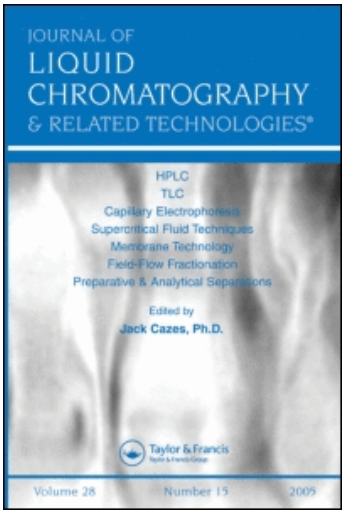

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Ion-Pair Isolation and Liquid Chromatographic Determination of Albendazole, Oxfendazole, Oxibendazole, and Thiabendazole Residues in Milk

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ION-PAIR ISOLATION AND LIQUID CHROMATOGRAPHIC DETERMINATION OF ALBENDAZOLE, OXFENDAZOLE, OXIBENDAZOLE, AND THIABENDAZOLE RESIDUES IN MILK

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ABSTRACT

A liquid chromatographic [HPLC] method for the determination of Albendazole (ABZ), Oxfendazole (OFZ), Oxibendazole (OXBZ), and Thiabendazole (TBZ) residues in milk at levels below 3 ppb has been developed. Samples were deproteinized with acetonitrile, defatted with hexane partition, and evaporated to constant volume. Following addition of acidified aqueous octane-1-sulfonate solution, the target benzimidazoles were selectively extracted as ion pairs into chloroform and analyzed on a reversed-phase C_{18}, 5 μm, column. Overall recoveries were found to be 93.5 ± 2.6% for OFZ, 90.0 ± 1.3% for OXBZ, 85.7 ± 3% for ABZ, and 74.0 ± 2.6% for TBZ. Limited data on the depletion of OXBZ and ABZ in sheep milk were also generated.

INTRODUCTION

Benzimidazoles have become an integral part of the animal producing industry for the prevention and control of internal worm parasites. Among the benzimidazoles most widely used in farm animals are ABZ, OFZ, OXBZ, and TBZ. As some members of the benzimidazole class of anthelmintics have shown teratogenic and embryotoxic effects in a variety of animal species [1], quantitation of residues remaining in tissues and milk of treated animals intended for human consumption is of major interest.

There have been many multiresidue methods for analyzing benzimidazoles in animal matrixes but only two [2, 3] are available for milk. They are both useful HPLC procedures, the former one [2] utilizing a modern matrix solid-phase dispersion methodology for effective extraction and cleanup whereas the latter employing classic liquid-liquid partitions based on the weakly basic nature of the target residues, and solid-phase extraction. These methods, although useful for monitoring benzimidazole residues in milk, either lack the sensitivity required for trace residue analysis [2] or are time, labor and materials intensive [3].

This paper deals with the development of a sufficiently sensitive, accurate, precise, and rapid method for the determination of ABZ, OXBZ, OFZ, and TBZ residues in cow and sheep milk. Using a new ion-pair extraction approach, many of the limitations of the extraction and cleanup procedures outlined above are overcome.

EXPERIMENTAL

Instrumentation

HPLC was carried out on Gilson system consisting of a Model 805 manometric module, a Model 305 piston pump, a Model HM/HPLC dual-beam variable-wavelength spectrophotometer set at 292 nm, and a model N1 variable-span recorder (Villiers-le-Bel, France). A Model TC 831 HPLC-Technology column oven (Macclesfield, UK), set at 40 °C, permitted temperature regulation. Injections were made on a Hichrom, 250x4.6 mm, stainless-steel column, packed with Nucleosil 120 C_{18} 5-μm, through a Rheodyne 7125 sample injector equipped with a 100-μl loop.

Chemicals

Octanesulfonate sodium salt and HPLC grade methanol were obtained from Merck-Schuchard (Munchen, Germany). Benzimidazole standards included ABZ and OXBZ which were obtained from Smith and Kline (Westchester, PA, USA), and OFZ and TBZ which were purchased from Hoechst (Frankfurt, Germany) and Merck (Rahway, NJ, USA), respectively. All other reagents and solvents used were of analytical reagent grade. Deionized water was distilled before use.

Standard Solutions

Stock solutions of the individual benzimidazoles (100 μg/ml) were prepared by dissolving 10 mg of each standard in 10 ml dimethylsulfoxide and diluting to 100-ml volume with methanol.

Aliquots of these stock solutions were mixed and diluted with mobile phase to give mixed standard working solutions in the range 10-500 ng/ml.

Mobile Phase and HPLC Conditions

A 0.01 M ammonium phosphate buffer was prepared by dissolving 1.15 g $NH_4H_2PO_4$ in 950 ml deionized water, adjusting to pH 7.0 with 50% NH_4OH solution, and diluting to 1 l final volume.

Mobile phase was prepared by combining 600 ml methanol with 400 ml ammonium phosphate buffer, and passing through 0.45 μm filter before use. The mobile phase was degassed using helium and delivered at a rate of 0.7 ml/min. Recordings were made at chart speed of 2 mm/min and 0.020 a.u.f.s. sensitivity setting.

Extraction and Cleanup Procedure

A 5-ml sample was transferred to a 25-ml centrifuge tube and accurately weighed.. A volume (15 ml) of acetonitrile was added, and the tube was vortexed for 15 s and centrifuged for 3 min at 1000g. The clear supernatant liquid formed was decanted into another 50-ml tube, rinsing inner walls of first tube with additional 3 ml of extracting solvent and combining the extracts. A volume (10 ml) of hexane was added to the extracts, and partitioning was effected by vortexing for 15 s at high speed. After discarding the top layer, the remaining bottom layer was decanted into a 50-ml evaporating flask and rotary-evaporated under vacuum to constant volume at 40 °C to be further mixed with 5 ml chloroform and 3 ml of 0.005 M octane-sulfonate in 0.1% phosphoric acid. Following vigorous shaking, flask

content was decanted into a 10-ml centrifuge tube, vortexed for 5 s, and centrifuged for 10 min at 1000g. A 4-ml volume of the bottom layer was pipetted into another 10-ml tube and evaporated to dryness with gentle nitrogen stream at 40 °C. The residue remaining was redissolved in 500 µl of mobile phase, and a 100-µl aliquot was used for HPLC analysis.

Calculations

Calibration curves were constructed by running 100-µl aliquots from the series of working solutions and plotting the recorded peak heights versus quantity of each benzimidazole injected. The concentrations of ABZ, OXBZ, OFZ, and TBZ in samples were calculated by reference to calibration curves and multiplication by appropriate dilution factor.

RESULTS AND DISCUSSION

The extraction of milk with ethyl acetate caused a persistent emulsion. Emulsion formation was also observed when chloroform was used. To eliminate this problem, acetonitrile was used as an extracting solvent. Treatment of samples with 3 volumes of acetonitrile was effective in precipitating milk proteins, since clear extracts were consistently taken after centrifugation.

Some purification of the extracts was effected by partitioning them with hexane. Further purification was initially attempted by evaporating the extracts to constant volume, adding a pH 10 phosphate buffer, and partitioning the analytes into diethylether.

Although the weakly basic nature of the target benzimidazoles favors such a partition, a significant loss of OFZ recovery (<65%) was noted.

To increase the recovery of OFZ, a different purification scheme was tested. Benzimidazoles were first converted, under acidic conditions, to ion pairs with octanesulfonate anions to be further extracted with chloroform. This ion-pair extraction enhanced the recovery of OFZ, and resulted also in efficient cleanup.

The effectiveness of the cleanup procedure permitted chromatographic analysis of milk samples under isocratic conditions. Using the ammonium phosphate/methanol mobile phase, OFZ was eluted in 6.9 min, TBZ in 7.8 min, OXBZ in 11.5 min, and ABZ in 16.6 min. Peak heights, although quite reproducible at a given mobile phase flow rate, varied with it at different extent for each analyte. By gradually reducing the flow rate from 1.0 to 0.5 ml/min, peak heights of OFZ, TBZ, OXBZ, and ABZ were increased 8%, 6%, 14%, and 24%, respectively.

Due to absence of any interfering peaks in samples chromatograms (Figure 1), concentrations as low as 1 ppb for OFZ, TBZ and OXBZ, and 3 pp. for ABZ could be readily determined (peak to noise ratio, 3/1). Regression analysis of the data obtained by running a series of mixed standard working solutions showed the response to be linear for all compounds in the range examined ($y=0.51+3.14x$, $r=0.9996$ for OFZ; $y=1.04+5.01x$, $r=0.9990$ for TBZ; $y=0.89+3.40x$, $r=0.9983$ for OXBZ; $y=0.94+1.05x$, $r=0.9989$ for ABZ,

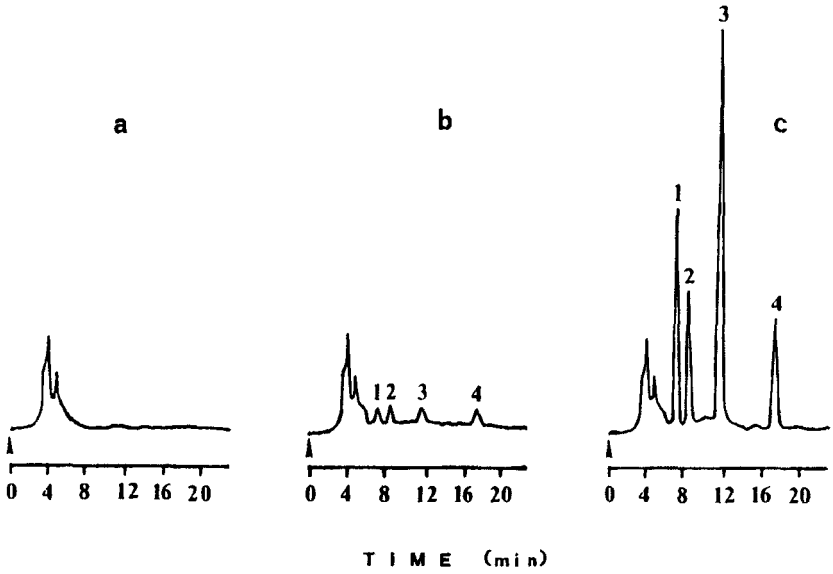

FIGURE 1. Typical chromatograms of (a) a blank milk sample, (b) a milk sample fortified with 1 ppb of OFZ (1), TBZ (2), OXBZ (3), and 3 ppb of ABZ, and (c) a milk sample fortified with 18 ppb of OFZ (1), 9 ppb of TBZ (2), 31 ppb of OXBZ (3) and 29 ppb of ABZ (4). Conditions: mobile phase, methanol-0.01 M ammonium phosphate buffer, pH 7.0 (60:40, V/V); column 250x4.6 mm, C_{18} (5 μm); temperature, 40 °C; flow rate, 0.7 ml/min; wavelength, 292 nm; recorder sensitivity, 0.020 a.u.f.s.; chart speed, 2 mm/min; injection volume, 100 μl.

where y represents peak height in mm and x the quantity in ng of the compound injected).

The accuracy and the precision of the method were studied by spiking milk samples at 6 fortification levels with mixed standard working solution, and analyzing 5 replicates. Least-squares and regression analysis of the data presented in Tables 1 and 2 showed that the relationship between "added" and "found" was

TABLE 1

Precision and Accuracy Data for the Determination of OFZ, and TBZ in Milk

Concn. added, ppb	OFZ Mean concn[a] found, ppb	OFZ Mean rec., %	TBZ Mean concn[a] found, ppb	TBZ Mean rec., %
15	13.7 ± 0.6 (4.2)[b]	91.1	12.0 ± 1.0 (8.3)[b]	80.0
60	58.6 ± 3.0 (5.1)	97.7	44.6 ± 4.0 (8.9)	74.3
120	110.6 ± 2.5 (2.3)	92.2	89.2 ± 1.8 (2.0)	74.3
180	168.0 ± 6.4 (3.8)	93.3	131.2 ± 4.4 (3.3)	72.9
240	219.0 ± 4.0 (1.8)	91.3	178.7 ± 1.1 (0.6)	74.4
300	283.4 ± 3.8 (1.3)	94.5	222.8 ± 3.9 (1.7)	74.3

[a]Mean of 6 replicates ± SD.
[b]Values in parenthesis represent RSD %.

TABLE 2

Precision and Accuracy Data for the Determination of OXBZ, and ABZ in Milk

Concn. added, ppb	OXBZ Mean concn[a] found, ppb	OXBZ Mean rec., %	ABZ Mean concn[a] found, ppb	ABZ Mean rec., %
15	13.0 ± 1.0 (7.7)[b]	86.7	12.7 ± 0.6 (4.6)[b]	84.4
60	54.0 ± 3.2 (5.8)	90.0	49.4 ± 2.5 (5.1)	82.3
120	108.4 ± 4.4 (4.1)	90.3	100.4 ± 2.9 (2.9)	83.7
180	163.2 ± 6.3 (3.9)	90.7	152.4 ± 4.5 (2.9)	84.7
240	215.3 ± 3.5 (1.6)	89.7	209.0 ± 2.6 (1.3)	87.1
300	270.0 ± 1.9 (0.7)	90.0	254.2 ± 4.7 (1.8)	84.7

[a]Mean of 6 replicates ± SD.
[b]Values in parenthesis represent RSD %.

adequately described, for all compounds, by a linear regression ($y=0.054+0.935x$, $r=0.9988$ for OFZ; $y=0.076+0.740x$, $r=0.9991$ for TBZ; $y=0.099+0.900x$, $r=0.9992$ for OXBZ; $y=-1.43+0.857x$, $r=0.9991$ for ABZ). Therefore, the slopes of these regression lines could be used as estimates of the overall recovery for OFZ (93.5±2.6%), TBZ (74.0±2.2%), OXBZ (90.0±1.3%), and ABZ (85.7±3.0%) determination in milk.

Since other antibiotics or drugs might interfere with the analysis, an interference test was evaluated. Several compounds that are, frequently, added in feeds and/or used for treatment of mastitis, such as penicillin G, cloxacillin, furazolidone, nitrofurazone, oleandomycin, ampicillin, sulfathiazole, erythromycin, streptomycin, chlortetracycline, tetracycline, and oxytetracycline were dissolved in mobile phase at 1 ppm level and submitted to HPLC. It was found that none of the compounds tested interfered with the analysis.

To validate the method with real samples, a trial was undertaken to quantitate residues in milk of two dairy sheep each given orally a single dose of 10 mg OXBZ/kg of body weight and 20 mg ABZ/kg of body weight, respectively. The control milk samples, which had been collected before treatment, and all other samples taken during the trial at 12 h milking intervals were stored at -25 °C, until analyzed. The analysis data, presented in Table 3, showed that both compounds could be detected in milk for up to 48 h after dose. Extractable metabolites could also be seen in all positive chromatograms of the milk samples analyzed (Figure 2). The chromatographic behavior of these metabolites indicated a substantial increase in polarity over the parent compounds.

TABLE 3

Levels of OXBZ and ABZ Residues in Sheep Milk After Single Dosing with the Respective Benzimidazole

Hours after dosing	Concn of residue found[a], ppb	
	OXBZ	ABZ
12	13.6	1805.0
24	4.6	135.3
36	2.0	35.3
48	2.5	7.0
60	<1.0	<3.0
72	<1.0	<3.0
84	<1.0	<3.0

[a]Values corrected for recovery.

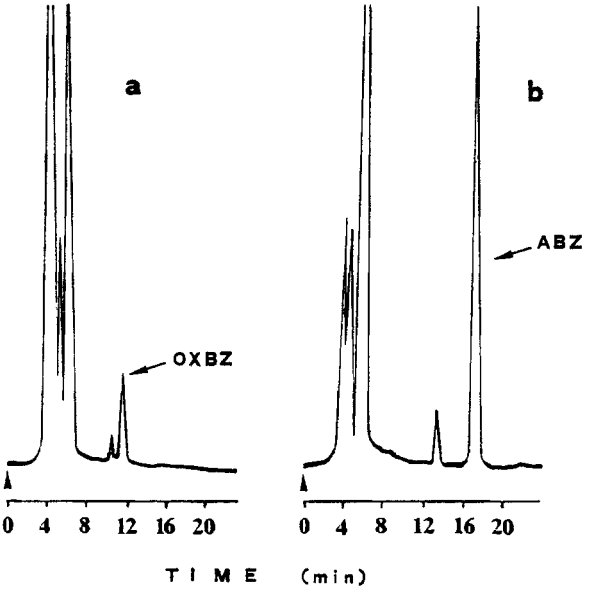

FIGURE 2. Chromatograms of milk samples from a sheep dosed with OXBZ (a) and a sheep dosed with ABZ (b). HPLC conditions as in Figure 1.

In conclusion, the results of the present study show that the developed HPLC procedure offers acceptable recoveries, high precision, increased sensitivity, and minimal background interference. It uses small sample size without any need for several pH adjustments, back washings, additional solvent partitioning steps, and evaporation of large volumes of extracting solvents. This results in considerable savings in terms of time, labor and materials requirements. One analyst can easily process 6 samples in 2 h. These advantages make the procedure an attractive alternative to published methods.

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