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Note

Determination of flumequine in fish by high-performance liquid chromatography and fluorescence detection

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With the development of fish farms several antibiotics and antibacterials have been used for the prevention and treatment of various infectious diseases in fish. Concern has arisen about the presence of drug residues in fish tissues, and the need for rapid, simple and sensitive analytical methods of determining them has increased. Flumequine (fluoro-6,7-dihydro-5-methyl-1-oxo-1*H*,5*H*-benzo[*ij*]quinolizine-2-carboxylic acid) has been used against furunculosis [1,2] and, in Norway, for superficial skin infections in salmon and cod caused by *Flexibacter* sp. [3]. Harrison et al. [4] and Decolin and Nicholas [5] reported high-performance liquid chromatographic (HPLC) methods for determination of flumequine in human plasma and urine, but no HPLC method for the determination of flumequine in fish has been reported. Although flumequine is chemically similar to oxolinic acid, none of the methods reported for oxolinic acid also worked well for flumequine. The methods were either tedious and insufficiently sensitive, or interfering substances occurred in the chromatogram [6,7]. This paper describes a simple and rapid HPLC method for the determination of flumequine in fish, using fluorimetric detection and oxolinic acid as an internal standard.

EXPERIMENTAL

Chemicals and reagents

Flumequine and oxolinic acid were kindly supplied by Prof. E. Egidius (Institute of Marine Research, Bergen, Norway). Methanol, acetonitrile (HPLC

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grade), dichloromethane (DCM), tetrahydrofuran (THF) (p.a. grade), citric acid, sodium carbonate, sodium hydrogencarbonate and disodium hydrogenphosphate dihydrate were all from Merck (Darmstadt, F.R.G.).

Chromatography

The HPLC system used consisted of a Spectra-Physics SP 8800 ternary HPLC pump (San Jose, CA, U.S.A.) connected to a Spectra-Physics SP 8780 XR autosampler and a Shimadzu RF-535 fluorescence detector (Kyoto, Japan) operating at an excitation wavelength of 324 nm and an emission wavelength of 363 nm. The integrator was the Model SP-4270 from Spectra-Physics. The analytical column (100 mm × 5 mm I.D.) was packed with 3-μm ODS-Hypersil (Shandon Southern Products, Astmoore, Runcorn, U.K.) in our laboratory using a Shandon column-packing machine. A short pre-column was operated at room temperature. The mobile phase was citric acid (0.1 *M*, pH 3.2)–methanol–acetonitrile–THF (60:30:5:5). The flow-rate was 1 ml/min and the pressure ca. 16.5 MPa.

Standards

Stock solutions of flumequine and oxolinic acid were prepared at a concentration of 1 mg/ml in acetonitrile and stored in the dark at −20°C. Working standards were prepared by dilution from the stock solutions.

Sample preparation

The edible muscle tissues of Atlantic salmon (*Salmo salar*) served as samples. To 2.0 g sample, 0.2 μl of oxolinic acid dissolved in 100 μl of acetonitrile was added as internal standard prior to homogenization with two 20-ml portions of McIlvaine buffer (pH 3.6)–methanol (55:45) at high speed for 1 min, and centrifugation for 10 min at 7996 *g* in a Sorwall RC-5B refrigerated superspeed centrifuge (Du Pont Instruments, Newtown, CT, U.S.A.). The combined supernatants were evaporated under reduced pressure at a temperature of 40°C. Evaporation was halted when ca. 15 ml of solution remained in the flask. Using 5 ml of McIlvaine buffer (pH 3.6) for washing, the flask contents were transferred to a separating funnel already containing 25 ml of DCM. The funnel was shaken gently for ca. 60 s. The two layers were allowed to separate and the DCM fraction was transferred to a new separating funnel and rinsed with 10 ml of McIlvaine buffer. The DCM phase containing the drugs was transferred in portions to a small beaker and evaporated to dryness, using a water-bath at 40°C and a stream of nitrogen. The residue was dissolved in 250

μl of 0.1 *M* sodium carbonate–0.1 *M* sodium hydrogencarbonate buffer (pH 9.0, 5:95), and centrifuged for 3 min at 17 400 *g* in a Biofuge A centrifuge (Heraeus Sepatech, Osterode am Harz, F.R.G.). This solution (10 μl) was used for HPLC.

Calibration

Standard calibration curves for flumequine in the range 0.01–0.05 μg/g were prepared in four replicates using drug-free muscle tissue and oxolinic acid as internal standard. Standard curves were drawn by plotting the known flumequine concentrations against the peak-height ratio of flumequine to internal standard. This curve was also used to calculate the detection limit.

Recovery and precision studies

The extraction recoveries of flumequine and oxolinic acid were determined by comparing peak heights from the analysis of tissue samples spiked with 0.05 μg/g flumequine and 0.1 μg/g oxolinic acid with peak heights resulting from direct injection of the standards.

To determine the within-run precision, five replicates of a muscle tissue sample spiked with 0.05 μg/g flumequine and 0.1 μg/oxolinic acid were analysed.

RESULTS AND DISCUSSION

It is known that oxolinic and nalidixic acid, which have similar chemical structures to flumequine, give broad or tailing peaks in reversed-phase chromatography. Neither methylation [8] nor paired-ion techniques [7,9,10] gave suitable results with respect to separation or sensitivity for residual analysis. However, both Horie et al. [6] and Hamamoto [11] achieved good results using a Kaiseisorb LC-ODS 300-5 column (Tokyo Kasei Kogyo, Tokyo, Japan) and a Nucleosil C_8 5-μm column (Macherey-Nagel, Düren, F.R.G.), respectively. In the present study, an ODS-Hypersil 3-μm column (Shandon Southern Products) was compared with a Spherisorb Hexyl 5-μm column (Chrompack, Middelburg, The Netherlands): the former gave both more ef-

TABLE I

ACCURACY AND PRECISION OF THE HPLC ASSAY FOR FLUMEQUINE IN FISH MUSCLE

Concentration added (ng/g)	Concentration found (mean ± S.D., $n=5$) (ng/g)
25	22.1 ± 1.7
50	42.9 ± 0.9

ficient separation of the chromatographic peaks of interest, as well as less tailing.

The mobile phase initially chosen had been employed earlier for a similar analysis [11]. This proved to be unsatisfactory. Subsequently several mobile phases were tested. The one that gave the best results was 0.1 *M* citric acid (pH 3.2)–methanol–acetonitrile–THF (60:30:5:5). The THF prevented tailing of the interesting peaks, and the peak shapes were therefore markedly improved.

Fluorometry was chosen because of its sensitivity and specificity. UV detection was tried at several wavelengths but the sensitivity was either too low or interfering peaks occurred in the chromatogram. The best sensitivity on the fluorescence detector was achieved by using an excitation wavelength of 323 nm and an emission wavelength of 363 nm.

Several mixtures of methanol and McIlvaine buffer were tried for extraction. Too high a methanol content gave interfering peaks, and an increasing buffer content lowered the recovery.

A linear calibration graph for flumequine in salmon muscle tissue was obtained from at least 10 to 50 ng/g ($y=0.49x+0.01$; $r=0.997$), and the limit of detection was calculated to be 5 ng/g with a signal-to-noise ratio of 4. The recovery of the drugs from five samples spiked with 50 ng/g flumequine and 100 ng/g oxolinic acid were 83.5–87.1% and 89.5–92.6%, respectively. The within-run coefficient of variation of the flumequine peak height was 2.8% and that of oxolinic acid 1.2%. The accuracy and precision of the method were determined for two concentrations (Table I).

Fig. 1 shows typical chromatograms for muscle tissue (A), muscle tissue

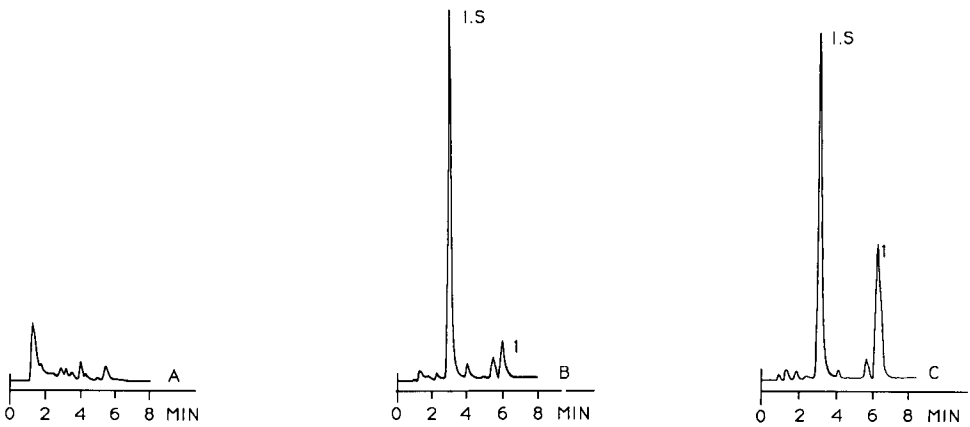

Fig. 1. Chromatograms of Atlantic salmon muscle tissue. (A) Untreated muscle sample; (B) muscle sample spiked with 0.025 μg/g flumequine and 0.1 μg/g oxolinic acid as internal standard; (C) muscle sample from fish treated with flumequine (12 mg/kg fish per day) for ten days. Peaks: I.S. = internal standard; 1 = flumequine.

spiked with flumequine and the internal standard (B) and muscle tissue from a fish treated with flumequine (C). No interfering peaks were present in the area where flumequine appeared, and in most cases baseline separation from endogenous compounds was achieved. An interfering peak was observed in the area of oxolinic acid, but it is too small to have any disturbing effect on the peak height.

In conclusion, the HPLC method described is simple and sensitive and therefore suitable for pharmacokinetic and residue studies on flumequine in fish muscle.

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