Trace Multiresidue Analysis of Fenbendazole and Its Sulfoxide, Sulfone, and *p*-Hydroxylated Metabolites in Milk by Liquid Chromatography

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A liquid chromatographic method for trace analysis of fenbendazole and its sulfoxide, sulfone, and *p*-hydroxylated metabolites in milk has been developed. Samples are deproteinized with acetonitrile, and the supernatants are extracted with dichloromethane. The separated bottom organic layers are washed with alkaline buffer and evaporated to dryness, and the residues are partitioned between water and ethyl acetate. The separated top organic layers are evaporated to dryness, reconstituted in mobile phase, and submitted to reversed-phase ion-pair chromatography. Overall recoveries ranged from 82% for the sulfoxide and *p*-hydroxylated metabolites to 91% for fenbendazole and its sulfone metabolite. The linearity of the assay was quite satisfactory, whereas the limit of detection was in the range 2−5 ng/mL. Precision studies based on within- and between-days variation suggested overall precision values better than 4.3% for all analytes. The method was successfully applied to quantitate incurred fenbendazole residues in milk from a lactating dairy cow treated with a fenbendazole formulation.

Keywords: *Fenbendazole; fenbendazole metabolites; benzimidazoles; residues in milk; ion-pair chromatography*

INTRODUCTION

Fenbendazole, a benzimidazole anthelmintic, has approved indications for use in cattle for prevention and control of internal worm parasites (FDA, 1988). Following its administration to animals, fenbendazole is eliminated mainly as a function of oxidative metabolism. The parent drug, a sulfide, is rapidly oxidized to the sulfoxide metabolite, oxfendazole, which is also a very effective anthelmintic (Prichard et al., 1985). The sulfoxide can be oxidized further to the sulfone, a considerably less active metabolite (Averkin et al., 1975). Another metabolite that is the product of oxidation of the 4-position of the phenyl ring, the *p*-hydroxyfenbendazole, is also considered to have little anthelmintic activity (Short et al., 1987). The widespread use of fenbendazole in cattle increases the risk of such drug-related residues appearing in the milk of treated animals, posing a health hazard to consumers (Roberson, 1982; Delatour, 1986). In this regard, a maximum residue limit of 10 ng/mL for total fenbendazole residues in milk has been set by regulatory agencies (FAO/WHO, 1991; Commission Regulation, 1995). To ensure, therefore, the safety of the marketed milk from the presence of violatile residues, analytical methods capable of trace level monitoring of total fenbendazole residues are of value.

Published methods for trace analysis of fenbendazole residues in milk are limited to either the parent drug (Fletouris et al., 1994) or/and its sulfoxide metabolite (Tai et al., 1990). A multiresidue method that can analyze the different fenbendazole metabolites in milk has also been described (Long et al., 1989) but lacks generally the sensitivity required to ensure that samples do not contain violatile residue levels. At present, no method is available for the extraction, separation, and quantification of the major fenbendazole metabolites in milk.

The main objective of this study was to develop and validate an analytical method for the determination of fenbendazole residues in milk in a single procedure. The simple liquid−liquid partition procedures for extraction and cleanup and the efficient ion-pair chromatographic separation applied can provide the means for an inexpensive, sensitive, and reliable determination of fenbendazole residues in cow milk. The method was successfully applied to determine fenbendazole residues in milk of a dairy cow orally given a fenbendazole formulation.

MATERIALS AND METHODS

Instrumentation. Liquid chromatography was carried out with a Gilson system (Gilson Medical Electronics, Villiers-le-Bel, France) consisting of a Model 805 manometric module, a Model 305 piston pump, a Model 119 UV−vis detector, and a Model TC 831 column heater. Injections were made using a Rheodyne Model 7725 injection valve (Cotati, CA) equipped with 100-μL sample loop. Recordings were made with a Kipp & Zonen Model BD 111 pen recorder (Delft, Holland).

A Model G-560E vortex mixer (Scientific Industries, Bohemia, NY), a Centra-MP4 centrifuge (IEC, Needman Heights, MA), a Model ReactiTherm heating/stirring thermoblock (Pierce Chemicals, Rockford, IL), a Model 522 pH meter (WTW, Weilheim, Germany), and a 0.2-μm Nylon-66 syringe filter (Alltech Associates, Deerfield, IL) were used for sample treatment. A Model D7402 EasyPure UV compact ultrapure water system (Barnstead/Thermolyne Corp., Dubuque, IA) was also used for purification of tap water.

Reagents. Analytical-grade dichloromethane, dimethylsulfoxide, ethyl acetate, toluene, phosphoric acid, and disodium hydrogen phosphate were from Merck (Darmstadt, Germany), while dichlorodimethylsilane was from Sigma (St. Louis, MO). Lichropur-grade octanesulfonate sodium salt and tetrabutylammonium hydrogen sulfate and LC-grade acetonitrile were also from Merck (Darmstadt, Germany).

Fenbendazole sulfoxide, *p*-hydroxyfenbendazole, fenbendazole sulfone, and fenbendazole (Figure 1) reference standards were from Hoechst Hellas (Athens, Greece).

Chromatographic Conditions. The analytical column used in this study was a Hichrom (Reading, UK), 250 × 4.6 mm i.d., column packed with Nucleosil 120, C_{18}, 5 μm. The

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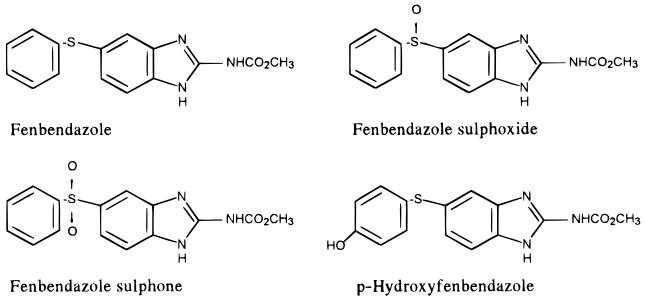

Figure 1. Chemical structures of the investigated benzimidazoles.

mobile phase consisted of acetonitrile and 0.01 M phosphoric acid, (30:70, v/v) and contained 2.5 mM and 5 mM octanesulfonate and tetrabutylammonium ion-pair reagents, respectively. Following its preparation, the mobile phase was passed through 0.2 μm Nylon-66 filter (Anachem, Luton, UK) and degassed using helium. The mobile phase was delivered in the system at a rate of 1 mL/min.

The Nucleosil 120 C_{18} stationary phase was thoroughly equilibrated with the mobile phase each time before use. Reproducible capacity factors could be realized after passage through the column of at least 150 mL of mobile phase. During runs, the column was kept thermostatted at 50 °C. After use, successive column washings with at least 200-mL volumes of water and acetonitrile were quite indispensable for removing the adsorbed pairing ions. Detection was made at 290 nm at a sensitivity setting of 0.005 AUFS. Chart speed was set at 5 mm/min.

Standard Solutions. Individual stock solutions of each reference standard were prepared in 10-mL volumetric flasks by weighing ca. 5 mg of the compound and dissolving to volume with dimethyl sulfoxide. A mixed standard intermediate solution containing all four analytes was prepared by combining appropriate aliquots (120−500 μL) from each of the stock solutions in a 10-mL volumetric flask and diluting to volume with acetonitrile. A concentrated mixed standard working solution was prepared by transferring 150 μL from the intermediate solution into a 10-mL volumetric flask and diluting to volume with mobile phase. Successive dilution of this solution with mobile phase gave the series of mixed standard working solutions in the desired concentration range (7.0−89.2 ng/mL for fenbendazole sulfoxide, 14.1−180.0 ng/mL for *p*-hydroxyfenbendazole, 10.2−129.6 for fenbendazole sulfone, and 23.5−300.0 ng/mL for fenbendazole).

A mixed standard spiking solution was also prepared by transferring 200 μL from the mixed standard intermediate solution into a 10-mL volumetric flask and diluting to volume with acetonitrile. Both stock and mixed standard intermediate solutions were stable for at least 6 months in a freezer at −25 °C. Mixed standard working solutions were prepared fresh weekly.

Milk Samples. All control milk samples were obtained from a local farm. For accuracy and precision studies, control milk samples were spiked at the desired concentration level by pipetting portions (25−300 μL) of the mixed standard spiking solution into separate 15-mL centrifuge tubes, evaporating to dryness under N_2, and adding under vortexing 1 mL of milk. Fortified milk samples were allowed to stand for 30 min prior to extraction.

Incurred milk samples were collected from a dairy cow orally administered with a single dose of fenbendazole formulation (Panacur, Hoechst, Germany) corresponding to approximately 10 mg of fenbendazole/kg of body mass. The control milk samples, which were taken before treatment, and all other samples collected during the trial at 12-h milking intervals were stored at −25 °C until analyzed.

Analytical Procedure. Analysis was carried out using a sample size of 1 mL. Both extraction and cleanup steps were performed in 15-mL screw-capped centrifuge tubes pretreated with 5% (v/v) dichlorodimethylsilane in toluene in order to minimize adsorption of the analytes during sample preparation. Following sample introduction, 2 mL of acetonitrile

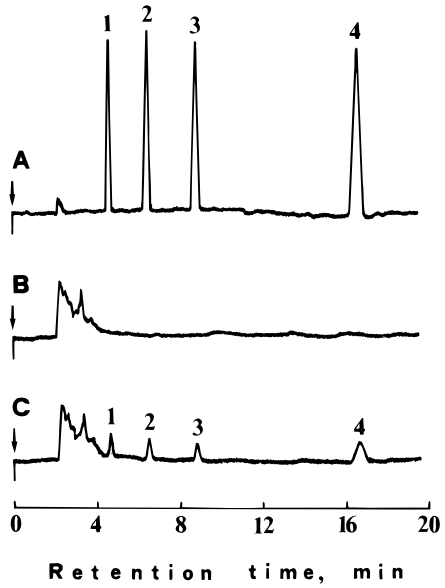

Figure 2. Chromatograms of a mixed standard working solution (A), a blank milk sample (B), and a milk sample fortified with standard benzimidazoles at a level close to their detection limit (C). Chromatographic conditions: acetonitrile−0.01 M phosphoric acid (30:70, v/v) mobile phase containing 2.5 mM octanesulfonate and 5 mM tetrabutylammonium reagents; column 250 × 4.6 mm, Nucleosil 120, $C_{18,}$ 5 μm; temperature, 50 °C; flow rate, 1 mL/min; wavelength, 290 nm; detection sensitivity, 0.005 AUFS; chart speed, 5 mm/min; injection volume, 100 μL. Peak identification: fenbendazole sulfoxide (1), *p*-hydroxyfenbendazole (2), fenbendazole sulfone (3), and fenbendazole (4).

was added, and the tube was vortexed for 15 s and centrifuged for 1 min at 4000*g*. The clear supernatant was decanted into another tube where 3 mL of dichloromethane and 2 mL of water were also added. The mixture was vortexed for 15 s and centrifuged for 1 min at 2000*g*. The separated top aqueous layer was discarded, while the bottom organic layer was vortex-mixed for 15 s with 4 mL of 0.1 M phosphate buffer, pH 10, and centrifuged for 1 min at 2000*g*. The bottom organic layer was transferred into another tube to be further evaporated to dryness under nitrogen at 50 °C. The residue was vortexed for 30 s with 3 mL of water and 5 mL of ethyl acetate and centrifuged for 1 min at 2000*g*. The separated top ethyl acetate layer was transferred into another tube, and evaporated to dryness under nitrogen at 50 °C. The remaining residue was reconstituted in 0.5 mL mobile phase and filtered through a 0.2 μm syringe filter, and a 100-μL aliquot was injected into the LC.

Determination. Calibration curves were generated by running working solutions, plotting recorded peak heights versus known quantities of analytes in injected volumes, and computing slope, intercept, and least squared fit of standard curves. Calibration curve slopes and intercept data were used to determine the mass of analytes in injected sample extracts. Quantification of fenbendazole and its metabolites in milk samples was realized according to equation

$$\text{analyte concentration (ng/mL)} = QV \times 5$$

where Q is the mass (ng) of the individual benzimidazole determined in injected extract (100 μL), and V is the dilution factor, if any, applied.

RESULTS AND DISCUSSION

Many of the LC methods for benzimidazoles specify an octadecylsilane column that uses the reversed partition mode. However, application of such a separation mode resulted generally in broad and tailing peaks. Considering that this poor chromatographic perfor-

Table 1. Calibration Curves for Assay of Fenbendazole Residues in Milk

benzimidazole	quantity injected (ng)	data points	slope ± SD	intercept	correlation coefficient
fenbendazole sulfoxide	0.7−8.9	7	14.29 ± 0.12	0.40	0.99997
p-hydroxyfenbendazole	1.4−18.0	7	7.24 ± 0.04	0.38	0.99999
fenbendazole sulfone	1.0−13.0	7	9.38 ± 0.04	0.15	0.99999
fenbendazole	2.3−30.0	7	4.15 ± 0.04	0.49	0.99996

mance might be due to interactions of the weakly basic analytes with the residual silanol groups on the silica-based C_{18} packing material, a study was made of the separation conditions using acetonitrile−phosphoric acid as the mobile phase. To inactivate the action of the residual free silanol groups while achieving an acceptable degree of separation between all analytes in a minimum time, modification of the LC partitioning process through addition to the mobile phase of various pairing agents (Botsoglou et al., 1994) was investigated. Optimum results for detecting the analytes were obtained by addition to the mobile phase of octanesulfonate anions, which formed more hydrophobic ion pairs with the positively charged benzimidazoles, and also tetrabutylammonium cations, which could efficiently mask residual silanols on the stationary phase. Increasing column temperature also had a favorable effect on chromatography. At 50 °C, the late eluted peaks became sharper and more symmetric, due possibly to better mass transfer of the compounds between the stationary and the mobile phase. Under the established conditions, fenbendazole sulfoxide eluted at 4.6 min, *p*-hydroxyfenbendazole elution at 6.5 min, fenbendazole sulfone eluted at 8.8 min, and fenbendazole eluted at 16.5 min (Figure 2A).

Analyses at several wavelength settings showed that the most selective wavelength and best response for the simultaneous detection of fenbendazole and its metabolites was at 290 nm (Barker et al., 1986; Long et al., 1989). Regression analyses of the data obtained by running mixed standard working solutions showed the response to be linear for all analytes in the range examined. The established calibration curves that corresponded to the equation $y = A + Bx$ (y represents peak height in mm, x is the quantity in ng of the compound injected, A is the intercept point, and B is the slope of the curve) are presented in Table 1.

As sample extraction and cleanup have become major rate-limiting procedures in residue analysis, simple analytical schemes for the isolation of fenbendazole and its metabolites from milk were investigated. Fenbendazole and its metabolites are weakly basic hydrophobic compounds with appreciable solubility in polar organic solvents such as ethyl acetate and dichloromethane. Preliminary experiments on the partition of the analytes between these organic solvents and acidic, neutral, and alkaline phosphate buffers showed that the benzimidazoles could be quantitatively partitioned into the ethyl acetate or dichloromethane. Further testing, however, on fortified milk samples revealed a different partitioning profile for fenbendazole which, unlike its metabolites, was poorly partitioned into the test organic solvents. This behavior indicated substantial binding of fenbendazole to milk constituents. To disrupt these interactions and recover fenbendazole from the milk matrix, a debinding process (Fletouris et al., 1994) was investigated. It was found that, using acetonitrile as a deproteinization agent, fenbendazole losses due to binding could be eliminated.

Direct injection of the aqueous acetonitrile extracts into the LC gave chromatograms with many interfering peaks. To purify the extracts prior to injection, a series of consecutive liquid−liquid partition cleanups was evaluated. Significant purification could be made possible by first diluting the extracts with water and then adding dichloromethane. By this step, some hydrophilic constituents of the organic phase could be efficiently removed. Further purification was attained by washing the organic phase with a pH 10 phosphate buffer. By this treatment, a number of remaining acidic interferents could be removed. Additional purification could be achieved by submitting the organic phase to evaporation and partitioning the residue between ethyl acetate and water. By this step, some very polar matrix constituents could be eliminated through the aqueous phase. Analyses of a series of various blank milk samples provided information concerning the cleanliness of the extracts and the presence of interfering peaks (Figure 2B).

Table 2. Accuracy Data for Assay of Fenbendazole Residues in Milk

benzimidazole	added (ng/mL)	found[a] (ng/mL)	relative standard deviation (%)	recovery (%)
fenbendazole sulfoxide	3.0	2.5	6.0	83.3
	6.0	5.0	3.0	83.3
	18.0	14.8	2.4	82.2
	36.0	29.3	1.1	81.4
p-hydroxyfenbendazole	6.0	5.1	2.0	85.0
	12.0	10.2	7.0	85.0
	36.0	29.3	2.4	81.4
	72.0	59.3	1.5	82.4
fenbendazole sulfone	4.3	3.9	1.5	90.7
	8.6	7.8	1.9	90.7
	25.8	23.4	2.8	90.7
	51.6	46.9	1.2	90.9
fenbendazole	10.0	9.1	1.6	91.0
	20.0	18.1	0.9	90.5
	60.0	54.5	1.2	90.8
	120.0	108.3	0.7	90.2

[a] Mean of five samples analyzed.

The accuracy of the method was studied by spiking control milk samples with the target analytes at four fortification levels and analyzing five replicates. Table 2 presents the spiking levels and the individual mean recoveries found at each level for each analyte. Least-squares and regression analysis of the raw data showed that the relationships between "added, x" and "found, y" were described by linear regressions. The practically zero intercept points and the excellent linearity of these regression lines shown in Table 3 could permit estimation of the overall recovery of the method for each analyte on the basis of the slope of the corresponding regression. All correlation coefficients were excellent, reflecting high linearity of recovery and response. The high overall recoveries found coupled with the high response at 290 nm, the cleanliness of the extracts, and the efficiency of the ion-pair chromatographic system could allow very low limits of detection (peak to noise ratio, 3/1), also shown in Table 3, to be realized (Figure 2C).

The precision of the method was also studied by analyzing on each of three different days five replicates from control milk that had been spiked with benzimidazoles at a certain level for each analyte. To estimate

Table 3. Linearity Data for Assay of Fenbendazole Residues in Milk

benzimidazole	spiking range (ng/mL)	spiking levels[a]	slope ± SD	intercept	correlation coefficient	overall recovery (%)	detection limit (ng/mL)
fenbendazole sulfoxide	3.0−36.0	4	0.814 ± 0.018	0.07	0.99979	81.4 ± 1.8	2
p-hydroxyfenbendazole	6.0−72.0	4	0.820 ± 0.025	0.17	0.99961	82.0 ± 2.5	3
fenbendazole sulfone	4.3−51.6	4	0.910 ± 0.022	-0.02	0.99976	91.0 ± 2.2	2
fenbendazole	10.0−120.0	4	0.902 ± 0.011	0.11	0.99993	90.2 ± 1.1	5

[a] Five samples at each spiking level.

Table 4. Precision Data Based on Repetitive Analysis on Each of Three Different Days of Five Milk Samples Fortified with Fenbendazole and Its Metabolites

benzimidazole	spiking level (ng/mL)	within-day precision (RSD %)	between-days precision (RSD %)	overall precision (RSD %)
fenbendazole sulfoxide	6.0	4.2	3.3	4.0
p-hydroxyfenbendazole	12.0	4.7	2.6	4.3
fenbendazole sulfone	8.6	3.0	0.7	2.6
fenbendazole	20.0	0.8	0.3	0.7

Table 5. Benzimidazole Residues in Milk (ng/mL) from a Dairy Cow Administered Orally a Fenbendazole Formulation

hours after administration	fenbendazole[a]	fenbendazole sulfoxide[a]	fenbendazole sulfone[a]	*p*-hydroxyfenbendazole[a]
12	36.1	187.5	26.5	3.9
24	48.0	164.5	42.3	<3.0
36	30.5	118.0	61.7	<3.0
48	12.5	55.5	71.8	
60	<5.0	16.1	47.1	
72	<5.0	4.1	23.0	
84		2.5	8.1	
96		<2.0	<2.0	
108		<2.0	<2.0	

[a] Values have not been corrected for recovery.

the components of variance, the concentrations found were subjected to "analysis of variance and expected mean squares for the one way classification-balanced design" (Wernimont, 1987). Statistics showed that the within-day precision was better than the between-day one in all cases (Table 4). They also suggested that the overall precision of the method, which is in fact the overall uncertainty of a single determination, was well within acceptable limits.

Since other drugs or antibiotics that are frequently administered to cattle might interfere with the analysis, an interference test was evaluated. Several compounds such as thiabendazole, 5-hydroxythiabendazole, albendazole-2-aminosulfone, albendazole sulfoxide, oxibendazole, albendazole sulfone, mebendazole, levamisole, triclabendazole, penicillin G, penicillin V, oxacillin, cloxacillin, ampicillin, furazolidone, oleandomycin, sulfathiazole, erythromycin, streptomycin, neomycin, gentamicin, chlortetracycline, tetracycline, and oxytetracycline were added to milk samples at the 0.5 μg/mL level, and all samples were submitted to analysis. It was found that none of the tested compounds interfered with the analysis.

To validate the method with real samples, a trial was undertaken to quantitate residues in milk of a dairy cow orally administered with fenbendazole. The results of the analysis of all samples collected during the trial, at 12-h milking intervals, are presented in Table 5. Fenbendazole showed its highest concentration in milk 24 h after dosing, whereas it declined below the detectable level 60 h after dosing. The found concentration−time profile lends support to previous findings (Fletouris et al., 1994) on the elimination of fenbendazole with cow milk. It becomes clear that fenbendazole is readily oxidized to the sulfoxide metabolite as the latter was already at its highest concentration 12 h after dosing. This facile oxidation of the sulfide moiety of fenbendazole to the sulfoxide has been also demonstrated in

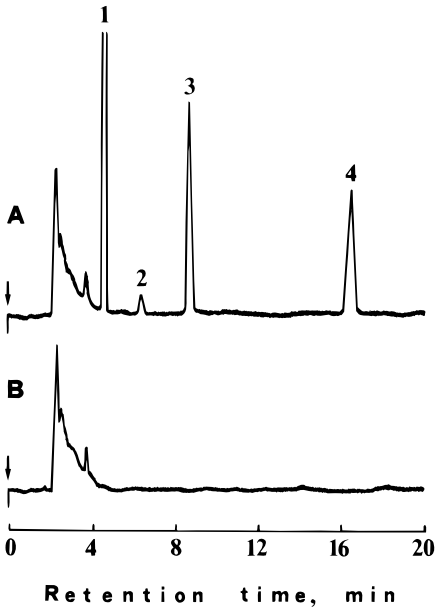

Figure 3. Chromatograms of a milk sample collected 12 h after dosing a cow with fenbendazole (A) and a control milk sample (B). LC conditions as in Figure 2.

cattle by other workers (Ngomuo et al., 1984; Prichard et al., 1985). The sulfoxide metabolite declined below detectable levels by 96 h, while the sulfone metabolite, which is the end product of the oxidation of fenbendazole, although it attained its highest level more slowly (48 h), also disappeared at 96 h. The somewhat delayed appearance of the highest level of sulfone metabolite can be accounted for by its two-step oxidization production that requires fenbendazole sulfoxide as an intermediate substrate (Averkin et al., 1975; Short et al., 1987). The elimination profile shown in Table 5 indicates that the predominant metabolite in the milk collected by 36 h is

the sulfoxide whereas in the milk collected from 48−84 h it is the sulfone. The *p*-hydroxy metabolite occurs at trace residue levels only in the milk collected at 12 h (Figure 3).

In conclusion, the results of the present study show that the developed method is an efficient and reliable means for quantitating fenbendazole residues in milk. The method has excellent linearity with respect to response and recovery; is simple, sensitive, and selective; and has low within- and between-day variability and good accuracy. One analyst familiar with the method can easily process 20 samples in a 8-h working day. Due to these advantages, the method might be particularly useful in residue monitoring programs.

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