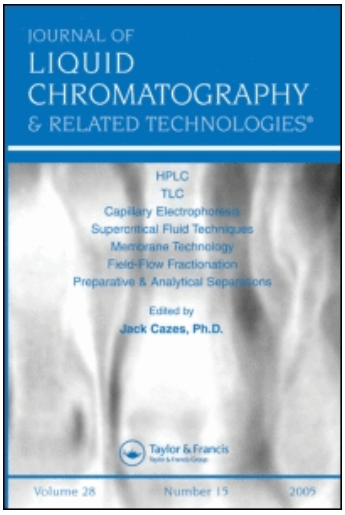

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RAPID ASSAY FOR THE SIMULTANEOUS DETERMINATION OF RESIDUES OF OXOLINIC ACID AND FLUMEQUINE IN FISH TISSUES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A simple and rapid method for the simultaneous extraction and determination of residues of oxolinic acid (OX) and flumequine (FQ) in fish tissue, muscle and liver, is presented. The samples were extracted with acetonitrile and ammonia. The drugs were then extracted into a water phase, acidified, and extracted with $CHCl_3$. After evaporation, the sample was redissolved in mobile phase and filtered through a spin-X centrifuge filter. The filtrate was injected onto the HPLC. The calibration curves were linear, and the recovery of oxolinic acid was 101-104 % while the recovery of flumequine was 88-94 %. The detection limits were 5 ng/g for oxolinic acid and 10 ng/g for flumequine.

INTRODUCTION

In Norwegian fish farming, oxolinic acid was the most used drug in 1989. And a small amount of flumequine was also employed. Oxolinic acid has also been recommended for use in Japanese fish farming. For some years, flumequine has been applied against furunculosis in French fish farming (1,2).

There is a need for analytical methods to detect residues of oxolinic acid (OX) and flumequine (FQ) in fish tissues, so as to be able to ascertain safe withdrawal period, as well as monitor residues in slaughtered fish. Several analytical methods have been developed for the determination of OX in body fluids using microbiological and high performance liquid chromatographic procedures (3,4,5,6). Kasuga et al. (7,8) developed a procedure for the HPLC determination of OX in fish tissues which was applied in a residue study on OX in rainbow trout (9). The method is however time-consuming and insensitive. Horie et al. (10) developed a more sensitive method using fluorescence detection and solid phase extraction in the clean-up steps, with a detection limit of 10 ng/g. Ueno et al. (11,12) used a slight modification of the method of Kasuga et al. (13) for the determination of oxolinic acid in tissue and body fluids from fish. The method is based on UV-detection. Average recovery was 81% and the detection limits 5-100 ng/g. The method is time consuming and requires large amounts of chemicals. Ikai et al. (14) presented a method for the determination of OX in fish with a detection limit of 50 ng/g.

Analytical methods for the determination of flumequine in plasma and urine have been developed by Harrison et al. (15) and, more recently, by Decolin et al. (16), and Rasmussen et al. (6). Tao et al. (17) published a microbiological method for residue analysis of flumequine in fish tissues, with a sensitivity of 250 ng/g, while Ellerbroek & Bruhn (18) presented a rapid and simple method for determination of flumequine in biological fluids and meat by HPLC and fluorometric detection. The detection limits of this latter method were ca. 50 ng/ml in urine, 90 ng/ml in serum and 90 ng/g in kidney. Though recovery from urine was in the range 72-84 %, the recovery from serum and kidney was as low as 32-41 %. Samuelsen (19) presented a method for the analysis of flumequine in fish muscle that is sensitive (detection limit 5 ng/g), but the preparation of the samples was tedious.

Rogstad et al. (20) published a method for the simultaneous determination of OX and FQ in fish tissues that is sensitive but time-consuming, the detection limits being 0.5 ng/g for OX and 2 ng/g for FQ.

The purpose of the present study was to develop a rapid, yet sensitive, method for the simultanous determination of oxolinic acid and flumequine,which required only small quantities of chemical reagents.

MATERIALS AND METHODS

Materials and Reagents

Muscle and liver tissues of salmon and rainbow trout served as samples.

All chemicals were of analytical or HPLC grade. Oxolinic acid was supplied by Sigma Co. (St. Louis, MO, USA), and flumequine by Solchem Italiana S.p.a. (Mulazzano, Italy).

Solvents were of analytical and HPLC grade. Stock solutions (1 mg/ml) of OX and FQ were prepared in 0.03 M sodium hydroxide, and working standards were prepared by dilution with mobile phase. The solutions were stored in the refrigerator in dark stoppered flasks.

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 equipped with a cooler (14 °C) Lauda RMT6 from Messgeräte Werk Lauda, (Lauda-Königshafen, Germany), and a LS 4 fluorescence detector (Perkin Elmer, Norwalk, Conn., USA). The detector was operated at an excitation wavelength of 260 nm and emission wavelength of 380 nm. The analytical column (stainless steel, 150 x 4.6 mm I.D.) and guard column (stainless steel, 5.0 x 3.0 mm I.D.) were packed with 5 µm particles of PLRP-S polymer adsorbent (Polymer Laboratories, Amherst, MA, USA).

The mobile phase was 0.002 M phosphoric acid-acetonitrile-tetrahydrofurane (64:21:15) at a flow rate of 0.7 ml/min. Aliquots of 10 µl and 20 µl were injected onto the column for the determination of OX and FQ, respectivly.

Sample Preparation and Clean-up (Figure 1)

When oxolinic acid residues were to be determined flumequine was used as internal standard, while oxolinic acid was used as the internal standard when determining flumequine.

Oxolinic Acid

The tissue sample, 3 g ground muscle or liver, was weighed into a 50 ml centrifuge tube with screw cap (NUNC). Internal standard 900 µl FQ (10 µg/ml), 1 ml NH_3 and 5.1 ml CH_3CN were added. The sample was mixed for 1 min. at whirlimixer, and the homogenate then centrifuged for 3 min. at 3000 rpm.

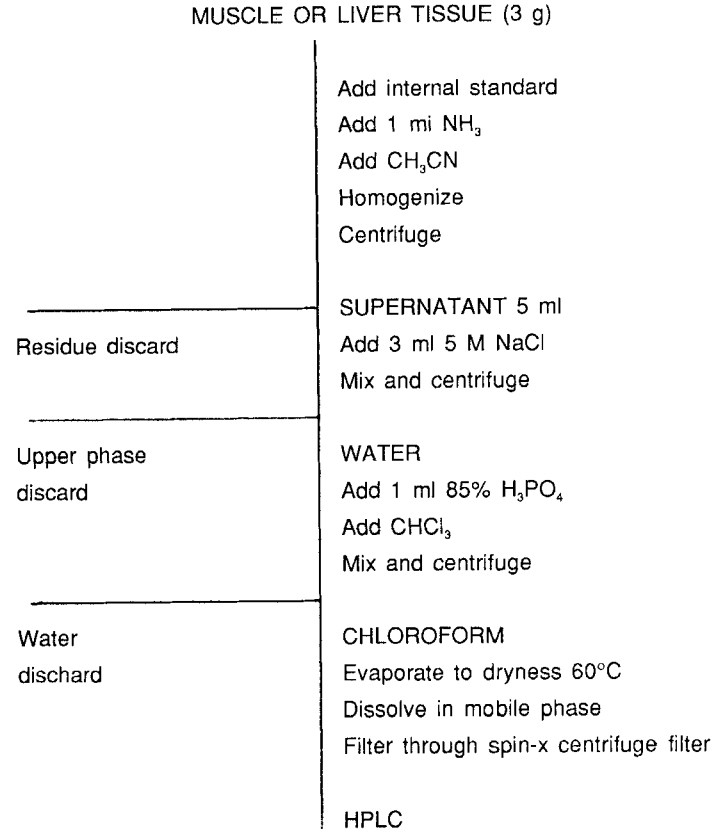

FIGURE 1
Extraction and clean-up procedure for oxolinic acid and flumequine from fish muscle and liver.

5 ml of the supernatant was pipetted into a glass stoppered centrifuge tube and 3 ml 5 M NaCl added. The sample was mixed for 5 sec., and centrifuged for 1 min. at 3000 rpm. The upper acetonitrile layer was discharged, or the lower layer transferred to another centrifuge tube. 1 ml 85 % H_3PO_4 and 4 ml $CHCl_3$ were added. The sample was shaken vigorously for 10-15 sec. followed by centrifugation for approximately 30 seconds. The upper aqueous layer and any solid residues between the two phases were discharged. The chloroform phase was evaporated at 60 °C under a stream of nitrogen. The residue was then

redissolved in 3 ml mobile phase, and approximatly 0.5 ml filtered through a Costar$_R$ spin-X^{TM} centrifuge filter unit (low type) with 0.45 µm cellulose acetate binding. Aliquotes of the filtrate were injected onto the HPLC.

Flumequine.

The tissue sample, 3 g ground muscle or liver, was weighed into a 50 ml centrifuge tube with screw cap (NUNC). Internal standard 300 µl OX (1 µg/ml), 1 ml NH_3 and 5.7 ml CH_3CN were added, after which the sample was mixed for 1 min. in a whirlimixer, and the homogenate centrifuged for 3 min. at 3000 rpm. Further preparation procedures were as for oxolinic acid.

Calibration Curves and Recovery Studies.

The calibration curves for OX were made by spiking tissue samples with standard solutions of OX and internal standard (FQ) to yield 5, 10, 25, 50, 100, 150 and 400 ng OX pr gram in muscle and liver samples. The calibration curves for FQ were similarly made by spiking tissue samples with FQ standard solution and internal standard (OX) to yield 10, 15, 25, 50, 100, 150, 200 and 400 ng FQ pr gram in muscle and liver samples. Duplicate samples were used. The recovery rates were determined by comparing analysis of spiked tissues, muscle and liver, with those of standard solutions.

RESULTS AND DISCUSSION

Chromatograms of muscle and liver samples and spiked samples are shown in Figure 2 for oxolinic acid and in Figure 3 for flumequine. When 20 µl was injected onto the HPLC, for the determination of flumequine, interference would sometimes be observed at the oxolinic acid peak (Figure 3 A,C). When 10 µl was injected onto the HPLC, for the determination of oxolinic acid, no such interference was seen (Figure 2).

The linearity of the standard curves for oxolinic acid and flumequine in muscle and liver were tested using peak height measurements and the internal standard. The standard curves were linear in the investigated areas, 5-400 ng/g for oxolinic acid and 10-400 ng/g for flumequine. The correlation coefficients for oxolinic acid in muscle and liver were r=0.9993 and r=0.9964 respectivly, while those for flumequine were r=0.9996 and r=0.9989 in muscle and liver, respectivly.

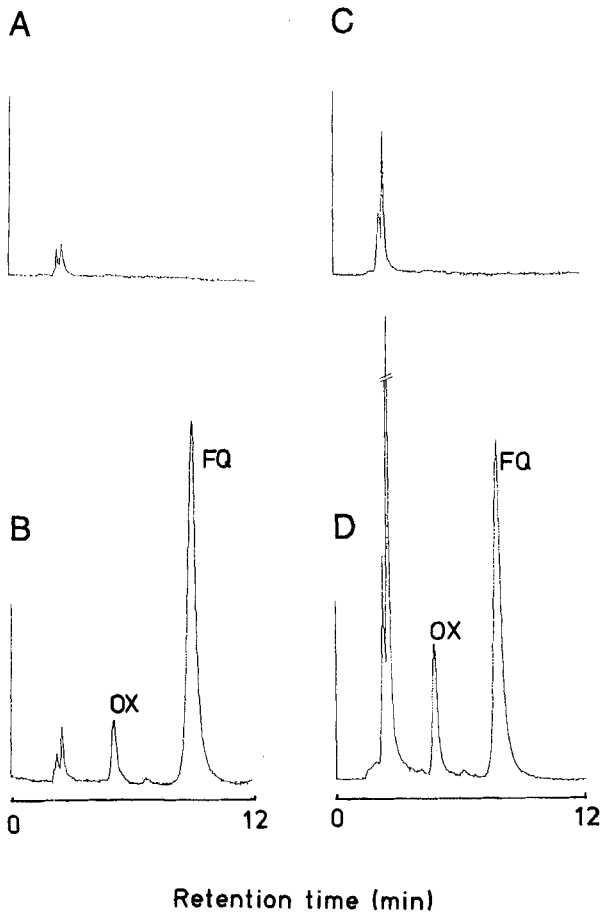

FIGURE 2
Chromatograms of extracts from 3 g muscle and liver for the determination of oxolinic acid (10 µl injected onto the HPLC).
A - Unspiked muscle tissue.
B - Muscle tissue spiked with 50 ng oxolinic acid (OX) and 3 µg flumequine (FQ) pr gram sample.
C - Unspiked liver tissue.
D - Liver tissue spiked with 100 ng oxolinic acid (OX) and 3 µg flumequine (FQ) pr gram sample.

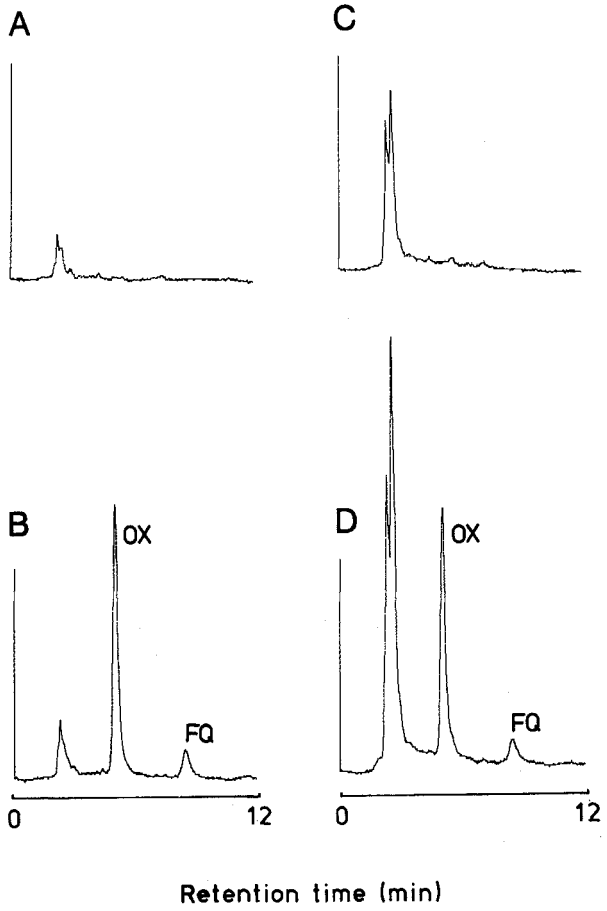

FIGURE 3
Chromatograms of extracts from 3 g muscle and liver for the determination of flumequine (20 µl injected onto the HPLC).
A - Unspiked muscle tissue.
B - Muscle tissue spiked with 50 ng flumequine (FQ) and 100 ng oxolinic acid (OX) pr gram sample.
C - Unspiked liver tissue.
D - Liver tissue spiked with 50 ng flumequine acid (FQ) and 100 ng oxolinic acid (OX) pr gram sample.

TABLE 1.
Recovery of Oxolinic Acid and Flumequine from Spiked Samples of Fish Muscle and Liver Tissue.

Tissue	No. of samples	Amount in spiked samples (µg/g)	Recovery % OX Mean	OX SD	FQ Mean	FQ SD
Muscle (3 g)	8	0.15	104	6.7		
	8	0.01	103	5.7		
	8	0.4			94	8.0
	8	0.03			94	7.0
Liver (3 g)	8	0.15	103	7.7		
	7	0.01	101	8.0		
	8	0.4			90	5.4
	8	0.03			88	5.8

Table 1 shows the recoveries and repeatabilities of OX and FQ from muscle and liver. The recovery of oxolinic acid from muscle and liver tissue varied from 101 to 104 %, while the recovery of flumequine from the same tissues varied between 88 and 94 %. The standard deviation varied from 5.4 to 8.0. The detection limit for oxolinic acid in muscle and liver is 5 ng/g, the corresponding figure for flumequine being 10 ng/g.

The method presented by Rogstad et al. (20), while also determines OX and FQ simultanously, is more sensitive with detection limits of 0.5 ng/g and 2 ng/g for oxolinic acid and flumequine, respectivly, compared with the 5 ng/g and 10 ng/g for OX and FQ, respectivly, achieved by the present method. However the method of Rogstad et al. (20) is more time-consuming and requires the use of larger quantities of chemicals. The methods of Kasuga et al. (8,9), Horie et al. (10) and Ueno et al. (11,12) for the determination of oxolinic acid in fish tissue are not any more sensitive and are much more time-consuming than the method presented in this paper. The method of Ikai et al. (14) for the determination of OX is rapid but less sensitive. The method of Samuelsen (1989) for the determination of flumequine is also much more time-consuming than the present method, though the sensitivity is the same. The method of Ellerbroek & Bruhn (18) for the determination of flumequine is rapid, but recovery from serum and kidney is as low as 32-41%.

The method presented in this paper should be useful for most work on residues of OX and FQ in farmed fish. The method is selective, sensitive and robust, and should be commonly applicable for monitoring drug levels in fish tissues. The method is very rapid, a technichen could easily do 30 samples a day. In addition the use of chemicals is low.

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