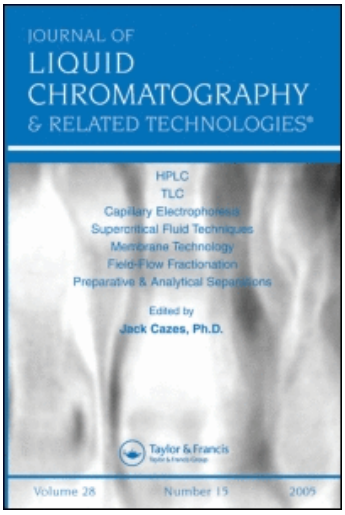

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RAPID ASSAY FOR MONITORING RESIDUES OF ENROFLOXACIN AND SARAFLOXACIN IN FISH TISSUES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A simple and rapid method for extraction and HPLC-analysis of residues of enrofloxacin and sarafloxacin in muscle and liver from Atlantic salmon is presented. After homogenization of the tissue the fat was separated by extraction into organic solvents and the aqueous phase was analysed on the chromatograph. The method is selective and robust. The sensitivity is 5 ng/g for enrofloxacin and 10 ng/g for sarafloxacin. The method was validated and correlated with a more sensitive, but time-consuming pretreatment procedure for monitoring enrofloxacin. The method is also applicable for monitoring residues of the drugs oxolinic acid and flumequine, respectively, in Atlantic salmon.

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INTRODUCTION

In fish farming several antibiotics and chemotherapeutics have been tested for prevention and treatment of infectious diseases in fish. In Norwegian aquaculture the quinolone analogues of nalidixic acid were the most used drugs in 1990 and through the first nine months a total amount of about 5,300 kg of oxolinic acid and flumequine was applied (1). The two compounds enrofloxacin and sarafloxacin are at the moment being evaluated for treatment of infections caused by gram-negative bacteria. Recently, we developed a sample clean-up procedure for analysing residues of enrofloxacin in tissues of Atlantic salmon (2). The sensitivity of the method was 1 ng/g. Sarafloxacin is a new quinolone having a potent wide-spectrum antibacterial activity (3,4) and is now being tested in treatment of infections in aquaculture.

In order to control the problem of drug residues in fish analytical methods having good sensitivity and a high sample throughput must be available. Furthermore, the methods must have a good selectivity, accuracy and be robust. False negative results should not occur and the number of false positive results has to be low.

Traditionally, microbiological methods have been used for trace analyses of antimicrobial drug residues, whereas chromatographic analytical methods have been applied for verification of positive results. Due to tedious sample clean-up procedures of the biological material prior to the chromatographic analysis these methods have a low sample throughput. Norwegian Fishery Authorities require both a pre-slaughtering and post-slaughtering control of farmed fish (5), and more than 20,000 samples are tested per year using microbiological test assays.

For some years we have been engaged in developing methods for chromatographic determination of residues of the quinolone derivatives flumequine and oxolinic acid (6-8) and more recently, enrofloxacin (2). This study is a continuation of our recent work on flumequine and oxolinic acid with the purpose to develop a rapid, yet sensitive method for simultaneous determination of

enrofloxacin and sarafloxacin. The challenge has been to improve the pretreatment procedure to obtain methods of acceptable accuracy, having high sample throughput in order to replace the non-specific microbiological methods with chemical methods.

EXPERIMENTAL

Chemicals and Reagents

All chemicals were of analytical grade. Enrofloxacin (EF) was supplied by Bayer Norge A.S (Oslo, Norway), whereas sarafloxacin (SF) was donated by Abbott Laboratories (Chicago, IL, USA).

Solvents were of analytical or HPLC grade. Stock solutions (1 mg/ml) of EF and SF were prepared in 0.03 M sodium hydroxide and were stored in the fridge in dark stoppered flasks. Working standards were prepared by dilution with mobile phase.

Spin-X™ centrifuge filter units from Costar (Cambridge, MA, USA).

Samples of Fish

Muscle and liver tissues of Atlantic salmon was obtained from Ewos Aqua (Skårer, Norway).

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 cooler (14°C) Lauda RMT6 from Messgeräte Werk, Lauda-Königshafen, Germany), and an LS 4 fluorescence detector (Perkin Elmer, Norwalk, Conn., USA). The integration was carried out by use of the software program Omega-2 (Perkin Elmer), which was operated on the personal computer Olivetti M300 connected to a Star LC24-10 printer. The detector was operated at excitation

wavelength of 278 nm and emission wavelength of 440 nm. The analytical column (stainless steel, 15 cm x 4.6 mm ID) and guard column (stainless steel, 5.0 x 3.0 mm ID) were packed with 5 µm particles of PLRP-S polymer adsorbent (Polymer Laboratories, Amherst, MA, USA).

As mobile phase was applied the mixture 0.002 M phosphoric acid/acetonitrile/methanol (73:19:8) at a flow rate of 0.9 ml per minute. Aliquots of 20 µl were injected onto the column.

Sample Pretreatment

The sample pretreatment is shown schematically in Figure 1. The procedure was developed using both internal and external standard. As internal standard was applied sarafloxacin when determining enrofloxacin and vica versa. Spiked sample of ground muscle or liver tissue (3 g) was first mixed mechanically with a mixture of 1 ml ammonia and 5-6 ml acetonitrile and then on a whirlimixer for one minute. The total volume of added solvents should amount to 7 ml. After centrifugation 2.5 ml of the supernatant was pipetted into a glass stoppered centrifuge tube and 1 ml NaCl-solution (1 M), 3 ml diethyl ether and 2 ml hexane were added. The mixture in the tube was blended well and centrifuged. The upper layer of organic solvents was discarded, and the aqueous solution was acidified using 0.5 ml phosphoric acid (5 M). A portion of the extract was centrifuged using Spin-X™.

Validation of the Pretreatment Procedure

The within-day precision, the recovery of the sample clean-up relative to direct injection of standard solutions and the linearity of the calibration curve of the above assay were determined by analyses of spiked samples of muscle and liver in the concentration range 15-400 ng/g for SF and 5-400 ng/g for EF. Each concentration level was assayed in triplicate. Both internal standard method and external standard method were validated.

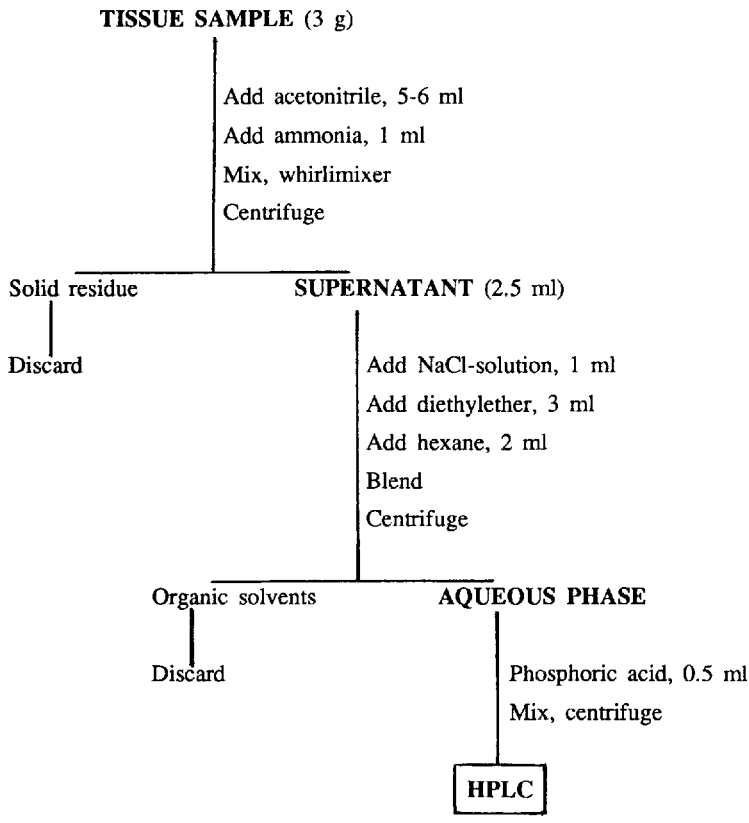

FIGURE 1

Extraction and Clean-up Procedure for Enrofloxacin and Sarafloxacin from Fish Tissue.

RESULTS AND DISCUSSION

Chromatograms of drug-free samples of muscle and liver, spiked samples and samples from medicated fish are shown in Figure 2. The chromatographic system for enrofloxacin has been tested previously (2) and demonstrated to separate enrofloxacin, ciprofloxacin and difloxacin. Minor modifications of the mobile phase appeared necessary to analyse enrofloxacin with sarafloxacin as internal standard and vica versa.

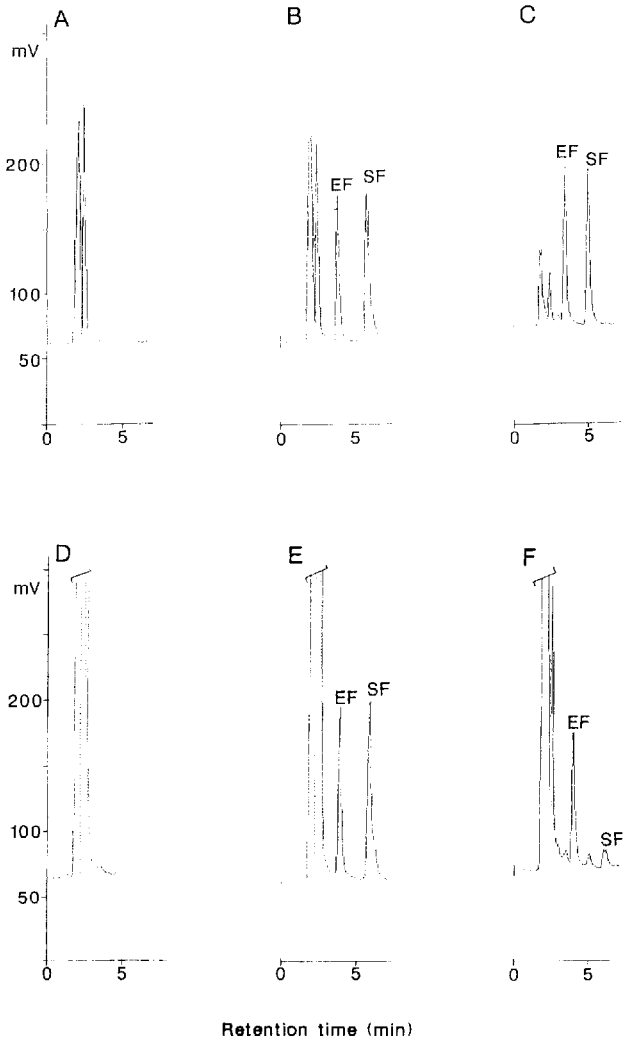

FIGURE 2

Chromatograms of extracts from fish muscle and liver.
A - drug-free muscle, B - muscle spiked with enrofloxacin (EF, 250 ng/g) and sarafloxacin (SF, 500 ng/g), C - muscle from fish treated with sarafloxacin, D - drug-free liver, E - liver spiked with enrofloxacin (EF, 250 ng/g) and sarafloxacin (SF, 500 ng/g), F - liver from fish treated with sarafloxacin

The published assay for monitoring residues of enrofloxacin in Atlantic salmon (2), applied organic solvents for extraction of fat from the tissue homogenate and then a solid phase extraction using C18 SPE-column to purify the aqueous phase. It was obtained chromatograms of the extracts having no impurity peaks and the sensitivity was 1 ng/g. However, only 12-16 samples were analysed per day and the cost of chemicals was high.

By reducing the demand on sensitivity it has been possible to simplify the sample pretreatment procedure for monitoring flumequine and oxolinic acid in fish tissue (8). That experience was applied in the present study. Previously, we have high-speed blended the sample three times. By applying whirlimixing only once on the tissue sample with extraction solvents good recovery of the drug was obtained. Fat and acetonitrile were removed using a mixture of the organic solvents hexane and diethyl ether. The aqueous phase was acidified and filtered and then injected onto the analytical column. This simplified clean-up procedure was applicable on residues of both EF and SF in fish tissue. It was found a limit of quantification of 5 ng/g for EF and 10 ng/g for SF in both muscle and liver tissues.

The precision of the assay was found to vary from 1.8-4.9% and the linearity of the standard curve was 0.9996-0.9999 for both EF and SF when applying internal standard. The calculations were also performed without internal standard. The results are listed in Table 1.

It was found that the precision of the sample pretreatment, the accuracy and the linearity of the standard curve were acceptable without applying internal standard. The tissue extraction for assaying EF and SF was the same as for flumequine (FQ) and oxolinic acid (OX) (8). It was therefore developed a combined method for analysis of EF, SF, FQ and OX in the same tissue sample. After the initial extraction step using acetonitrile and ammonia two 2.5 ml portions of the supernatant were applied for analysis of EF/SF and FQ/OX, respectively. FQ and OX were determined using the method of I. Steffenak et al.(8) with slight modifications, whereas the assay described in this paper was applied for determination of EF amd SF. The precision of the external standard determination

TABLE 1

Analysis of Residues of Enrofloxacin and Sarafloxacin in Fish Tissues.

Sample	No. of samples	Amount of drug added (ng/g)	ENROFLOXACIN		SARAFLOXACIN	
			CV# %	Linearity (IS or ES)*	CV# %	Linearity (IS or ES)*
Muscle (3g)	8	15	4.9 7.9	0.9998 (IS) 0.995 (ES)		
	8	150	1.8 3.7	(IS) (ES)		
	12	30			4.0 6.2	0.9998 (IS) 0.996 (ES)
	11	400			1.8 3.1	(IS) (ES)
Liver (3g)	8	15	3.1 3.9	0.9994 (IS) 0.9997 (ES)		
	8	150	2.0 3.0	(IS) (ES)		
	8	30			4.2 4.5	0.9999 (IS) 0.9996 (ES)
	8	400			2.1 3.6	(IS) (ES)

* IS = internal standard method, ES = external standard method

CV = coefficient of variation

of OX varied from 7.5-9.0%, whereas the linearity of the calibration curve was 0.997 for OX in liver and 0.9992 for OX in muscle. For FQ the precision was 6.0-8.5%, and the linearity of the calibration curve with external standard was 0.9991 for liver and 0.9990 for muscle.

The combined method is demonstrated to be efficient for screening and quantification of residues of drugs of the 4-quinolone group. The simplified

TABLE 2
Concentration of Enrofloxacin (EF) in Muscle of Salmon Monitored Using Two Different Methods

Sample no.	1	2	3	4	5	6	7	8	9	10
This method conc. EF (ng/g)	70	81	117	4	6	20	13	75	176	5
Method ref. 2 Conc. EF (ng/g)	69	79	116	4	5	17	10	72	176	3

extraction and clean-up procedure makes it possible to monitor the drug concentration of approximately 30 samples per day. The cost of chemicals is also reduced compared to previously published methods. This method is specific, sensitive and robust and should replace the less selective microbiological methods for monitoring drug residues in fish tissue.

The method (internal standard) was correlated to that published previously by analysing muscle of salmon treated with EF. The results were compared by using linear regression and the correlation coefficient was 0.9998. The individual concentration values are given in Table 2.

CONCLUSION

This study has shown that residues of the two antibacterial compounds enrofloxacin and sarafloxacin in tissues of Atlantic salmon can be analysed after some very simple clean-up steps of the samples. Good recovery, precision and sensitivity

were obtained. The assay is particularly applicable in screening of drug residues of oxolinic acid, flumequine, enrofloxacin and sarafloxacin, respectively, in Atlantic salmon.

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