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Note

Extraction of praziquantel from fish tissue and its determination by high-performance liquid chromatography*

ASTRI ROGSTAD*, VICTOR HORMAZABAL and MAGNE YNDESTAD
Department of Food Hygiene, Norwegian College of Veterinary Medicine, N-0033 Oslo 1 (Norway)
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The fish farming industry in Norway has grown rapidly during the last decade and has achieved a considerable production of trout and salmon. The fish farms are located in the coastal waters of Norway. A variety of bacterial, viral and parasitic infections which affect fish has become a problem to the farmers and may cause considerable economic loss. Treatment of these infections involves an increasing use of antibiotics, sulphonamides and other drugs. Concerning parasites, cestode infections are a growing problem in fish farming. Praziquantel (Droncit vet. "Bayer"®) is highly active against cestodes. Its pharmacokinetic behaviour, mode of action and toxicity are known from a large number of studies in human and veterinary medicine[1]. However, this drug has not previously been used in food-producing animals.

The pharmacokinetics of praziquantel in fish are unknown. The elimination from fish tissue is assumed to be dependent on water temperature. The drug level is therefore unpredictable. The public health authorities require safe drug withdrawal periods before slaughter. In order to ascertain this and to monitor residues in fish foods a sensitive assay for detecting praziquantel in tissues is needed. Various assay procedures for studying praziquantel levels in serum and other body fluids have been reported to date: radiometry[2], fluorimetry[3], gas chromatography[4] and biological assay[5]. More recently, a high-performance liquid chromatographic (HPLC) method for determination of praziquantel in serum was published[6].

The HPLC method described in this paper was developed to provide a routine tissue assay with a detection limit of 5 ng praziquantel per gram of tissue. The use of extraction columns in the cleanup procedure appeared to be efficient and time-saving. Several types of extraction columns were tested to arrive at a selective procedure.

EXPERIMENTAL

Chemicals and reagents

Praziquantel (PQ) (2-cyclohexylcarbonyl 1,2,3,6,7,11*b*-hexahydro-4H-pyra-

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zino [2,1-*a*]isoquinoline-4-one) and the internal standard, a hippuroyl derivative of isoquinoline [2-(N-formylhexahydro-hippuroyl)-1,2,3,4-tetrahydroisoquinoline-1-one], were donated by Bayer Kjemi A.S. (Oslo, Norway). Solvents were of analytical and HPLC grade. Stock solutions (1 mg/ml) of PQ and internal standard were prepared in methanol and stored in the freezer in dark stoppered flasks. Extraction columns Bond Elut C_{18}, C_8, C_2, 2OH, and NH_2 (size 200 mg, 3 ml) and SI (size 500 mg, 2.8 ml) were purchased from Analytichem International (Harbor City, CA, U.S.A.). The sorbent materials C_2 (40 μm), SI (40 μm), empty columns (4 and 8 ml) and appropriate frits for packing of columns in our laboratory were also supplied by Analytichem, whereas SI-type sorbent material, particle size 63–200 μm was purchased from E. Merck (Darmstadt, F.R.G.). Empty reservoirs of 125 ml (Analytichem) were combined with the columns when large volumes were applied.

Chromatographic conditions

The analyses were performed on a Perkin-Elmer HPLC system, consisting of a Series 400 solvent delivery system, an ISS 100 sampling system, an LC 85 UV detector, and an LCI 100 laboratory computing integrator. The detector wavelength was set 210 nm. The analytical column (stainless-steel, 15 cm × 4.6 mm I.D., and guard column (2 cm × 4.6 mm I.D.) were packed with 5 μm Supelcosil® LC-18 (Supelco, Bellefonte, PA, U.S.A.). The mobile phase was water–acetonitrile (50:50) at a flow-rate of 0.9 ml/min. Between each 25 μl injection the column was washed for 15 min with 100% acetonitrile.

Extraction and cleanup (Fig. 1)

To a sample of ground fish tissue (10 g muscle, 5 g liver, or 5 g kidney) was added internal standard (2 μg). The sample was allowed to stand for 15 min. Dry sodium sulphate (3.5 g) was added and the tissue was extracted twice with 20-ml portions and once with a 10-ml portion of ethyl acetate by using a M.S.E. homogeniser (Measuring and Scientific Equipment, London, U.K.). The homogenate was centrifuged at 3000 *g* for 5 min. The collected supernatants were evaporated to dryness with a rotary evaporator. Three portions of 5 ml hexane were added to the dry residue and evaporated off. The dry residue was dissolved in hexane (50 ml), and the solution was loaded onto a conditioned SI column using a Vac Elut™ system (Analytichem International).

The flask was rinsed with 15% dichloromethane in hexane which also was loaded onto the column. The column was washed thoroughly with diethylether-hexane mixtures (15%, 30% and 35%). PQ and internal standard were eluted from the column with 9 ml of 60% acetone in hexane. The collected eluate was evaporated to dryness under a stream of nitrogen using a Reacti-Therm™ heating module at 60°C and Reacti-Vap™ evaporating unit (Pierce, Rockford, IL, U.S.A.).

The residue was dissolved in 70% methanol in water. After centrifugation the supernatant was diluted with water until 75% water in the solution. The solution was loaded onto a conditioned C_2 column. The column was washed with 25% methanol in water. PQ and internal standard were eluted from the column with five 300 μl portions of 50% acetonitrile in water. The fractions were collected and evaporated to dryness under a stream of nitrogen. The drug residue was dissolved in 150 μl of mobile phase, and portions of 25 μl were injected into the chromatograph.

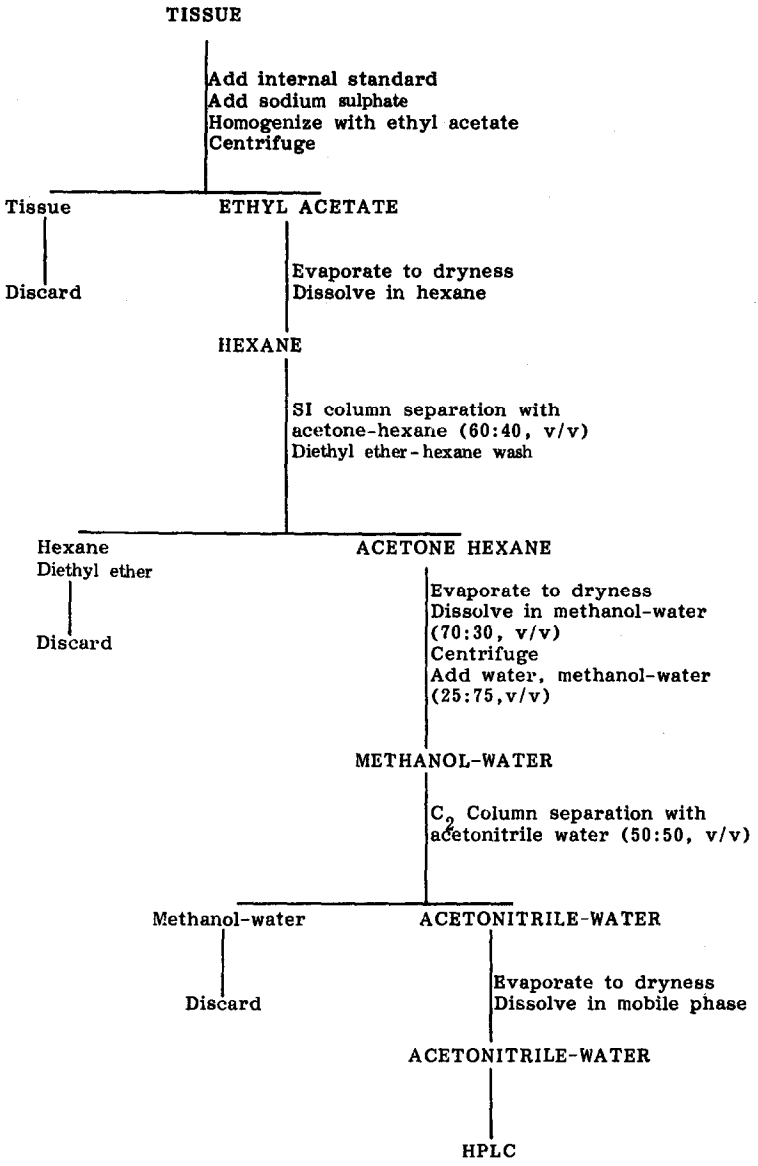

Fig. 1. Extraction and clean-up procedure for praziquantel (PQ) from fish tissues.

Calibration curve and recovery studies

The calibration curve was made by spiking tissue with standard solutions of PQ and internal standard to yield concentrations of 10, 25, 50, 100, 200, 300, 500 ng/g of PQ and 200 ng/g of the latter. The tissue samples were extracted by the above procedure. Each level was assayed in triplicate. The HPLC analyses of tissues spiked with PQ (10 ng/g and 200 ng/g, respectively) were compared with those of standard solution to calculate recovery rates. Also the recovery of the internal standard was examined. Muscle tissue was spiked with 100, 200 and 300 ng/g, respectively.

RESULTS AND DISCUSSION

Typical chromatograms of fish muscle spiked with 10 and 200 ng/ng PQ and 200 ng/g IS are shown in Fig. 2. The retention time of PQ varied from 5.4 to 5.6 min and that of the internal standard from 6.0 to 6.2 min. A chromatogram of unspiked tissue is also shown in Fig. 2.

The retention and elution properties of PQ, internal standard and the tissue matrix were studied on bonded-phase extraction columns of polar and non-polar character. Both PQ and the internal standard are fat-soluble and residue of fat in the extracts strongly influenced the recovery of the two components. It was possible to remove the fat from the muscle extracts by tedious liquid–liquid extractions with hexane and acetonitrile. However, experiments with SI and NH_2 columns to remove the fat, showed that PQ and IS were strongly retained on the SI column under non-polar conditions. An hexane solution of the tissue homgenate was applied onto the column. Due to the high fat content PQ and IS dissolved well in that solvent, and impurities were removed by thorough washing with 20% diethylether in hexane. The compounds were eluted with 5% methanol in chloroform. PQ and the internal standard were also retained on the NH_2 column under similar conditions. Their elution properties were, however, poor and the chromatogram showed a distorted peak pat-

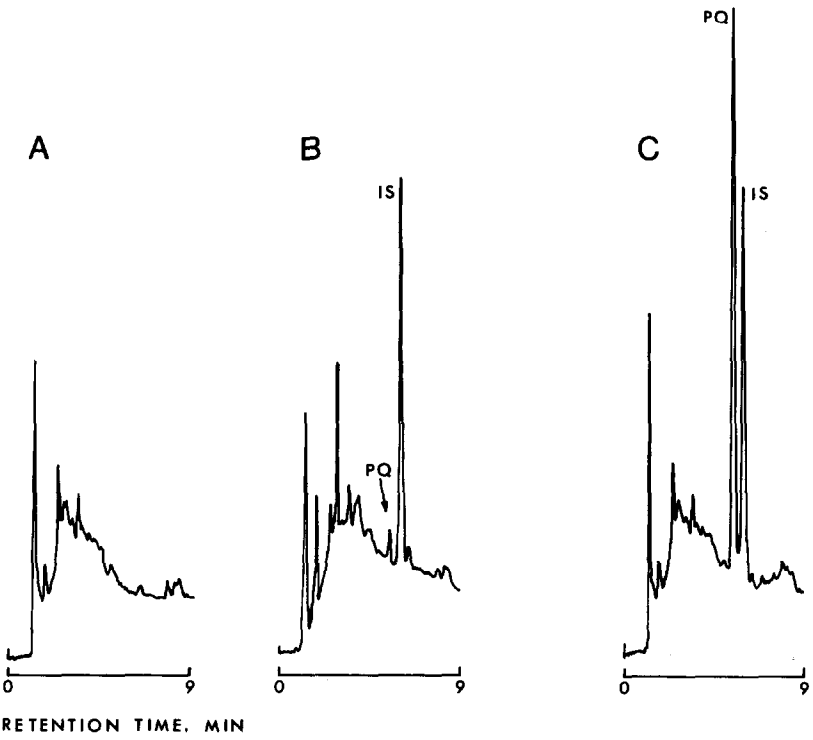

Fig. 2. Chromatograms of extracts from 10 g fish muscle. (A) Unspiked tissue; (B) tissue spiked with praziquantel (PQ), 10 ng/g and IS (200 ng/g); (C) tissue spiked with PQ, 200 ng/g and IS (200 ng/g).

tern, probably, due to decomposition of the compounds. Two different types of silica material were tested. When using Merck particles the impurities were more efficiently washed out with the diethyl ether–hexane mixture.

The same cleanup procedure was applied to liver and kidney tissues. Unfortunately, impurities in the liver extracts interferred with PQ and IS in the chromatogram. The cleanup on the SI column was modified and improved by changing the polarity of the washing solvent and the eluting solvent slightly. A variety of solvent mixtures were tried. The impurities were efficiently removed by washing with diethylether–hexane mixtures of increasing ether content. Clean eluates were obtained using 60% acetone in hexane. Also the capacity of the SI-sorbent material had to be tested. On columns containing only 500 mg sorbent material the recovery of PQ and IS from liver homogenates was variable. When increasing the amount of SI material in the columns to 1.0 g, the analyses of spiked tissue showed good reproducibility.

Further purification of fish tissue before the HPLC analysis was studied on less polar extraction columns. The drugs were retained on C_{18}, C_8 and C_2 columns and only partly retained on the 2OH column using methanol–water (25:75). However, more than ten bed volumes of a 50% mixture of acetonitrile in water were required for complete elution from the two former columns whereas only 1.5 ml of the above solvent was required for complete elution from the C_2 column.

The calibration curves of PQ in fish muscle, liver and kidney were linear over the range of 5 to 500 ng/g with correlation coefficients of 0.998 for muscle, 0.997 for liver and 0.998 for kidney. The recovery rates of PQ in muscle, liver and kidney at 10 ng/g and 200 ng/g were also calculated and are listed in Table 1. The recovery of the internal standard varied from 93 to 95% at the different concentration levels with a coefficient of variation of 5%.

The method was applied to determine PQ residues in muscle, liver and kidney from rainbow trout, weighing 500 g, each given a single oral dose (10 mg/kg) of "Droncit" and slaughtered 4, 7, 24 and 48 h after medication. Serum was also analysed using the method of Xiao *et al.*[6]. Preliminary results showed that the highest

TABLE I

RECOVERY OF PRAZIQUANTEL (PQ) FROM MUSCLE, LIVER AND KIDNEY OF FARMED FISH

Tissue	*No. of samples*	*Amount (ng/g)*	*Recovery (%)*	
			Mean	*C.V.**
Muscle	6	10	79	7
	10	200	93	3
Liver	8	10	78	7
	10	200	93	6
Kidney	6	10	79	6
	8	200	93	3

* C.V. = Coefficient of variation.

residue level was obtained 7 h after treatment in muscle, liver, kidney and serum. Forty-eight h after medication residues were not found in muscle and serum, whereas liver still contained small amounts. A complete residue study will be published elsewhere.

CONCLUSION

The method described above meets the generally accepted requirements for a tissue residue monitoring system. Use of SI and C_2 mini columns in the clean-up steps resulted in removal of interfering tissue components. The procedure is sensitive and time-saving and only standard analytical equipment and commercially available reagents are used. An experienced technician can process 8–10 samples per day.

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