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Note

High-performance liquid chromatographic determination of furazolidone in Atlantic salmon (*Salmo salar*) tissue

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Certain antibacterial nitrofurans are used in clinical and veterinary medicine in many countries. One of these, furazolidone, [N-(5-nitro-2-furfurylidene)]-3-amino-2-oxazolidone (Fig. 1), is a widely used growth promotor and antimicrobial feed additive for disease control in poultry, swine and other animals [1]. In Japan, this nitrofuran is also used in fish breeding, to stimulate growth and to prevent infection [2]. In Norwegian aquaculture, furazolidone is strictly used as a therapeutic antibacterial agent. Many nitrofurans are mutagenic [3,4] and some are carcinogenic [5,6]. Furazolidone was found to be mutagenic in *Escherichia coli* WP2 [3], *Salmonella typhimurium* TA100 [7] and *Drosophila* [8].

In order to ascertain safe withdrawal periods and to monitor residues in fish foods, a sensitive assay for detecting furazolidone is needed. Bioassays are most often used for determination of residual furazolidone in fish liver. However, their precision appears to be variable and the specificity is poor. When apply-

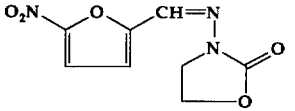

Fig. 1. Structure of furazolidone.

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ing microbiological tests, there is always a possibility for non-specific inhibition zones. Several high-performance liquid chromatographic (HPLC) methods for the quantitative determination of furazolidone residues in feed, animal tissues and plasma have been described in the literature [9–13]. Horie et al. [14] described an HPLC method for the determination of furazolidone in rainbow-trout using a Sep-Pak C_{18} cartridge for the clean-up step. However, the limit of detection was 0.05 μg/g and the recovery 69.7 ± 4%. The method reported here is capable of detecting 0.005 μg/g furazolidone in both muscle and liver tissue of Atlantic salmon (*Salmo salar*) and gave recoveries of 94.5 ± 5.1% for muscle and 94.2 ± 3.2% for liver tissue at the 0.1 μg/g level.

EXPERIMENTAL

Chemicals and reagents

Furazolidone (99% pure) was supplied by Norsk Medisinaldepot (Bergen, Norway). Methanol, acetonitrile (HPLC grade), dichloromethane, *n*-hexane, tetrahydrofuran, chloroform, citric acid monohydrate, Na_2EDTA dihydrate, nitric acid, potassium nitrate, disodium hydrogenphosphate dihydrate and sodium chloride (all p.a. grade) were from Merck (Darmstadt, F.R.G.). The Bond Elut NH_2 and C_{18} columns (100 mg) were supplied by Analytichem International (Harbor City, CA, U.S.A.).

Chromatography

The HPLC system 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 Spectra-Physics SP-8480 XR UV detector operating at 400 nm and with the range set to 0.001. The integrator was a Model SP-4270 from Spectra-Physics. The analytical column used was operated at room temperature, had dimensions of 100 mm × 5 mm I.D. and was packed with ODS-Hypersil (3 μm particle size, Shandon Southern Products, Cheshire, U.K.) in our laboratory using a Shandon column-packing machine. A short pre-column (25 mm × 4.8 mm I.D.) packed with C_{18} pellicular material (40 μm particle size) was used. The mobile phase was acetonitrile–water (16:84, v/v) containing 0.001 *M* Na_2EDTA and 0.1 *M* potassium nitrate, and was adjusted to pH 3.2 with diluted nitric acid [15]. The flow-rate was 1.5 ml/min and the pressure ca. 16.4 MPa.

Standards

A stock solution of furazolidone was 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 solution.

Sample preparation

Furazolidone is light-sensitive. Samples must not be exposed to fluorescent light or direct sunlight. All samples were processed in subdued incandescent light throughout the procedure and kept frozen when not in use. Liver and the edible muscle tissue of *S. salar* served as samples. To 2.0 g of sample, 5 g of sodium chloride were added prior to homogenization with two 20-ml portions of McIlvaine buffer (pH 3.6)–methanol (70:30, v/v) at high speed for 1 min and centrifugation for 10 min at 7996 *g* in a Sorvall RC-5B refrigerated super-speed centrifuge (DuPont Instruments, Newtown, CT, U.S.A.). The combined supernatants were evaporated under reduced pressure at 40°C until ca. 15 ml of the solution remained. Using 5 ml of McIlvaine buffer for washing, the flask contents were transferred to a separating funnel already containing 25 ml of dichloromethane. The funnel was shaken gently for ca. 60 s. The two layers were allowed to separate and the dichloromethane fraction was transferred to a new separating funnel and rinsed with a 10-ml portion of the McIlvaine buffer. The dichloromethane phase containing the drug was transferred to a suitable dark bottle and evaporated to 2 ml, using a water-bath at 40°C and a stream of nitrogen. To the 2-ml dichloromethane fraction, 6 ml of *n*-hexane were added, prior to loading on a conditioned Bond Elut NH_2 column.

Clean-up on Bond Elut NH_2 column

The column, 100 mg, was conditioned with 3 ml of tetrahydrofuran and 5 ml of *n*-hexane prior to loading the fish tissue extract. The column was washed with 5 ml of *n*-hexane–dichloromethane (50:50, v/v) and 1 ml of *n*-hexane–chloroform (50:50, v/v) prior to elution of the furazolidone with 1 ml of 15% (v/v) methanol in chloroform. The eluate was evaporated to dryness using a water-bath (40°C) and a stream of nitrogen, and dissolved in 1 ml of McIlvaine buffer and centrifuged for 3 min at 16 900 *g* in a Biofuge A centrifuge (Heraeus Sepatech, Osterode am Harz, F.R.G.). This solution (20 µl) was used for HPLC.

Calibration curves, recovery and precision studies

The calibration curve for furazolidone was prepared in three replicates by spiking muscle and liver tissue (2 g) with standard solutions of furazolidone to yield 0.025, 0.05, 0.1 and 0.2 µg/g furazolidone per sample. The samples were extracted using the above procedure. These curves were also used to calculate the detection limits. The analyses of spiked tissues (0.025 and 0.1 µg/g) were compared with those of standard solutions to calculate recovery values (Table I).

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

TABLE I

RECOVERY OF FURAZOLIDONE FROM FORTIFIED SALMON TISSUE

Tissue	n	Amount (ng/g)	Recovery (mean ± S.D.) (%)
Muscle, 2 g	6	25	95.1 ± 3.3
Muscle, 2 g	6	100	94.5 ± 5.1
Liver, 2 g	6	25	93.7 ± 4.4
Liver, 2 g	6	100	94.2 ± 3.2

RESULTS AND DISCUSSION

The UV spectrum of furazolidone dissolved in the mobile phase showed absorption maxima at 255 and 364 nm. In a preliminary study, Bond Elut C_{18} and NH_2 columns from Analytichem were tested. A standard solution of furazolidone was 100% retained on the C_{18} column from McIlvaine buffer (pH 3.6) when the column had been conditioned with 3 ml of methanol, 5 ml of distilled water and 5 ml of McIlvaine buffer. When the C_{18} column was washed with 5 ml of water and eluted with 2 ml of methanol, the recovery of furazolidone was ca. 89%. When unspiked samples of liver and muscle tissue were extracted using methanol–McIlvaine buffer (30:70, v/v), loaded on a conditioned C_{18} column after evaporation of the methanol fraction and eluted with 2 ml of methanol, the eluate was coloured and seemed impure. The eluate was evaporated to dryness, dissolved in 1 ml of McIlvaine buffer and analysed under the chromatographic conditions described above. The chromatograms of both liver and muscle extract showed interfering peaks when the UV detector was adjusted successively to 255, 364, 385 and 400 nm. Using an NH_2 column as described above, the retention on the column of a standard from the *n*-hexane–dichloromethane (75:25, v/v) mixture and the recovery from the column was ca. 100%, the recovery being enhanced by washing the column with a 1-ml portion of chloroform–*n*-hexane (50:50, v/v) prior to elution. Chromatograms of unspiked muscle and liver samples (detector at 364 nm) showed a very small interfering peak corresponding in peak height to 0.3 ng of furazolidone. With the detector set at 400 nm, this peak disappeared. Several mixtures of methanol–McIlvaine buffer were tried for extraction in an attempt to minimize the small interfering peak at 364 nm. However, decreasing the methanol content stepwise from 30 to 5% (v/v) lowered the extraction recovery from 96 to 85% and the interfering peak was still present. When using 25% (v/v) methanol or less it was necessary to filter the extract, after evaporation of the methanol fraction, even when sodium chloride was added as a precipitating agent. A larger methanol content (50%, v/v) increased the height of the interfering peak at 364 nm and occasionally introduced the peak also at 400

nm. Therefore, to avoid filtration of the extract and to prevent the appearance of the interfering peak, methanol–McIlvaine buffer (30:70, v/v) was chosen as the extracting solvent.

The results of the recovery study are listed in Table I. With a signal-to-noise ratio of 3, the sensitivity of the method was found to be 0.005 μg of furazolidone per gram of both muscle and liver tissue. The calibration curves were linear over the range of 0.025 to 0.2 μg/g ($y=0.72x$ for muscle tissue and $y=0.67x+0.01$ for liver tissue) with correlation coefficients of 0.995 and 0.991, respectively. The limit of detection of standard furazolidone was 0.2 ng. The within-run relative coefficient of variation of the furazolidone peak height was 1.4%.

Fig. 2 shows typical chromatograms of untreated liver tissue, liver tissue spiked with furazolidone and liver tissue from fish treated with furazolidone. No interfering peaks were present in the area where furazolidone appeared. Similar chromatograms were obtained from the muscle tissue analysis.

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

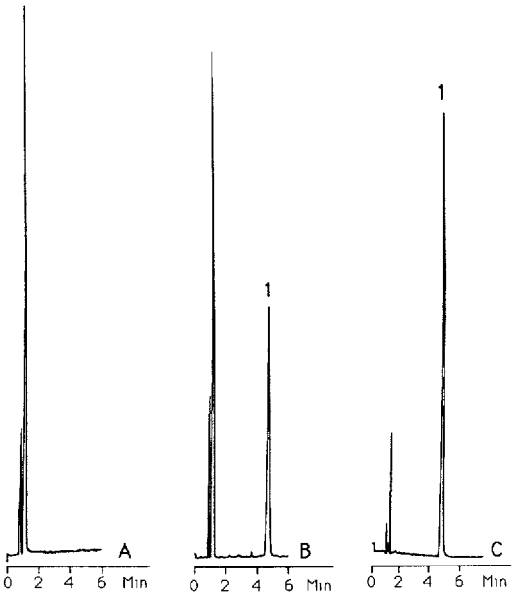

Fig. 2. Chromatograms of *S. salar* liver tissue. (A) Untreated liver sample; (B) liver sample spiked with 0.2 μg of furazolidone per gram of liver; (C) liver sample from fish treated with 75 mg of furazolidone per kg fish per day for ten days. Peak 1 = furazolidone. The integrator sensitivity for chromatograms A and B was $\times 64$ and $\times 128$ for chromatogram C.

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