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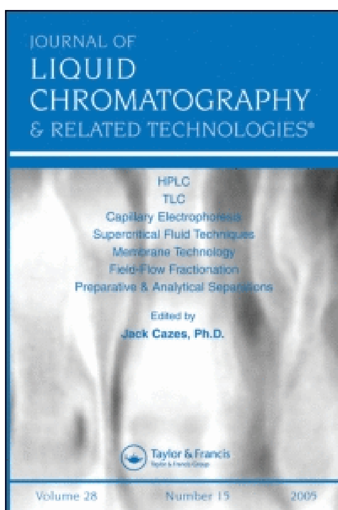

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RAPID EXTRACTION AND ION-PAIR LIQUID CHROMATOGRAPHIC DETERMINATION OF FENBENDAZOLE IN POULTRY FEEDS

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ABSTRACT

A liquid chromatographic method is described for the determination of the fenbendazole anthelmintic in poultry feeds at levels as low as 0.5 µg/g. Sample is extracted with acetonitrile, and an aliquot of the extract, after partitioning with isooctane, is evaporated to dryness, reconstituted with mobile phase, and analyzed by ion pair liquid chromatography with detection at 300 nm. The method presents quite acceptable analytical characteristics yielding recoveries in the range of 95.8-99.8% for broilers, laying hens, and turkeys diets. Precision data, based on within- and between-days variation, suggested an overall relative standard deviation of 1.9%.

INTRODUCTION

Fenbendazole, a broad-spectrum benzimidazole anthelmintic, has been shown to be highly efficacious against nematode and cestode parasites that commonly occur in poultry, especially breeding stock maintained on deep litter or in outdoor systems.[1,2] The fenbendazole treatment, which often requires to be prolonged and at high concentrations to be fully effective, includes administration of the drug with the feed in concentrations ranging from 30 to 180 µg/g for periods of 3 to 6 days.[3-6]

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As a result of this use, availability of analytical methodology capable to determine the actual level and to ensure homogeneity as well as stability of fenbendazole in medicated feeds is of concern. However, a literature survey shows that such methodology has not been yet reported.

Previous work in this laboratory has dealt with the development of liquid chromatographic methods for analyzing residues of fenbendazole and its metabolites in edible animal products.[7-9] The objective of this work was to develop the analytical method needed for quantitation of this anthelmintic in poultry feeds. Primary target in developing this method was minimization of the time, labor, glassware, and cost of the materials required.

The performance of the developed method was studied in terms of precision, accuracy, linearity, and sensitivity, in a variety of poultry feeds.

EXPERIMENTAL

Equipment

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 TC 831 column heater, and a Model 119 UV-vis detector. Injections were made using a Rheodyne, Model 7725, injection valve (Cotati, CA) equipped with 20-µL sample loop.

The analytical column was a Hichrom (Reading, UK), 250 x 4.6 mm i.d., column packed with Nucleosil 120, C_{18}, 5 µm. 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), and a Model ReactiTherm heating/stirring thermoblock (Pierce Chem., Rockford, IL), were used for sample treatment. A Model D7402 EasyPure UV compact ultrapure water system (Barnstead/Thermolyne Corp., Dubuque, I) was also used for purification of tap water.

Reagents

Analytical-grade phosphoric acid, isooctane and dimethylsulphoxide, LC-grade acetonitrile, and lichropur-grade octane-1-sulphonic acid sodium salt were from Merck (Darmstadt, Germany). Fenbendazole reference standards was from Hoechst (Frankfurt, Germany).

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Chromatography Conditions

The mobile phase consisted of acetonitrile and 0.01 M phosphoric acid, (50/50, v/v), and contained 5 mM octane-1-sulphonic acid sodium salt. The flow rate of the mobile phase was set at 1 mL/min.

The Nucleosil 120 C_{18} stationary phase was heated at 50°C and thoroughly equilibrated with the mobile phase prior to analysis. Reproducible capacity factors could be realized after passage through the column of at least 150 mL of mobile phase. After use, column washing with at least 200 mL volume of water followed by 200 mL volume of acetonitrile were quite indispensable for removing the adsorbed pairing ions. Detection was made at 300 nm at a sensitivity setting of 0.02 a.u.f.s. Chart speed was set at 5 mm/min.

Standard Solutions

A fenbendazole stock solution was prepared by weighing ca 5 mg of the compound in a 10-mL volumetric flask and dissolving to volume with dimethylsulphoxide. The stock solution was stable for at least 6 months in a freezer at -25°C. An intermediate standard was then prepared by transferring an aliquot of the stock solution in a 25-mL volumetric flask and diluting to volume with acetonitrile. Working standard solutions in the range of 0.2-1.6 μg/mL were finally prepared by transferring aliquots from intermediate solution in 10-mL volumetric flasks and diluting to volume with the mobile phase. Working solutions were prepared fresh weekly.

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Feed Samples

Typical commercial feeds formulated for laying broiler breeders hens, broilers, and turkeys were used in this study. As these feeds were free of fenbendazole, they did provide information concerning the cleanliness of the extracts and the presence of interfering peaks. Following spiking with standard fenbendazole, these feeds were also used for carrying out accuracy and precision experiments. Following mixing with a fenbendazole premix, these feeds were further used for examining the applicability of the method in medicated feeds.

Analytical Procedure

A 2-g finely ground (1 mm sieve screen) feed sample was weighed into a 50 mL screw-capped centrifuge tube. A volume of 20 mL of acetonitrile was added, and the tube was vortexed at high speed for 30 s and centrifuged for 1 min at 2000*g*. An aliquot (0.1-1.0 mL) of the clear supernatant, its volume

depending on the expected level of fenbendazole in the analyzed feed, was transferred into another tube, and acetonitrile was added to a final volume of 1 mL. Following volume adjustment, 3 mL isooctane were added, and the tube was vortexed for 15 s, and centrifuged. The top layer was discarded, and the remaining was evaporated to dryness under N_2 at 40°C. The residue was reconstituted in 0.5 mL of mobile phase, and 20 μL was analyzed by LC.

Determination

Calibration curves were generated by running working standard solutions, plotting recorded peak heights versus the corresponding mass of the analyte 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 the analyte in injected sample extracts.

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RESULTS AND DISCUSSION

Extraction/Cleanup

Considering that the best solvent for a selective extraction procedure should be the most apolar solvent with which the analyte could be extracted with sufficient efficiency, ethyl acetate and dichloromethane were initially tested as extraction solvents. Results showed that neither ethyl acetate nor dichloromethane could achieve complete recovery of fenbendazole. More rigorous partitioning though the use of an ultra-turrax homogenizer also failed to improve the recovery despite the high solvating power of the employed organic solvents. One possible reason for this incomplete recovery might be the irreversible adsorption of fenbendazole to the feed matrix. Thus, acetonitrile, an organic solvent with appreciable de-binding properties,[10] was used to compensate for losses due to adsorption. Results showed quantitative recoveries when pure acetonitrile was used, but incomplete recoveries when aqueous acetonitrile was employed. As co-extracted lipids caused emulsions when the evaporated feed extracts were reconstituted in mobile phase prior to injection, a defatting procedure was evaluated. It was found that single partitioning of the feed extracts with isooctane could remove fat without dissolving fenbendazole.

Liquid Chromatography

Analyte isolation in injected feed extracts was performed by ion-pair liquid chromatography. Without addition of the octanesulfonate ion-pair reagent, the peak of fenbendazole was severely distorted. Increasing the eluting strength of

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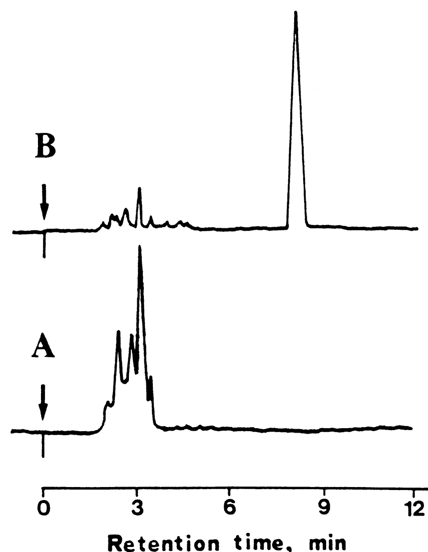

Figure 1. Typical chromatograms of a blank broiler feed sample (A), and a sample containing 30 mg/g of fenbendazole (B).

the mobile phase and/or the column temperature in order to favor a better mass transfer of the analyte between the stationary and the mobile phase, peak tailing was not reduced.[7] As this indicated adsorptive interaction of the protonated analyte with the silica-based stationary phase,[8] 5 mM of octanesulfonate were added in the mobile phase. Upon this addition, peak tailing was totally eliminated and the height of the peak that appeared at 8.3 min was almost twofold increased (Figure 1).

Calibration

Regression analysis of the data obtained by running a series of working solutions showed the response to be linear in the range examined (y=0.03+2.87x, r=0.99997, where y represents peak height in mm and x the quantity in ng of the compound injected).

Accuracy and Linearity

The accuracy of the method was studied by analyzing three sets of four 2-g samples from each of the broiler, laying hen, and turkey feeds, each set being

Table 1

Accuracy Data for the Determination of Fenbendazole in Poultry Feeds

Feed Type	Spiking Level, μg/g	Mean Concn. Found,[a] μg/g	Recovery, % (Mean ± SD)
Broilers Diet	20.00	19.84	99.2 ± 1.5
	100.00	97.30	97.3 ± 2.2
	180.00	177.48	98.6 ± 0.7
Laying Hens Diet	20.00	19.24	96.2 ± 2.2
	100.00	99.80	99.8 ± 1.6
	180.00	177.84	98.8 ± 2.0
Turkeys Diet	20.00	19.16	95.8 ± 1.4
	100.00	98.20	98.2 ± 1.6
	180.00	174.78	97.1 ± 1.1

[a] Mean of 4 replicates.

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previously spiked with fenbendazole at 20, 100, and 180 μg/g levels, respectively. Spiked samples were flushed with a gentle nitrogen stream for 1 min so the spiking solvent could be removed, and allowed to stand for 1 h prior to analysis. Table 1 presents the spiking levels and the individual mean recoveries found at each level for each feed. Least-squares and regression analysis of the data showed that the relationship between "added, x" and "found, y" could be adequately described by linear regressions ($y = -0.32 + 0.985\ x$, r=0.99995 for broilers diet; $y = -0.16 + 0.991\ x$, r=0.99996 for laying hens diet; $y = 0.12 + 0.973\ x$, r=0.99996 for turkeys diet). The almost quantitative recoveries found in all instances and the excellent linearity of the regression lines demonstrated the efficiency of the method. Overall mean recovery was estimated at 97.9 ± 1.3%.

When 4 samples from each of the broiler, laying hen, and turkey feeds, that had been medicated with fenbendazole at a claimed level of 100 μg/g, were submitted to analysis, the recovery values found were 98.1 ± 4.3%, 97.5 ± 3.9%, and 99.4 ± 1.8%, respectively.

Precision

Within- and between-days precision was determined by analyzing, on each of three different days, six 2-g samples from a total of 18 samples from a broiler

Table 2

Day	Conc. of Fenbendazole, Found µg/g	Mean Value, µg/g ± SD	Rel. SD, %
1	58.20, 58.82, 59.52, 58.10, 59.28, 58.82	58.79 ± 0.52	0.9
2	55.76, 57.72, 58.20, 57.19, 57.80, 56.82	57.25 ± 0.80	1.4
3	57.60, 58.10, 55.82, 58.20, 58.82, 59.52	58.01 ± 1.15	2.0
	Overall mean:	58.02 ± 1.07	1.8

Variance Estimates

Source	Rel. SD, %
Between-Days	3.2
Within-Day	1.6
Overall	1.9

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feed, all samples being spiked with standard fenbendazole at the 60 µg/g level. Spiked samples were flushed with a gentle nitrogen stream for 1 min so the spiking solvent could be removed, and allowed to stand for 1 h prior to analysis. To estimate the components of variance, the concentrations found (Table 2) were subjected to "analysis of variance and expected mean squares for the one way classification-balanced design."[11] Analysis data showed that the within- and between-days precision values, expressed as percent relative standard deviation, were 1.6% and 3.2%, respectively. The overall precision, which is in fact the overall uncertainty of a single determination, was estimated to be 1.9%.

Limit of Detection and Limit of Determination

The efficiency of the ion-pair chromatographic system coupled with the cleanliness of the extracts could allow a very low limit of detection to be realized in feed samples. The detection limit that corresponded to the concentration which yielded a signal that could be clearly distinguished from the signals obtained from the blank (ratio of peak height of the analyte to average peak-to-peak amplitude of the blank equals to 3/1) was estimated at 0.3 µg/g.

To determine the limit of determination, analysis was carried out on a series of 4 blank feed samples spiked with the analyte at the estimated detection limit, on a second series spiked at 0.5 µg/g, and on a third series spiked at 1.0 µg/g. Analysis results showed that the limit at which the method was sufficiently precise to yield a satisfactory estimate of the unknown concentration (RSD ≤ 10%) was that of the second series.

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Interferences

The efficiency of the employed chromatographic system coupled with the selectivity of the sample preparation procedure did not allow any interference to fenbendazole analysis from flavonoids, carotenoids, xanthophylls, and other pigments that usually occur in large amounts in poultry feeds. Since other drugs that are frequently incorporated in feeds might interfere with the analysis, an interference test was also evaluated. Analysis of a series of broiler feed samples each containing 100 μg/g of each of monensin, virginiamycin, avilamycin, nicarbazine, ethoxyquine, tetracycline, oxytetracycline, amprolium, ethopabate, mebendazole, albendazole, oxfendazole, oxibendazole, and febantel, showed that none of these compounds interfered with the analysis.

CONCLUSION

The results of the present study show that the developed method is an efficient and reliable means for the determination of fenbendazole in medicated feeds. All the criteria for a routine quantitative test that is rapid (24 samples in a 8-h working day is realistic), simple, and economical are met in this method.

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