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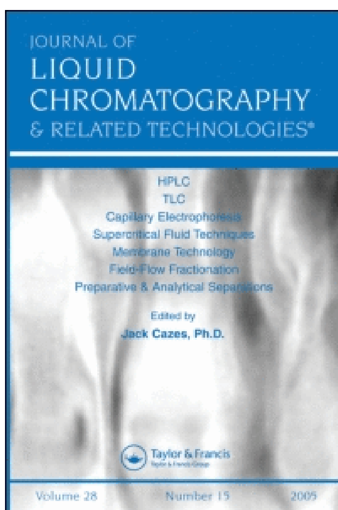

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EXTRACTION AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF ENROFLOXACIN IN FISH SERUM AND TISSUES

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ABSTRACT

The extraction of enrofloxacin from fish serum and tissues using solid phase extraction columns has been studied. Good recoveries were obtained by using C2 columns for clean-up of serum and C18 columns for clean-up of tissue samples. The sample pretreatment procedures are selective and robust having a sensitivity of 1 ng/ml and 1 ng/g from serum and tissues, respectively, making them particularly useful for monitoring drug residue levels. The recoveries were 97-100% from serum and 96-99% from tissues. The method was tested on fish treated with enrofloxacin.

INTRODUCTION

Infectious diseases have become a serious problem to the fish farming industry and the use of chemotherapeutics for treatment is growing. Oxytetracycline has been used with success for many years. However, its slow elimination makes it less applicable for slaughter fish. As less than 10% of the compound is absorped and distributed when administered

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with feed (1) large amounts are excreted into the sea and may cause problems to the marine organisms and environment. The 4-quinolones are a group of compounds showing antimicrobial activity against a borad spectrum of gram-negative bacteria. The compounds are well absorped following oral administration and the elimination is rapid. The two compounds flumequine and oxolinic acid have to a certain extend replaced oxytetracycline for treatment of infections in Atlanic salmon in Norwegian fish farming (2,3). Enrofloxacin (Baytril) is a new compound of the 4-quinolone group having a broad antimicrobial activity on both gram-negative and gram-positive bacteria as well as mycoplasmas (4). The compound is under investigation concerning its application in the treatment of infectious diseases in cultured fish. Its MIC_{90} is found to be in the range 100-200 ng/ml (5). In order to carry out pharmacokinetic and toxicological studies of the compound specific and robust methods for monitoring drug levels in serum and tissues are needed. In Norway the Fishery authorities require methods, both screening and reference for rapid and accurate determination of drug residues at slaughtering (6). By use of a screening method (ex. microbiological method) fish samples shall be controlled for drug residues and positive results have to be verified by chemical method.

Few methods for determination of enrofloxacin in biological samples have been published. Recently, Waggoner at al. (7) published a method for spectrofluorometric determination of enrofloxacin residues in poultry tissues and Tyczkowska et al. (8) an HPLC-method for simultaneous determination of enrofloxacin and its metabolite ciprofloxacin in canine serum and its prostatic tissue. In this paper is presented a rapid and sensitive assay for accurately monitoring drug levels of enrofloxacin in serum and tissues from Atlantic salmon. Solid phase extraction was applied in the pretreatment of extracts of the biological materials. The chromatographic system was based on our experience with the two quinolone derivatives flumequine and oxolonic acid (9, 10). Fish administered with enrofloxacin has been analysed to determine residues of the drug and the possible metabolite ciprofloxacin (11).

EXPERIMENTAL

Chemicals and Reagents.

All chemicals were of analytical grade. Enrofloxacin (EF) and ciprofloxacin (CF) were supplied by Bayer Norge A.S (Oslo, Norway). Difloxacin (DF) was donated by Abbot Labs (Abbot Park, IL, USA) and applied as internal standard.

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

Solid phase extraction (SPE) columns were prepared in our laboratory. Empty Bond Elute R columns, appropriate frits and sorbent materials Bondesil™ type C18, C8, C2, CH and PH were purchased from Analytichem International (Harbor City, CA, USA).

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

Samples of fish and medication

Drug-free serum and tissues were sampled from Atlantic salmon and rainbow trout obtained from Norwegian Institute of Water Research, Biological Research Center at Solbergstrand (Norway) and National Veterinary Institute (Oslo, Norway), respectively.

A study of the absorption, distribution and elimination of EF in Atlantic salmon has been carried out at the Biological Research Center (11). The sea temperature was 6°C . Healthy fish were given a dose of 10 mg EF per kg body weight of fish for ten consecutive days. Plasma sampled from two fish on day 6, 9 and 12 after start of medication and muscle sampled from 4-6 fish on day 20 and 30 after start of medication were analysed for detection of metabolites. CF was added to some of the pretreated sample extracts and analysed.

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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 (Lauda - Köningshafen, Germany), an LS 4 fluorescence detector and an LCI 100 laboratory computing integrator (Perkin Elmer, Norwalk, Conn., USA). The detector was operated at excitation wavelength of 278 nm and emission wavelength of 440 nm. The LC 95 UV detector was operated at 289 nm. The analytical column (stainless steel, 15 cm x 4.6 mm I.D.) and guard column (stainless steel, 5.0 cm x 3.0 mm I.D.) 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 (80:20) at a flow rate of 0.8 ml/min. The mixture was varied slightly, from 78:22 to 82:18 of 0.002 M phosphoric acid/acetonitrile, to be able to separate ciprofloxacin from peaks of unknown impurities/metabolites in the chromatograms of pretreated sample extracts. Aliquots of 20 µl were injected onto the column.

Clean-up by Solid Phase Extraction

Serum: Spiked fish serum was prepared by addition of working standard of EF. Extraction column packed with 0.1 g C2 sorbent material was conditioned with two column volumes of methanol and three column volumes of phosphoric acid (0.002 M). The column was loaded with phosphoric acid (0.1 M, 300 µl), serum (250 µl), 100 µl difloxacin (1 µg/ml), and distilled water (250 µl). The mixture was sucked through at 3 psi. The column was rinsed with distilled water (100 µl) and phosphoric acid (0.1 M, 100 µl), then eluted with five portions of 100 µl phosphoric acid (0.5 M)/methanol, 3:7 v/v. The collected eluates were diluted to 1.0 ml with distilled water.

Tissue. Spiked sample (1-3 g) of minced muscle (or liver) with internal standard (3.5 µg difloxacin) added was homogenized in 10 ml of a mixture of sodium hydroxide (1 M) and acetone (1:10, v/v). After centrifugation (3000 rpm, 3 min) the supernatant was collected. The solid residue was blended twice more in a mixture of 1 ml sodium hydroxide and 5 ml acetone. The collected supernatants were extracted twice with 10 ml hexane, which was discarded.

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Residues of acetone and hexane in the aqueous phase were evaporated off in a rotary evaporator (50°C). The water phase was transferred to a centrifuge tube, acidified with phosphoric acid (0.1 M, 5 ml) and extracted once more with hexane. Sodium chloride(s, 2 g) was applied and the water phase was extracted once more with hexane (10 ml). The organic phases were discarded. Column packed with 0.2 g C18 sorbent material was activated in the same way as the C2 column for serum pretreatment. The aqueous tissue extract was applied onto the column and slowly sucked through (3 psi). The column was washed with distilled water (300 µl) and H_3PO_4 (1 M, 200 µl) and eluted with five portions of 500 µl methanol/phosphoric acid (1 M), 9:1 v/v. The collected eluates were diluted with distilled water to 10 ml. A portion of the eluate was centrifuged using Spin-X™ filter unit.

Validation of the Extraction Methods

The precision, recovery and linearity of the extraction methods were determined by analyses of spiked serum and muscle in the concentration range 10-400 ng/ml and ng/g, respectively.

The spiked samples were extracted using the above procedures. Each concentration level was assayed in triplicate. The analyses of spiked samples were compared with those of standard solution to calculate recovery rates.

RESULTS AND DISCUSSION

Chromatographic System

Enrofloxacin, ciprofloxacin and the internal standard, difloxacin, show strong fluorescence in acidic aqueous solutions. The intensity of the fluorescence appeared constant in the pH-range 2.0-2.8. The pretreated samples after elution from the SPE-column and dilution had a pH ≈ 2.0-2.2, whereas the pH of the mobile phase was 2.8.

The UV absorption of EF was also determined and appeared to be approximately 1/20 of the intensity of the fluorescence of the molecule.

The chromatographic system that had appeared efficient for analysis of some other derivatives of quinolone carboxylic acid, was applied with minor modifications (9,10).

Sample Pretreatment of Serum

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Our previous study (9) on sample clean-up of plasma spiked with flumequine and oxolinic acid showed that solid phase extraction columns were efficient for sample pretreatment and good recovery was obtained. EF which is soluble in both alkaline and acidic solutions, has a polarity similar to that of flumequine. Non-polar sorbent materials such as C18, C8, C2, CH and PH were tested and acceptable recoveries were obtained for EF in serum pretreated on C2 and CH columns. Also extraction by use of C8 columns gave acceptable recovery, but the precision was poor, 10-12%. EF showed good affinity to the C18 sorbent material and was therefore difficult to elute by use of strong acidic eluting agent. The recovery was approx. 70%.

Based on these preliminary studies further optimization of the washing and elution steps were performed on the C2 column containing 0.1 g sorbent material. Washing with water and 0.1 M H_3PO_4 prior to elution gave "clean" extracts. As eluting agents were tested various mixtures of methanol and chloroform, respectively, with acids such as formic acid, glacial acetic acid and phosphoric acid, respectively. The eluting solvents chloroform/formic acid (7:3, v/v) and methanol/acetic acid (1:1, v/v) gave ≈100 % recovery. It appeared, however, necessary to reduce the acidity of the eluate before injection onto the analytical column. The eluate therefore had to be evaporated to dryness in a stream of nitrogen gas and reconstituted in the mobile phase. Various combinations of methanol and phosphoric acid were also tested and elution with five portions of 100 µl 30% H_3PO_4 (0.5 M) in methanol give 96-100 % recovery of EF. The evaporation step appeared to be redundant and the precision of the analyses varied from 5 to 9% (Table 1).

TABLE 1

Recovery of Enrofloxacin, Difloxacin and Ciprofloxacin from Serum and Tissues of Fish.

Sample	No of samples	Amount EF added (ng/g or ml)	Recovery (%)					
			EF		DF		CF	
			Mean	SD*	Mean	SD*	Mean	SD*
Serum	8	10	100	9.0	99	5.2	88	8.6
"	9	200	96	5.1	99	5.2	90	5.1
Muscle, 3 g	8	10	98	5.6	94	4.9		
"	8	400	97	4.8	98	4.3		
Liver, 3 g	8	10	99	5.8	96	3.2		
"	8	400	96	6.3	93	6.1		

*SD - relative standard deviation.

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The recovery by stepwise elution was examined to determine the optimal elution volume. The results demonstrated that 95.0 % of both EF and DF were eluted in the first three eluates of 100 µl. When applying five portions of 100 µl approx. 100 % was eluted of both analytes. Standard curves based on peak height measurements were linear in the concentration range 10-500 ng/ml of EF in serum (r = 0.9998). The limit of determination was 1 ng/ml. The recovery of CF was also studied by the standard addition method and approx. 88-90% was eluted from the SPE-column. Chromatograms of drug-free fish serum and serum from fish treated with enrofloxacin are shown in Fig. 1. The latter chromatogram showed a peak, RRT=0.74 (retention time relative to EF) not present in that of drug-free serum, which might be a metabolite. The serum concentrations in treated fish are quoted in Table 2.

CF was added to extracts of serum samples from Atlantic salmon medicated with EF. The chromatographic peak of CF (RRT=0.80) appeared to be well separated from the unknown peak at RRT=0.74. The analyses showed that CF, being the de-ethylated compound of EF, does not occur in serum sampled on the sixth, ninth and twelfth day after start of medication. Tyczkowska et al. (8) pointed out this metabolic step in animal species such as the dog.

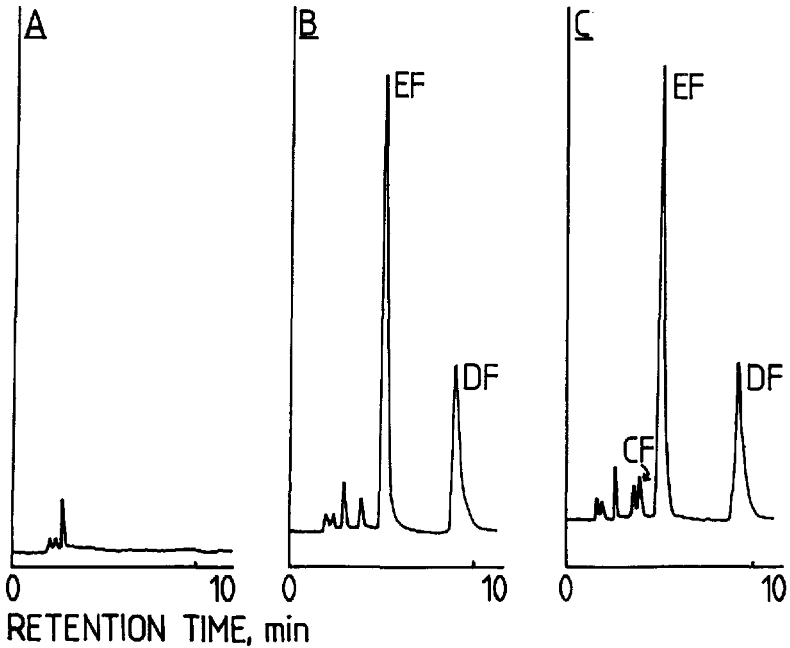

FIGURE 1

Chromatograms of extracts from fish serum and muscle cleaned on SPE-columns.
A - drug-free serum, B - serum from fish treated with enrofloxacin (EF), internal standard difloxacin (DF) added, C - serum from treated fish, ciprofloxacin (CF) and internal standard (DF) added.

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TABLE 2

Serum and Muscle Concentration of Enrofloxacin in Atlantic Salmon. Dose 10 mg EF/kg Body Weight for Ten Days. Sea Temperature 6°C.

Sample	Time after start of medication (days)	Concentration (ng/ml or g)		
		Number of samples	Range	Average
Serum	6	2	440 - 1110	775
Serum	9	2	391 - 629	510
Serum	12	2	359 - 523	441
Muscle	20	4	69 - 116	84
Muscle	30	6	3 - 17	7

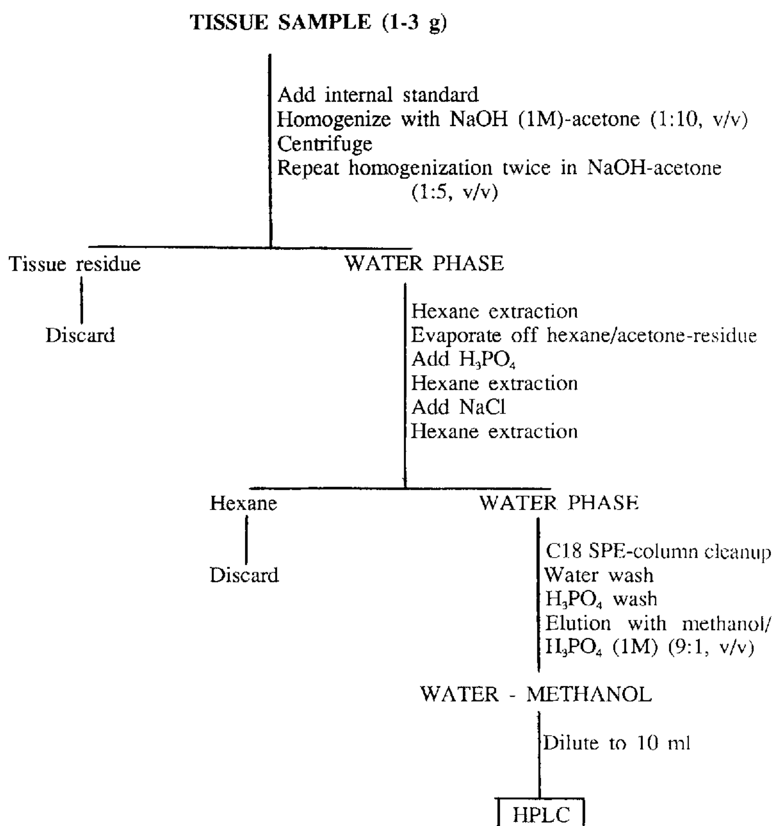

FIGURE 2

Extraction and Clean-up Procedure for Enrofloxacin (EF) from Fish Tissue

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Sample Pretreatment of Muscle and Liver Tissues

The pretreatment procedure of fish tissues is shown in Fig. 2. Tissue samples were homogenized in a mixture of sodium hydroxide and acetone, and hexane was added to remove lipids and other impurities. The extraction procedure is identical to that applied for determination of the two quinolone derivatives oxolinic acid and flumequine in fish tissues. After acidifying the collected waterphases further washing with hexane appeared necessary and to avoid gelatinization solid sodium chloride had to be added.

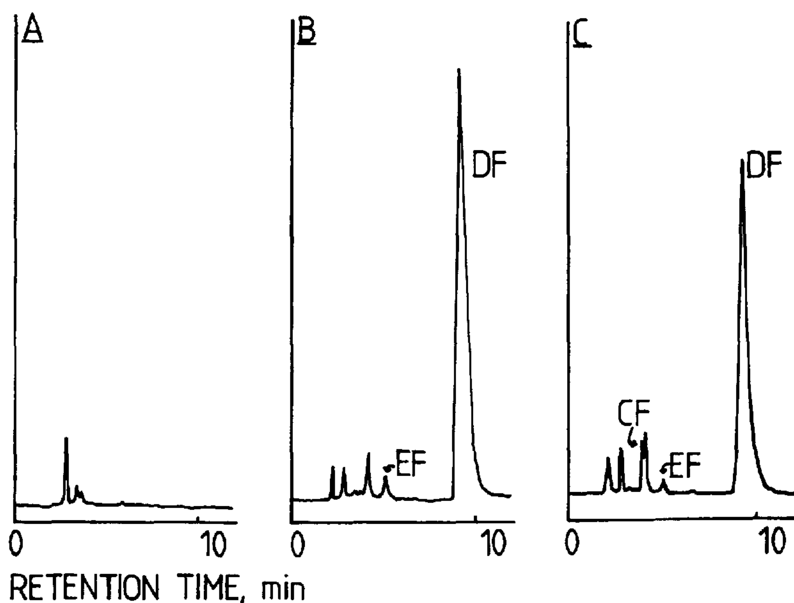

FIGURE 3

A - drug-free muscle, B - muscle from fish treated with enrofloxacin (EF), internal standard difloxacin (DF) added, C - muscle from treated fish, ciprofloxacin (CF) and internal standard (DF) added.

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Further clean-up was based on application of SPE-columns, and our experiences from sample pretreatment of serum were applied. Good recovery was obtained by use of C2 columns. However, leakage of the analyte was observed when loading 400 ng or more of EF per g tissue onto a column of 0.1 g sorbent material. An increase of the amount of sorbent material to 0.3-0.4 g gave optimal recovery.

This experience demonstrates that interaction between sorbent material and tissue matrix is of significance and influence the affinity between analyte and sorbent. It was considered worthwhile to evaluate other non-polar sorbent materials. C18 and C8 were tested, and acceptable recoveries were obtained on both column materials. The optimal quantity of sorbent material was studied, and only 0.2 g of C18 material had to be applied in the column. Based on these results the C18 column was chosen for clean-up of tissue extracts for determination of EF.

The washing and eluting conditions of the column that were developed for serum, were also tested for tissue samples. However, the eluting agent had to be changed from phosphoric acid (0.5M)/ methanol, 3:7 v/v to phosphoric acid (1M)/methanol, 1:9 v/v to obtain good recovery.

It was found a recovery in the range 93-99% of both drugs (EF and DF) in spiked muscle and liver, respectively (Table 1). The coefficients of variation were satisfactory, i.e. 3.2-6.3%. The linearity of the standard curves based on peak height measurements was tested in the range 10-400 ng/g, r = 0.9998. The sensitivity of the method appeared to be good with a limit of determination equal to 1 ng/g. Chromatograms of extracts from drug-free muscle tissue and tissue from treated fish are shown in Fig. 3. The concentrations of EF in muscle tissue of salmon sampled on day 20 and 30 after start of medication are listed in Table 2.

The chromatograms of muscle extracts from treated fish showed a slightly different peak profile than the serum samples. A peak having relative retention time (RRT=0.83, relative to EF) almost identical to that of CF in a standard solution (RRT=0.80) was observed in chromatograms of muscle extracts from treated salmon. CF was added to the sample extracts and it was observed that the unidentified peak separated from that of CF (Fig. 3). When using a mobile phase of 82% phosphoric acid and 18% acetonitrile the two peaks overlapped, whereas the best separation was obtained using a mixture of 78:22 of the two solvents.

The results indicate different metabolites in serum and muscle, respectively, after treatment of salmon with EF, when sampled at different points of time. CF has not been detected which indicates that the metabolism of EF in fish may be different from that in animal species such as the dog and the canine.

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CONCLUSIONS

This study has shown that the antibacterial drug enrofloxacin in Atlantic salmon can be determined by HPLC equipped with fluorescence detection after extraction of serum and tissue, respectively, and further clean-up by solid phase extraction. Good recovery, precision and sensitivity of the assays make them particularly applicable in the drug residue control work. Unknown metabolites were detected in serum and tissue from salmon treated with EF when applying the same method, and CF does not seem to be a metabolite of EF in Atlantic salmon as in other animal species.

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