBZ), 4-butyl-1,2-diphenyl-3,5-pyrazolidenedione, is a non-steroidal anti-inflammatory drug (NSAID) with antipyretic and analgesic activity. In 1949, PBZ became available for use in humans for the treatment of rheumatoid arthritis and gout. However, because it is known to induce blood dyscrasias, including aplastic anemia, leukopenia, agranulocytosis, thrombocytopenia, and deaths in humans, it is no longer approved nor marketed for any human use in the United States. PBZ was approved only for oral and injectable use in dogs and horses by the United States Food and Drug Administration (FDA) for the treatment of bone and joint inflammation.[1] Use in horses is limited to horses not intended for food and there are currently no approved uses of PBZ in food-producing animals. Yet, a survey study conducted in 2000–2002 of US slaughter cattle revealed the presence of residues of PBZ in female dairy cattle 20 months of age or older.[2] As a result, FDA issued an order in February 2003 prohibiting the extra-label use of PBZ human and animal drugs in female dairy cattle 20 months of age or older.

While the literature is replete with methods for the determination of PBZ in plasma, urine, interstitial fluids, and bovine milk, there is a paucity of published methods for the determination of PBZ in animal tissues. An LC-MS/MS method was developed by Clark et al.[3] and used to confirm presumptive positive test results obtained from an enzyme-linked immunosorbent assay (ELISA) they had developed, to screen PBZ residues in bovine kidney tissues collected from US slaughter houses. In order to determine the prevalence of use or misuse of NSAIDs in food animal production in Canada, a project was set up to develop analytical methods that would be suitable for the determination of the NSAIDs including flunixin, PBZ, dipyrone, etodolac, carprofen, vedaprofen, and ketoprofen in food animal tissues. In this regard, methods were developed for flunixin[4] and dipyrone[5] that were used to demonstrate that these veterinary drugs were not being used, to any

extent, in food animal production in Canada. This paper describes the analytical method that was developed and validated for the determination of PBZ residues in injection sites and non-injection site bovine muscle tissues, and the application of the method to the analysis of tissues of Canadian slaughter animals over a 3-year period.

EXPERIMENTAL

Apparatus

Solid phase extraction cartridges, 2 g florisil (12 mL capacity), were obtained from Varian (Harbor City, CA). The solvent evaporator, a Zymark Turbo LV, was obtained from Zymark Ltd. (Mississauga, ON, Canada).

HPLC System

The system consisted of a Waters 2695 LC system with a diode array detector. Chromatographic separation was achieved by isocratic analysis on a waters symmetry C_8 analytical column (150 × 3.9 mm 5 m plus guard, Waters Canada, Toronto, ON, Canada), held at 40°C with a mobile phase flow rate of 1.2 mL/min. PBZ was monitored at a wavelength of 270 nm.

Reagents

All solvents used in this study, including acetonitrile, methanol, ethyl acetate, and methylene chloride were of HPLC grade obtained from Caledon (Georgetown, ON, Canada). Ammonium acetate, ammonium hydroxide, glacial acetic acid, and sodium acetate were of reagent grade obtained from Fisher Scientific (Pittsburg, PA). DL-dithiothreitol, 99% purity, and trifluoroacetic acid were obtained from Sigma (St. Louis, MO). Diethyl ether, anhydrous, was obtained from BDH (Toronto, Canada). Water was obtained from a Barnstead, reverse osmosis Nanopure Ultra Filtration unit (Dubuque, IA).

A 0.05 M ammonium acetate solution (pH 5.0) was prepared by dissolving 1.95 g ammonium acetate in approximately 450 mL water and adjusting the pH to 5.0 with trifluoroacetic acid. It was mixed and made up to volume with water in a 500 mL volumetric flask, and filtered prior to use.

Stabilizing solution A was prepared by dissolving 125 mg DL-dithiothreitol in 500 mL ethyl acetate. Extraction solution B was prepared by mixing 210 mL

stabilizing solution A with 30 mL methanol. It must be prepared fresh daily. The SPE conditioning and rinse solution C was prepared by mixing 1 mL methanol, 1 mL ammonium hydroxide, and 70 mL methylene chloride with 70 mL stabilizing solution A. It must be prepared fresh daily.

Elution solution D was prepared by measuring approximately 60 mL methylene chloride into a 100 volumetric flask, adding 2 mL glacial acetic acid, 4 mL methanol, mixing and diluting to volume with methylene chloride. A fresh 1 : 1 (v/v) mixture of this solution and diethyl ether should always be prepared for use.

The mobile phase was prepared by mixing 0.05 M ammonium acetate solution (pH 5.0), methanol, and acetonitrile (53 : 35 : 12, v/v/v).

Preparation of Standard Solutions

Stock standard solutions of diclofenac (DCF) and PBZ (50 g/mL) were prepared by dissolving 0.0050 g DCF sodium [analytical standard 99% + purity (Sigma-Aldrich Canada, Oakville, ON)], or PBZ [analytical standard 99% + purity (Sigma-Aldrich Canada, Oakville, ON)], in 80 mL methanol and 5 mL stabilizing solution A in two separate 100 mL volumetric flasks. The solution was thoroughly mixed and brought to volume with methanol. Stock solutions were prepared every 3 months and stored at 2–4°C. Note that PBZ is toxic and a suspected cancer causing agent. It must, therefore, be handled with care.

From the stock solutions, working standard solutions of 2.0 μg/mL of DCF and PBZ were prepared weekly by appropriate dilution with methanol and used for the preparation of calibration standard solutions. Working standard solutions must be stored at 2–4°C.

Sample Preparation

Portions (2.00 ± 0.05 g) of thawed test sample were accurately weighed into individual 50 mL polypropylene centrifuge tubes. Six other 2.00 ± 0.05 g drug free (control) tissues were weighed into individual 50 mL polypropylene centrifuge tubes. Of the 2 μg/mL DCF working solution, 100 μL were added to each weighed sample (DCF serves as an internal standard and retention time marker for the analytical method). To the control tissues were added 0, 10, 25, 50, 100, and 200 μL of the 2.0 μg/mL PBZ working standard solution, to prepare tissue equivalent calibration standards at 0, 10, 25, 50, 100, and 200 ng/g PBZ, respectively, each containing 100 ng/g of DCF. Each test or fortified control sample was mixed and allowed to sit for 15 min. Extraction

solution B (8 mL) was added to each sample tube and homogenized for 15 sec. The samples were mechanically shaken for 2 min and centrifuged at 5°C for 5 min at 2850 × *g*. The supernatant was decanted into a second labeled centrifuge tube and the extraction steps repeated twice, combining the supernatants in the second tube. The pooled tissue extracts were centrifuged at 5°C for 10 min and 2850 × *g*, the clear extract was transferred into a labeled 50 mL polypropylene centrifuge tube, and evaporated to near dryness with nitrogen at 55°C. The residue was re-constituted in 2 × 2.5 mL portions of solution C, combined, and loaded onto a Florisil SPE cartridge that had been conditioned with 5 mL of solution C. After the SPE cartridge had been washed with 1 mL solution C, the retained PBZ and DCF were eluted with 12 mL of solution D into a 15 mL glass centrifuge tube, and the extract evaporated to dryness with nitrogen at 55°C. The dried extract was reconstituted in 400 μL mobile phase, vortex-mixed at high speed for 20 sec, filtered into an autosampler vial, and 50 μL injected into the HPLC system.

Liquid Chromatographic Analysis

Peak heights of the peak responses of PBZ and DCF in the extracts from the fortified control tissue standards and the test samples were measured. Using regression analysis, a calibration curve of response ratio of PBZ (i.e., peak height of PBZ/peak height of DCF) vs. concentration of PBZ (ng/g) added to control tissue was derived. If PBZ was detected in the test extract, its response ratio was calculated and its concentration determined by interpolation from the regression curve.

Validation

Selectivity and Specificity

The selectivity of the method (i.e., the ability of the test method to detect truly negative samples as negative) was demonstrated by analyzing control muscle tissues (injection site or non-injection site) obtained from two beef cows with a known history of not having been treated with any NSAID, using the developed analytical method. The specificity of the method was demonstrated by analyzing six sets of negative control bovine muscle tissues obtained from six different geographical locations in Canada using the method. Figure 1(a) is a typical chromatogram of an extract obtained from a control bovine muscle tissue fortified with the internal standard,

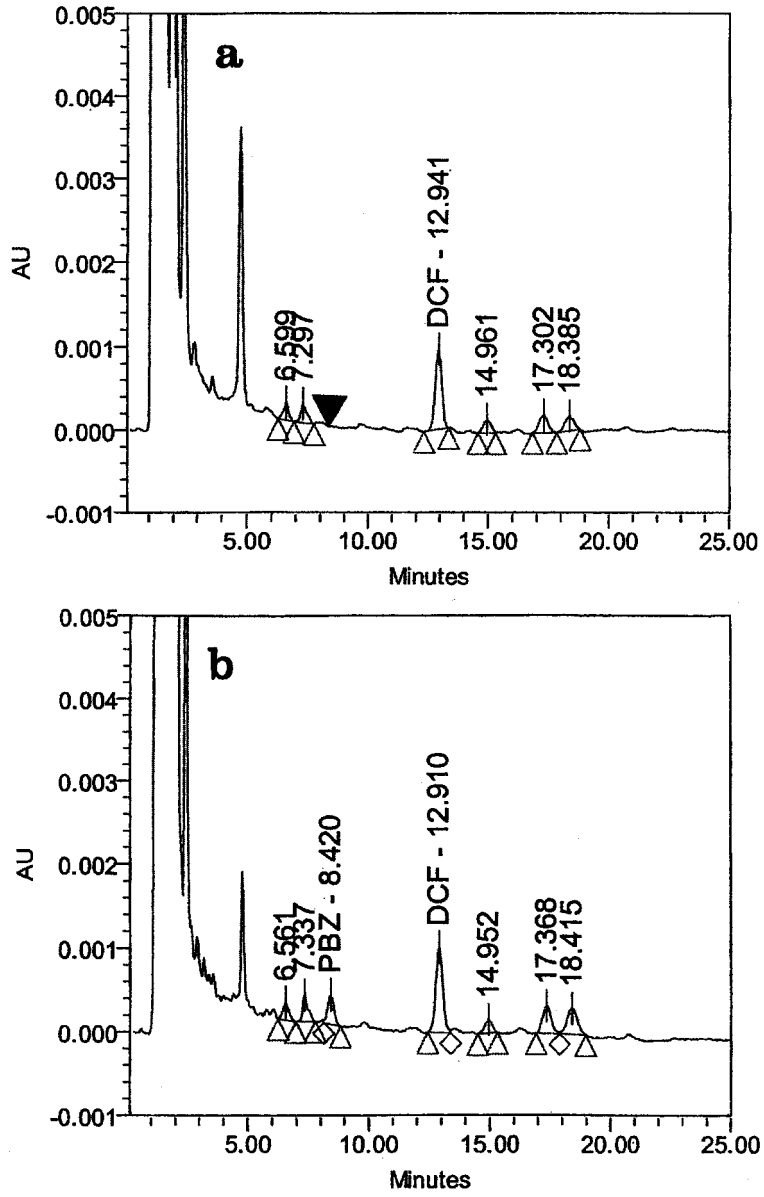

Figure 1. A typical chromatogram of (a) an extract of a negative control (drug-free) bovine muscle tissue containing the internal standard, DCF, and (b) an extract of a negative control bovine muscle tissue fortified with PBZ at 15 ng/g and containing DCF. Details of the chromatographic conditions are provided in the text.

DCF, processed and analyzed according to the described procedure. Figure 1(b) is a chromatogram of a tissue extract obtained from a control muscle tissue sample fortified with PBZ at a concentration of 15 ng/g and the same fixed amount of the internal standard, processed and analyzed using the described method.

To evaluate whether other NSAIDs such as flunixin and dipyrone, and antibiotics including tetracyclines (oxytetracycline, tetracycline, and chlortetracycline) and penicillin G, likely to be used in combination with NSAIDs in veterinary practice would interfere with the analysis of PBZ, control tissue extracts fortified with PBZ at 50 ng/g were injected together with the above named drugs at a concentration of 50 ng/g.

Quantitative Analysis

Standard calibration curves were generated from five 2.00 g portions of negative control tissues individually fortified with 10, 25, 50, 100, and 200 μL of a 2.0 μg/mL PBZ standard working solution and analyzed as previously described. DCF was added as an internal standard to each sample at a fixed concentration of 100 ng/g. An unweighted least squares regression curve was generated by plotting the response ratio (i.e., detector response [peak height] of PBZ/detector response [peak height] of DCF) vs. concentration of PBZ added to control tissue. Response ratios obtained for any test samples found to contain PBZ were measured and used to calculate the concentrations of PBZ in those test samples.

Recovery, Inter-Assay, and Intra-Assay Precision

The absolute recovery of PBZ from tissue was calculated by comparing the detector responses obtained from six sets of negative control tissues fortified at 10, 25, 50, 100, and 200 ng/g and analyzed on different days according to the procedure described, with the detector responses obtained from chemical standards that had not been taken through the extraction procedure (Table 1). The intra-assay precision and accuracy of the analytical method were determined by analyzing six sets of control tissues fortified with PBZ at 15, 30, 75, and 150 ppb on the same day (Table 2). These concentrations covered the analytical range of the method. Inter-assay precision and accuracy were determined by analyzing two sets of control tissues fortified with PBZ at 15, 30, 75, and 150 ppb on each of 3 consecutive days (Table 3). The accuracy of the analytical method was verified by using the described procedure to analyze tissue samples prepared by the laboratory quality manager (QM) or her designate, randomized, coded, and presented to an analyst familiarized with the method (Table 4).

Table 1. Recovery of PBZ from bovine muscle tissues.

Amount of PBZ added per gram of control tissue (ng)	Mean detector response ± SD in arbitrary units for		Absolute mean recovery (%)
	Chemical standard	Tissue standard	
10.0	2.2 ± 0.2	1.3 ± 0.2	59
25.0	5.4 ± 0.3	3.1 ± 0.6	57
50.0	10.8 ± 0.7	6.0 ± 0.6	56
100.0	22.1 ± 0.6	13.3 ± 2.1	60
200.0	44.6 ± 1.2	25.4 ± 5.1	57

Note: Mean recovery = 58%; SD, standard deviation ($n = 6$); RSD, relative standard deviation.

Limit of Quantification and Limit of Detection

The limit of detection (LOD) of the method for PBZ from muscle tissues was determined by analyzing three sets of matrix calibration standards over 2 days in the region around the LOD (i.e., at 3, 5, 8, 10, 12, and 15 ppb). An unweighted linear least square regression equation of mean response ratio = 0.0187 ± 0.0002 [PBZ] − (0.0208 ± 0.0199) was generated from which a detection limit of 3 ng/g was calculated; this being the concentration

Table 2. Intra-assay precision of the analytical method.

	Concentration of PBZ in ng/g found in control tissues fortified with PBZ			
Sample replicate	15.0	30.0	75.0	150.0
1	10.8	30.8	76.0	163.6
2	13.5	24.8	85.0	154.6
3	12.0	26.5	74.5	155.0
4	12.2	27.4	67.0	148.8
5	13.4	29.2	70.3	147.6
6	14.3	28.0	72.0	—
Mean concentration (ng/g)	12.7	27.8	74.1	153.9
SD	1.3	2.1	6.2	6.4
RSD (%)	10	8	8	4
Accuracy (%)	−15	−7	−1	+3

Note: Accuracy (%) = [(amount found − amount added)/amount added] × 100. SD, standard deviation ($n = 6$); RSD, relative standard deviation.

Table 3. Inter-assay precision of the analytical method.

	Concentration of PBZ in ng/g found in control tissues fortified with PBZ			
Day of analysis	15.0	30.0	75.0	150.0
1	12.9	29.8	75.8	123.5
	13.4	29.6	72.9	135.2
2	10.7	21.8	66.6	131.6
	11.3	25.8	76.2	155.8
3	14.5	27.5	69.8	150.6
	12.8	26.4	71.6	148.9
Mean concentration (ng/g)	12.6	26.8	72.2	140.9
SD	1.4	2.9	3.7	12.7
RSD (%)	11	11	5	9
Accuracy (%)	−16	−11	−4	−6

Note: Accuracy (%) = [(amount found + amount added)/amount added] × 100. SD, standard deviation; RSD, relative standard deviation.

at which the signal-to-noise ratio (S/N) was equal to 3. The limit of quantitation (LOQ), also the lowest point on the tissue matrix calibration curve and defined as the concentration at which the S/N ratio was equal to 10, was found to be 10 ng/g.

Stability Studies

Stability of Standard Solutions and Tissue Extracts During Analysis

Figure 2 shows a plot (non-linear) of the detector response of PBZ to the concentration of PBZ in the absence of DTT. It was demonstrated at the very onset of method development that standard solutions of PBZ were not stable. In Fig. 2 is also shown the equivalent plot after the PBZ standard solution was stabilized with DTT. These results prompted us to stabilize all PBZ standard solutions used in the study with DTT prior to use. Under these conditions, they were stable for 3 months at 2–4°C. The stability of the standard solutions and tissue extracts during analysis under typical laboratory room temperature conditions, were evaluated by analyzing chemical standard solutions and tissue extracts over about three cycles of analysis time (i.e., about 14 hr).

Stability of Fortified Negative Control Tissues Under Freezer Storage Conditions

The stability of control muscle tissues fortified with PBZ at 100 ng/g and frozen at −25°C, was also monitored over a period of 28 days to evaluate the

Table 4. Verification of the accuracy of the analytical method on blind-fortified samples.

Sample ID	PBZ concentration in ng/g added to control tissue (ng/g)	Concentration of PBZ found in control tissue fortified with PBZ (ng/g)	Accuracy (%)
VM 1001	15.0	13.6	−9
VM 1003	15.0	10.8	−28
CG 2	15.0	12.9	−14
CG 4	15.0	13.1	−13
CG 1	30.0	25.7	−14
CG 3	30.0	27.8	−7
CG 9	30.0	26.5	−12
CG 11	30.0	25.0	−17
VM 1000	30.0	26.1	−13
VM 1004	30.0	24.9	−17
RF 03	50.0	43.3	−13
RF 04	50.0	44.2	−12
VM 1002	60.0	57.1	−5
VM 1005	60.0	52.8	−12
CG 5	60.0	59.5	−1
CG 7	60.0	62.1	+4
CG 10	75.0	63.9	−15
CG 12	75.0	65.4	−13
RF 02	100.0	109.4	+9
RF 05	100.0	108.7	+9
CG 6	100.0	100.7	+1
CG 8	100.0	119.0	+19
RF 01	160.0	173.1	+8
RF 06	160.0	168.8	+6

Note: Accuracy (%) = [(amount found − amount added)/amount added] × 100.

effect of freezing storage and freeze/thaw cycles on the stability of PBZ (Table 5).

Ruggedness Study

The effects of small changes on the analytical performance of the method were investigated to evaluate the ruggedness of the analytical method. The perturbations listed in Table 6 were imposed on the method and a 2^3 experiment conducted as follows: 2.00 g portions of control

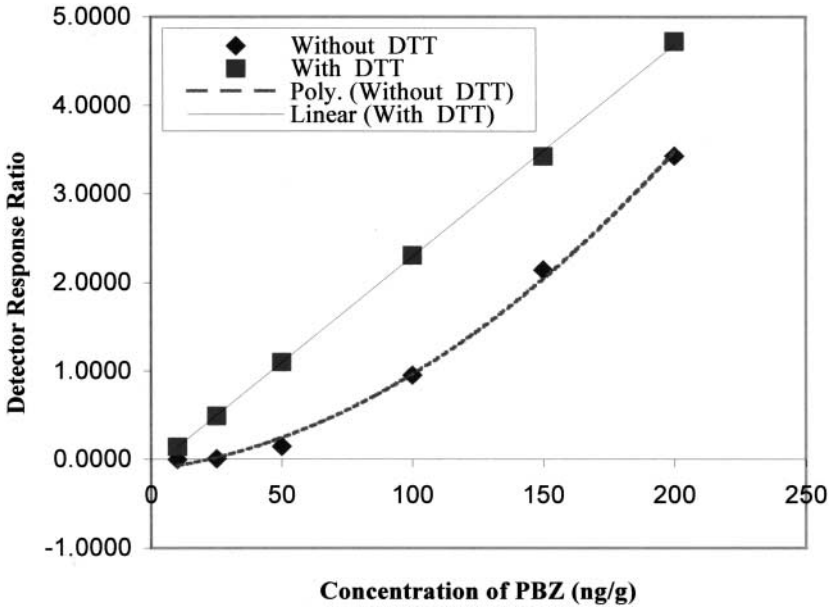

Figure 2. Calibration curve showing the relationship between detector response ratio and concentration of PBZ in the absence of DTT (curvilinear plot) and in the presence of DTT (linear plot).

tissues were weighed into each of 8, 50 mL polypropylene centrifuge tubes and fortified with PBZ at a concentration of 100 ng/g together with the internal standard at a fixed concentration of 100 ng/g. The samples were then all processed as described in Table 6 and analyzed using the described procedure.

Table 5. Bovine tissue storage stability study.

Length of storage days	Concentration of PBZ ± SD in ng/g found in control bovine tissues fortified with PBZ		
	25.0	80.0	120.0
0	24.0 ± 1.7	78.5 ± 12.1	113.4 ± 10.6
14	22.3 ± 1.1	82.4 ± 1.9	110.0 ± 15.2
28	17.9 ± 1.3	69.9 ± 5.8	85.1 ± 3.7

Table 6. Ruggedness study.

Experiment	Nominal activity in procedure	Perturbation imposed on system
1	Add 8.0 mL of extraction solution.	Add 7.0 mL of extraction solution.
2	Mechanically shake for 2 min.	Mechanically shake for 10 min.
3	Centrifuge at 5°C.	Centrifuge at 20°C.
4	Evaporate on N-evap at 55°C.	Evaporate on N-evap at 30°C.
5	Evaporate to dryness.	Evaporate to dryness and 5 more min
6	Wash SPE with 1 mL solution C.	Wash SPE with 0 mL solution C.
7	Dry SPE cartridge after elution.	Do not dry SPE cartridge after elution.

RESULTS AND DISCUSSION

Figure 1(a) shows a typical chromatogram of an extract of a negative control tissue sample fortified with the internal standard, DCF, at a concentration of 100 ng/g, extracted according to the procedure, and injected into the LC-UV system. Figure 1b is a chromatogram of an extract of a negative control tissue fortified with PBZ at a concentration of 15 ng/g and DCF at 100 ng/g, extracted according to the procedure described, and injected into the LC-UV system. It can be seen from Figure 1(a) and (b), that PBZ and DCF eluted from this analytical column with a retention time of 8.4 ± 0.3 and 12.9 ± 0.4 min, respectively. Both compounds were resolved from all other tissue co-extractives and there were no endogenous tissue components that were likely to interfere with the analysis of PBZ. The results of the analysis of the negative control tissues indicated that the analytical method was able to detect truly negative samples as negative (selective), and that it is able to detect negative samples as negative, regardless of what geographical location in Canada the sample was taken from (specific). In addition, other veterinary drugs including flunixin, dipyrone, oxytetracycline, tetracycline, chlortetracycline, and penicillin G were not detected when they were co-injected with PBZ extracts on the LC system. It was, therefore, concluded that the method was selective and specific for the determination of PBZ residues in bovine muscle (non-injection site) and injection site muscle tissues.

Figure 2 shows the non-linear detector response vs. concentration of PBZ that was observed for standard solutions of PBZ during the early stages of method development. This effect was similar to what Gowik et. al.[6] had observed in 1998, while developing a multi-residue method for NSAIDs in serum where PBZ and its metabolite, oxyphenbutazone, readily underwent partial to complete irreproducible degradation in an apparently random manner, even in the presence of ascorbic acid as a stabilizer. Addition of dithiothreitol ($C_4H_{10}O_2S_2$), a stronger reducing agent,[7,8] as a stabilizer to all the standard solutions prevented this phenomenon. Under these conditions, linear detector responses over the concentration range of interest were obtained (Fig. 2), and stock solutions of PBZ were stable for 3 months when stored at 2–4°C.

Table 1 summarizes the results of the experiments conducted to determine the recovery of PBZ added to negative control tissue using the described method. It is seen from Table 1, that the method is able to recover about 58% of PBZ added to muscle tissue. Matrix fortified calibration curves generated for PBZ were found to be linear within the analytical range of 10–200 ng/g. The LOD for the analytical method was determined to be 3 ng/g, estimated as the concentration at which the S/N for the detector response was equal to 3. Results of replicate analysis conducted over three separate days, to estimate within day (intra-assay) and between day (inter-assay) precision and accuracy characteristics of the method, are presented in Tables 2 and 3. The results demonstrate that the analytical method is precise with intra-assay and inter-assay RSDs of $<12\%$. The accuracy of the replicate determinations was $\leq 20\%$ from the true amounts of PBZ added to control tissue matrices. Additionally, the results shown in Table 4 of blind-fortified samples prepared by the laboratory quality manager, and/or her designate, confirm that the method is suitable for quantifying PBZ residue levels in "real unknown samples" at concentrations ≥ 10 ng/g. Because PBZ is not approved for use in food animal production in Canada and USA, any residues of PBZ detected in any edible tissue of a food animal will be sufficient justification for classifying tissues from the animal as unfit for human consumption.

PBZ tissue extracts were shown to be stable under analysis conditions. Control tissue samples fortified with PBZ at 25, 80, and 120 ng/g and stored at -20°C began to show significant losses in PBZ concentration after 28 days of storage (Table 5). The results suggest that tissue samples obtained for such analysis must not be stored for longer than this period of time at this temperature, if the analytical results obtained at the time of analysis are to reflect the true concentrations of drug residues in the tissues at the time they were first received.

Table 7. Survey study to establish the prevalence of PBZ residues in Canadian slaughter animals.

Year	Animal species tested	# Animals tested	# Samples found negative	# Samples found positive[a]
2001	Beef	3	3	0
	Cow	277	275	2
	Horse	291	291	0
	Pork	293	293	0
	Veal	311	311	0
2002	Beef	0	0	0
	Cow	253	251	2
	Horse	285	284	1
	Pork	280	280	0
	Veal	265	265	0
2003	Beef	277	277	0
	Cow	101	101	0
	Veal	177	177	0
	Total	2,813	2,808	5

[a][PBZ] found in the four cows were 205, 942, 36, and 88 ng/g and 25 ng/g in the horse sample.

Before the method was transferred to other analysts, a ruggedness experiment was conducted to determine whether there were any critical control points in the procedure that had not been previously uncovered/identified by using an experimental design based on a seven factor, two-level factorial plan (Table 6). Analysis of the results of the ruggedness study did not reveal any other critical control points.

The simple, rugged, and validated method was applied to the analysis of a total of 2813 equine, bovine, and porcine tissue samples collected as part of the Canadian National Chemical Residues Monitoring Program from April 2001–November 2003. The results of the survey study summarized in Table 7, indicate that with the exception of two cow samples that tested positive in 2001 and two in 2002 and one horse in 2002, PBZ was not detected in the remaining 2808 animals tested over that 3 year period.

In the light of the assumptions that went into initiating this project, it would seem that the results already obtained on the other NSAIDS, such as flunixin[4] and dipyrone,[5] for which we have developed sensitive analytical methods, and now PBZ in this study, indicate that the use/misuse of these NSAIDs is not prevalent in food animal production in Canada.

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