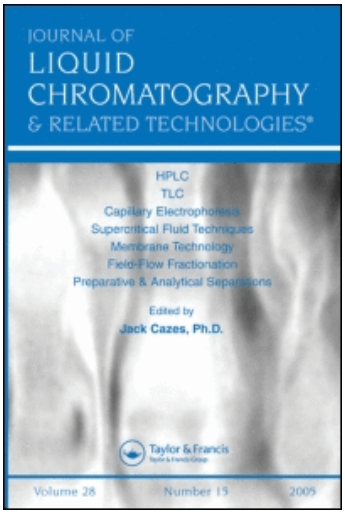

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF TEFLUBENZURON AND DIFLUBENZURON IN FISH TISSUES OF FARMED FISH FOR RESIDUE STUDIES

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ABSTRACT

A method for the determination of teflubenzuron and Diflubenzuron in fish tissues by HPLC is presented. The samples were extracted with acetone - tetrahydrofuran. Employing traditional liquid-liquid extraction and using a Si and C_8 solid phase extraction column, a clean extract was obtained. The lower limit of quantification for teflubenzuron was 20 ng/g (mL) for muscle, liver and plasma and for diflubenzuron in muscle and liver, the limit of quantification was 25 ng/g.

INTRODUCTION

Infestation with sea lice, *Lepeophtheirus salmonis* and *Caligus elongatus*, is a growing problem in fish farming. The lice damages the skin, causing unthriftiness, and in severe cases the lice may even result in death of the fish. The parasite may also transmit microbial pathogens. Farmed fish are usually more commonly and more heavily infested compared to wild fish[1,2,3]

A range of methods (chemical, physical and biological) has been introduced for controlling sea lice. Treatment with chemotherapeutics has included the use of dichlorvos, trichlorfon, azamethiphos, carbaryl, ivermectin, pyrethrum and hydrogen peroxide.[2] These drugs are administered by bathing or dipping, except for ivermectin which is applied orally (in feed).[2]

The pesticides teflubenzuron (TFB) from Cyanamid and diflubenzuron (DFB) from Solvay Duphar which can be given orally (in feed) to salmon, are two of the newest drugs used in the treatment of sea lice in salmon.

Although the pharmacokinetics of TFB and DFB in fish is unknown, their elimination from fish tissue is assumed to be dependent on water temperature. Drug levels in different tissues are therefore, to some extent, unpredictable. In order to establish safe withdrawal periods to protect the consumer, a sensitive assay for detecting TFB and DFB in tissues is needed.

This paper describes simple and rapid extraction and clean-up procedures for the HPLC determination of TFB in fish plasma and tissues and for the determination of DFB in muscle and liver. The methods are suitable for pharmacokinetic studies and residue analysis of these compounds in Atlantic salmon and rainbow trout. The method is reliable and sensitive and requires only small quantities of chemical reagents. There is no interference from the tissue matrix in the chromatographic analysis.

MATERIALS AND METHODS

Materials and Reagents

Samples of muscle, liver, and plasma from salmon and rainbow trout were used. All chemicals and solvents were of analytical or HPLC grade. Teflubenzuron (Cyanamid), was donated by Skretting (Stavanger, Norway) and diflubenzuron (Solvay Duphar), was donated by Ewos (Skårer, Norway). Stock solutions (1mg/mL) of TFB and DFB were prepared by dissolving the compound in tetrahydrofuran.

Working standards were prepared by dilution with acetonitrile-water (1 : 1) and dichloromethane - hexane (60 : 40). The solutions were stored in the refrigerator. Extraction columns Bond Elut (3cc/500mg, 1cc/100mg) SI and C_8 (1cc/100 mg) were purchased from Varian (Harbor City, CA, USA). Empty reservoirs of 125 mL (Analytichem) were combined with the SI columns.

Chromatographic Conditions

The analyses were performed on a Perkin-Elmer HPLC system, consisting of a Series 410 Bio solvent delivery system, an ISS 100 sampling system (with hexane as flushing liquid) equipped with a Lauda RMT6 cooler (10 °C) from Messgeräte Werk Lauda (Lauda Königshafen, Germany), and a LC 235C diode array detector (Perkin-Elmer, Norwalk, CT, USA). The detector was operated at 250 nm (fixed wavelength). The integration was carried out using the software programme Turbochrom 4.0 (Perkin-Elmer), which was operated on a Brick personal computer connected to a BJ-330 printer (Canon).

The analytical column (stainless steel, 25 cm x 4.6 mm. ID) and guard column (stainless steel , 2 cm x 4.6 mm. ID), were packed with 5 μm particles of Supelcosil LC-ABZ+Plus (Supelco, Bellefonte, PA, USA). The guard column was connected with an A.318 precolumn filter with an A-102X frits (Upchurch Scientific, USA).

For TFB, the mobile phase was a mixture of dichloromethane- hexane (60: 40). The flow rate was 0.8 mL/min for 6 min, followed by 1.5 mL/min for 7 min (5 min for plasma). For DFB, the mobile phase was a mixture of hexane-dicloromethane (60 : 40). The flow rate was 0.9 mL/min. for 6 min., followed by 1.5 mL/min. for 8 min.

Sample Pretreatment

Muscle and liver

The tissue sample, (3 g) was weighed into a 50 mL centrifuge tube with a screw cap (NUNC, Roskilde, Denmark). Volumes of 300 μl water (or standard dissolved in acetonitrile-water) and 9 mL acetone-tetrahydrofurane (6 : 4) were added. The mixture was homogenized for approx. 6 sec. in an Ultra-Turrax TP 18/2 (Janke & Kunkel KG, Ika Werk, Staufen, F. R. G.), and then centrifuged for 3 min. (5000 rpm). 6.15 mL of the supernatant (corresponding to 1.5 g) were transferred into a glass-stoppered centrifuge tube. Volumes of 2 mL CH_2Cl_2 and 3 mL hexane were added and mixed for approx. 5 sec .

After centrifugation for 3 min (3000 rpm), the upper layer (organic layer) was transferred to another glass-stoppered tube (the water layer was discarded), mixed with 3 mL hexane, and centrifuged after 5 min (3 min., 3000 rpm). The supernatant was then transferred into another glass-stoppered tube and evaporated to dryness under a stream of nitrogen using a Reacti-Therm heating module at 35 °C, and a Reacti-Vap evaporating unit (Pierce, Rockford, IL, USA). The dry residue was dissolved in 6 mL hexane, and loaded onto a conditioned SI column (500mg).

Clean-up on SPE-column

The SI column was conditioned with 2.5 mL hexane, and 2 mL hexane was added to the column. The sample extract was loaded onto the columm without being suctioned through. The glass-stoppered tube was rinsed with 3 x 1 mL hexane and the empty reservoir washed with 2 x 1.5 mL hexane, which was also loaded onto the column. The column was washed with 3 x 2.5 mL hexane, 2 x 2.5 mL hexane-diethyl ether (95 : 5) and 2.5 mL hexane-diethyl ether (90 : 10). The column was then eluted with 2 x 2.5mL hexane-diethyl ether (60 : 40), with full vacuum, the collected eluate being evaporated to dryness. The dry residue was dissolved in 300 µl CH_3OH, and 1.5 mL water then added. The mixture was loaded into a conditioned C_8 column (with 1 mL CH_3OH and 2 x 1 mL H_2O) and slowly suctioned through (0.2 mL/ min.). The glass-stoppered tube was rinsed with 0.5 mL H_2O - CH_3CN (70 : 30), which also was loaded onto the column . The column was washed with 0.5 mL H_2O-CH_3CN (70 : 30), and 2 x 0.5 mL H_2O-CH_3CN (55 : 45). The column was suctioned to dryness for 5 sec. at a vacuum of -5 in. Hg. and then eluted with 2 x 200µl CH_3CN with full vacuum using a VacMaster system (International Sorbent Technology). The collected eluate was evaporated to dryness. The dry residue was dissolved in 0.5 mL CH_2Cl_2 - hexane (60 : 40). After centrifugation (2 min. 3000 rpm.), aliquots of 20µl were injected into the HPLC at intervals of 16 min. for the determination of TFB and DFB.

Plasma

The pretreatment of plasma samples was as follows: To 500µl plasma were added 200 µl water (or standard dissolving in acetonitrile-water) and 6 mL acetone-tetrahydrofurane (6 : 4). The mixture was shaken for 10 sec. Volumes of 2 mL CH_2Cl_2 and 3 mL hexane were added, mixed for 5 sec. and then centrifuged for 3 min. (3000 rpm.). The upper organic layer was transferred to another glass-stoppered centrifuge tube, mixed with 3 mL hexane, and centrifuged after 5 min. The supernatant was transferred into another glass-stoppered tube and then evaporated to dryness.

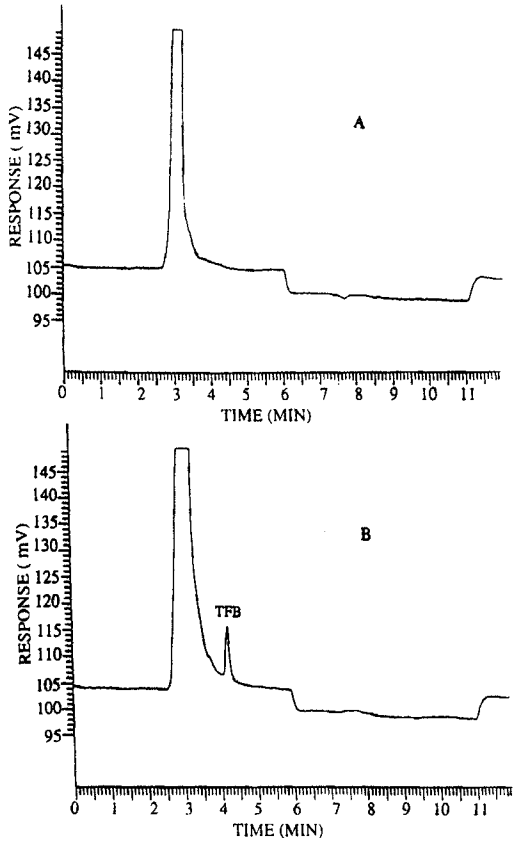

Figure 1. Chromatograms of extracts from fish plasma. A: drug-free plasma, B: plasma spiked with teflubenzuron (200 ng/ mL).

The dry residue was dissolved in 200 µl dichloromethane, and mixed with 3 mL hexane, and loaded (without being suctioned through) onto a conditioned 100 mg SI column (conditioned with 1 mL hexane and 0.6 mL hexane added to the column). The glass tube was rinsed with 3 x 1 mL hexane and the empty reservoir washed with 2 x 1 mL hexane, which also was loaded onto the column. The column was washed with 1mL hexane, 1 mL hexane-diethyl ether (95 : 5) and 0.5 mL hexane-diethyl ether (90 : 10). The column (with full vacuum) was eluted with 2 x 1 mL hexane - diethylether (60 : 40). The collected eluate was evaporated to dryness and the dry residue dissolved in 300 µl CH_3OH and 1.5 mL H_2O then added. Further preparation procedures were as for muscle and liver. Aliquots of 40 µl were injected into the HPLC at intervals of 12 min. for the determination of TFB.

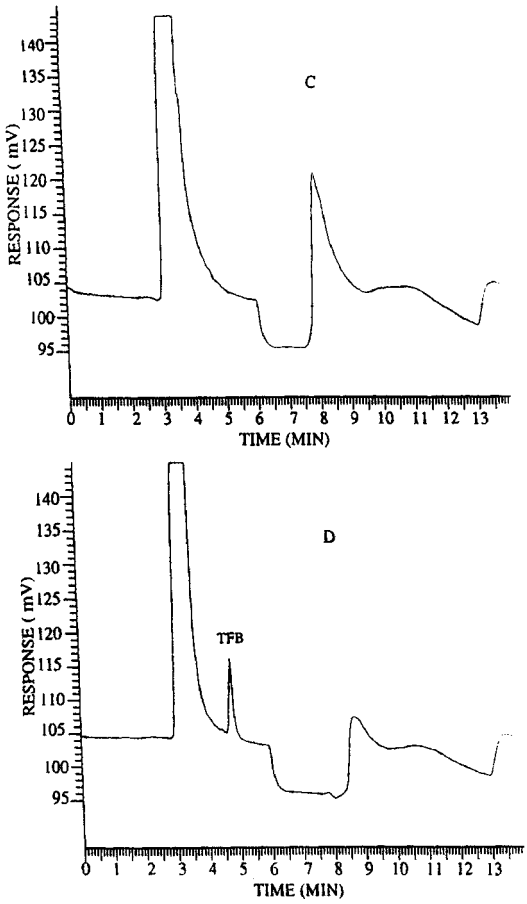

Figure 2. Chromatograms of extracts from fish muscle. C: drug-free muscle, D: muscle spiked with teflubenzuron (200 ng/g).

Calibration Curves and Recovery Studies

The calibration curves for TFB and DFB were obtained by spiking plasma, muscle and liver tissue samples with standard solutions. These were prepared by dilution with acetonitrile - water to yield 20, 30, 50, 100, 200, 500, 1000 and 2000 ng TFB/mL for plasma, and 20, 25, 30, 50, 100, 200, 400 and 500 ng/g both for muscle and liver. For DFB, the standard solutions were prepared to yield 25, 30, 50, 100, 200, 300, and 500 ng/g for muscle and liver.

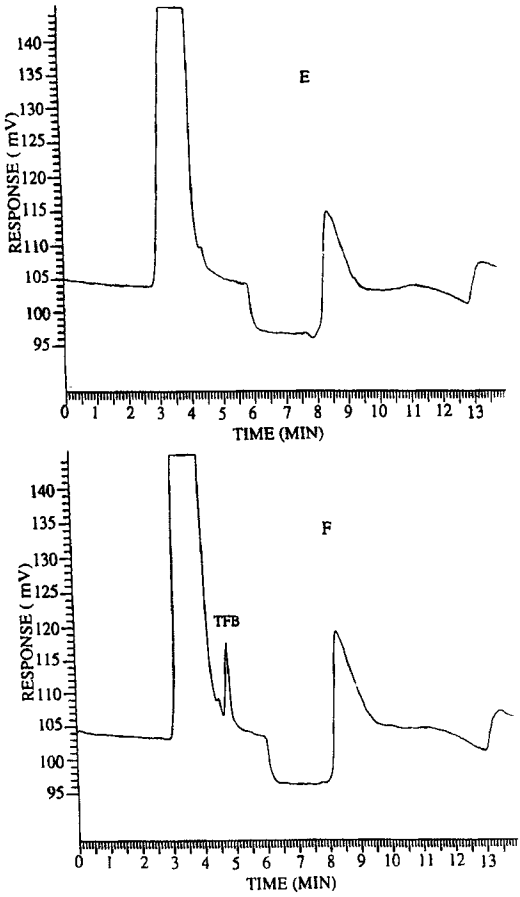

Figure 3. Chromatograms of extracts from fish liver. E: drug-free liver, F: liver spiked with teflubenzuron (200 ng/g).

Duplicate samples were used. The recovery rates were determined by comparing results of analysis of the spiked plasma, muscle and liver samples with those of standard solution (prepared by dilution with dichloromethane - hexane). The linearity of the standard curves for TFB in plasma, muscle and liver and for DFB in muscle and liver was tested using peak-height measurements.

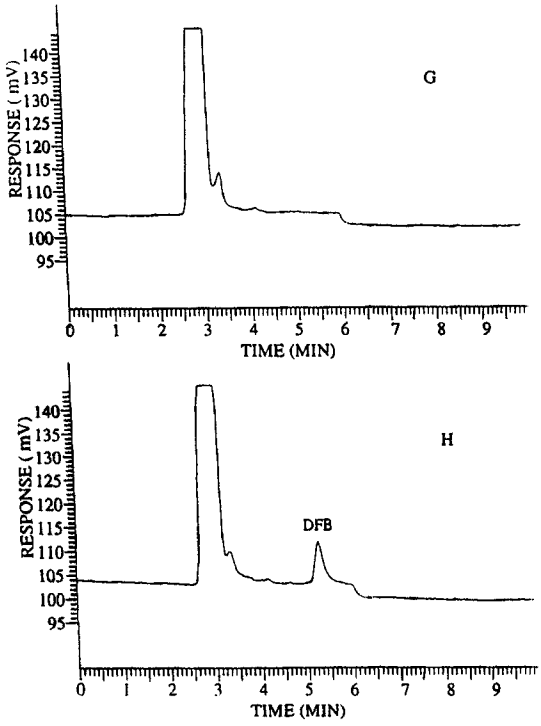

Figure 4. Chromatograms of extracts from fish muscle. G: drug-free muscle, H: muscle spiked with diflubenzuron (200 ng/g).

RESULTS AND DISCUSSION

Chromatograms of clean plasma, muscle and liver, and spiked samples are shown in Figures 1, 2, 3, 4, and 5. The standard curves were linear in the investigated areas; 20 - 2000 ng/mL for TFB in plasma and 20 - 500 ng/g for TFB in muscle and liver, and 25 - 500 ng/g for DFB in muscle and liver.

The linearity of the standard curves for TFB was 0.9995 for plasma and liver and 0.9997 for muscle, the corresponding figures for DFB being 0.9998 for muscle and 0.9996 for liver, when using the external standard method of calculation.

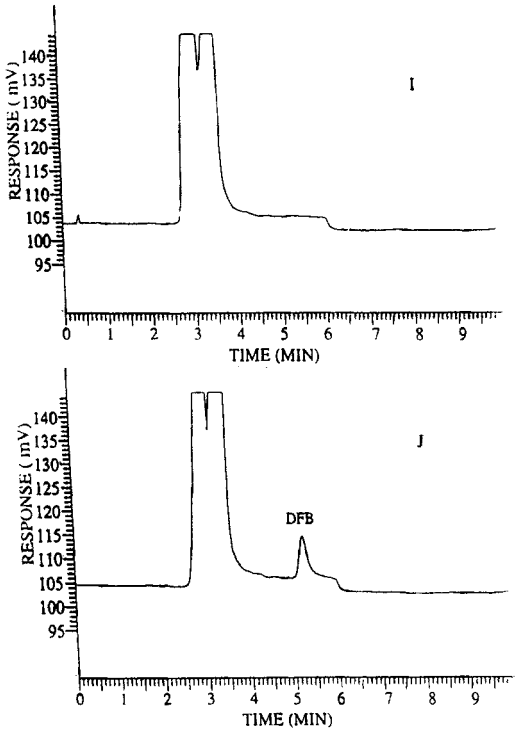

Figure 5. Chromatograms of extracts from fish liver. I: drug-free liver, J: liver spiked with diflubenzuron (200 ng/g).

The precision and recovery for TFB from plasma, and for TFB and DFB from muscle and liver were also calculated and are shown in Table 1. The extraction procedures were validated, and showed good recovery of TFB and DFB. The recovery of TFB was 96% for muscle, and varied from 89 to 90%, and 92 to 96 %, for plasma and liver, respectively. The recovery of DFB varied from 81 to 83% and 83 to 84% for muscle and liver, respectively. The precision of these recovery studies varied from 3.0 to 3.1, 0.8 to 1.5, and 0.5 to 1.6% for TFB in plasma, muscle and liver, respectively. For DFB, the precision varied from 1.0 to 1.9 and 1.2 to 1.5% in muscle and liver, respectively. For TFB, the limit of quantification was 20 ng/g (mL) and the limit of detection 15 ng/g (mL) for plasma, muscle and liver. The limit of quantification for DFB was 25 ng/g and the limit of detection 17 ng/g for muscle and liver. No interference was seen during analysis when calibrating the curves, nor when performing recovery studies.

Table 1

Tissue	No. of samples	Amount in spiked samples (μg/mL-g)	Recovery (%) TFB Mean	TFB S.D.	DFB Mean	DFB S.D.
Plasma	8	0.05	89	3.0		
500μL	8	1.00	90	3.1		
Muscle	8	0.05	96	1.5		
(3g)	8	0.40	96	0.8		
Liver	8	0.05	92	1.6		
(3g)	8	0.40	96	0.5		
Muscle	8	0.05			83	1.0
(3g)	8	0.30			81	1.9
Liver	8	0.05			84	1.2
(3g)	8	0.30			83	1.5

S.D. = standard deviation.

The described method for determination of TFB in plasma can also be used for detection of DFB in plasma, but the procedure has not yet been evaluated. The method was tested under practical conditions by analysing about 60 samples from different rainbow trout, no interfering peaks being observed.

The retention and elution properties of TFB and DFB and the tissue matrix, were studied on bonded-phase extraction columns of polar and non-polar character. Both TFB and DFB are fat-soluble, and residues of fat in the extract strongly influenced the recovery of the two components. It was possible to remove the fat from the tissue extracts by liquid-liquid extractions with hexane and acetonitrile, but this procedure was tedious and, in addition, the recovery was variable.

However, experiments with SI columns to remove the fat, showed that TFB and DFB were strongly retained on the SI column under non-polar conditions. A hexane solution of the tissue homogenate was applied onto the column. Due to the high fat content, TFB and DFB dissolved well in that solvent, and impurities were removed by thorough washing with hexane, and 5 and 10% diethylether in hexane. The compounds were eluted with 40% diethylether in hexane. Also the capacity of the SI-sorbent material had to be

tested. On columns containing only 100 mg sorbent material, the recovery of TFB and DFB from muscle and liver homogenates was variable. On increasing the amount of SI material in the columns to 0.5g, the analyses of spiked tissue showed good reproducibility.

Further purification studies of fish tissue before the HPLC analysis was carried out on less polar extraction columns, and acceptable recovery of TFB and DFB was obtained on C_8 using water-acetonitrile (70 : 30 and 55 : 45).

When a new analytical column (ABZ+Plus) was taken into use, we observed poor peak height and asymetric peak shape. We have found that this can be avoided by removing the organic solvents and conditioning the columns twice as follows:

Time (min.)	H_2O %	CH_2CN %	CH_3OH %	Flow (mL/min.)
20	90	10		1.0
10	50	50		1.0
20	40		60	1.2
15	10	90		1.2
5	15		85	1.0
30	15		85	1.5
5			100	1.5
20			100	1.5

After this procedure, the HPLC system was flushed with dichloromethane for 15 min (1.5 mL/min), prior to the mobile phase. We assume that the combination and the variation of the viscosity and flow, influence column particles in such a way that the result was a good peak shape and good separation. The mobile phase was a mixture of dichloromethane-hexane (85 : 15 for TFB and 60 : 40 for DFB). The flow rate was 0.8 mL/min. for 6 min., followed by 1.5 mL/min. for 7 min. After use, the analytical column was washed for 25 min (1 mL/min) with a mixture of dichloromethane - methanol (80 : 20), followed by dichloromethane for 15 min (1.5 mL/min) and stored in hexane - dichloromethane (90 : 10) for a maximum period of one day. If the column is stored for more than one day, it should be flushed with 100 to 200 mL methanol. New conditioning is not necessary.

An experienced technician can carry out sample clean-up of about 18 samples per day. The assay shows good precision when using the external standard method. The method is robust and sufficiently sensitive, with good recovery.

The method is efficient for quantification of residues of the drugs TFB and DFB. The quantification is linear over a wide concentration range. Only small amounts of solvents are required.

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