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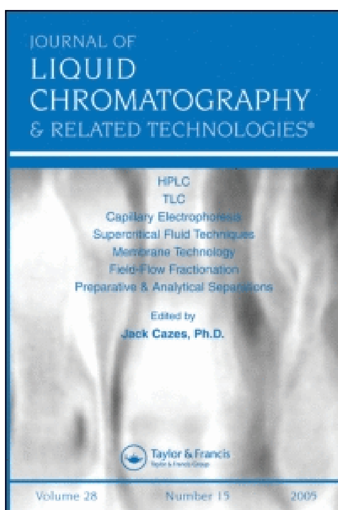

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High-Performance Liquid Chromatographic Determination of Praziquantel in Plasma and Tissues of Cultured Fish for Residue and Pharmacokinetic Studies

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF PRAZIQUANTEL IN PLASMA AND TISSUES OF CULTURED FISH FOR RESIDUE AND PHARMACOKINETIC STUDIES

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ABSTRACT

A simple method for the determination of praziquantel in fish plasma and tissues by HPLC is presented. The samples were extracted with acetone, the organic layer then being separated with diethylether-hexane and evaporated to dryness. The lower limit of quantification was 5, 15, and 20 µg/kg, for muscle, liver, and plasma, respectively.

INTRODUCTION

The pseudophyllide cestode Eubothrium crassumw occurs in the intestine of salmonids in both fresh and sea water (1). The presence of this cestode in farmed, Atlantic salmon (Salmo salar) and rainbow trout (Oncorhynchus mykiss) has become an increasing problem in Norwegian fish farming (2, 3) and presumably also in

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many other countries. Drug treatment against this parasite therefore plays an important role in fish farming. Praziquantel (PQ) is a drug known for its broad spectrum activity against trematodes and cestodes (4, 7). In Norway, appropriate withdrawal periods after end of treatment have been established for this drug, and the fishery control authorities carry out both pre-slaughter and post-slaughter control of drug residues.

Various assay procedures for studying praziquantel levels in serum and other body fluids have been reported: radiometry (8), fluorimetry (9), gas chromatography (10), and biological assay (11). A HPLC method for the determination of praziquantel in serum (12) and tissues (13) has also been published. The latest method (13) is based on UV detection, and is time-consuming and requires rather large amounts of reagents.

This paper describes simple and rapid extraction and clean-up procedures for the determination of PQ in fish plasma and tissues. The method is suitable for pharmacokinetic studies and residue analyses of this (antiparasitic) compound in Atlantic salmon and rainbow trout. This method is reliable and sensitive. There is no interference from the tissue matrix in the chromatographic analysis, which requires only small quantities of chemical reagents.

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MATERIALS AND METHODS

Materials and Reagents

Samples of muscle, liver, and plasma of salmon and rainbow trout were used.

All chemicals and solvents were of analytical or HPLC grade. Praziquantel (Droncit vet. "Bayer"), 2-cyclohexylcarbonyl-4-oxo-1,2,3,6,7,11b-hexahydro-4H-pyrazino[2,1-a] isoquinoline, was donated by Bayer Kjemi A.S. (Oslo, Norway). Stock solutions (1mg/ml) of PQ were prepared by dissolving the compound in a small amount of acetone and diluting to volume with water. Working standards were prepared by dilution with distilled water. The solutions stored in refrigerator are stable for five days.

Spin-X centrifuge filter units from Costar (Cambridge, MA, USA) were also employed.

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 equipped with a Lauda RMT6 cooler (12°C) from Messgeräte Werk Lauda, (Lauda Köningshafen, Germany), and a LC 235C Diode Array detector (Perkin-Elmer, Norwalk, CT, USA). The detector was operated at 205 nm. The integration was carried out using the software programme Omega-2 (Perkin-Elmer), which was operated on an Olivetti M300 personal computer connected to a BJ-330 printer (Canon).

The analytical column (stainless steel, 15 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 (Supelco, Bellefonte, PA, USA).

The mobile phase was a mixture of water-acetonitrile (60:40 for muscle and 61:39 for liver and plasma). The flow rate was 1.0 ml/min for 3 min followed by 0.8 ml/min for 6 min. Between each 25 µl injection, the column was washed for 5 min with 100% acetonitrile with a flow rate of 1.5 ml/min, followed by the mobile phase with a flow rate of 1.8 ml/min for 7 min and 1.0 ml/min for 3 min. The samples were injected at intervals of 26 min.

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Sample pretreatment

Plasma. The pretreatment of plasma samples was as follows: to 250 µl plasma were added 100 µl water (or standard) and 1 ml acetone. The mixture was homogenized for approx. 10 sec., and 50 µl 1 M NaOH and 2 ml diethylether-hexane (3:2) were added. The sample was mixed for approx. 10 sec. and then centrifuged for 3 min. (3000 rpm.). The upper layer was transferred to another glass-stoppered tube. The organic layer was evaporated to dryness at 60°C under a stream of nitrogen. The dry residue was dissolved in 200 µl methanol - solution A (70:30). Solution A was 0.02 M 1-heptane sulfonic acid sodium salt (Supelco, USA)-0.01 M di-sodium hydrogenphosphate-2-hydrate (Ferax, Germany), made by dissolving 4.45 g/l heptane sulphonate and 1.779 g/l di-sodium hydrogen phosphate 2-hydrate in c. 750 ml of water when preparing 1 litre of solution. The pH was then adjusted to 6 with 2 M phosphoric acid and the solution made up to volume with water. After 0.5 ml of hexane had been added, the sample was again whirlimixed. After centrifugation for 3 min, the hexane layer was discharged. To 100 µl of the methanol-based phase was added 100 µl of 0.01 M phosphoric acid (corresponding to 400 µl dilution). The sample was then mixed. The water based phase was filtered through a Costar Spin-X centrifuge filter unit with a 0.2 µm nylon membrane by centrifugation for 4 min. at 10000 rpm. (5600g). Aliquots of the filtrate (25 µl) were injected into the HPLC system.

Muscle. The stepwise procedure for the pretreatment of muscle samples is shown in Fig.1.
The tissue sample, 3 g of muscle, was weighed into a 50 ml centrifuge tube with a screw cap (NUNC, Roskilde, Denmark). Volumes of 300 µl water (or standard) and 4.7 ml acetone 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). Four ml of the supernatant (corresponding 1.5 g muscle) were transferred into a glass-stoppered centrifuge tube and 5 ml diethylether-hexane (3:2) added. The sample was shaken vigorously for 10 sec., and centrifuged for 3 min. at 3000 rpm. The upper layer was transferred to another glass-stoppered tube. The organic layer was evaporated to dryness at 60°C under a stream of nitrogen. The dry residue was dissolved in 400 µl methanol-solution A (70:30), and kept in a freezer (-20°C) for 5 min. After centrifugation for 3 min. (3000 rpm.), 300 µl water, was added to 300 µl of the methanol-based phase which was then mixed (corresponding to 800 µl dilution). The water-based phase was filtered through a Spin-X centrifuge filter. Aliquots of the filtrate (25 µl) were injected into the HPLC system.

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Liver. The samples of liver tissue (3g) were homogenized and extracted as described for muscle tissue. The upper layer (acetone, diethylether, hexane) was transferred to another glass-stoppered tube, and 50 µl 1 M NaOH were added. The sample was shaken vigorously for 5 sec., and centrifuged for 2 min. The upper layer was transferred to another glass-stoppered tube. The organic layer was evaporated to dryness. The dry residue was dissolved in 600 µl methanol-solution A (70:30). After 1 ml of hexane had been added, the sample was again whirlimixed. After centrifugation for 3 min, the hexane layer was discharged, and 160 µl of 0.02 M phosphoric acid was added to 240 µl of the methanol-based phase (corresponding to 1 ml dilution). The sample was again mixed for 3 sec. and filtered through a Spin-X centrifuge filter.
Aliquots of the filtrate (25 µl) were injected into the HPLC.

Calibration curves and recovery studies

The calibration curves for PQ were obtained by spiking plasma, liver and muscle tissue samples with standard solutions to yield 20, 50, 100, 200, 300, 500, 1000, and 2000 ng/ml, 20, 50, 100, 150, and 200 ng/g, and 5, 10, 20, 50, 100, 150 and 200 ng/g of PQ for plasma,

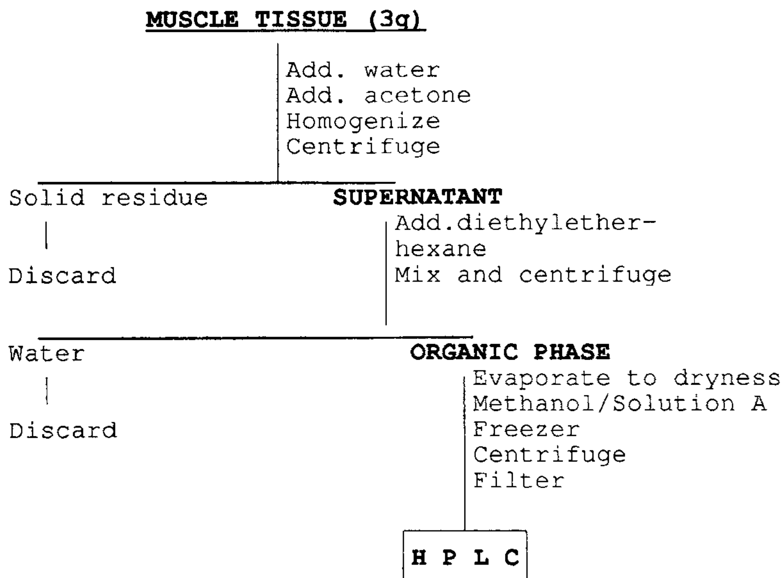

FIGURE 1

Extraction and clean-up procedure for praziquantel from fish muscle.

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liver, and muscle, respectively. Duplicate samples were used. The recovery rates were determined by comparing results of analysis of the spiked plasma, liver and muscle samples with those of standard solution. The linearity of the standard curves for PQ in plasma, liver and muscle were tested using peak-height measurements.

RESULTS AND DISCUSSION

Chromatograms of clean plasma, liver, muscle and spiked samples are shown in Figure 2. The standard curves were linear in the investigated areas; 20 - 2000ng/ml, 20 - 200 and 5 - 200 ng/g PQ in plasma, liver and muscle respectively. The linearity of the standard curves was 0.9997 for plasma and 0.9996 for liver and muscle, respectively, when using the external standard method of calculation. The precision and recovery for PQ from plasma, liver and muscle were also calculated and are shown in Table 1.

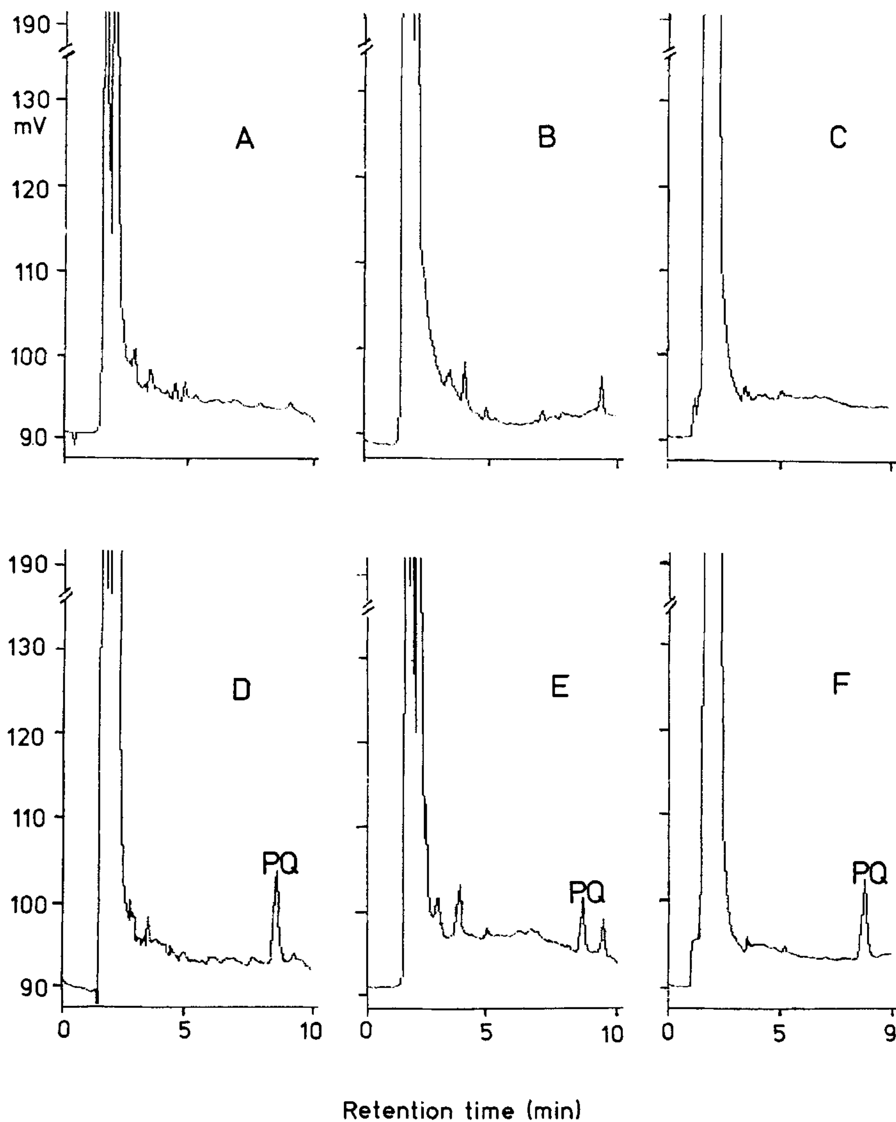

FIGURE 2

Chromatograms of extracts from fish plasma, liver and muscle.

A: drug-free plasma, **B**: drug-free liver, **C**: drug-free muscle, **D**: plasma spiked with PQ (500 ng/ml), **E**: liver spiked with PQ (200 ng/g), **F**: muscle spiked with PQ (200 ng/g).

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TABLE 1.

Recovery and repeatability for PQ from spiked samples of plasma, liver and muscle.

Tissue	No. of samples	Amount in spiked samples (µg/ml-g)	Recovery % PQ Mean	S.D.
Plasma	8	0.05	92	1.3
(250 µl)	8	2.00	91	1.6
Liver	8	0.02	82	0.9
(3g)	8	0.10	81	0.8
Muscle	8	0.02	99	0.8
(3g)	8	0.10	100	0.5

S.D.= standard deviation

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The extraction procedures were validated, and showed good recovery of PQ. The recovery of PQ varied from 91 to 92, 81 to 82, and 99 to 100% for plasma, liver and muscle, respectively. The precision of these recovery studies varied from 1.3 to 1.6, 0.8 to 0.9, and 0.5 to 0.8% for PQ in plasma, liver and muscle, respectively, the results also show that the precision and accuracy of the quantification of PQ are good.
The muscle clean-up procedure was applied to liver and plasma. Unfortunately, minor residues of endogenous compounds in the liver and plasma extracts interfered with PQ in the chromatogram. These impurities were efficiently removed from plasma by washing with NaOH. However, although this procedure also removed most impurities from liver, some remained, and the liver samples were therefore diluted to 1 ml, with somewhat reduced sensitivity as a consequence. Minor modifications of the mobile phase were therefore necesary. The limit of quantification was 5 and 15 ng/g PQ for muscle and liver, respectively, and 20 ng/ml PQ for plasma. No interference was seen during analysis, when calibrating the curves, and when performing recovery studies.
PQ, is a pyrazino isoquinoline derivative with low solubility in water. Its solubility increases in acetone, but residues of the endogenous compound also

increase. In the event of insufficient samples of liver or muscle (3 g) it is advantageous supplement the difference with water.
The method was tested under practical conditions by analysing about 100 samples from different rainbow trout, with no interfering peaks being observed.
This study has shown that residues of the antiparasitic compound PQ in plasma, liver and muscle may be determined using minimal sample manipulation. The cost of chemicals and the manual work-up procedures are also reduced compared to previously published methods. An experienced technician can carry out sample clean-up of about 30 plasma and muscle samples, and about 18-24 liver samples, per day. The assay shows good precision when using the external standard method. The method is robust, simple, and sufficiently sensitive, with good recovery. The quantification is linear over a wide concentration range. The amount of solvents required is minimized, and pretreatment of tissues by liquid-liquid extractions combined with centrifugation filters, is preferable to solid-phase extraction columns when performing the pretreatment manually. The chromatographic system was specific with regard to PQ.

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