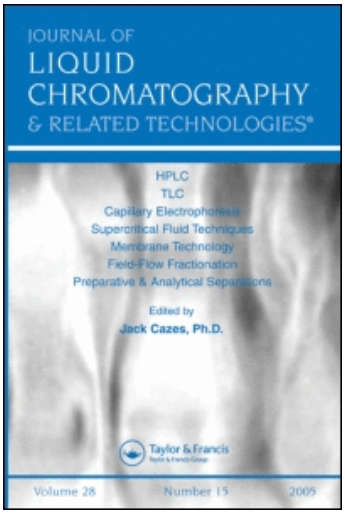

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OXOLINIC ACID AND FLUMEQUINE IN FISH TISSUES: VALIDATION OF AN HPLC METHOD; ANALYSIS OF MEDICATED FISH AND COMMERCIAL FISH SAMPLES

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ABSTRACT

This paper describes a simple method for residue analysis of oxolinic acid and flumequine in fish tissues by HPLC and fluorometric detection. The quinolones were extracted with ethyl acetate, purified with hexane and then analyzed using a RP-8 Lichrosorb reversed phase column. The method was validated with satisfying results. The high sensitivity of the fluorometric detection allowed to reach a quantification limit of 2 µg oxolinic acid and 5 µg flumequine/kg fish tissue and an absolute detection limit of 20 pg and 50 pg respectively. Medicated fish as well as commercial cultured fish samples were analyzed with the described method. Oxolinic acid and flumequine were detected in both of them.

INTRODUCTION

During the last decades commercial aquaculture has been developed and widely extended. For the prevention and the treatment of infectious diseases of cultured fish chemotherapeutic agents are used. Oxolinic acid and flumequine are synthetic antibacterial drugs, structurally related to each other and belonging to the group of quinolones. They are very active against gram-negative microorganisms even at low concentrations and frequently used by fish farmers. The antimicrobial activity of quinolones, their efficacy and toxicity (1) as well as pharmacokinetics and bioavailability of flumequine and oxolinic acid in Atlantic Salmon were studied (2).
Recently I. Steffenak and al.(3) showed that "oxolinic acid and flumequine seem to be especially entrapped in bone..... This reservoir seems to act as a depot from which the drug is slowly released into other tissues." Both drugs are extensively used in Europe. Neither EEC reglementation nor legislation do exist. The proposed waiting time between the end of a treatment and the moment when the fishes are killed may not be less than 6 days if oxolinic acid is used and 3 days if flumequine is used as drug (4). To protect the consumers health, we were interested to know, whether or not, residues of oxolinic acid and flumequine may be found in commercial fish tissues. Several methods using HPLC were published concerning the determination of oxolinic acid and flumequine alone or simultaneously with other antibacterials (5-10). Simple analytical methods with high sensitivity are necessary to detect residues. Lyse Larocque and al. (11) determined oxolinic acid in salmon muscle tissue using a very simple and rapid extraction procedure. We extended this method for the extraction of flumequine as well and worked out new chromatographic conditions suitable for both quinolones.

EXPERIMENTAL

Apparatus

A Stomacher Lab-Blender 80 from L.E.D. Techno (Belgium) was used for the extraction procedure. Centrifugations were achieved with a centrifuge GLC-2B from Sorvall (Dupont, USA). Ethyl acetate was evaporated using a Reacti-Therm heating module from Pierce (Illinois, USA) and nitrogen. A Vortex super mixer from Lab-Line Instruments (Illinois, USA) was used for the purification procedure. HPLC analyses were performed with a 5000 liquid chromatograph from Varian (USA). Oxolinic acid and flumequine were detected with an LS-4 fluorescence spectrometer from Perkin-Elmer (USA). The chromatograms were registered with a recorder A 41 from Ankersmit (The Netherlands) (paper speed: 2 mm/min).

Solvents and reagents

Ethyl acetate, acetonitrile and hexane were delivered by Labscan (Ireland), sodium sulphate anhydrous by UCB (Belgium) and oxalic acid G.R. by Merck (Germany). For the extraction of oxolinic acid and flumequine an oxalic acid solution 0.01 M, pH 3 was prepared. The eluent for the HPLC analyses consisted of acetonitrile and a solution of oxalic acid 0.025 M, pH 3.2 (32 + 68; v/v), both were filtered and degassed by helium before use.

Standards and standard solutions

Oxolinic acid (5-ethyl-5,8-dihydro-8-oxo-1,3-dioxolo[4,5-g]quinoline-7-carboxylic acid) (figure 1) and flumequine (9-fluoro-6,7-dihydro-5-methyl-1-

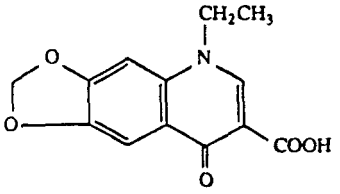

FIGURE 1. Oxolinic acid

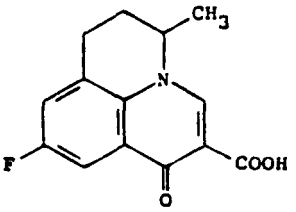

FIGURE 2. Flumequine

oxo-1H,5H-benzo[ij]quinolizine-2-carboxylic acid) (figure 2) were supplied by Sigma (USA). The standard stock solutions contained 1 mg/ml NaOH 0.1 M. The working standard solution contained both quinolones, each at a concentration of 1 µg/ml water.

Sample preparation

Oxolinic acid and flumequine were extracted from 2 g minced fish tissue, dried by 2 g sodium sulphate anhydrous, with 24 ml ethyl acetate in a plastic bag by means of a Stomacher Lab-Blender 80 during 1 minute. The mixture was poured into a 100 ml glass tube and centrifuged at 2000 rpm during 5 minutes. The solvent was transferred into a small glass tube

and evaporated under a stream of nitrogen at 45 °C. The residue was rinced with 5 ml ethyl acetate, centrifuged and the solvent was added to the first portion of solvent to be evaporated. An oily residue of approximately 0.5 ml was left to which were added successively: 2 ml 0.01 M oxalic acid, pH 3 and 2 ml hexane. After each addition the tube was shaken vigourously on a Vortex during 1 minute. The mixture was then centrifuged at 2000 rpm during 5 minutes and the upper organic layer discarded. The lower aqueous layer was the final extract from which a 100 µl portion was injected into the liquid chromatograph.

Chromatography

The liquid chromatograph was fitted with a 5 µm RP-8 Lichrosorb reversed phase column (125 mm x 4 mm, cat. Nr. 50432) from Merck (Germany). The flow rate was 1 ml/minute, the analyses were performed at room temperature. Oxolinic acid and flumequine were detected with a fluorescence detector (excitation wavelength: 327 nm; emission wavelength: 369 nm; slits: 15 and 20; fixed scale: 2 or 5).

RESULTS AND DISCUSSION

Validation of the HPLC - method

The described method was validated using cultured rainbow trout tissues. For the extraction procedure we used a Stomacher Lab-Blender 80 which homogenizes very well sample, solvent and sodium sulphate, a required condition to extract efficaciously residues. For the HPLC analyses we have chosen a 5 µm RP-8 Lichrosorb reversed phase column and a mixture of acetonitrile and oxalic acid 0.025 M, pH 3.2 (32 + 68; v/v) as

eluent. A very good elution and separation of both quinolones could be achieved. The analyses of oxolinic acid and flumequine standards resulted in a linear response within a range of 1 to 100 ng. The correlation coefficients were 0.999 for both quinolones. Table 1 summarizes the recoveries, standard deviations and coefficients of variation of oxolinic acid and flumequine. The recoveries were checked on spiked rainbow-trout tissues (50, 25, 12.5 and 6.25 µg of each quinolone/kg tissue). Each result is the mean of 5 extractions. Even 6.25 µg/kg gave satisfying results with maximum recoveries of 89 % for oxolinic acid and 67 % for flumequine. This allowed us to fix the quantification limit at 2 µg oxolinic acid and 5 µg flumequine/kg fish tissue. The absolute detection limit was 20 pg for oxolinic acid and 50 pg for flumequine (fixed scale: 20), which is shown in figure 3 A. A typical chromatogram of a standard solution is shown in figure 3 B, a

TABLE 1

Recovery Data of Oxolinic Acid and Flumequine in Fish by HPLC and Fluorometric Detection

Added drug	Added quantity (µg/kg)	Mean recovery (n=5) (µg/kg)	Standard deviation	Coefficient of variation (%)	Recovery (%)
Oxol. acid	50.00	41.33	2.21	5.35	82.67
Flumequine	50.00	34.88	2.43	6.97	69.77
Oxol. acid	25.00	19.04	1.08	5.70	76.17
Flumequine	25.00	13.32	0.93	7.02	53.27
Oxol. acid	12.50	8.74	0.83	9.50	69.95
Flumequine	12.50	6.36	0.83	13.11	50.85
Oxol. acid	6.25	5.81	0.17	2.84	89.43
Flumequine	6.25	4.41	0.07	1.51	67.86

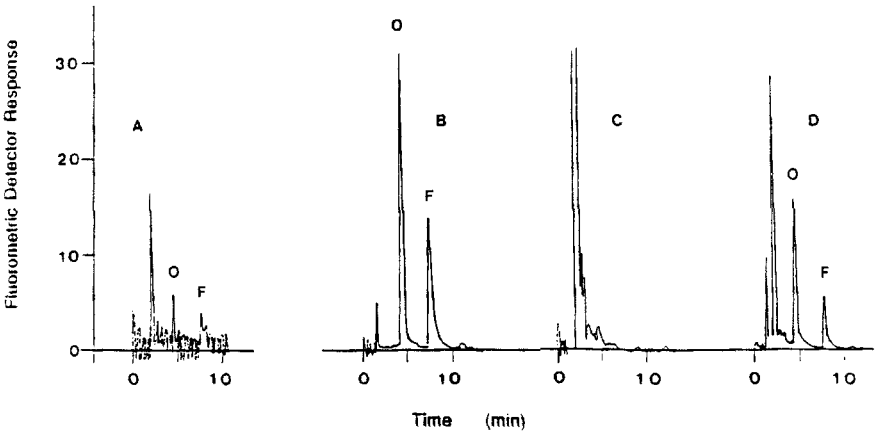

FIGURE 3. Chromatograms of Oxolinic Acid (O) and Flumequine (F). -A. Absolute Detection Limit (20 pg Oxolinic Acid. 50 pg Flumequine); -B. Standard Solution (12.5 ng Oxolinic Acid and 12.5 ng Flumequine); -C. Blank Fish Tissue Sample; -D. Spiked Fish Tissue Sample (12.5 µg Oxolinic Acid and 12.5 µg Flumequine/kg Fish Tissue); Injection Volume: 100 µl; Fixed Scale of the Fluorescence Spectrometer: 20 (A), 5 (B,C,D).

blank fish tissue sample in figure 3 C and a spiked fish tissue sample in figure 3 D. The retention time for oxolinic acid was 4.5 minutes and for flumequine 7.5 minutes under the described LC conditions.

Other quinolones are used as well for the prevention and the treatment of infectious diseases of cultured fish, such as enrofloxacin, sarafloxacin and difloxacin. We did not include their analysis in this method, since their detection wavelengths are different. Their extraction procedures have to be adapted as well, except for difloxacin, for which we obtained very good results, but at an excitation wavelength of 278 nm and an emission wavelength of 440 nm.

Analysis of medicated fish and commercial fish samples

13 rainbow trouts were treated with flumequine (12 mg/kg weight/day) (4), 3 other-ones were fed without any additive and were used as blank

samples. The trouts (> 50 g) were hold in a pool with constant temperature (10 - 11 °C) and a flow of 6 litres water/minute approximately. Flumequine was mixed to the feed and distributed automatically during 12 hours a day. The quantity of feed was 1 % of fish weight. The trouts were treated during 6 days and then killed successively, 4 trouts 1 day after the end of the treatment, 2 trouts 4 days after it, 4 trouts 6 days after it and 3 trouts 7 days after it. The trout tissues were analyzed using the experimental conditions described. The results are given in table 2. Each result is the mean of (n) analyzed trouts. These results are similar to those obtained by I. Steffenak and al.(3). Flumequine is very slowly eliminated and it seems to be clear that the proposed waiting time of 3 days after the end of the treatment with flumequine is far to short to get commercial fish free of residues.

The method has been applied to the analysis of commercial samples of different fish tissues coming from different fish farms. 84 samples have been analyzed until now. 2 samples contained flumequine at a concentration of 46 and 75 µg/kg fish tissue. 4 samples contained oxolinic acid ranging from 17 µg/kg to 1.1 mg/kg fish tissue.

TABLE 2

Recovery Data of Flumequine in medicated Rainbow Trouts

Days after end of medication	Concentration of flumequine (µg/kg fish tissue)
1	20 (n=4)
4	8.75 (n=2)
6	2.81 (n=4)
7	1.41 (n=3)

CONCLUSION

The developed method has proved to be suitable as a routine analysis method for the detection of residues of oxolinic acid and flumequine in fish tissues. More than 20 samples can be analyzed a day. Fishes were medicated with flumequine. Considering the fact that these fishes contained still flumequine 7 days after the end of the medication as well as commercial cultured fishes contained residues of flumequine and oxolinic acid, it seems to be necessary to study more precisely the waiting time after the end of a medication and the moment when the fishes are free of residues. This study has to cover flumequine and oxolinic acid. Modern analytical methods, such as HPLC and fluorometric detection, are very sensitive and able to detect low residue levels of drugs. Maximum residue levels (MRL) may be adapted to it.

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