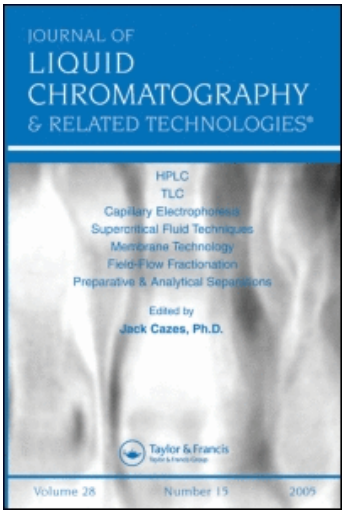

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ABSTRACT

A simple method for the simultaneous determination of flumequine and oxolinic acid in fish muscle with attached skin is presented. Samples of mass 3.0 g were extracted with NH_3 and acetone, acidified, diluted, and analyzed by high-performance liquid chromatography. The limit of quantification was 30 μg/kg for flumequine and 20 μg/kg for oxolinic acid. The recoveries were 75–76% and 79–82% for flumequine and oxolinic acid, respectively.

INTRODUCTION

The extensive use of flumequine (FQ) and oxolinic acid (OX) by the fish farming industry for treatment of bacterial infections in fish has created a demand for a rapid and simple analytical method for residue control of these drugs.

Several methods have been published describing the determination of FQ and OX in different fish tissues without attached skin, using high-performance liquid chromatography (HPLC) (1–9). All of these methods involve extraction of

the compounds with organic solvents, and manual workup procedures, including liquid-liquid extraction (1–7) or solid-phase extraction (7,8). One method (9) is very simple, but treatment with trichloroacetic acid (TCA) during the extraction, leads to clumping of muscle protein. This problem increases to some extent if skin is present in the matrix (sample). In addition, TCA is not especially efficient for extracting FQ and OX from skin.

Residues of FQ and OX have been shown to be especially bound to bone and skin (10), and can be detected in these organs long after termination of treatment and also after the concentration in muscles has fallen below the detection level.

The European Community Committee for Veterinary Medicinal Products (EUEA) has set the provisional maximum residue limit (MRL) at 150 and 300 μg/kg for FQ and OX, respectively. The committee also recommends that muscle with a naturally occurring amount of skin be analyzed for drug residues. However, none of the published methods appear to be effective for combined muscle and skin, apart from one that has been developed for fish silage, and that is rather time-consuming (11).

The purpose of the present study is to develop a simple method for the simultaneous determination of FQ and OX in muscle and skin, which meets the MRL requirements for FQ and OX as specified by EUEA.

EXPERIMENTAL

Materials and Reagents

Samples of muscle with attached skin of salmon and rainbow trout were used.

All chemicals and solvents were of analytical or HPLC grade. FQ and OX were supplied by Sigma Chemical Co. (St. Louis, MO, USA). Stock solutions (1.0 mg/mL) of FQ and OX were prepared in 0.1 *M* sodium hydroxide. Working standards were prepared by dilution of the stock solution with solution A, which consisted of 0.002 *M* H_3PO_4/MeCN/tetrahydrofuran (64:21:15). All standard solutions were stored in a refrigerator (+4°C). Spin-X centrifuge filter units (0.22 μm, nylon type) from Costar (Cambridge, MA, USA) were used for filtration.

Chromatographic Conditions

The analyses were performed with a Perkin-Elmer HPLC system, consisting of a Series 410 Bio-solvent delivery system, an ISS 100 sampling system (with 85% CH_3CN/15% 0.002 *M* H_3PO_4 as flushing liquid) equipped with a Lauda RMT6

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cooler (11°C) from Messgeräte Werk Lauda (Lauda Königshafen, Germany), and a LC 240 fluorescence detector (Perkin-Elmer, Norwalk, CT, USA). The detector was operated at an excitation wavelength of 325 nm and emission wavelength of 360 nm and with a response of 5 and a factor of 512. The integration was carried out using the software program Turbochrom 4.1 (Perkin-Elmer), which was operated on a Compaq personal computer connected to a BJ-330 printer (Canon).

The analytical column [stainless steel, 150 × 4.6 mm inside diameter (I.D.)] and guard column (stainless steel, 5.0 × 3.0 mm I.D.) were packed with a 100 A PLRP-S, 5 μm (Polymer Laboratories, Amherst, MA, USA).

The mobile phase was 0.02 *M* phosphoric acid/acetonitrile/tetrahydrofuran (64:21:15), and the pump was operated isocratically at a flow rate of 0.7 mL/min. The samples were injected at intervals of 10 min.

Sample Pretreatment

Each sample of 3.0 g of muscle with attached skin (2.7 g of muscle and 0.3 g of skin) was cut into small pieces with scissors, and weighed into a 50-mL of centrifuge tube with screw cap (NUNC, Roskilde, Danmark), after which 1.0 mL of solution A or standard (the total volume should always be 1.0 mL), 1.0 mL of NH_3 (25%), and 5.0 mL of acetone were added. The mixture was homogenized for approximately 6 s with an Ultra-Turrax TP 18/10 (Janke & Kunkel KG, Ika Werk, Staufen, Germany) and left in an ultrasonic bath for 15 min, whereafter the sample was left for a minimum of 5 h and maximum of 20 h at room temperature. The sample was then mixed on a vortex and centrifuged for 5 min at 5000 rpm. One milliliter of supernatant was transferred to a glass-stoppered centrifuge tube, followed by 500 μL of 3 *M* H_3PO_4. After mixing, 1.0 mL of acetone was added and the tube mixed on a vortex and centrifuged for 3 min at 3500 rpm. To 1.0 mL of supernatant, 1.0 mL of water was added and mixed. Approximatly 500 μL of this solution (suspension) was filtered through a Spin-X centrifuge filter and centrifuged for 5 min at 10000 rpm. Aliquots of the filtrate (50 μL) were injected into the high-performance liquid chromatograph.

Extraction from Skin

The extraction procedure was tested on samples of pure skin, of weiging approximately 0.5 g, in three parallel experiments from fish treated with FQ or OX. To each sample (finely divided into small pieces using scissors), 1.0 mL of solution A, l.0 mL of NH_3 (25%), 2.0 mL of water, and 5.0 mL of acetone were added, sequentially. The mixture was homogenized in the Ultra-Turrax and left in an ultrasonic bath for 15 min.

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The samples were then allowed to stand for 0, 1, 2, 5, 20, or 24 h (room temperature). At the conclusion of each time interval, each sample was mixed on a vortex and centrifuged for 5 min (5000 rpm). Subsequently, 0.6 mL of 3 *M* H_3PO_4 was mixed with 1.0 mL of supernatant, followed by addition of 1.0 mL of acetone, mixing, and centrifugation for 3 min. Approximately 0.5 mL of supernatant was filtered through a Spin-X filter unit, and aliquots of 10 μL were injected into the chromatograph.

Calibration Curves and Recovery Studies

The calibration curves for FQ and OX were established by spiking muscle with attached skin samples with standard solutions to produce concentrations of 30, 50, 100, 200, 400, 800, and 1000 ng/g for FQ, and 20, 50, 100, 200, 400, 800, and 1000 ng/g for OX. Duplicate samples were used.

The recovery rates were determined by comparing recoveries from spiked muscle and standard solutions.

In this procedure, each corresponding standard solution was first diluted into 10.0 mL of solution A and blended. To 1.0 mL of this sample, 1.5 mL of solution A was added and blended. To 1.0 ml of this mixture 1.0 mL of water was added and blended.

The linearity of the standard curves for FQ and OX was calculated using peak-height measurements.

Stability of OX and FQ During Extraction

The stability of OX and FQ during the extraction procedure was also tested. To six glass-stoppered tubes, a 1.0-mL standard of mixed OX and FQ (1 μg/mL), 1.0 mL of NH_3, 2.0 mL of water, and 5.0 mL of acetone, were added and mixed (stock mixture). To 1.0 mL of the stock mixture, 1.0 mL of acetone, and 600 μL of 3 *M* H_3PO_4 were added and blended. After centrifugation for 3 min, ca. 500 μL of supernatant was filtered through a Spin-X centrifuge filter.

Aliquots of the filtrate (10 μL) were injected into the chromatograph (time 0). The stock mixture was stored at room temperature for 24 h and then reanalyzed.

RESULTS AND DISCUSSION

Chromatograms of drug-free and spiked muscle/skin samples are shown in Figure 1. The standard curves in the areas investigated, from 30 to 1000 ng/g for FQ and from 20 to 1000 ng/g for OX, were linear. The correlation coefficient (r)

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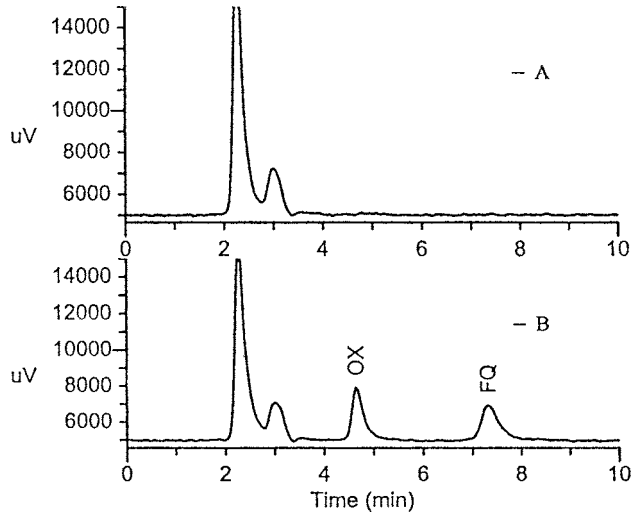

Figure 1. Chromatograms of extracts from fish muscle with attached skin. A) Drug-free muscle/skin; B) Muscle/skin spiked with oxolinic acid and flumequine (200 ng/g, respectively).

for FQ in muscle with attached skin was 0.998 and for OX was 0.999, using the external standard method. Table 1 shows the recovery and repeatabilities for FQ and OX from muscle/skin.

The recoveries of FQ and OX from muscle/skin were 75–76% and 79–82%, respectively, with standard deviations of 1.7–2.2% for FQ and 1.8–2.0% for OX. Our chromatographic system appears to be efficient for the determination of FQ and OX from fish muscle and skin. The limits of quantification were 30 and 20 ng/g and the detection limits were 15 and 10 ng/g for FQ and OX, respectively. The detection limit of the assay was calculated to be 3 times the baseline noise from

Table 1. Recovery and Repeatability for Flumequine and Oxolinic Acid from Spiked Samples of Fish Muscle/Skin Tissue

Tissue	No. of Samples	Amount in Spiked Samples (μg/g)	Recovery (%)[a]	
			FQ	OX
Muscle/Skin	8	0.1	76±2.2	
(2.7 ± 0.3 g)	8	0.4	75 ± 1.7	
	8	0.1		82 ± 2.0
	8	0.4		79 ± 1.8

[a] Values are means ± relative standard deviation.

Table 2. Amount of Oxolinic Acid and Flumequine in Skin Detected at Different Times of Exposure to the Extractant

Hours at Room Temperature	Average Content of OX and FQ (Peak Height)		
	No. of Samples	OX (mV)	FQ (mV)
0	3	3037	2821
1	3	3086	2884
2	3	3660	2900
5	3	3946	2994
20	3	4134	3250
24	3	4020	3228

a drug-free tissue. No interference was seen during analysis, when calibrating the curves, or when performing recovery studies.

Our experience from other studies is that the extraction of drugs from skin is difficult. When developing the present method, we tested the effect of the duration of exposure of a pure skin sample to different extraction chemicals. The most efficient system seems to be a mixture of NH_3 and acetone, and the results from this extraction are shown in Table 2.

To obtain complete extraction of the drugs it was necessary to add water to the skin sample. Without the water the results varied excessively. When we used muscle and skin in natural proportions, it was not necessary to add water, since 2.7 g of muscle and 0.3 g of skin contain ca. 2.0 mL of water.

Concerning the stability of the two drugs during extraction, the average sample peak height at time 0 was 638 mV for FQ and 969 mV for OX. After 24 h, the averages from the six samples were 644 and 961 mV for FQ and OX, respectively. Thus, the extraction procedure and temperature described seem to have no influence to the amount of FQ and OX detected.

Under the sample pretreatment acetone was added. For this purpose, automated pipettes with tips produced irregular volumes. This problem was avoided with a bottletop dispenser, which gave good results.

In summary, the method described is specific and robust. It has been demonstrated to be efficient for screening and quantification of residues of FQ and OX in muscle and skin in natural proportions.

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