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Short communication

High-performance liquid chromatographic method with fluorescence detection for the screening and quantification of oxolinic acid, flumequine and sarafloxacin in fish

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Abstract

A previously published liquid chromatographic method for determining residues of nine quinolones in chicken, porcine, bovine and ovine muscle was adapted and applied to fish tissue for simultaneous determination of three quinolones (flumequine, oxolinic acid and sarafloxacin). The analytes were extracted from homogenised muscle using an acetonitrile basic solution. After centrifugation, partial evaporation and cleaning with hexane, direct injection was possible. Separation was achieved on PLRP-S column and detection was performed with a programmable fluorescence detector. Chromatographic conditions were optimised to be compatible with the determination of the three quinolones in a single run. The linearity, recovery, accuracy and precision of the method were evaluated from fortified tissue samples at concentration levels ranging from 15 to 120 μg kg^{-1} for sarafloxacin and 75 to 600 μg kg^{-1} for oxolinic acid and flumequine according to the EU maximum residue limit of each quinolone. The limits of detection were estimated to be 2, 5 and 7 μg kg^{-1}, respectively, for sarafloxacin, oxolinic acid and flumequine. The limits of quantification were validated at 15 μg kg^{-1} for sarafloxacin and 75 μg kg^{-1} for oxolinic acid and flumequine. Mean extraction recoveries of quinolones in fish ranged from 56.9 to 71.0%. This simple and rapid method is suitable for residue control.
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Keywords: Oxolinic acid; Flumequine; Sarafloxacin

1. Introduction

Quinolones are often used in livestock and fish farm industries because of their high potency against Gram-negative bacteria through inhibition of bacterial DNA-gyrase. There is now a strict legislative framework controlling the use of such substances, with the aim of minimising the risk to human health associated with their residue consumption. Therefore, to ensure human food safety the European Union has set maximum residue limits (MRL) for these compounds. Recently the MRL has been changed for flumequine. The MRL in fish muscle with a naturally occurring of skin is presently fixed to 600 μg kg^{-1} for flumequine, 300 μg kg^{-1} for oxolinic acid and 30 μg kg^{-1} for sarafloxacin.

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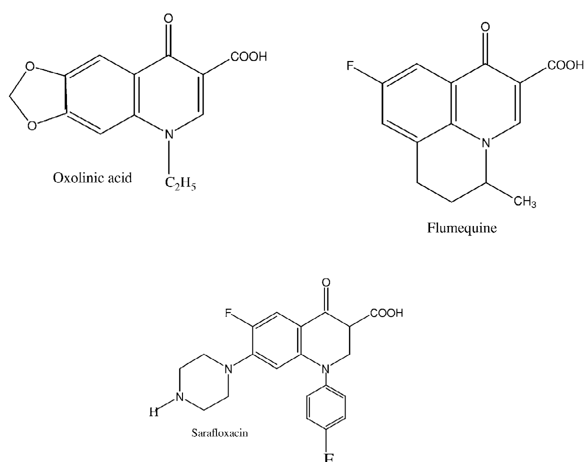

Fig. 1. Chemical structures of oxolinic acid, flumequine and sarafloxacine.

Many HPLC methods (monoresidue or multiresidue) with fluorescence detection have been published for the determination of quinolones in fish tissue [1–9]. None of the published methods described the simultaneous determination of the three quinolones (flumequine, oxolinic acid and sarafloxacin) in fish tissues.

A multiresidue method with three chromatographic runs has been developed for monitoring quinolones (ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, flumequine, marbofloxacin, nalidixic acid, oxolinic acid, and sarafloxacin) in animal muscles [10] in our laboratory. The aim of this study was to apply this methodology to fish sample and to include sarafloxacin with pyridonecarboxylic acid quinolones (Fig. 1) for the determination, in a single run, of the three quinolones at the MRL level.

2. Experimental

The extraction procedure used to extract the quinolone residues from fish tissues (salmon and trout) is essentially the same as the extraction developed for the previous method [10] with a few modifications. A more detailed description of reagents, apparatus and extraction procedure was previously reported [10]. An abbreviated description of the extraction procedure follows.

2.1. Standard solutions

Stock solutions at 0.75 g l^{-1} were prepared by dissolving each reference compound with a solution of methanol sodium hydroxide 0.001 mol l^{-1} in amber volumetric flasks. The stock solutions were stable for at least 3 months when stored at +4 °C. An intermediate standard solution (75 μg ml^{-1}) was prepared by diluting stock solution of sarafloxacin with the buffer pH 9.0. Another intermediate standard solution was prepared by diluting individual stock solution of oxolinic acid and flumequine and the intermediate standard solution of sarafloxacin with the buffer pH 9.0. The working solutions (600, 300, 150 and 75 μg l^{-1} for oxolinic acid and flumequine; 120, 60, 30 and 15 μg l^{-1} for sarafloxacin) obtained by dilution with the buffer pH 9.0 were stable for at least 2 weeks when stored at +4 °C.

2.2. Chromatographic apparatus

The chromatographic system consisted of a ThermoQuest CLHP P4000 pump (Les Ulis, France), a ThermoQuest autosampler, with a 200-μl injection loop and a fluorescent detector model 821-FP Jasco obtained from Prolabo (Nogent sur Marne, France). The analytical column, maintained at 50 °C, was a commercial polymeric PLRP-S column (150×4.6 mm, 5 μm, 100 Å) obtained from Polymer Laboratories (Marseille, France) equipped with a precolumn packed with RP18E, 4×4 mm (Merck, Darmstadt, Germany). A linear gradient of acetonitrile in 0.02 mol l^{-1} phosphoric acid, pH 2.2 was used: 20–45% acetonitrile (12 min), then 45% acetonitrile (5 min), 45–20% acetonitrile (2 min) and 20% acetonitrile (3 min) before the next injection. The flow-rate was 0.8 ml min^{-1}. The fluorescence detector was programmed as indicated in Table 1. Data were acquired with a PC1000 computer data system through a SP4510 ThermoQuest interface (Les Ulis, France).

2.3. Analytical method

2.3.1. Extraction procedure

Salmon or trout muscle was thawed and minced with a Moulinette mixer. Then 0.50±0.01 g of

Table 1
Wavelengths program for the fluorescent detection of the quinolones

Time (min)	Excitation wavelength (nm)	Emission wavelength (nm)
0	280	450
8.5	320	380
14	320	360

thawed muscle was weighed accurately into a 2-ml microcentrifuge tube into which 300 μl of buffer solution pH 9.0 (250 μl in case of fortified samples) were added. Sample was vortexed for 1 min and left to stand for 15 min. Then 200 μl of acetonitrile were added and muscle was pulverised during 20 s with an ultrasonic probe (power 60 W, frequency 20 kHz). The probe was rinsed with 2×400 μl of acetonitrile and the solvent was collected into the microcentrifuge tube. The homogenate was vortexed for 1 min and centrifuged for 3 min at 17 000 *g* and +4 °C. The supernatant was transferred to a microcentrifuge tube and evaporated under a gentle stream of nitrogen at 50 °C until the volume was less than 500 μl. Buffer solution pH 9.0 was added until the total content weight was 500 mg. The extract was washed with 300 μl of hexane, vortexed for 20 s and centrifuged for 3 min at 17 000 *g*. Then 400 μl of the aqueous phase were transferred into the HPLC vial and 100 μl injected into the chromatographic system.

2.3.2. Validation of the analytical method

The linearity of the detector response was checked by analysing four series standard solutions with four calibration points ranging in concentration from 15 to 120 μg l^{-1} for sarafloxacin, and from 75 to 600 μg l^{-1} for oxolinic acid and flumequine. Calibration curves were prepared by plotting the peak area versus the analyte concentration. Linearity and goodness of fit were tested by analysis of variance [11]. The linearity of the analytical procedure and goodness of fit were also tested by using fortified salmonidae muscle with known amounts of quinolones (50 μl of the working solutions to 0.50±0.01 g of minced blank tissue) to cover the concentration range: 15, 30, 60 and 120 μg kg^{-1} for sarafloxacin and 75, 150, 300 and 600 μg kg^{-1} for oxolinic acid and flumequine. Each level was assayed in three replicates for 4 days. The analyses of fortified samples were compared with those of standard solutions to calculate the extraction recoveries. Dose independence of the recovery was also checked with a Student's *t*-test. Accuracy was estimated by the bias, i.e. the percentage difference between the calculated values and the theoretical concentrations. Intra-day and inter-day repeatability were calculated as described in ISO standard 5725-2. The limits of detection in the HPLC system using the fluorescence detector were determined from 20 representative blank samples, at three times signal-to-noise, as recommended in EC Commission Decision 93/256/EEC. Fortified muscle samples at the MRL level were also prepared for a stability study. They were immediately frozen and stored for more than 6 months at a temperature of −18 °C.

3. Results and discussion

Typical chromatograms of standard solutions, blank, incurred or fortified muscles to a level of 60 μg kg^{-1} for sarafloxacin, 300 μg kg^{-1} for oxolinic acid and flumequine are shown in Fig. 2. The retention times of sarafloxacin, oxolinic acid and flumequine were, respectively, about 6.0, 11.2 and 15.5 min. Quinolones were chromatographically well resolved under gradient conditions. Other quinolones (danofloxacin, difloxacin, enrofloxacin ciprofloxacin, and nalidixic acid) were also tested. Difloxacin was partially resolved from sarafloxacine, likewise nalidixic acid from flumequine.

A defatting step with hexane prior to the HPLC run has been added like for pig muscle. The lack of interferences in the separation suggests a high specificity of the chromatographic method and a good selectivity of the extraction procedure.

The method was validated on fortified salmonidae muscle samples and successfully tested on fortified muscle and skin samples (in the proportion: 90%

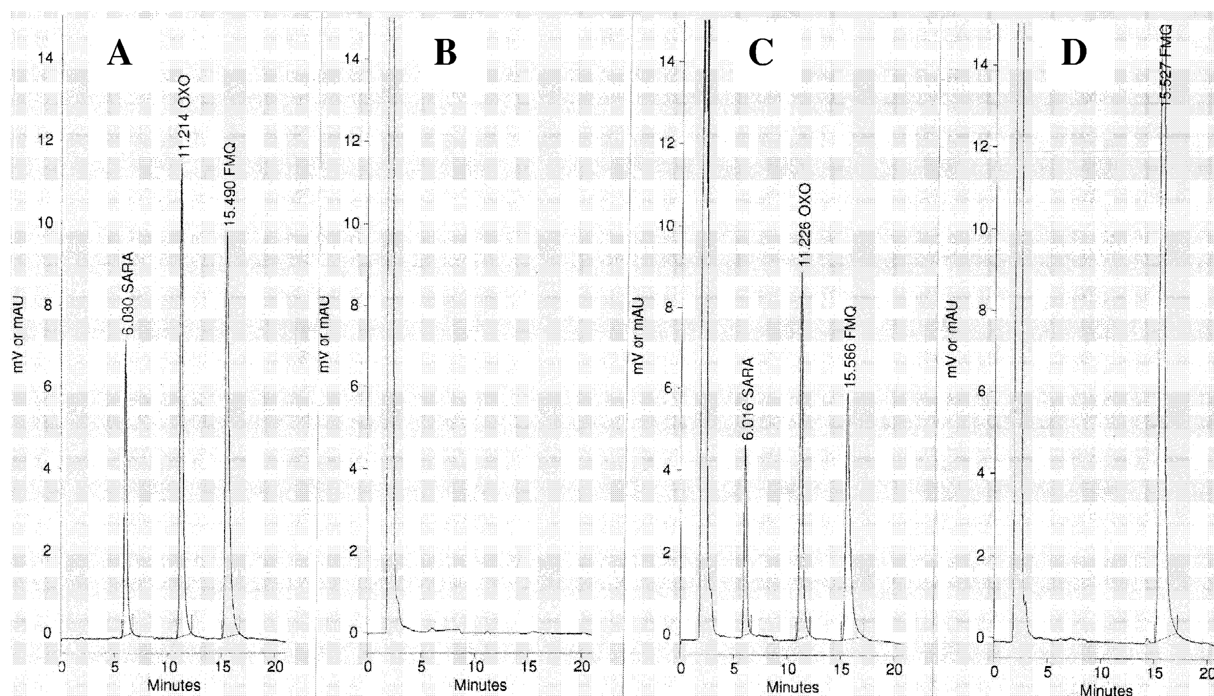

Fig. 2. Chromatograms of the three quinolones obtained with (A) standard solutions containing sarafloxacin (60 μg kg^{-1}), oxolinic acid, and flumequine (300 μg kg^{-1}), (B) blank fish tissue, (C) extract from fish tissue fortified at 60 and 300 μg kg^{-1}, and (D) incurred fish tissue with flumequine (>600 μg kg^{-1}).

muscle, 10% skin). A linearity test and regression analysis were performed for each calibration and matrix curve. The values of the correlation coefficients (>0.990) indicated high correlation between peak areas and quinolone concentrations in the range of concentrations. Moreover, the F-values for the linearity test are statistically significant ($P<0.001$) and the F-values for the lack of fit are not statistically significant indicating a good linearity.

The mean recoveries of quinolones in fish ranged from 56.9 to 71.0% (Table 2). Student's t-test proved that the recoveries are independent of the concentrations. The mean RSDs of the within- and between-day repeatabilities and accuracy (−4.6 to 6.5%) of the method gave results in accordance with EC Commission Decision 93/256/EEC in the range of concentrations. The limits of quantification were validated at 15 μg kg^{-1} for sarafloxacin and 75 μg kg^{-1} for oxolinic acid and flumequine. The limits of detection were estimated to be 2, 5 and 7 μg kg^{-1}, respectively, for sarafloxacin, oxolinic acid and flumequine. Good stability of fortified tissues at −18 °C for 6 months was shown.

This method has been used for monitoring quinolone residues in fish tissues (salmon and trout) before confirmation by a liquid chromatography–mass spectrometry method [12]. It has been transferred successfully as a screening method for quinolones in the French field laboratories. Moreover, this method was applied in a collaborative study for the determination of oxolinic acid in incurred fish.

4. Conclusion

This paper describes an HPLC method for the screening and quantification of quinolone residues in fish tissues. This assay, which has been designed to achieve a high throughput of samples with a short time for the preparation step, could be used for screening purposes.

Table 2
Recovery, accuracy and precision of the method for the three quinolones analysed in fish muscle

Analyte	Level (μg/kg)	Recovery (%)[a]		Accuracy (%)	RSD_r[b] (%)	RSD_R[c] (%)
		Mean	S.D			
Sarafloxacin	15	63.5	7.4	6.5	12.5	12.5
	30	58.2	4.5	−2.5	7.9	7.9
	60	56.9	5.6	−4.6	9.8	9.9
	120	60.0	7.3	0.7	6.2	13.1
Oxolinic acid	75	70.4	7.0	1.5	10.3	10.3
	150	68.4	4.6	−1.4	6.8	6.8
	300	67.7	5.8	−2.4	8.1	8.7
	600	71.0	7.4	2.3	5.6	11.2
Flumequine	75	69.1	7.3	5.3	9.3	10.9
	150	63.4	5.4	−3.3	6.3	8.8
	300	63.4	5.5	−3.3	8.7	8.7
	600	66.4	6.6	1.3	5.5	10.7

[a] Three replicates were conducted on 4 days for each concentration ($n=12$).
[b] Relative standard deviation of the intra-day repeatability.
[c] Relative standard deviation of the inter-day repeatability.

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