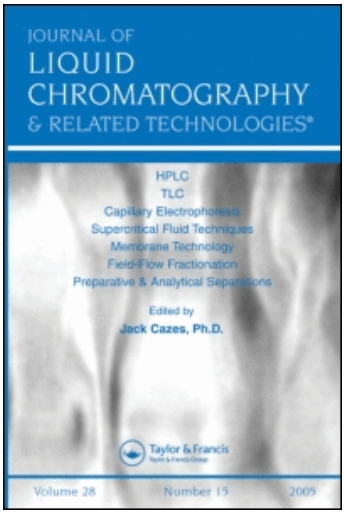

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DETERMINATION OF SARAFLOXACIN RESIDUES IN GILTHEAD SEABREAM (*SPARUS AURATA* L.) TISSUES BY HPLC-SFD-PDA

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ABSTRACT

The aim of this study was to develop a validated analytical method for sarafloxacin residues determination in tissues of cultured fish gilthead seabream (*Sparus aurata* L.), an antimicrobial chemical compound of fluoroquinolone family. Tissue samples were extracted with acidified absolute ethanol and cleaned up on SPE cation exchange mini-column. Sarafloxacin was analysed on a ZORBAX SB-C18 column at a temperature of 60°C, with the

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mobile phase being 30% acetonitrile–methanol (3 + 2 v/v) – 70% trifluoroacetic acid 0.1% (pH 2.15), delivered isocratically. Detection was performed using a Scanning Fluorescence Detector (SFD) with excitation at 278 nm and emission at 450 nm. The mean validated recovery was 82.1 ± 4.2% (RSD = 5.1%) in muscle plus skin and ranged from 76.6% to 78.68% in the other tissues. The limits of detection (LOD) and quantification (LOQ) were 1 μg/kg and 5 μg/kg, respectively, in all tissues examined.

INTRODUCTION

Fluoroquinolones, and in particular 6-fluorinated piperazinyl derivatives, have been shown to be very active with antibacterial activity approaching 1,000 times that of nalidixic acid. They are not only highly active against gram-negative bacteria but also moderately active against gram-positive bacteria.(1) The addition of either fluorine or a piperazino moiety or both, to the basic quinolone molecule enhances the overall antibacterial activity.(2) They act by inhibiting DNA-gyrase, a key enzyme in DNA replication, which is the essential topoisomerase that is found in all bacteria and it is also the target of potent antibiotics such as the quinolones. Also, by creating DNA lesions and inducing the bacterial SOS response, these drugs are not only highly cytotoxic but also mutagenic.(3–5) Although several fluoroquinolones are available, many more are being developed.

Today there is a strict legislative framework controlling the use of these substances, with the aim of minimizing the risk to human health associated with their residues consumption. Sarafloxacin has been proposed for use in the drinking water of poultry to treat bacterial diseases, and in fish feed to treat diseases such as furunculosis, vibriosis, and enteric redmouth. The recommended dose rate is 10 mg/kg b.w., administered in the feed as the hydrochloride for 5 consecutive days, and is the only one currently approved for salmonidae in the USA and EU with a Maximum Residue Limit (MRL) of 30 μg/kg in the target tissue of muscle plus skin in natural proportions.(6)

A review of the literature revealed several methods for chromatographic determination of sarafloxacin in other fish species (7–11), but no method for gilthead seabream (*Sparus aurata* L.). Most of them are based on HPLC with various pre-treatment procedures to achieve better and cleaner extracts. Moreover, the very long run time with gradient mode (7) for better resolution between sarafloxacin and matrix peaks, was undesirable for a regulatory routine method.

This study describes a high performance liquid chromatographic method for sarafloxacin residue determination in tissues of cultured gilthead seabream (*Sparus aurata* L.) by fluorescence and UV/vis detection (HPLC-SFD-PDA).

EXPERIMENTAL

Reagents and Chemicals

HPLC grade methanol, acetonitrile, and n-hexane p.a. were obtained from LabScan (UK). Absolute ethanol (0.05% water) was obtained from J. T. Baker (Holland). Ammonium hydroxide 29.1% from Sigma (USA) and trifluoroacetic acid and glacial acetic acid 100% from Merck (Germany). HPLC grade water was from Ultrapure Water RiOs™-Milli-Q® system, Millipore (USA). The extracting solution used was ethanol–water–acetic acid 100% (98 + 1 + 1 v/v/v), the SPE equilibrating solution consisted of the extracting solution-1% acetic acid (35 + 20 v/v). The eluting solution was ammonium hydroxide 29.1%-methanol (1 + 4 v/v) which is stable and active for 12–24 hours at 25°C.

Mobile phase filters type HV 0.45 μm and FH 0.5 μm and syringe filters ∅13 mm Millex® GV Hydrophilic PVDF 0.22 μm were from Millipore (USA). SPE mini columns Bond Elut® LRC, propylsulfonic acid (PRS) bonded phase, 500 mg, 10 mL volume, and Bond Elut Reservoirs w/Frit 75 mL with 20 μm pore polyethylene frit were from Varian (USA).

The analytical standard of sarafloxacin hydrochloride (Figure 1), 99.5% (Lot No: 23-336-CE) was provided by Abbott Laboratories (North Chicago Illinois). Stock solution of 0.2 g/L was prepared in HPLC methanol using vortex mixer and sonication and stored in the refrigerator in amber volumetric flask for two months. Working standard solutions were prepared from the stock solution by diluting aliquots in the mobile phase to obtain concentrations of 2 μg/L, 5 μg/L, 10 μg/L, 20 μg/L, 40 μg/L, 80 μg/L, 160 μg/L, and 320 μg/L. Spiking solutions were prepared in methanol from the stock solution to obtain fortification levels of 10 ng/g, 20 ng/g, 40 ng/g, 60 ng/g and 120 ng/g.

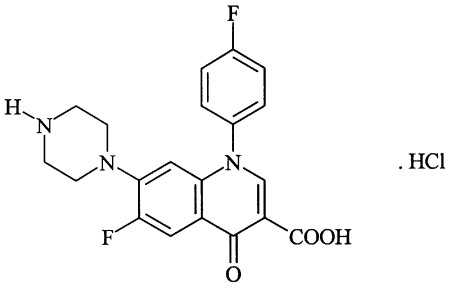

Figure 1. Chemical structure of sarafloxacin hydrochloride.

Apparatus and Chromatographic Conditions

The apparatuses used were homogenizer Mulinette S (Mulinex, Italy), aggregator model PCU Polytron-Aggregate® (Kinematica AG, Switzerland), vortex mixer Gennie 2 (Scientific Industries, USA), ultrasonic bath Transsonic 460 (Elma, Germany), reacti-therm heating module (Pierce, UK), centrifuge model 102B-K-UT (Runne, Germany), analytical balance model AE240 (Mettler, Switzerland), ultrapure water RiOs™-Milli-Q® (Millipore, USA), SPE manifold (Millipore, USA), pH meter mode PHM210 (Radiometer, Denmark), and automated pipettes (Gilson, France).

The liquid chromatographic system used was the ALLIANCE 2690 MX, Revision 1.21 Separation Module, (Waters) equipped with a Scanning Fluorescence Detector 474 and a UV/vis Photodiode Array Detector 991 (Waters). Injections were performed automatically on a ZORBAX® SB-C18, 5 μm (250 × 4.6 mm) stainless steel column (Hewlett Packard) with a guard column Lichrospher RP-select B (Merck) and the volume injected was 20 μL and 50 μL for samples and standards, respectively.

Sarafloxacin detection was performed by fluorescence monitoring with excitation at 278 nm and emission at 450 nm and by UV/vis PDA at 280 nm. Column temperature was set at 60°C. The mobile phase used was 30% acetonitrile–methanol (3 + 2) v/v – 70% trifluoroacetic acid 0.1% (pH adjusted to 2.15) and delivered at a rate of 0.9 mL/min in isocratic mode. Autosample's compartment temperature was adjusted at 15°C. The control of the LC system, data acquisition, and peak integration was performed by the software Millennium^{32} Chromatography Manager (rev.1.21) (Waters, USA).

Sample Preparation

The frozen tissue was kept at room temperature until semi-frozen. Then the tissue was blended until the entire sample became a thick and uniform paste. From this homogenous fish tissue, a test portion (2 g of muscle plus skin and 0.250 g of liver, kidney and vertebra) was accurately weighted in a 22 mL glass vial with a screw cup, and was aggregated with 15 mL of the extracting solution consisting of ethanol–water–acetic acid (98 + 1 + 1 v/v/v), and then extracted for 1 min with vortex and for 2 min with sonication. Centrifugation was followed at 3000 rpm and 10°C and the supernatant was decanted to a 100 mL glass beaker. Another 15 mL of the extraction solution was added to the glass vial and the pellet was re-extracted, collecting the supernatant into the same glass beaker. To the combined extracts, 20 mL of 1% acetic acid were added and the whole mixture was transferred to the reservoir for SPE. After conditioning of the SPE Bond Elut PRS mini column with 2 mL methanol and 2 mL of the SPE

equilibrating solution, with 1% acetic acid (35 + 20 v/v), serving as the extracting solution, tissue extract was passed through the SPE mini column at a flow rate of 2 drops per second.

Three washing steps, with 2 mL methanol, 5 mL ultra pure water, and 2 mL methanol were followed, and excess methanol was removed by vacuum aspiration. Sarafloxacin was eluted by 3 mL freshly prepared eluting solution consisting of ammonium hydroxide 29.1% – methanol (1 + 4 v/v) in a 22 mL glass vial. A volume (5 mL) of hexane was added to the elute and partitioning was effected by mixing for 1 min with vortex mixer. Following centrifugation at 3000 rpm for 10 min at 10°C, the supernatant was aspirated by vacuum and rejected. The final extract was evaporated to dryness with a gentle stream of nitrogen at 55°C and the remaining residue was redissolved in 1 mL of mobile phase by the aid of vortex and sonication. This final extract was passed through a 0.22 μm filter in the auto sampler's vial and a volume of 20 μL was injected on the top of the chromatographic column for the analysis.

Quantification

Fortified and incurred seabream tissue samples were quantified by regression analysis using the external standard calibration curve and by plotting peak areas versus sarafloxacin concentrations from 50 mL injections of working standard solutions.

RESULTS AND DISCUSSION

Chromatography

For the HPLC of sarafloxacin, three columns such as Hypersil BDS-C18, Symmetry C8 and ZORBAX SB-C18 were tested with two mobile phases such as 2% glacial acetic acid–acetonitrile (86 + 16) and 30% acetonitrile–methanol (3 + 2 v/v) – 70% trifluoroacetic acid 0.1% (pH 2.15), described in the literature (7,8). All column systems were usable, but the problems encountered with peak broadening, tailing, and long retention time were overcome by using ZORBAX SB-C18 at a temperature of 60°C, with the mobile phase of 30% acetonitrile–methanol (3 + 2 v/v)/70% trifluoroacetic acid 0.1% (pH 2.15) delivered isocratically, instead of gradient elution described by Meinertz et al. (7). Under the established chromatographic conditions, sarafloxacin eluted at 6.9 min ($Rt = 6.983 \pm 0.008$ with $RSD = 0.11\%$), instead of 21 min retention time of Meinertz's method.

Sarafloxacin was detected by Scanning Fluorescence Detector (SFD) with excitation at 278 nm and emission at 450 nm. The identification and confirmation

was done by UV spectra overlay produced by the PDA detector monitored at $\lambda_{max} \sim 280$ nm, which also contributed to check, peak purity.

A characteristic chromatogram of a sarafloxacin standard solution with the above chromatographic conditions is shown in Figure 2.

Method Validation

The analytical method was validated for linearity, accuracy, precision, sensitivity, and specificity. The linearity of sarafloxacin response was calculated using an external standard calibration curve with 9 points (zero included) and with standard concentrations of 2 μg/kg, 5 μg/L, 10 μg/L, 20 μg/L, 40 μg/L, 80 μg/L, 160 μg/L, and 320 μg/L. The standard curve was linear with $r^2 = 0.999988$, slop–0.006366738, and y-intercept 0.00000134. Accuracy was determined by recovery data from fortified control samples. Recovery for each sample was determined and a mean sarafloxacin recovery for each of the five concentrations of 10 μg/kg, 20 μg/kg, 40 μg/kg, 60 μg/kg, and 120 μg/kg and 6 replicates for each concentration ($n = 6$) was calculated. Mean recovery for over all concentrations was determined. The results are presented in Table 1. A good recovery at all levels investigated and an acceptable standard deviation for repeatability were attained. Recovery experiments were also performed for liver, kidney, and vertebra at fortification levels of 15 μg/kg, 30 μg/kg, and 60 μg/kg and 4 replicates. The results are presented in Table 2. Good recoveries with acceptable standard deviation can be observed. To determine within laboratory day to day variability (inter-assay precision), each concentration of 10 μg/kg, 20 μg/kg, 40 μg/kg, 60 μg/kg, and 120 μg/kg, along with standards and quality samples, were analyzed on five different days.

The overall inter-assay variability was RSD = 4.46%. (Table 1). Intra-assay precision (within day) was determined using six replicate injections of the 40 μg/kg. The intra-assay variability was RSD = 2.35%. (Table 3). In this study, RSD gave values 9.2, 3.9, 2.2, 0.2, 6.8% for 10, 20, 40, 60, and 120 μg/kg, respectively, with a mean value of 4.46%. For sensitivity determination, the chromatographic resolution (Rs = 1.25), the tailing factor (Tf = 1.25) and Signal-to-Noise ratio S/N = 2 : 1 were calculated.

Specificity was determined by the analysis of solvent blanks and tissue blank samples. No matrix associated interferences at the retention time of sarafloxacin and of related compounds, such as oxolinic acid and flumequine were observed. At this point, PDA contributed with its mathematical spectra comparison gained along the sarafloxacin peak, as purity confirmation. The Limit of Detection (LOD) based on a S/N = 2 : 1 was 1 μg/kg and the Limit of Quantification (LOQ) was 5 μg/kg in all tissues examined.

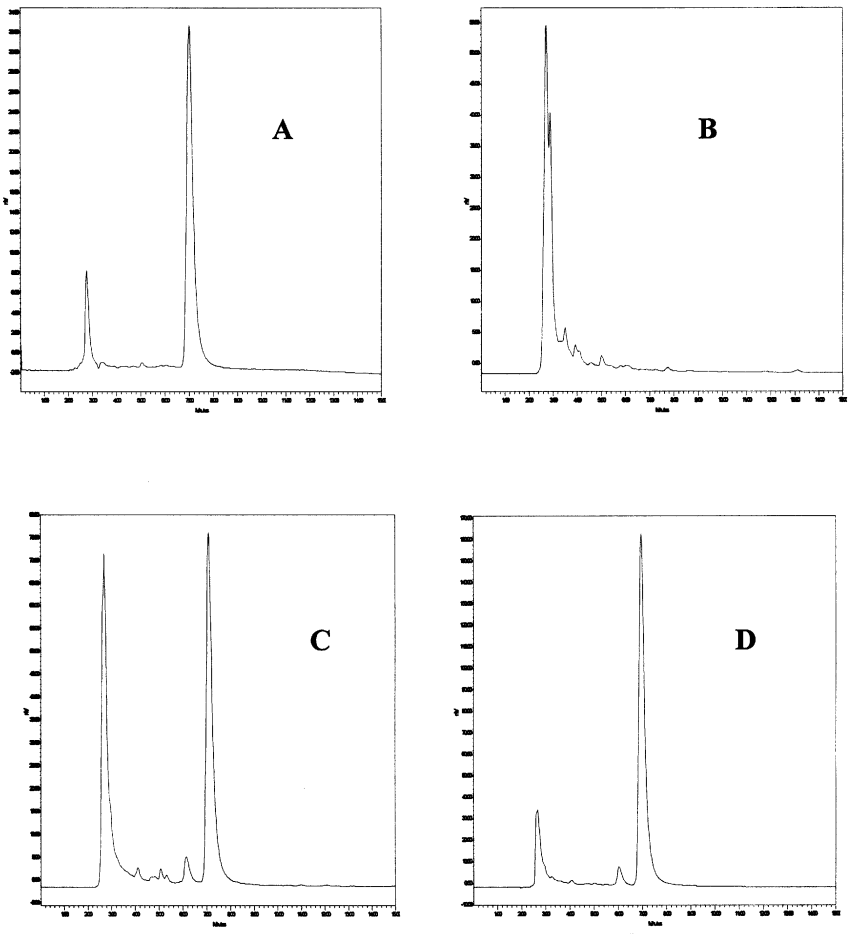

Figure 2. Chromatograms of (A) standard sarafloxacin of 1 ng. (B) a control gilthead seabream muscle + skin sample. (C) a gilthead seabream muscle + skin extract fortified with sarafloxacin at 50 μg/kg. (D) Real incurred muscle + skin sample containing 106.25 μg/kg of sarafloxacin. Chromatographic conditions: column ZORBAX® SB-C18, 5 μm (250 × 4.6 mm); column temperature: 60°C; mobile phase: 30% acetonitrile–methanol (3 + 2) v/v – 70% trifluoroacetic acid 0.1% with the pH adjusted to 2.15; flow rate: 0.9 mL/min; wavelengths: excitation $\lambda_{ex} \sim 278$ nm and emission $\lambda_{em} \sim 450$ nm.

Table 1. Recovery Data and Inter-Assay (Between-Day) Variability for Sarafloxacin in Muscle Tissue plus Skin of Gilthead Seabream (*Sparus aurata* L.)

Fluoroquinolone	Amount Added (μg/kg)	Mean* Amount Found (μg/kg)	Standard Deviation SD	Relative Standard Deviation RSD %	Recovery %
Sarafloxacin	10	8.7	0.8	9.2	87.0
	20	15.3	0.6	3.9	76.5
	40	31.7	0.7	2.2	79.3
	60	50.9	0.1	0.2	84.8
	120	99.5	6.8	6.8	82.9
Overall inter-assay variability, RSD = 4.46%					
Mean recovery, R% = 82.1 ± 4.2 (RSD = 5.1%)					

*Six (6) replicates.

Sample Preparation

It is known that ion exchange extraction is the most selective extraction mechanism, which yields the cleanest extracts (12). Since examination of the sarafloxacin structure indicated that cation exchange might be an appropriate means of cleanup, the propylsulfonic acid (PRS) solid phase extraction (SPE) clean-up procedure, proposed by the Laboratory Information Bulletin method No. 4046 of the US FDA (8), was tried with some modifications.

In order to optimise the extraction yield, the extraction duration was increased from 10–20 sec to 3 min, while the extracting solvent was decreased from 18 to 15 mL. Moreover, the extraction was performed in two steps, firstly by aggregation and vortex mixing with a solvent volume of 15 mL of the extracting solution and secondly, by sonication. In addition, SPE eluting volume was optimally increased to 3 mL and it has to be prepared just before the elution, because it was found that it is active and stable for 12–24 hours at 25°C. Also, a partitioning step with 5 mL of n-hexane was added to the cleanup procedure, to remove the nonpolar compounds of the column elute.

Sarafloxacin, as a basic quinolone and a zwitterion with pKa (predicted) of the acid side of 6.15 and that of basic side of 10.17 (13), caused unstable recoveries, which were overcome by following, exactly, the validated SPE steps. Following these steps, such as SPE flow rate (2 drops/sec), pump strength (550 mm Hg), the pH of the SPE equilibrating solvent (pH 3.5) for retention to occur, and the freshly prepared eluting solvent (1 + 4 v/v) at a high pH (pH = 12)

Table 2. Recovery Data for Sarafloxacin in Liver, Kidney, and Vertebra of Gilthead Seabream (*Sparus aurata* L.)

Fluoroquinolone	Tissues	Amount Added (μg/kg)	Mean* Amount Found (μg/kg)	Standard Deviation SD	Relative Standard Deviation RSD %	Recovery %
Sarafloxacin	Liver	15	12.23	0.68	5.66	81.25
		30	23.63	0.78	3.3	78.75
		60	46.46	1.42	3.06	77.44
	Kidney	15	12.5	0.54	4.32	82.91
		30	22.42	1.23	5.49	73.96
		60	47.5	2.61	5.49	79.17
	Vertebra	15	11.3	0.75	6.6	76.66
		30	22.0	1.67	7.6	73.24
		60	47.94	2.49	5.19	79.9
Liver		Mean Recovery, R% = 79.15 ± 1.9 [RSD = 2.4%]				
Kidney		Mean Recovery, R% = 78.68 ± 4.5 [RSD = 5.72%]				
Vertebra		Mean Recovery, R% = 76.6 ± 3.33 [RSD = 4.35%]				

*Four (4) replicates.

Table 3. Intra-Assay (Within-Day) Variability for Sarafloxacin in Muscle Tissue plus Skin of Gilthead Seabream (*Sparus aurata* L.)

Fluoroquinolone	Amount Added (μg/kg)	Amount Found (μg/kg)	Standard Deviation of the Mean SD	Relative Standard Deviation RSD %
Sarafloxacin	40	34.81	0.841	2.35
	40	35.47		
	40	36.19		
	40	37.16		
	40	36.19		
	40	35.25		

to neutralize the charge of isolate, sarafloxacin was always quantitatively eluted from the PRS sorbent.

In addition, it is essential to mention that, during the whole procedure, direct day and artificial light should be avoided as much as possible because of sarafloxacin photosensitivity.(14) It was found, that this property resulted in a small peak in front of the main sarafloxacin peak.

Real Samples

An experiment was also undertaken to obtain real samples and to test the applicability of the described method. Sarafloxacin hydrochloride was incorporated into feed and administered orally to a few gilthead seabreams with a mean weight of 163 ± 21 g. They were fed for 5 days at the therapeutic dose of 10 mg/kg b.w./day and a sea water temperature of $25.19 \pm 1.4^{\circ}$C. Immediately after the end of the treatment, three fish were sacrificed and muscle plus skin, liver, kidney, and vertebra were sampled and analyzed with the method described. The levels of the incurred sarafloxacin residues in muscle plus skin individual samples and liver, kidney, and vertebra pooled samples of the three treated seabreams, are presented in Table 4. Characteristic chromatograms of a sarafloxacin standard, a blank, a spiked, and a real sample are shown in Figure 2. An unknown peak observed before the sarafloxacin peak in some of the incurred samples, could possibly be a metabolite of sarafloxacin.

In conclusion the method is suitable to be used, with safety and accuracy, for the control of sarafloxacin residues in cultured seabreams, and a trained analyst could carry out, ready for chromatography, 16–20 samples per working day.

Table 4. Tissue Levels (μg/kg) of Sarafloxacin Residues in Three Individual Gilthead Seabream (*Sparus aurata* L.) After Oral Administration*

Fish	Drug	Muscle + Skin	Liver[a]	Kidney[a]	Vertebra[a]
1	Sarafloxacin	178.08	335.2	180.1	83.8
2		214.08			
3		181.98			

*10 mg/kg. b.w./day for 5 days at 25.19 ± 1.4°C.
[a] Pooled samples.

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