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Determination of Albendazole and Its Major Metabolites in the Muscle Tissues of Atlantic Salmon, Tilapia, and Rainbow Trout by High Performance Liquid Chromatography with Fluorometric Detection

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A liquid chromatographic procedure for the determination of albendazole ([5-(propylthio)-1H-benzimidazol-2yl]carbamic acid methyl ester) and its major metabolites, albendazole sulfoxide, albendazole sulfone, and albendazole-2- aminosulfone in rainbow trout, tilapia, and salmon muscle with adhering skin tissue is described. The muscle tissue samples are made alkaline with potassium carbonate and extracted with ethyl acetate. The extracts are further subjected to cleanup by utilizing a number of liquid−liquid extraction steps. After solvent evaporation, the residue is reconstituted in mobile phase and chromatographed. The chromatography is carried out on a reversed phase Luna C_{18} column, using acetonitrile/methanol/buffer as a mobile phase and a fluorescence detector. The average recoveries from the fortified muscle tissue of the three fish species for albendazole (25−100 ppb), albendazole sulfoxide (15.5−62 ppb), albendazole sulfone (1−10 ppb), and albendazole-2- aminosulfone (10−100 ppb) were 94, 77, 82, and 67%, respectively. The average CV for each compound was ≤10%. The procedure was validated and then applied to the determination of albendazole and its three major metabolites in the muscle tissue of the three fish species obtained after orally dosing with albendazole.

KEYWORDS: Albendazole; HPLC; tilapia, rainbow trout, Atlantic salmon, muscle tissue; depletion

INTRODUCTION

Albendazole ([5-(propylthio)-1H-benzimidazol-2yl]carbamic acid methyl ester) is a potent broad-spectrum benzimidazole anthelmintic agent widely used against intestinal helminth infections in mammals (*1*). The metabolism of albendazole has mainly been studied in mammalians. Albendazole (ABZ) is metabolized reversibly to its major active metabolite albendazole sulfoxide (ABZ−SO) by liver microsomal enzymes (*2*). Albendazole sulfoxide is further oxidized irreversibly by gut flora to an inactive metabolite albendazole sulfone (ABZ−SO_2) (*3*−*4*). The carbamate group of ABZ−SO_2 is further deacytylated to form a polar and inactive metabolite, albendazole-2-aminosulfone (ABZ−$2NH_2SO_2$) (*5*). Like parent ABZ, ABZ−SO is also marketed as ricobendazole, because it has significant anthelmintic activity (*6*). Toxicological studies in both farm and laboratory animals have shown ABZ and its active metabolite ABZ−SO to be teratogenic (*7*).

Numerous methods for analyzing benzimidazoles in bovine milk (*8*−*10*), plasma (*11*), ovine plasma (*12*), mouse plasma (*13*), and human plasma (*14*−*16*) have been developed. A few liquid chromatography and GC/MS methods have been used for the determination of benzimidazole residues in swine muscle (*17*); ovine, bovine and swine muscle and liver (*18*), and poultry meat and eggs (*19*).

Methods for the quantitation of benzimidazole residues in fish matrix are less numerous, and for ABZ in particular, are not available in the literature. Liquid chromatography was used to determine fenbendazole and its metabolites from trout and eel muscle (*20*) and mebendazole in eel muscle (*21*). The procedure, in both cases, involved extraction of the muscle tissue with ethyl acetate, and the extract, after addition of hexane, was concentrated and cleaned up on a silica gel solid-phase extraction column. The benzimidazoles were eluted with 3% acetic acid in methanol, and the eluate was reconstituted in mobile phase. The analysis was carried out on an RP-8 column using ammonium phosphate/acetonitrile mobile phase and UV detection.

This paper describes an accurate, precise, and sensitive method for the determination of ABZ and three of its major metabolites, ABZ−SO, ABZ−SO_2, and ABZ−$2NH_2SO_2$ in muscle, with adhering skin tissues of trout, tilapia, and salmon. The sample preparation for cleanup involved simple liquid−liquid extraction steps, followed by reversed phase high-performance liquid chromatography (HPLC) analysis, using fluorometric detection. The method had sufficient sensitivity to determine albendazole and its metabolites in incurred muscle tissue of Atlantic salmon, tilapia, and rainbow trout.

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MATERIALS AND METHODS

Apparatus. The liquid chromatographic (LC) system consisted of Hewlett-Packard (HP) (Palo Alto, CA) Model 1050 quaternary pump and autosampler and Agilent series 1100 fluorescence detector (290 and 330 nm excitation and emission wavelengths, respectively), HP ChemStation software, HP Laser Jet 5000N printer, and a Dell Optiplex GX1 computer. Analytical (150 × 4.6 mm) and guard (4 × 3 mm) columns were 5-Fm Luna C18 and ODS C18, respectively (Phenomenex, Torrence, CA). Both the analytical and guard columns were used at ambient temperature.

Centrifugations were carried out for 15 min at 4100*g* using a swing-out rotor (M4) in a Jouan CR 422 refrigerated centrifuge (Jouan Inc., Winchester, VA) at 4 °C, unless otherwise indicated. Polypropylene tubes with conical bottoms (15 mL) were used (Corning Glass Works, Corning, NY). All liquid transfers were made with Eppendorf digital pipets.

An Eberbach shaker (Eberbach Corp., Ann Arbor, MI), a Zymark Turbo Vac LV evaporator (Zymark Corp., Hopkinton, MA), and a Tru-Sweep Ultrasonic cleaner (Crest Ultrasonic Corp, Trenton, NJ) were used during cleanup steps.

Chemicals and Reagents. Glass distilled organic solvents (Burdick and Jackson Laboratories, Muskegon, MI) and water from Milli-Q plus Ultra-Pure Water System (Millipore Corporation, Bedford, MA) were used. Dimethyl sulfoxide (DMSO), sodium metabisulfite, and ABZ were purchased from Sigma Chemical Company (St. Louis, MO). Ammonium acetate was from Aldrich (Milwaukee, WI); glacial acetic acid and potassium carbonate were from J. T. Baker (Phillipsburg, NJ). Albendazole sulfoxide, albendazole sulfone, and albendazole aminosulfone were gifts from Pfizer (Groton, CT). ABZ-SO_2 was also procured from Lancaster Synthesis (Pelham, NH).

HPLC Mobile Phase. A stock solution of 0.5 M ammonium acetate, pH 5, was prepared by weighing 38.6 g of ammonium acetate and transferring with water to a half water filled 1-L glass volumetric flask. A 10 mL portion of acetic acid was added, and the flask was brought to the mark with additional water and mixed. A mobile phase buffer of 0.05 M ammonium acetate was prepared by transferring 50 mL of stock 0.5 M solution into a 500-mL flask; additional water was added to reach the mark, and the contents of the flask were mixed. Isocratic mobile phase composed of acetonitrile/methanol/0.05M buffer in ratios of 30:15:55 and 17:8:75 were used for the analysis of albendazole and metabolites, respectively.

Muscle Tissue Samples. Tissue samples of muscle with adhering skin were used in this study and are referred as "muscle samples" throughout this manuscript. Incurred muscle tissue samples of rainbow trout, tilapia, and salmon were generated in pilot studies by orally dosing each species of fish with albendazole at 10 mg/kg body weight.

Preparation of Gel-Food and Dose. Gel-food was prepared by mixing gelatin (unflavored Knox), 40 g of pulverized fish chow, and one sheet of Nori with 50 mL of cold water. A 60 mL portion of hot water was added to the solution, mixed and poured into a plastic mold and refrigerated until solid. Gel-food was cut into 1-cm square pieces. A hole was made in the center of the gel-food squares with a cork borer, the appropriate amount of albendazole powder was transferred, and the hole was plugged with gel-food.

Oral Treatment. Gel-food squares containing the drug were transferred into a Living Stream or glass tanks holding the fish. Rainbow trout and tilapia accepted these as food, but Atlantic salmon declined to accept. Therefore, for salmon, albendazole was weighed into gel-capsules (Torpak #4) and administered via stomach tube (Tygon) with manual restraint. One fish from each species was sacrificed at 8, 12, 24, 48, 72, 96, and 120 h after dosing. Muscle fillets with adhering skin were collected and stored at −80 °C until analyzed.

Blending of Muscle Tissue Samples. The dry ice homogenization was based on the procedure by Benville and Tindle (*22*). Muscle fillets, stored at −80 °C, were semi-defrosted and cut into small pieces (∼1 × 1 cm). About $^1/_2$ cup of dry ice pellets were transferred into a Warring blender jar and powdered. The cut-up tissue was transferred into the blender jar and run at high speed for ∼30 s intermittently, until the tissue was well blended with dry ice powder. The blended tissue was transferred into wide mouth plastic bottles, covered loosely, and stored at −20 °C overnight, to release the CO_2. The sample bottles were then capped tightly and stored at −80 °C until assayed.

Sample Extraction and Cleanup Procedure. One gram aliquots of finely ground frozen tissue were weighed into tarred 15-mL polypropylene centrifuge tubes. Samples were thawed at room temperature, fortified with albendazole or its metabolites when necessary, and 0.1 mL of 4 M potassium carbonate (pH 11), 0.5 mL of DMSO, 0.4 mL of sodium metabisulfite (4 mg/mL), and 5 mL of ethyl acetate were added. Samples were vortex mixed, and extracted for 10 min on an Eberbach shaker set at high speed. Samples were then centrifuged, and supernatants were transferred to 15-mL tubes. An additional 5 mL of ethyl acetate was added to sample pellets, vortex mixed, extracted, and centrifuged. Resulting supernatants were combined with corresponding supernatants from the first extractions, 0.5 g of sodium sulfate was added, then samples were vortex mixed, and centrifuged for 5 min. Aliquots (9 mL) of each extract were transferred to another tube and evaporated to dryness under nitrogen in a water bath (50 °C). To the dried residue, 2.0 mL of hexane was added and vortex mixed. Subsequently, 0.5 mL of ethanol-0.2 N HCl (2:1) was added, vortex mixed, and centrifuged for 5 min. The upper hexane layer was removed and discarded. An additional 2.0 mL of hexane was added to the remaining extract, vortex mixed and centrifuged again. The upper hexane layer, along with emulsion, if any, was removed and discarded. The lower layer was evaporated to dryness and reconstituted in 1.0-mL mobile phase for HPLC analysis. Portions of the extract were transferred to auto sampler vial insert, and 100 μL injections were made into the LC column.

Preparation of Standard Solutions. Solid reference standards (1−10 mg) of ABZ, ABZ−SO, ABZ−SO_2, and ABZ−2NH_2SO_2 were weighed into small glass boats using a Mettler Toledo AG245 analytical balance fitted with an LC P43 printer (Greifensee, Switzerland). Reference standards were transferred into 25−100-mL actinic glass volumetric class A flasks and dissolved in acetonitrile/DMSO (9 + 1) to give approximately 40 to 100 ppm stock solutions. Intermediate solutions (10 ppm) of each compound were prepared in 10−25-mL actinic glass volumetric flasks by transferring appropriate volumes of stock solution and diluting with acetonitrile. Working solutions (1 ppm) were prepared by transferring 1 mL of the 10 ppm intermediate solutions into 10-mL actinic glass volumetric flasks and diluting with acetonitrile. Intermediate and working solutions were used for the preparation of standard curves and for fortification of recovery samples. Stock, intermediate, and working solutions were refrigerated at 4 °C when not in use.

Stability of 1 ppm standard solutions was determined over an eight week period, stored in both clear and amber glass at room and refrigerated temperatures. ABZ and its three metabolites were stable over the two week period under the above conditions. ABZ−SO solution appear to deteriorate by the third week and ABZ in clear glass was unstable at room temperature by the eighth week.

Standard Curves. Appropriate volumes of intermediate and working standards were transferred into 10-mL actinic glass volumetric flasks, diluted with HPLC mobile phase and stored at 4 °C when not in use. The range of calibration standards for each compound differed according to level of quantitation. For ABZ, the range was 20−200 ppb; for ABZ−SO, 1.6−46.5 ppb; for ABZ−SO_2, 0.5−20 ppb; and for ABZ−2NH_2SO_2, 5−100 ppb. Calibration standard solutions were transferred into 300-μL auto sampler vial inserts, and 100-μl aliquots were injected into the LC column.

RESULTS AND DISCUSSION

Analytical Method Development. The procedure described in this manuscript is simple, utilizing liquid−liquid extraction to remove endogenous interfering compounds. Although the procedure is based on the methods by Balizs and Wilson (*17*−*18*), it was simplified to avoid the use of solid-phase extraction. Additionally, the procedure was optimized to include DMSO and sodium metabisulfite in the initial extraction step. Inclusion of DMSO improved the recovery of ABZ from muscle tissue, and sodium metabisulfite inhibited the oxidation of ABZ−SO

to ABZ−SO_2, resulting in the increased recovery of ABZ−SO. The extraction efficiency of albendazole and its metabolites was evaluated by using potassium carbonate solution at pH 9, 10, and 11. No significant difference in analyte recovery due to pH was noted, and subsequent extractions were conducted at pH 11, using 4 M potassium carbonate solution. The procedure can be used to process 10−12 samples per day.

For quantitation, reversed phase liquid chromatography with fluorometric detection was chosen to separate and detect albendazole and its metabolites. The use of a fluorescence detector eliminated most of the endogenous interferences from conjugated and aromatic compounds that absorb at UV wavelengths. Initially, a mobile phase of acetonitrile and ammonium acetate at pH 5 was chosen. To keep the retention time (RT) under 20 min, albendazole required a higher percentage (35%) of acetonitrile to elute from the end capped reversed phase column than did its metabolites, which were eluted and resolved with 20% acetonitrile. This difference is perhaps due to differences in polarity of ABZ and its metabolites, as well as the binding of the protonated albendazole with the negatively charged silanol groups on the silica-based stationary phase.

To separate parent ABZ and its three metabolites in single analysis, a gradient mobile phase was evaluated. It consisted of an initial mobile phase combination of 20% ACN in buffer for 6 min, increased to 30% ACN in 30 min, and held for 40 min. A good separation of ABZ (RT, 33 min) and its three metabolites, ABZ−$2NH_2SO_2$ (RT, 6 min), ABZ−SO (RT, 8 min.), and ABZ−SO_2 (RT, 15 min.) was obtained. However, there was an increased baseline shift during the gradient run, therefore its use was abandoned and two isocratic modes of analysis, one for parent ABZ and the other for its metabolites, as described above, were adopted.

There was no interference from tissue matrix with the elution of albendazole; however, interference from early eluting material was sufficient to obscure the ABZ−$2NH_2SO_2$ in the LC chromatogram. Methanol was introduced as a third component of the mobile phase to resolve ABZ−$2NH_2SO_2$ interferences. The final mobile phase combination for parent albendazole was 30:15:55, and for metabolites was 17:8:75 acetonitrile/methanol/0.05 M ammonium acetate, respectively. Under these mobile phase conditions, the retention times for the parent ABZ was 16−17 min and for metabolites, ABZ−$2NH_2SO_2$, ABZ−SO, and ABZ−SO_2, retention times were 5−6, 8−9, and 16−17 min, respectively. Depending on the age and the batch of the column used, the mobile phase ratio was modified slightly to maintain the above retention times.

The ABZ−SO standard received was only 31% pure and contained 69% ABZ−SO_2 as an impurity. Therefore, the concentrations of its standard solutions were appropriately corrected in all calculations to compensate for its impurity.

Recoveries of Albendazole and Its Metabolites from Muscle Tissues of Rainbow Trout, Tilapia, and Salmon. **Figure 1** gives structures of albendazole and its metabolites, and **Figure 2** represents liquid chromatograms of a standard mixture of metabolites ABZ−$2NH_2SO_2$, ABZ−SO, and ABZ−SO_2 and control muscle tissue extracts of Atlantic salmon, tilapia, and rainbow trout. No significant endogenous interfering compounds are noted at the elution position of the three metabolites in the control samples. Similarly, **Figure 3** represents the chromatograms of the extracts of albendazole fortified salmon control muscle tissue and control muscle tissues of Atlantic salmon, rainbow trout, and tilapia. Again, albendazole is well resolved from the matrix peaks in the control tissue extracts of the three fish.

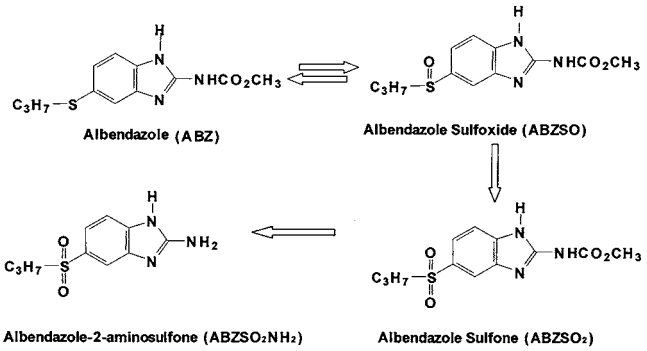

Figure 1. Structures of Albendazole and its metabolites.

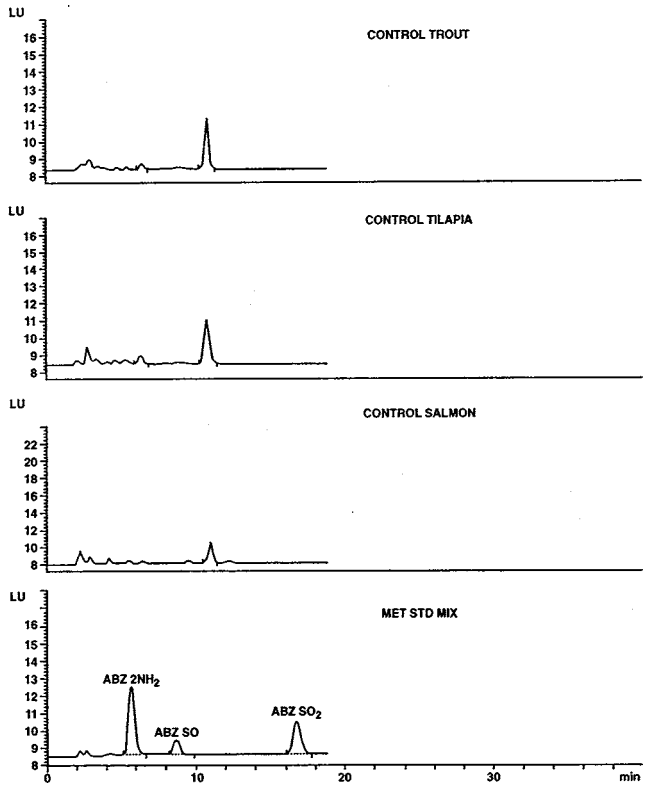

Figure 2. Chromatograms of 100-FL injection of control muscle extracts of trout, tilapia, salmon, and standard mixture of albendazole metabolites, using metabolite mobile phase combination.

A calibration curve for each compound was constructed before sample analysis and was used to quantitate the fortified and incurred samples. Typically, the calibration curves were generated from four different concentrations and were linear throughout the range for each compound. The correlation coefficient (r^2) for each compound was >0.995.

Tables 1, **2**, and **3** show recoveries of ABZ, ABZ−SO, ABZ−SO_2, and ABZ−$2NH_2SO_2$ from fortified muscle tissue of Atlantic salmon, tilapia, and rainbow trout, respectively. Good recoveries and low coefficient of variations (CVs) of ABZ and its metabolites from the muscle tissues of the three fish fortified at various concentration levels were obtained. Recoveries for ABZ, ABZ−SO, ABZ−SO_2 and ABZ$2NH_2SO_2$ ranged from 82 to 109, 61 to 85, 75 to 96, and 63 to 76%, respectively, with corresponding CV ranges of 2−11, 2−16, 1−19, and 2−12%. Recoveries and CVs were generally within the acceptable FDA/CVM guidelines for an analytical method (*23*) (i.e., for residues

Table 1. Accuracy and Precision Data for the Determination of ABZ, ABZ–SO, ABZ–SO_2, and ABZ–$2NH_2SO_2$ Residues from Fortified Muscle Tissue of Atlantic Salmon

albendazole		albendazole sulfoxide		albendazole sulfone		albendazole aminosulfone	
level-ppb (*n*)[a]	% recov[b] (CV)[c]	level-ppb (*n*)	% recov (CV)	level-ppb (*n*)	% recov (CV)	level-ppb (*n*)	% recov (CV)
25 (4)	90 (6)	15.5 (5)	82 (16)	1 (6)	82 (19)	10 (5)	74 (10)
50 (3)	82 (2)	31 (5)	78 (6)	5 (6)	77 (15)	25 (5)	67 (12)
100 (4)	82 (9)	62 (5)	78 (11)	10 (6)	75 (4)	50 (5)	63 (6)
						100 (3)	63 (2)

[a] *n* = number of replicates. [b] Recovery expressed as mean. [c] CV = % coefficient of variation.

Table 2. Accuracy and Precision Results for the Determination of ABZ, ABZ–SO, ABZ–SO_2 and ABZ–$2NH_2SO_2$ Residues from Fortified Muscle Tissue of Tilapia

albendazole		albendazole sulfoxide		albendazole sulfone		albendazole aminosulfone	
level-ppb (*n*)[a]	% recov[b](CV)[c]	level-ppb (*n*)	% recov (CV)	level-ppb (*n*)	% recov (CV)	level-ppb (*n*)	% recov (CV)
25 (3)	109 (8)	15.5 (3)	61 (11)	1 (3)	96 (11)	25 (3)	76 (3)
100 (3)	97 (11)	62 (3)	80 (3)	5 (3)	84 (12)	50 (3)	69 (10)
						100 (3)	63 (4)

[a] *n* = number of replicates. [b] Recovery expressed as mean. [c] CV = % coefficient of variation.

Table 3. Accuracy and Precision Data for the Determination of ABZ, ABZ–SO, ABZ–SO_2, and ABZ–$2NH_2SO_2$ Residues from Fortified Muscle Tissue of Rainbow Trout

Albendazole		Albendazole Sulfoxide		Albendazole Sulfone		Albendazole Aminosulfone	
level-ppb (n)[a]	% recov[b] (CV)[c]	level-ppb (n)	% recov (CV)	level-ppb (n)	% recov (CV)	level-ppb (n)	% recov (CV)
25 (3)	99 (2)	15.5 (3)	79 (2)	1 (3)	79 (1)	10 (3)	65 (4)
50 (3)	97 (8)	62 (3)	85 (2)	5 (3)	75 (9)	25 (3)	66 (9)
100 (3)	88 (4)					50 (3)	65 (3)

[a] *n* = number of replicates. [b] Recovery expressed as mean. [c] CV = % coefficient of variation.

below 100 ppb, recovery range 60−120% and CV ≤ 20%; for residues ≥ 100 ppb, recovery range 80−100% and CV ≤ 10%).

Application of the Method. To validate the method with the incurred samples, muscle tissue samples of albendazole treated salmon, tilapia, and trout were analyzed. **Figure 4** shows chromatograms of a control salmon and a 12-hr post dose muscle tissue sample containing major metabolites of albendazole. The three metabolites are well separated from the endogenous interfering peaks, and also, no interfering peaks were noted at the elution position of parent ABZ. Similarly, muscle tissue extracts from treated Atlantic salmon and rainbow trout were devoid of interferences at the retention times of albendazole and its metabolites. **Table 4** shows the result of a pilot study in which one fish from each of the three species was sacrificed at the indicated withdrawal times. Albendazole was converted by salmon, tilapia, and trout into three major metabolites found in other species, albendazole sulfoxide, albendazole sulfone, and albendazole aminosulfone. The biotransformation of albendazole into these metabolites in fish is consistent with other studies in cattle, sheep, rats, and mice (*24*). Studies on the residue depletion of albendazole and its major metabolites in muscles of a larger number of fish from each species are in progress and will be reported elsewhere.

Precision and Sensitivity. The within-day precision of the method for each analyte was determined by assaying five replicates of fortified salmon muscles. The CVs of ABZ and ABZ−SO, fortified at 15.5 and 31 ppb, were 2.8 and 1%, respectively; for ABZ−SO_2 and ABZ−$2NH_2SO_2$, each fortified at 10 ppb, the CVs were 0.4 and 2.6%, respectively. The between-day precision was determined by assaying albendazole and its metabolites on four different days over an 11-day period. The CVs for ABZ and ABZ−SO, fortified at 100 and 31 ppb, were 3.4 and 3%, respectively; for ABZ−SO_2, fortified at 5 ppb, the CV was 4.6%; and for ABZ−$2NH_2SO_2$, fortified at 10 ppb, the CV was 7.5%. The selectivity and sensitivity of the fluorescence detector coupled, with efficiency of the analytical column, afforded very low limits of detection, particularly toward the sulfoxide and sulfone metabolites. This made it possible to have lower concentration levels of these metabolites in the linear range of the standard curve. The limit of quantification (LOQ) of the method was considered to be the

Table 4. Concentrations of ABZ and Its Metabolites in the Muscle Tissues of Atlantic Salmon, Tilapia and Rainbow Trout, Obtained after Oral Dosing[a]

	albendazole			albendazole sulfoxide			albendazole sulfone			albendazole aminosulfone		
WD h	SA ppb	TL ppb	TR ppb	SA ppb	TL ppb	TR ppb	SA ppb	TL ppb	TR ppb	SA ppb	TL ppb	TR ppb
8	ND	68	ND	5	39	18	1	7	5	ND	18	ND
12	ND	ND	ND	26	31	47	2	6	14	5	15	10
24	22	ND	ND	69	7	21	5	1	8	9	51	7
48	ND	ND	ND	8	ND	ND	3	ND	8	6	34	3[b]
72	26	ND	ND	ND	7	ND	2	2	5	3[b]	100	4[b]
96	ND	ND	ND	3	ND	ND	1	1	ND	4[b]	69	ND
120	ND	ND	ND	7	ND	ND	2	1	ND	4[b]	53	ND

[a] ND = not detected, WD = withdrawal time, SA = salmon, TL = tilapia, TR = trout. [b] Below LOQ.

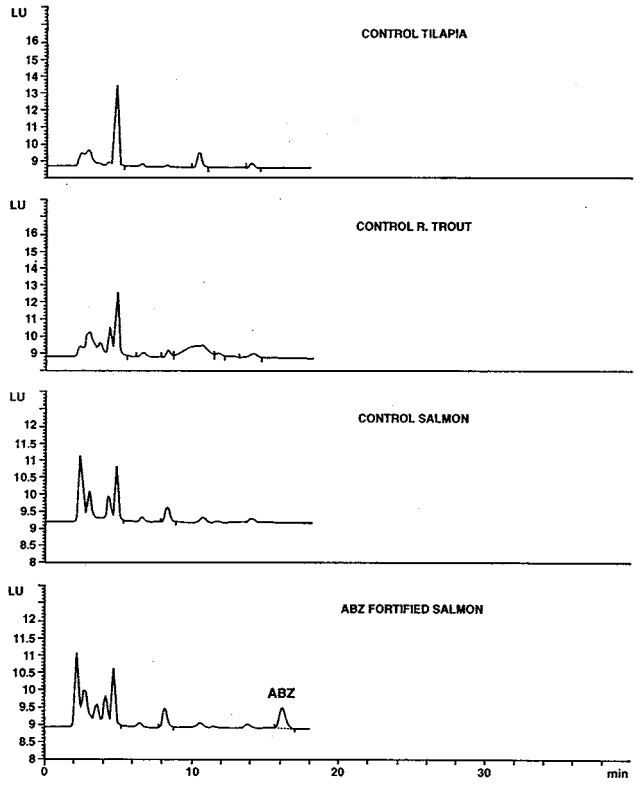

Figure 3. Chromatograms of 100-μL injection of control muscle extracts of tilapia, trout, salmon, and albendazole fortified salmon control, using albendazole mobile phase combination.

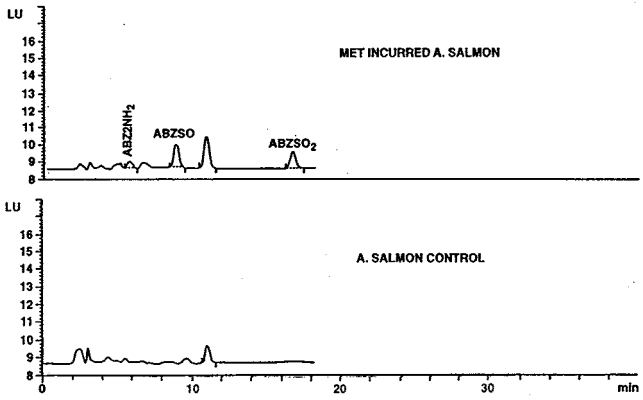

Figure 4. Chromatograms of 100-μL extract of control and 12-h post dose incurred muscle tissue of Atlantic salmon, using metabolite mobile phase combination.

lowest concentration on the calibration curve. Accordingly, the LOQ for ABZ, ABZ−SO, ABZ−SO_2, and for ABZ−$2NH_2$-SO_2 were 20, 1.5, 0.5, and 5 ppb, respectively. Limit of detection (LOD) estimates were based on the average response of the five-control sample plus three times the standard deviation (*25*). The LODs calculated for ABZ, ABZ−SO, $ABZSO_2$, and ABZ−$2NH_2SO_2$ were, 6, 1, 0.1, and 2 ppb, respectively.

Conclusion. An accurate, concise, precise and sensitive liquid chromatographic analysis method for the determination of albendazole and its major metabolites, albendazole sulfoxide, albendazole sulfone, and albendazole 2-aminosulfone, in muscle tissue of fish has been developed. The method is applicable to three species of fish, rainbow trout, tilapia, and Atlantic salmon, without modification. The method was validated by determining albendazole and its major metabolites in fortified and incurred muscle tissues of the three different species of fish.

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