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Short Communication

Simultaneous determination of residues of florfenicol and the metabolite florfenicol amine in fish tissues by high-performance liquid chromatography

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ABSTRACT

A simple and rapid high-performance liquid chromatographic method for the simultaneous extraction and determination of residues of florfenicol and its metabolite florfenicol amine in fish tissue, muscle and liver has been developed. The calibration curves were linear, the recovery of florfenicol was 99–107%, and the recovery of florfenicol amine was 94–100%. The detection limits for florfenicol and florfenicol amine were 20 ng/g in muscle and 50 ng/g in liver.

INTRODUCTION

Florfenicol (Sch 25298) is a fluorinated derivative of thiamphenicol. Experiments with cattle given florfenicol have shown the major metabolite to be florfenicol amine (Sch 40458) [1]. In addition, the metabolites florfenicol alcohol, florfenicol oxamic acid, monochloroflorfenicol and two unindentified metabolites were detected. Powers *et al.* [2] considered that florfenicol, because of its safety and high degree of efficacy, would become a major drug in veterinary medicine, with special value in food animals. The antibacterial activity of florfenicol against various fish pathogens has been determined by Fukui *et al.* [3], Yasunaga and Tsukahara [4], Yasunaga and Yasumoto [5], Kusuda *et al.* [6], and Inglis *et al.* [7]. The drug has been shown to be effective against a number of fish pathogens, and is therefore of potential value in fish farming.

Varma *et al.* [8] presented a high-performance liquid chromatographic (HPLC) method for the determination of florfenicol in serum and urine from veal calves. Adams *et al.* [9] modified this method to determine florfenicol in tissue samples for pharmacokinetic studies. Nagata and Saeki [10] presented a method for the determination of florfenicol residues in muscle of animals and cultured fish. Though sensitive, the method is time-consuming and requires the use of large volumes of chemicals. Lavy *et al.* [11] determined the concentration of florfenicol in milk and serum from goats by a microbiological assay, in addition to the HPLC method by Adams *et al.* [9].

The purpose of the present study was to devel-

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op an analytical method for the simultaneous determination of residues of florfenicol and its major metabolite florfenicol amine in fish muscle and liver.

EXPERIMENTAL

Materials and reagents

Muscle and liver tissues of both treated and untreated rainbow trout (*Salmo gairdneri*) served as samples.

All chemicals and solvents were of analytical or HPLC grade. Florfenicol (Schering) and florfenicol amine (Schering) were donated by Schering-Plough (Cranford, USA). Thiamphenicol (Sigma) was used as the internal standard (I.S.).

Stock 1 mg/ml standard solutions of florfenicol, florfenicol amine and thiamphenicol were prepared by dissolving each substance in 10 ml of methanol and diluting to volume with water. Working standards were prepared by diluting the stock standards with water.

Chromatographic conditions

The analyses were performed on a Perkin-Elmer HPLC system, consisting of a Series 400 solvent-delivery system, an ISS 100 sampling system equipped with a Lauda RMT6 cooler (14°C) from Messgeräte Werk Lauda (Lauda-Königshafen, Germany), and an LC 235C UV detector (Perkin-Elmer, Norwalk, CT, USA). The detector was operated at 220 nm. The integration was carried out using the software program Omega-2 (Perkin-Elmer) in an Olivetti M 300 PC connected to a Star LC24-10 printer. The analytical column (stainless steel, 25 cm × 4.6 mm I.D.) and guard column (stainless steel, 2.0 cm × 4.6 mm I.D.) were packed with 5-μm particles of Supelcosil-LC-18-DB (Supelco, Bellefonte, PA, USA).

The mobile phase was a mixture of two solutions, A and B (68:32). Solution A was 0.02 *M* heptanesulphonate–0.025 *M* trisodiumphosphate (pH 3.85), made by dissolving 4.45 g/l 1-heptanesulphonic acid sodium salt (Supelco) and 9.5 g/l trisodium phosphate 12-hydrate (Merck) in *ca.* 750 ml of water when making 1 l of the solution. The pH was then adjusted to *ca.* 5.0 with 5 *M* H_3PO_4 and then to pH 3.85 with 1 *M* H_3PO_4, and the solution made up to volume with water. Solution B was methanol containing 0.1% triethylamine. The mobile phase was degassed with helium before use.

The flow-rate was 0.6 ml/min for 14 min followed by 1.0 ml/min for 10 min. The samples were injected at intervals of 25 min. Aliquots of 10 μl were injected into the column for the determination of florfenicol and florfenicol amine.

Sample preparation and clean-up (Fig. 1)

The tissue sample, 3 g of ground muscle or liver, was weighed into a 50-ml centrifuge tube with a screw cap (NUNC, Roskilde, Denmark). Volumes of 100 μl of thiamphenicol (20 μg/ml) and 900 μl of water were added to muscle samples, and 200 μl of thiamphenicol (20 μg/ml) and 800 μl of water to liver samples. Acetone (4 ml) was added, and the sample was whirlimixed for 5 s. It was then left in the extraction fluid for 5 min before being again whirlimixed for 5 s. The homogeneate was then centrifuged for 5 min at 2800 *g*.

A 4-ml volume of the supernatant was pipetted into a graduated glass-stoppered centrifuge tube, and 3 ml of dichloromethane were added. The sample was shaken vigorously for 10 s, and centrifuged for 1 min at 1700 *g*. The upper aqueous layer was discharged, and the lower layer was transferred to another glass-stoppered centrifuge tube. The sample was evaporated under a stream of nitrogen (40°C), and 0.01 *M* Na_2HPO_4 (pH 2.8)–methanol (80:20) was added to the samples (600 μl to muscle samples, and 1200 μl to liver samples). Then 1 ml of hexane was added, and the sample again whirlimixed.. After centrifugation for 10 min, the hexane layer was discharged. The water-based phase was filtered through a Costar$_R$ Spin-X centrifuge filter unit (low type) with 0.22-μm cellulose acetate binding by centrifugation for 1 min at 5600 *g*. Aliquots of 10 μl of the filtrate were injected into the HPLC system.

Calibration curves and recovery studies

The calibration curves for florfenicol and florfenicol amine were obtained by spiking tissue samples (muscle and liver) with standard solu-

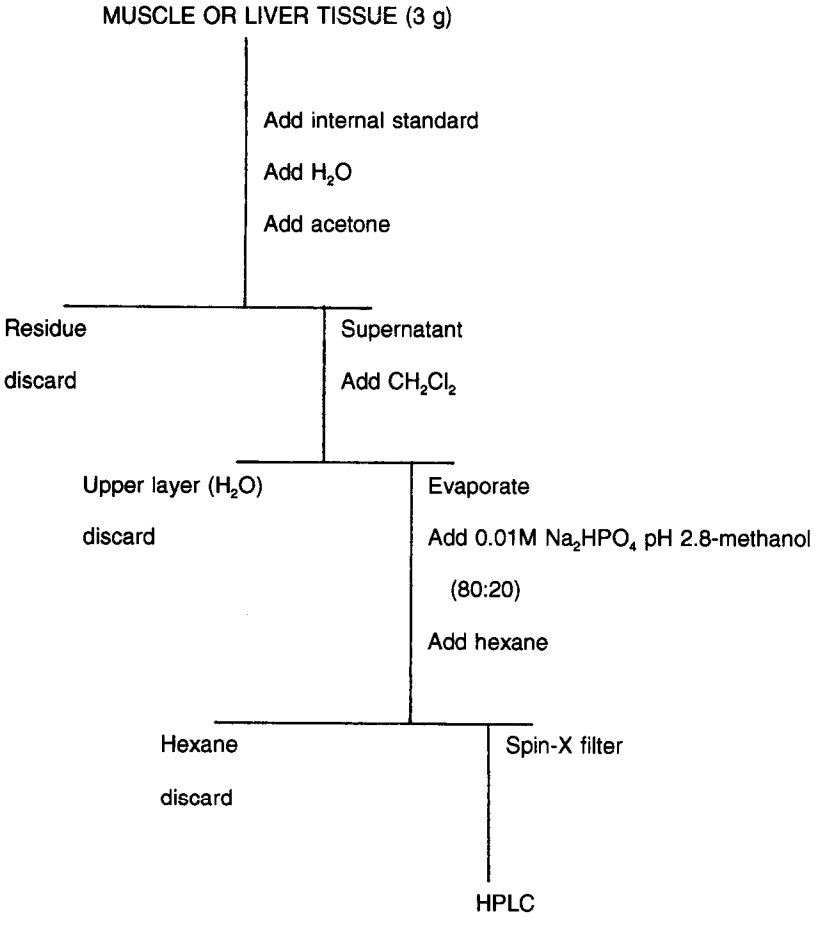

Fig. 1. Extraction and clean-up procedure for florfenicol and florfenicol amine from fish muscle and liver.

tions of florfenicol and florfenicol amine to yield 20, 25, 50, 100, 150, 200, 500, 800, 1000 and 1500 ng/g florfenicol and florfenicol amine in muscle, and 50, 100, 150, 200, 500, 800, 1000 and 1500 ng/g florfenicol and florfenicol amine in liver samples. Duplicate samples were used. The recovery rates were determined by comparing results of analysis of the spiked muscle and liver samples with those of standard solutions.

The linearity of the standard curves for florfenicol and florfenicol amine in muscle and liver was tested using peak-height measurements and the I.S.

RESULTS AND DISCUSSION

Chromatograms of muscle and liver samples and samples spiked with florfenicol and florfenicol amine are shown in Fig. 2. Chromatograms of "real" samples, *i.e.* samples of muscle and liver from fish treated with florfenicol, and therefore containing residues of florfenicol and florfenicol amine, are shown in Fig. 3.

The standard curves were linear in the investigated ranges, 20–1500 ng/g in muscle and 50–1500 ng/g in liver, for florfenicol and florfenicol amine. The correlation coefficients for florfenicol

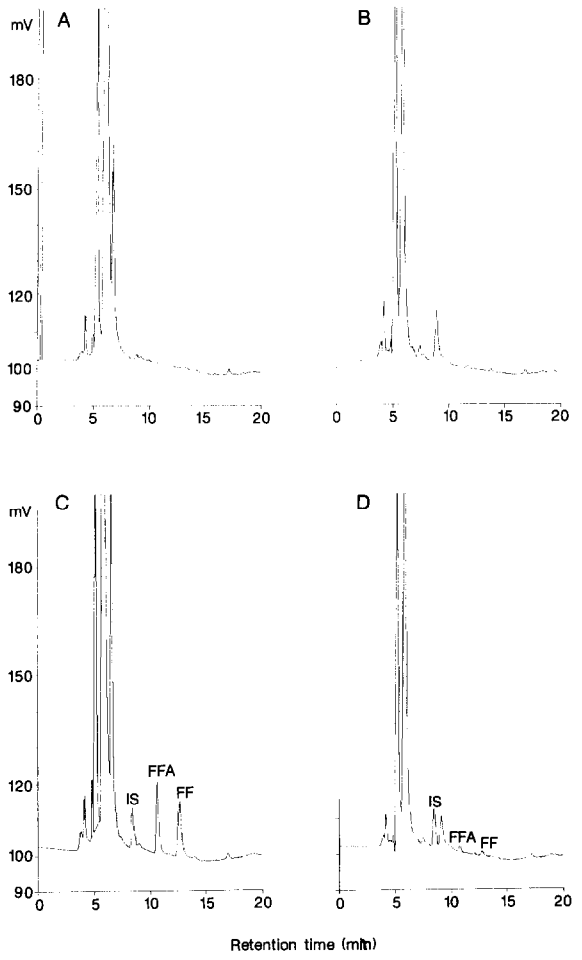

Fig. 2. Chromatograms of extracts from 3 g of muscle and liver for the determination of florfenicol (FF) and florfenicol amine (FFA) with thiamphenicol as internal standard (IS). (A) Unspiked muscle tisue; (B) unspiked liver tissue; (C) muscle tisue spiked with 1000 ng of FF and 1000 ng of FFA per gram of sample; (D) liver tissue spiked with 200 ng of FF and 200 ng of FFA per gram of sample.

in muscle and liver were $r = 0.9997$ and $r = 0.9989$, respectively; the corresponding figures for florfenicol amine were $r = 0.9994$ and $r = 0.9977$, respectively.

Table I shows the recoveries and repeatabilities of florfenicol and florfenicol amine from muscle and liver. The recovery of florfenicol from muscle and liver tissue varied from 99 to 107%, and the recovery of florfenicol amine from the same tissues varied between 94 and 100%. The S.D. varied from 4.4 to 8.7. The detection limits, for florfenicol and florfenicol amine were 20 ng/g in muscle and 50 ng/g in liver.

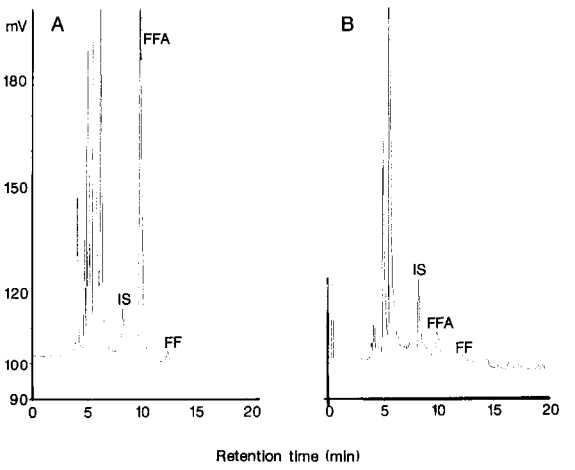

Fig. 3. Chromatograms of real samples, extracts from 3 g of muscle and 1 g of liver, for the determination of florfenicol (FF) and florfenicol amine (FFA) with thiamphenicol as internal standard (IS). (A) Muscle sample from rainbow trout treated with florfenicol: the sample contained 167 ng/g FF and 4262 ng/g FFA; (B) liver sample from rainbouw trout treated with florfenicol: the sample contained 293 ng/g FF and 1143 ng/g FFA.

TABLE I

RECOVERY OF FLORFENICOL AND FLORFENICOL AMINE FROM SPIKED SAMPLES OF FISH MUSCLE AND LIVER TISSUE

Amount in spiked samples (μg/g)	Recovery (%)			
	Florfenicol		Florfenicol amine	
	Mean	S.D.[a]	Mean	S.D.[a]
Muscle (3 g)				
0.1	99	5.5	98	5.8
0.5	105	8.6	100	8.7
Liver (3 g)				
0.2	100	4.7	94	4.8
1.0	107	4.4	98	5.3

[a] $n = 8$.

CONCLUSION

The method presented in this paper should be useful for most work on residues of florfenicol and florfenicol amine in farmed fish. The method is selective, sensitive and robust, and should be universally applicable for monitoring drug levels in fish tissues. The method is very rapid, a technician easily being able to deal with 24 samples a day. In addition, the consumption of chemicals is low.

ACKNOWLEDGEMENT

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