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High-performance liquid chromatographic analysis of oxytetracycline in chinook salmon following administration of medicated feed

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ABSTRACT

A high-performance liquid chromatographic assay was developed to detect oxytetracycline (OTC) in chinook salmon muscle tissue. A solid-phase extraction protocol was used to recover OTC and the internal standard, epitetracycline hydrochloride, from the salmon tissue samples. OTC was analyzed using a mobile phase of methanol–0.02 *M* phosphate buffer, pH 2.25 (60:190), an ultraviolet detection wavelength of 365 nm and a 250 mm × 4.6 mm I.D. Ultrasphere ODS column. A linear calibration curve ($r^2 = 0.999$) of OTC in salmon muscle tissue from 0.05 to 3.0 ppm was obtained. Using a signal-to-noise ratio of 5:1, the OTC detection limit was 0.05 ppm in salmon muscle tissue. OTC recovery (74.4%) and intra-assay variability (2.3%) were optimized for salmon muscle tissue. An *in vivo* feeding study was performed by administrating OTC-medicated feed for a period of 10 days, followed by a 42-day sampling period. The half-life for the elimination of OTC in chinook salmon muscle tissue was found to be 5.4 days.

INTRODUCTION

The raising of farmed salmon is an established, growing industry. Frequently antibiotics are used to treat diseases in salmon and the possible health risk to humans, presented by traces of antibiotics in salmon tissue, has generated considerable concern.

To ensure that levels of antibiotics in salmon tissue are within acceptable limits, monitoring of drug residues is an essential aspect of aquaculture. Thus, there is a demand for development of techniques which are specific and sensitive for the quantitative detection of antibiotic residues in salmon tissue and the determination of antibiotic wash-out times from tissue.

Oxytetracycline (OTC) is a widely used antibiotic in the aquaculture industry. It is used to treat a variety of diseases including coldwater disease, columnaris, enteric redmouth, fin rot, furunculosis, gill disease and vibriosis [1,2].

Previously reported methods for the analysis of OTC in fish include microbiological studies which investigated the clearance times of OTC from rainbow trout (*Salmo gairdneri*) [3–5] and another study which used a microbiological assay to determine the pharmacokinetics and tissue distribution of OTC in

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carp (*Cyprinus carpio*) [6]. However, chromatographic methods are generally preferred for their greater selectivity and sensitivity for antibiotic analysis. Many high-performance liquid chromatographic (HPLC) methods have been reported for the analysis of OTC in fish. The OTC concentrations of spiked serum, liver and muscle of rainbow trout and other fish species have been measured using HPLC analysis [7,8].

Rainbow trout have been used for studies of the absorption, elimination and tissue distribution of OTC. In one study, trout were fed medicated feed every day for 10 days and muscle and liver tissue were analyzed by HPLC for OTC [9]. In another study, trout were fed medicated freed every day for 8 days and OTC concentrations were determined for blood, kidney, muscle, skin and gutted trout using HPLC techniques [10].

This study employed an HPLC assay to measure the concentrations of OTC in muscle tissue of salmon administered medicated feed. To date, there have been no published reports of HPLC analysis of OTC in salmon. The wash-out time for OTC from salmon muscle tissue was also determined.

EXPERIMENTAL

Materials

Oxytetracycline dihydrate and trichloroacetic acid (TCA) were obtained from Sigma (St. Louis, MO, USA). The internal standard, epitetracycline hydrochloride, was obtained from the European Pharmacopeia (Strasbourg, France). HPLC-grade methanol was obtained from Fisher Chemicals (Vancouver, Canada). Disodium hydrogen orthophosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$) and analytical-grade phosphoric acid (H_3PO_4; 85%) were obtained from BDH Chemicals (Toronto, Canada). HPLC-grade water was produced using a Milli-Q water purification system [Millipore (Canada) Mississauga, Canada].

Apparatus

The HPLC system consisted of a Beckman Model 110A pump, a Model 210 sample injection valve fitted with a 20-μl loop, a Beckman Model 160 fixed-wavelength detector with a 365-nm filter, a mercury lamp and a Shimadzu C-R3 integrator. The column was a 250 mm × 4.6 mm I.D. Ultrasphere octadecylsilane (ODS) phase with a particle size of 5 μm. The mobile phase consisted of methanol–0.02 *M* phosphate buffer, pH 2.25 (60:190) and was delivered isocratically at a flow-rate of 1.0 ml/min. The mobile phase was filtered prior to use with a Millipore HPLC solvent filtration system and FP Vericel 47-mm, 0.45-μm membrane filters (Gelman Sciences, Ann Arbor, MI, USA).

Preparation of standard solutions and reagents

All OTC solutions were prepared immediately before use. A 500 μg/ml OTC stock solution was prepared by dissolving 5 mg in 10.0 ml of methanol. Varying volumes of stock solution were diluted with methanol to give concentrations of 0.5, 1.0, 4.0, 10, 20 and 30 μg/ml.

The internal standard, epitetracycline hydrochloride, was used for the calibration curve samples and the treated fish samples. A 200 μg/ml stock solution was prepared by dissolving 2.0 mg of epitetracycline hydrochloride in 10.0 ml of methanol. The 10 μg/ml working solution was prepared by diluting 0.5 ml of epitetracycline hydrochloride stock solution to a final volume of 10.0 ml with methanol. To each 5-g tissue sample, 0.5 ml of the internal standard working solution were added, resulting in a final concentration of 1.0 μg/g of tissue.

Extraction procedure

In a 50-ml polypropylene centrifuge tube, 15 ml of 0.02 *M* phosphate buffer, pH 2.0, 0.5 ml of the internal standard solution and 1.0 ml of 50% (w/v) TCA were added to 5.0 g of salmon muscle tissue. Each sample was homogenized three times for 30-s intervals at medium speed using a Brinkman Polytron Model PT 10/35 homogenizer (Brinkman Instruments, Rexdale, Canada). The samples were centrifuged for 20 min at 30 000 *g* at 4°C (JA-17 rotor, r_{avg}) in a Beckman Model J2-21 centrifuge (Beckman Instruments). The supernatant was transferred to a 50-ml erlenmeyer flask and stored in the dark at 4°C. An aliquot of 15 ml of phosphate buffer and 1.0 ml of TCA was added to the salmon muscle residue and the homogenization was repeated as before. The supernatants were combined and filtered through a Bakerbond solid-phase extraction (SPE) Sephadex G-25 gel disposable 6-ml column (J. T. Baker, Phillipsburg, NJ, USA). Three 2-ml

aliquots of phosphate buffer were used to rinse the supernatant onto the column. The Sephadex column was conditioned before use by adding 5 ml of phosphate buffer, pH 2.0, shaking well and drawing off the liquid under gentle vacuum until a 2-mm layer remained above the surface of the gel. The salmon tissue extract was collected in another 50-ml erlenmeyer flask and then passed through an activated Bakersbond SPE octadecyl (C_{18}) disposable 6-ml column (J. T. Baker) fitted with an adaptor and a 60-ml reservoir. The C_{18} column was activated by passing through 4 ml of 50% (v/v) methanol and then 4 ml of buffer. The OTC and internal standard were then eluted off the column with 7 ml of methanol into a 10-ml culture tube. The samples were evaporated under nitrogen in a 40°C water bath to dryness and reconstituted to 1.0 ml with methanol. The samples were vortex-mixed for 15 s and stored at −20°C until required for analysis. A 50-μl aliquot was injected onto the HPLC column. Since the sampling loop used had a volume of 20 μl, this excess volume of sample removed any residue from the previous analysis.

Calibration curve, assay precision and recovery

A calibration curve was prepared from salmon muscle tissue samples (5 g) to which 0.5 ml of the internal standard solution (1.0 μg/g of tissue) and 0.5 ml of the appropriate OTC standard solutions were added to give final concentrations of 0.05, 0.1, 0.4, 1, 2 and 3 ppm of tissue. The calibration curve was constructed by plotting the ratios of the peak areas of OTC to the peak areas of the internal standard against the known concentrations of OTC.

The recovery study was performed by adding 0.5 ml of 1.0, 10.0 and 20.0 μg/ml OTC solution to 5-g samples of salmon muscle tissue. One sample was prepared at each concentration. The samples were processed as described above, however, no internal standard solution was added to the samples. The area of the OTC peak was compared to the area of the peaks for identical amounts of the unextracted standard solutions.

Feeding study

Approximately 80 chinook salmon varying in weight from 222 to 1044 g were kept in a circular (4′ deep × 8′ diameter) flowing seawater tank at the West Vancouver Laboratory of the Department of Fisheries and Oceans (West Vancouver, Canada). The tank capacity was 3600 l and the flow-rate of seawater was 40–60 l/min. The water temperature varied from a minimum of 7.8°C to a maximum of 10.3°C during the 52-day study period. Prior to the start of the feeding study, three fish were removed and analyzed to confirm the absence of OTC as these fish were also used as control samples for the calibration curves, assay precision and recovery studies. The fish were fed medicated feed, Extruded New Age Salmon feed, 3.5 mm pellets (Moore-Clark, Vancouver, Canada) containing OTC at a concentration of 10 kg oxytetracycline per tonne of feed. The actual dosage of OTC was 80 mg per kg of biomass per day. Feeding was done twice daily until satiation, or until the 500-g aliquot of feed was consumed. The medication period was 10 days after which the fish were then fed a non-medicated feed formulation (Moore-Clark). Prior to the morning feeding, four fish were removed on days 2, 4, 6, 8, 11, 12, 14, 17, 20, 24, 28, 31, 34, 38, 45 and 52. The fish were sacrificed by a blow to the head, tagged and stored at −20°C. For analysis, three 5-g samples were removed from each fish at evenly spaced sites along the length of the fish.

RESULTS AND DISCUSSION

Fig. 1 shows representative chromatograms of a blank salmon extract and a salmon extract containing OTC and the internal standard. Epitetracycline hydrochloride was selected due to its elution properties and ultraviolet absorption characteristics. Other tetracycline analogues such as doxycycline and minocycline had unsuitable retention times, while chlorocycline and demeclocycline had impurities which eluted at the same retention time as OTC, thus precluding their use as internal standards.

The calibration curve for OTC extracted from salmon muscle tissue is shown in Fig. 2. A linear relationship was established over the concentration range 0.05–3.0 ppm of OTC (r^2 = 0.999).

OTC was recovered from salmon muscle tissue using the SPE procedure described. The mean recovery of OTC was found to be 74.4% over the concentration range 0.1–2.0 ppm. (Table I).

Protein precipitation with TCA and extraction into a buffer was found to be the most efficient method of recovering OTC from the salmon muscle

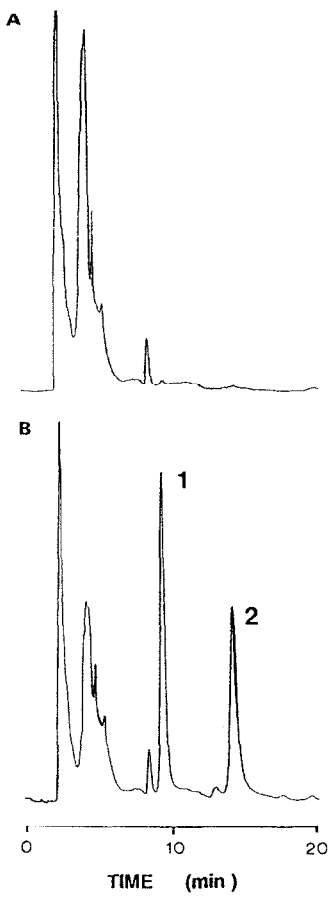

Fig. 1. Representative chromatograms from (A) a blank salmon muscle tissue extract and (B) salmon muscle tissue extract to which 2.0 ppm of OTC had been added. Chromatographic conditions: Ultrasphere ODS 5-μm (250 mm × 4.6 mm I.D.) column; mobile phase, methanol–0.02 *M* phosphate buffer, pH 2.25 (60:190); HPLC flow-rate, 1.0 ml/min; ultraviolet detection wavelength, 365 nm. Peaks: 1 = epitetracycline; 2 = OTC.

tissue. The double-extraction procedure improved recoveries over a single-extraction method. Using a signal-to-noise ratio of 5:1, the detection limit was 0.05 ppm. The quantitation limit was 0.1 ppm. The recovery of OTC compares favorably with a recovery of 69.9% in fish muscle reported by Rogstad *et al.* [8] and 61.3% in rainbow trout by Norlander *et al.* [9]. Other studies performed with rainbow trout muscle tissue [7] and yellow-tail and porgy [11] reported higher OTC recoveries. These differences may be explained by the high level of cholesterol and carotenoids found in salmon muscle tissue.

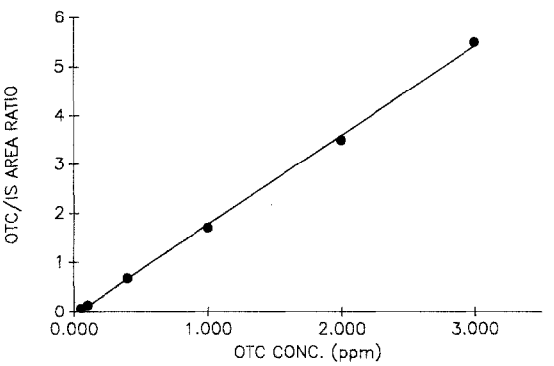

Fig. 2. Calibration curve for OTC extracted from salmon muscle tissue over the concentration range 0.05–3.0 ppm. r^2 = 0.999; *y*-intercept = −0.056; slope = 0.364. I.S. = internal standard.

Sheridan [12] found that cholesterol levels in coho salmon muscle tissue were two times higher than for muscle tissue from rainbow trout. Salmon tissue also has high levels of carotenoids. The high levels of carotenoids and cholesterol may contribute to the high total lipid content in the salmon muscle. These factors appeared to affect the efficiency of antibiotic extraction for chinook salmon muscle tissue. The intra-assay coefficient of variation was found to be 2.3% and is shown in Table II. A 0.4-ppm salmon extract was injected five times. The average calculated concentration was 0.410 ppm.

The feeding study was used in conjunction with the HPLC assay method to determine the wash-out time for OTC in salmon muscle tissue. The protocol for the administration of medication was the same as that used on a commercial salmon farm. The fish appeared to eat all of the feed aliquot. The flow-rate

TABLE I

RECOVERY OF OXYTETRACYCLINE FROM CHINOOK SALMON MUSCLE TISSUE

Each value represents a single determination at each sample concentration.

Sample concentration (ppm)	Recovery (%)
0.1	62.4
1.0	81.8
2.0	77.9
Mean	74.4

TABLE II

INTRA-ASSAY VARIABILITY OF OXYTETRACYCLINE IN CHINOOK SALMON MUSCLE TISSUE

Injection No.	Sample concentration (ppm)	OTC internal standard area ratio	Calculated concentration (ppm)
1	0.4	0.717	0.425
2	0.4	0.683	0.403
3	0.4	0.698	0.414
4	0.4	0.678	0.406
5	0.4	0.676	0.402
Mean		0.691	0.410
Standard deviation		0.017	0.009
Coefficient of variation (%)		2.30	2.30

of seawater resulted in a complete exchange in the tank in 60 to 90 min. The relatively low initial levels and short duration of OTC in seawater would suggest that the possibility of OTC uptake by the fish from the seawater was minimal.

There was some variation in concentration between different fish sampled on the same day. For samples from different sites of the same fish, the variation was only slightly above that of experimental error. There also appeared to be no correlation between the weight of the salmon and tissue concentrations of OTC. Fig. 3 shows the uptake and decline for OTC in salmon muscle tissue. The OTC concentrations are also given in Table III. Peak concentrations of 1.36 ppm were observed at day 14. The concentration of OTC then declined from day 17 to day 38. OTC could not be detected from day 45 to day 52 in any of the samples. Data points from the last eight sampling periods derived in Fig. 3 were used in the computer program Non-lin [13] to calculate a half-life of 5.4 days for OTC in salmon muscle tissue. Under the conditions of this study, the data show that OTC levels are below the detection limit of 0.05 ppm, 35 days after the last administration of medicated feed. Nordlander *et al.* [9] found that the levels of OTC in rainbow trout

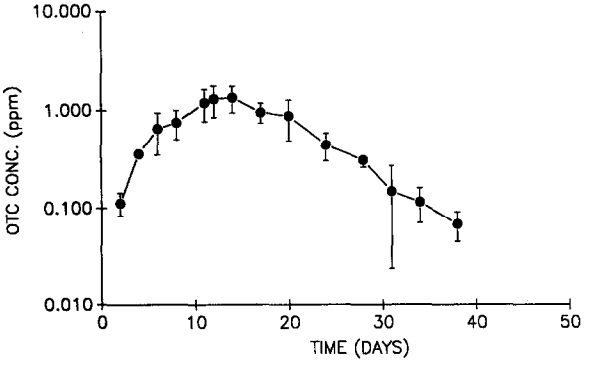

Fig. 3. Uptake and decline of OTC is muscle tissue from chinook salmon after administration of medicated feed. Each data point represents the average concentration of the samples assayed from four salmon on the sampling day ± standard error.

TABLE III

CONCENTRATION OF OXYTETRACYCLINE IN CHINOOK SALMON MUSCLE TISSUE OVER TIME

Day	OTC concentration (ppm)	Standard deviation (ppm) ($n=12$)[a]
2	0.050	0.020
4	0.359	0.024
6	0.650	0.300
8	0.752	0.252
11	1.203	0.432
12	1.317	0.463
14	1.360	0.411
17	0.959	0.221
20	0.885	0.396
24	0.444	0.140
28	0.311	0.048
31	0.148	0.124
34	0.116	0.045
38	0.069	0.023

[a] Four fish were sampled and triplicate analyses were performed for each day.

muscle tissue was 0.041 ppm, 23 days after last administration of medicated feed. Jacobsen [10] found that OTC could be detected in rainbow trout at the level of 0.05 ppm 22 days after the last dose of drug.

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