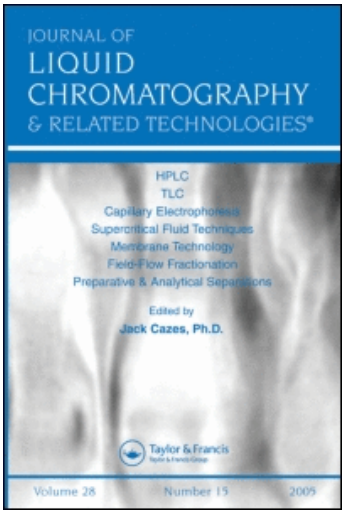

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High-Performance Liquid Chromatographic Determination of Fenbendazole and Its Metabolites, Sulphoxide and Sulphone, in Fish Muscle Tissue

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF FENBENDAZOLE AND ITS METABOLITES, SULPHOXIDE AND SULPHONE, IN FISH MUSCLE TISSUE

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ABSTRACT

An HPLC method for the determination of fenbendazole (FBZ) and its metabolites, fenbendazole sulphoxide (oxfendazole. FBZ-SO) and fenbendazole sulphone (FBZ-SO_2) in trout and eel tissue is described. The compounds are extracted with ethyl acetate and the extract, after addition of hexane, is concentrated and cleaned up on a silica gel solid-phase extraction column. After elution with 3% acetic acid in methanol the reconstituted eluate is analysed on a Lichrosorb RP8 column, the mobile phase being 0.05 M ammonium phosphate (pH=5): acetonitrile:methanol (55:28:17,v/v). Detection is performed at 297 nm. The average recovery in trout muscle tissue over the concentration range 10-500 μg/kg is 75.7±3.3 % and 77.5±4.1 % for FBZ-SO and FBZ-SO_2, respectively, and for FBZ the recovery is 76.3±5.5 % over the range 15-750 μg/kg.

The average recovery in eel muscle tissue over the concentration range 10-500 μg/kg is 83.5±4.5 % and 87.4±5.2 % for FBZ-SO and FBZ-SO_2 respectively and for FBZ the recovery is 81.4±5.3 % over the range 15-750 μg/kg.

The limits of detection are 3.0, 3.5 and 2.0 μg/kg for FBZ, FBZ-SO and FBZ-SO_2. respectively.

INTRODUCTION

Fenbendazole (FBZ) is an effective broad-spectrum anthelmintic belonging to the benzimidazole class of drugs. It is widely used in mammals to control internal worm parasites. More recently, FBZ is also applied in farmed fish to treat tape-worm infections.[1,2]

Metabolism studies in the goat,[3] in cattle[4] and in the rabbit[5] showed that the major metabolites of FBZ were fenbendazole sulphoxide (oxfendazole. FBZ-SO) and fenbendazole sulphone (FBZ-SO_2). These metabolites were also found in salmon using ^{14}C FBZ.[6] Short et al.[7] studied as well the oxidative metabolism of FBZ in hepatic fractions prepared from liver, among others, in catfish. In that study they could demonstrate the presence of FBZ-SO but not of FBZ-SO_2.

For the assessment of residue levels including these main metabolites in tissues of farmed fish an analytical procedure for the determination of FBZ, FBZ-SO and FBZ-SO_2 should be available. Methods of determination of FBZ, whether or not in combination with FBZ-SO, or with other benzimidazoles in tissue samples,[8,9,10,11,12] or in milk,[13,14,15,16] have been described.

For the determination of FBZ including the main metabolites FBZ-SO and FBZ-SO_2 only a few methods are described. These are in plasma, urine, faeces and tissue homogenates,[17] bovine liver,[18] in plasma[19,20] and in milk.[15,21] The methods for tissue are less or more laborious to be used as starting point for a method suitable for the determination of FBZ and its metabolites in fish muscle tissue.

This paper describes a rapid sample preparation method for the determination of FBZ in fish muscle tissue using solid-phase extraction (SPE) for clean-up and concentration. The method of Steenbaar et al.[22] developed for the determination of mebendazole in eel muscle tissue, was taken as starting point.

EXPERIMENTAL

Reagents and Chemicals

Water was purified via a Milli-Q system (Millipore, Bedford, MA, USA). Methanol, acetonitrile and anhydrous sodium sulphate were from Merck (Darmstadt. Germany). Acetic acid (99-100%), ammonium phosphate, dimethyl sulphoxide, potassium carbonate and silica gel (40 μm. 60 Å) were from J. T. Baker (Phillipsburg, NJ, USA). Ethyl acetate and n-hexane were from Rathburn (Walkerburn, UK). All organic solvents were HPLC grade.

Filter paper circles (S&S 589.1, diameter 90 mm) were from Schleicher and Schüll (Dassel, Germany). Filtration columns, (3 mL) were from J. T. Baker. FBZ was from Riedel-de Haën (Seelse, Germany). FBZ-SO and FBZ-SO_2 were a gift from Syntex (Palo Alto, California, USA).

Stock solutions of FBZ (1.5 mg/mL), FBZ-SO (1 mg/mL) and FBZ-SO_2 (1 mg/mL) were prepared by dissolving 30, 20 and 20 mg, respectively, in 20 mL of dimethyl sulphoxide. A working solution of FBZ (15 μg/mL), FBZ-SO (10 μg/mL) and FBZ-SO_2 (10 μg/mL) was prepared by diluting the stock solutions in methanol.

Standard solutions for HPLC were prepared in the range of 0.0225 - 2.25 μg/mL for FBZ and 0.015 - 1.5 μg/mL for FBZ-SO and FBZ-SO_2 by diluting the working solution in HPLC mobile phase.

A silica SPE column was prepared by weighing 1 g of silica gel in a filtration column with a fritted disk. The column was tapped for 30 s and a fritted disk was placed on the silica bed.

Just before use, the column was pretreated by passing 6 mL of ethyl acetate-hexane (1:4. v/v). After this the column should not allowed to run dry. The SPE elution solvent was 3 % (v/v) acetic acid in methanol.

An 0.05M ammonium phosphate buffer pH 5.0 was prepared by dissolving 5.75 g of ammonium phosphate ($NH_4H_2PO_4$) in 950 mL of water, adjusting the pH to 5.0 with 1 M NaOH and adjusting to 1000 mL with water.

The mobile phase for HPLC was methanol-acetonitrile-0.05 M ammonium phosphate buffer pH 5.0 (17:28:55, v:v) and was degassed before use.

Apparatus and Chromatographic Conditions

The instruments used were a Moulinette homogenizer (Moulinex, Gouda, The Netherlands), a KS 500 mechanical shaker (IKA-Labortechnik. Janke and Kunkel, Staufen, Germany), a Vibrofix VF 1 vortex mixer (IKA Labortechnik), a Centra-8R centrifuge (IEC, Needham, MA, USA), a 5414c Eppendorf centrifuge (Hamburg, Germany), a Reacti-Therm III heating module and a Reacti-Vap III evaporator (Pierce, Rockford, IL, USA), a SPE-21 column processor (J.T. Baker) and an ultrasonic bath (Branson, Soest, The Netherlands).

The HPLC system consisted of a 2150 HPLC pump (Pharmacia-LKB, Uppsala, Sweden), a 2153 autosampler (Pharmacia-LKB) with a 50-µL sample loop, and a 783A HPLC monitor with a 10-µL HPLC flow cell (Applied Biosystems, Foster City, CA, USA) operated at 297 nm.

A ChromSep cartridge holder system contained a stainless-steel guard column (10 mm x 2.1 mm I.D.) packed with pellicular 40-µm reverse phase particles and two coupled analytical glass columns (2 x 100 mm x 3.0 mm I.D.) packed with 5-µm Lichrosorb RP 8 (Chrompack, Bergen op Zoom, The Netherlands). The system was operated at ambient temperature. Peak areas were quantitated with an SP 4270 integrator (Spectra-Physics, San Jose, CA. USA).

Samples

For recovery studies blank trout and eel muscle tissue were spiked at levels of 15-750 µg/kg for FBZ, and 10-500 µg/kg for FBZ-SO and FBZ-SO_2 at least 15 min before extraction. When samples were not directly analysed they were stored at -20 °C.

Sample Preparation

Extraction. Ground trout or eel muscle tissue was accurately weighed (*ca.* 5 g) into a 50 mL disposable plastic centrifuge tube and 0.5 mL of 4 M potassium carbonate solution was added. The mixture was vortexed for 10 s and 10 mL of ethyl acetate was added. The tube was shaken again on a vortex mixer for 10 s. and placed on a mechanical shaker for 10 min (500 rpm). The suspension was centrifuged for 6 min at 3400 g. The supernatant was decanted. The extraction procedure was repeated with another 10 mL of ethyl acetate.

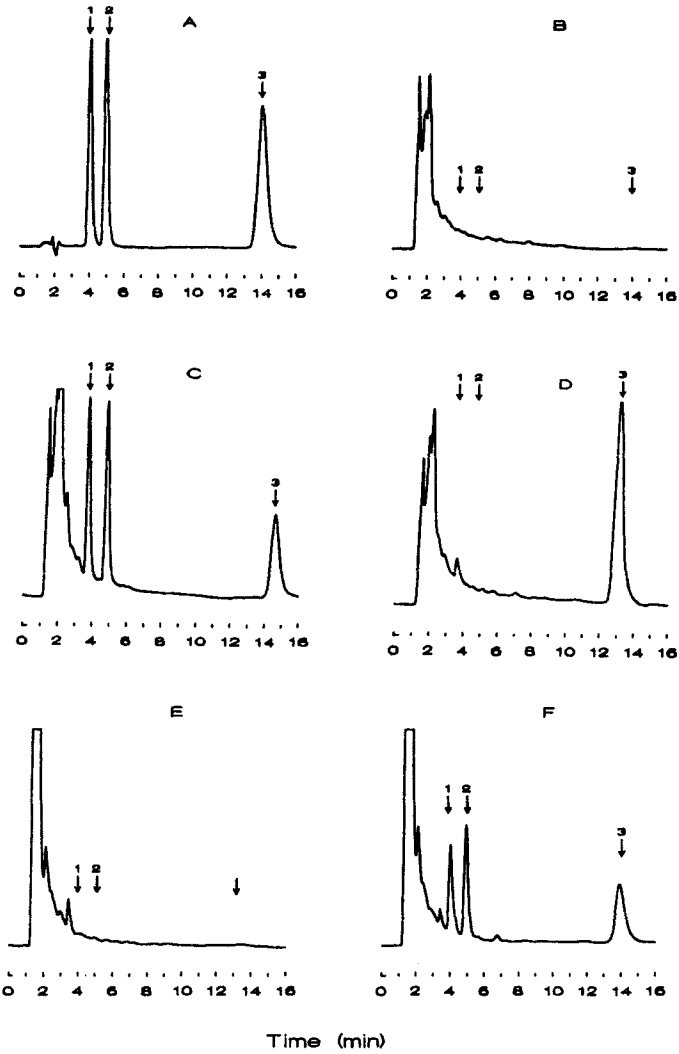

Figure 1: Chromatograms of (A) a standard solution of 500 µg/L of FBZ-SO (1), 500 µg/L FBZ of FBZ-SO_2 (2) and 750 µg/L of FBZ; (3) (B) a blank trout muscle tissue; (C) a trout muscle tissue sample spiked with 100 µg/kg of FBZ-SO,(1) 100 µg/kg of FBZ-SO_2 (2) and 150 µg/kg of FBZ (3); (D) a real trout muscle tissue sample containing 7 µg/kg of FBZ-SO (1) and 163 µg/kg of FBZ (3) ; (E) a blank eel muscle tissue sample; (F) an eel muscle tissue sample spiked with 50 µg/kg of FBZ-SO (1), 50 µg/kg FBZ-SO_2 (2) and 75 µg/kg of FBZ (3).

Clean up and concentration. 80 mL of hexane were added to the combined extracts (the ethyl acetate-to-hexane ratio should be *ca* 1:4 v/v). After the addition of 2 g of anydrous sodium sulphate, the solution was shaken and allowed to stand until it had become transparent. The solution was filtered over an S & S 589.1 filter paper circle, and the filtrate was passed through the pretreated silica gel SPE column via a 75-mL reservoir. The column was dried in a stream of nitrogen for 10 min. Then, FBZ, FBZ-SO and FBZ-SO_2 were eluted with 3 mL of 3% acetic acid in methanol. The eluate was evaporated to dryness in a stream of nitrogen at 37 °C.

The residue was dissolved in 550 μL of 0.05 M ammonium phosphate. Then, 280 μL of acetonitrile was added, followed by 170 μL of methanol. This solution was placed in an ultrasonic bath for 5 min, and centrifuged for at least 8 min at 15800 g.

Chromatography

Aliquots (50 μL) of the sample and standard solutions were injected. Samples were eluted isocratically at a flow rate of 0.6 mL/min.

RESULTS AND DISCUSSION

Chromatography

Chromatography of FBZ is usually carried out on reversed-phase columns. If metabolites are taken into account as well there is a problem with respect to retention. There is a significant difference in polarity of FBZ and FBZ-SO, resulting in a strong difference in retention times in most systems. If FBZ-SO has any retention then the retention time of FBZ is usually very high. To overcome this problem gradient elution was used[17,18,21] or different mobile phases.[9,13] As these systems are less or more complicated, attention was paid to the development of a suitable eluent including ion-pair systems in combination with different types of reversed phases. The optimal system proved to be a 20 cm Lichrosorb RP8 column with 0.05 M ammonium phosphate (pH=5): acetonitrile : methanol = 55:28:17 (v/v) at a flow rate of 0.6 mL/min, the retention times being *ca.* 4 min, 5 min, and 15 min for FBZ-SO, FBZ-SO_2 and FBZ, respectively.

Detection was performed at 297 nm. A typical chromatogram of a standard solution of FBZ, FBZ-SO and FBZ-SO_2 using the developed system is shown in Figure 1A.

Sample Preparation

In the method of Steenbaar *et al.*[22] extraction of mebendazole was performed with ethyl acetate after the addition of sodium sulphate and a potassium carbonate solution to the ground tissue. However, for FBZ low extraction recoveries were obtained, which were caused by the addition of sodium sulphate to the tissue. Therefore, sodium sulphate was added after extraction to remove the water originally present in the fish muscle tissue. This was necessary as the presence of water in the ethyl acetate extract has a negative effect on the retention of the analytes on the silica SPE column.

Next, the ratio ethyl acetate-to-hexane 1:2.5 (v/v) was changed to 1:4 (v/v) for complete retention of all three compounds on the silica SPE column. For the same reason the amount of silica sorbent in the SPE column has to be enlarged from 0.5 g to 1 g.

Further. the dissolution of the residue, prior to HPLC, proved to be very critical for the recovery of FBZ. The residue had to be dissolved into the buffer first, followed by addition of acetonitrile and methanol, instead of dissolution in mobile phase. Otherwise low recoveries were found. However, the obtained solution was not clear.

Attempts were made to make the solution transparent by extraction with n-hexane, iso-octane or petroleum ether. But there were losses of FBZ into the extractant being approximately 5-8%. Finally, centrifugation proved to be successfully.

Spiking Studies and Real Samples

Recovery experiments (6 replicates) were carried out on trout muscle tissue spiked at 10. 50 and 200 μg/kg for FBZ-SO and FBZ-SO_2, and 15, 75 and 300 μg/kg for FBZ. In addition some recovery experiments were carried out in triplicate. These results are presented in Table 1. The calibration curve is linear throughout the range 15-750 μg/kg for FBZ (r=0.998) and 10-500 μg/kg for FBZ-SO and FBZ-SO_2 (r=0.999 and 0.999, respectively). The same experiments were carried out on eel muscle tissue. These results are presented in Table 2.

The calibration curve is linear throughout the range 15-750 μg/kg (r=0.999) for FBZ and 10-500 μg/kg for FBZ-SO and FBZ-SO_2 (r=0.999 and 0.999, respectively). A good recovery at all the levels investigated and a low standard deviation (SD) for repeatability are attained for all three compounds.

Table 1

The Recovery of Analytes from Trout Muscle Samples Spiked at Different Levels

	FBZ		FBZ-SO		$FBZ-SO_2$	
N	Level Tg/kg	Recovery[a] (%)	Level Tg/kg	Recovery[a] (%)	Level µg/kg	Recovery[a] (%)
6	15	77.0±2.7	10	74.5±2.8	10	74.1±3.4
3	30	81.0±1.0	20	79.0±2.8	20	75.2±2.8
6	75	69.5±2.3	50	71.8±1.8	50	74.5±1.9
6	150	79.5±2.9	100	76.8±2.7	100	80.9±2.6
3	300	80.9±1.7	200	79.2±0.2	200	83.3±0.5
3	750	72.6±2.1	500	77.1±0.4	500	80.3±0.7

N = Number of replicates
[a] Mean ± S.D.

Table 2

The Recovery of Analytes from Eel Muscle Samples Spiked at Different Levels

	FBZ		FBZ-SO		$FBZ-SO_2$	
N	Level µg/kg	Recovery[a] (%)	Level µg/kg	Recovery[a] (%)	Level µg/kg	Recovery[a] (%)
6	15	79.1±4.3	10	86.3±6.7	10	89.6±6.6
2	30	75.6±0.3	20	75.3±2.3	20	75.7±2.3
6	75	79.8±3.1	50	82.7±1.7	50	86.8±2.4
6	150	93.4±2.0	100	80.9±4.8	100	84.1±0.5
3	300	81.7±2.2	200	85.3±2.1	200	90.7±1.6
3	750	83.9±0.5	500	85.5±1.8	500	90.8±1.0

N = Number of replicates
[a] Mean ± S.D.

Table 3

The Recovered Analyte Content of Spiked (75 μg/kg of FBZ and 50 μg/kg of FBZ-SO and 50 μg/kg of FBZ-SO_2) Trout Muscle Tissue Test Portions Analyzed on Different Days

		Analyte Content		
Day	**N**	**FBZ[a] (μg/kg)**	**FBZ-SO[a] (μg/kg)**	**FBZ-SO_2[a] (μg/kg)**
1	6	54.5±0.6	36.0±0.6	38.8±0.6
2	6	52.1±1.7	35.8±0.9	27.2±1.0
3	4[b]	57.9±2.5	40.2±4.6	41.6±5.0
4	6	53.8±3.3	40.2±0.9	41.7±1.9
5	6	58.7±2.2	39.4±0.6	40.5±0.8
6	5[c]	54.3±1.6	37.8±0.8	38.3±0.8
Overall mean	33	55.1±3.1	39.3±1.9	37.9±1.9

[a] Mean±S.D.
[b] Two samples lost
[c] One sample lost

The day-to-day variation was studied as well. Blank trout muscle tissue was spiked at 75 μg/kg of FBZ and 50 μg/kg of FBZ-SO and FBZ-SO_2. The spiked test portions were analysed in 6 replicates on 6 different days, following the procedure outlined above. The results are shown in Table 3. A reasonable SD of the mean for all compounds was attained, the relative SD being 5.6 %. 4.8 % and 5 % for FBZ, FBZ-SO and FBZ-SO_2, respectively.

Typical chromatograms of blank, and spiked trout and eel muscle tissue are shown in Figure 1B, 1C, 1E, 1F.

The absolute limits of detection of FBZ, FBZ-SO and FBZ-SO_2 were 0.5, 0.6 and 0.2 ng, respectively,on-column, at a signal-to-noise ratio of 3.0. As no interferences of endogeneous compounds from tissue at the retention time were found, the limits of detection were 3.0, 3.5, and 2.0 μg/kg for FBZ, FBZ-SO and FBZ-SO_2, respectively. in muscle tissue. Levels of 6.0, 7.0 and 4.0 μg/kg for FBZ. FBZ-SO and FBZ-SO_2. could easily be quantitated in these tissues.

As an illustration of the applicability of the described method muscle tissue of FBZ treated trout was analysed. To obtain real samples FBZ was administered orally. by intubation of the stomach, to two trouts *(Oncorhynchus mykiss)* with a mean body weight of 110 g. The dose was 6 mg/kg body weight, which is equivalent to 0.24 mL of Panacur suspension 2.5% w/v (Hoechst. Amsterdam, The Netherlands) per kg of fish weight. The contents of FBZ and FBZ-SO formed in tissue, were 139 μg/kg and 8 μg/kg, respectively after 24 h of the administration. FBZ-SO_2 was not detected. A chromatogram of a real sample is shown in Figure 1D.

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