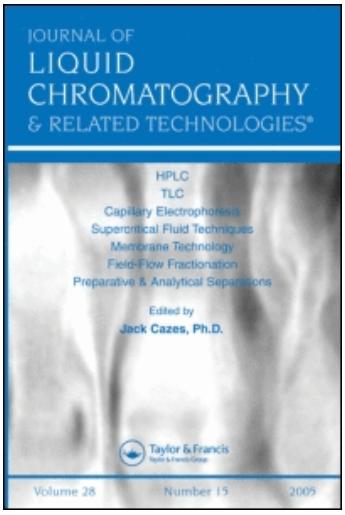

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A Rapid and Time-Effective Assay for Determination of Oxolinic Acid and Flumequine in Fish Tissues by HPLC

Váctor Hormazábal[a]; Magne Yndestad[a]
[a] Division of Food Hygiene, Norwegian College of Veterinary Medicine, Oslo 1, Norway

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A RAPID AND TIME-EFFECTIVE ASSAY FOR DETERMINATION OF OXOLINIC ACID AND FLUMEQUINE IN FISH TISSUES BY HPLC

VÍCTOR HORMAZÁBAL AND MAGNE YNDESTAD
Division of Food Hygiene
Norwegian College of Veterinary Medicine
P.O. Box 8146-Dep.
N-0033 Oslo 1, Norway

ABSTRACT

A simple method for analysis of oxolinic acid and flumequine in fish muscle and liver is described. The samples were extracted with trichloroacetic acid, neutralized and analysed by HPLC, with minimal sample manipulation. The limit of quantification was 30 µg/kg for oxolinic acid and 35µg/kg for flumequine.The simplified extraction and clean-up procedure makes it possible for one person to monitor the concentration of the drugs in approximately 80 samples per day.

INTRODUCTION

The extensive use of oxolinic acid (OX) and flumequine (FQ) 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 in fish.

Several methods based on high-performance liquid chromatography (HPLC) for the determination of OX and FQ in fish tissues have been published (1-8). All methods involve extraction of the compounds with organic solvents, and the manual work-up procedures include steps such as liquid-liquid extraction (1-7) or solid phase extraction (6-8). Microbiological methods for residue analysis of flumequine in fish tissues, with a sensitivity of 250ng/g, have been published (9). The purpose of the present study was to develop a rapid, simple and sufficiently sensitive method, for the simultaneous determination of OX acid and FQ, which required minimal sample manipulation and only small quantities of chemical reagents.

MATERIALS AND METHODS

Materials and Reagents

Samples of muscle and liver tissue of salmon and rainbow trout were used.
All chemicals and solvents were of analytical or HPLC grade. OX and FQ were supplied by Sigma Co. (St. Louis, MO, USA). 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 a refrigerator.
Trichloroacetic acid (TCA) was supplied by Ferax (Laborat GMBH Berlin-Germany), and ortho-phosphoric acid 85% by Merk, Germany.
Spin-X centrifuge filter units from Costar (Cambridge, MA, USA) were also used.

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 (14°C) from Messgeräte Werk Lauda, (Lauda Königshafen, Germany), and a LS 240 fluorescence detector (Perkin-Elmer, Norwalk, Conn., USA). The detector was operated at an excitation wavelength of 325 nm and emission wavelength of 360 nm, and with a Resp. of 5 and a Fctr. of 1024. The analytical column (stainless steel, 150 x 4.6 mm I.D.) and guard column (stainless steel, 5.0 x 3 mm I.D.), were packed with 5 µm particles of PLRP-S polymer adsorbent (Polymer Laboratories, Amherst, MA, USA).

The mobile phase was 0.02 M phosphoric acid-acetonitrile-tetrahydrofurane (65:20:15) at a flow rate of 0.7 ml/min. The samples were injected at intervals of 10 min. Aliquots of 25 µl were injected onto the column for the determination of OX and FQ.

Sample pretreatment

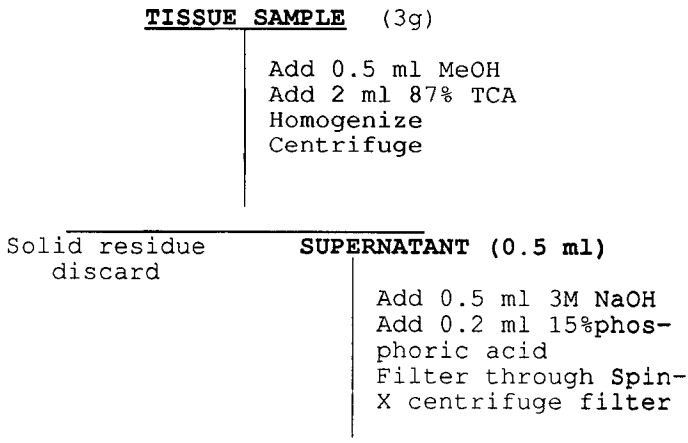
The sample pretreatment of tissues is shown in Fig. 1. Spiked samples (3g) of muscle and liver were mixed with 500 µl MeOH (or standard) and 2 ml 87% TCA in water. The mixture was homogenized for approx. 1 min. in an Ultra-Turrax TP 18/2 (Janke & Kunkel KG, Ika Werk, Staufen,F.R.G.). The mixture was then centrifuged for 3 min. (5000 rpm.). 500 µl of the supernatant were transferred to a centrifuge tube and 500 µl 3 M NaOH added. The sample was mixed for approx. 1 sec. and 200 µl 15% phosphoric acid in methanol then added. The sample was again mixed for 1 sec. Approximatly 500 µl were then filtered through a Costar Spin-X centrifuge filter unit (low type) with 0.22 µm cellulose acetate binding and centrifuged for 5 min. (10000 rpm.). Aliquots of the filtrate were injected onto the HPLC.

Calibration Curves and Recovery Studies

The calibration curves for OX were established by spiking muscle and liver tissue samples with standard solutions to yield 20,50,100,200,400 and 500 ng OX pr. gram, the calibration curves for FQ being similarly determined by spiking tissue samples with FQ standard solution to yield 25,50,100,200,400 and 500 ng FQ pr.gram. Duplicate samples were used. The recovery rates were determined by comparing analysis of spiked tissues, muscle and liver, with those of standard solutions. The linearity of the standard curves for OX and FQ was calculated using peak-heigth measurements.

RESULTS AND DISCUSSION

Chromatograms of clean muscle and liver samples, and spiked samples are shown in Figure 2. The standard curves were linear in the investigated areas; 20-500 ng/g for OX and 25-500 ng/g for FQ, in muscle and liver. The correlation coefficients for both OX and FQ in muscle and liver were r=0.999. Table 1 shows the recovery and repeatabilities for OX and FQ from muscle and liver.



F I G U R E 1
Extraction and Clean-up Procedure for Oxolinic acid and Flumequine from Fish Tissue.

The recovery of OX acid and FQ from muscle tissue varied from 67 to 71%, and from 61 to 64%, respectively. The corresponding figures for liver tissue were 56%, and from 51 to 53%, respectively. OX and FQ are acids with low solubility in water. Their solubility increases in alkaline solutions, and in TCA. The results presented in this paper show that OX and FQ can be extracted from samples of muscle and liver tissue with TCA. The extraction procedure appeared applicable to tissues of both Atlantic salmon and rainbow trout. The simplified extraction and clean-up procedure makes it possible to monitor drug concentrations in approximately 80 samples per day. The linearity of the standard curve for both OX and FQ in muscle and liver were 0.999, when using the external standard method. The limit of quantification was 30 ng/g for OX and 35 ng/g for FQ in muscle and liver. When 25 µl was injected onto the HPLC, no interfering peaks were observed. However, the sensitivity may be enhanced by using a larger sample amount. The cost of chemicals and the manual work-up procedures is also reduced compared to previously published methods. This method is specific and robust. The method is demonstrated to be efficient for screening and quantification of residues of drug of OX and FQ, and should replace the less specific microbiological

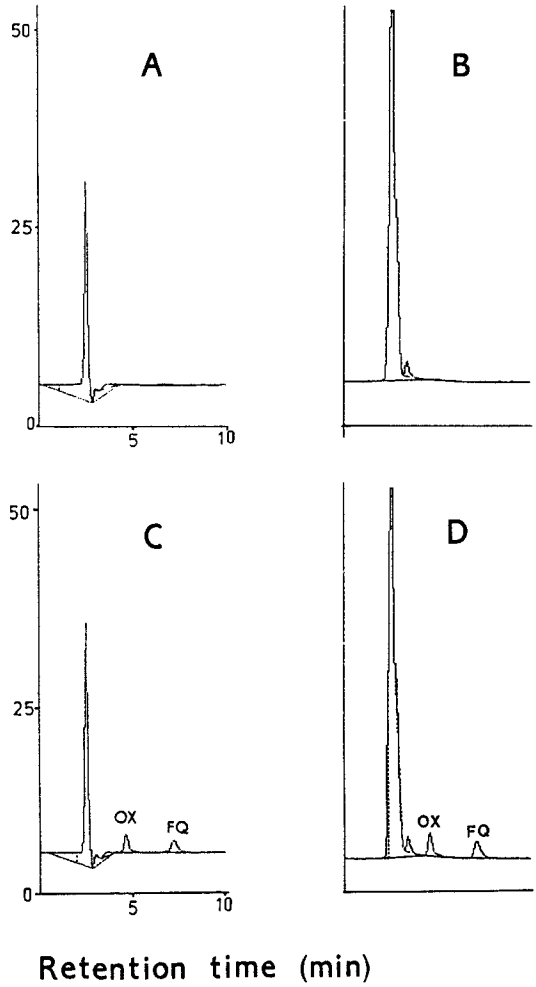


FIGURE 2

Chromatograms of extracts from fish muscle and liver. A: drug-free muscle, B: drug-free liver, C: muscle spiked with oxolinic acid and flumequine (300 ng/g respectively), D: liver spiked with oxolinic acid and flumequine (400 ng/g respectively).

T A B L E 1.

Recovery and Repeatability for 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
	8	0.1	71	3.0		
Muscle	8	0.4	67	1.3		
(3g)	8	0.1			64	1.3
	8	0.4			61	1.0
	8	0.1	56	1.9		
Liver	8	0.4	56	1.3		
(3g)	8	0.1			51	1.7
	8	0.4			53	1.1

SD = relative standard deviation

T A B L E 2.

Concentration of FQ in muscle of salmon monitored using two different methods

Sample no.	**1**	**2**	**3**	**4**	**5**	**6**
This method conc. FQ (ng/g)	79	366	774	501	502	588
Method ref.5 conc. FQ (ng/g)	68	340	786	492	514	564

methods for residue control of these drugs in fish tissue.
The method was correlated to that (5) published previously by analysing muscle of salmon treated with FQ. The results were compared by using linear regression and the correlation coefficient was 0.998. The individual concentration values are given in Table 2.

CONCLUSION

This study has shown that residues of the two antibacterial compounds oxolinic acid and flumequine in tissues of Atlantic salmon and rainbow trout can be analysed after samples have been subjected to some very simple clean-up steps. The method can replace the less sensitive microbiological method.

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