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Short communication

Simultaneous determination of ormethoprim and sulphadimethoxine in plasma and muscle of Atlantic salmon *(Salmo salar)*

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Abstract

A rapid clean-up and high-performance liquid chromatographic method for the simultaneous determination of ormethoprim and sulphadimethoxine in plasma and muscle of Atlantic salmon (*Salmo salar*) has been developed. Sample preparation is based on protein precipitation using trichloroacetic acid or methanol for plasma and muscle, respectively. The drugs are separated using a reversed-phase C_{18} analytical column and phosphate buffer–acetonitrile (80:20, v/v) containing 1-heptanesodiumsulphonate and triethylamine, as mobile phase. Detection was performed at 270 nm. The average recovery of ormethoprim was 97.2% in muscle and 95.7% in plasma, whereas the average recovery of sulphadimethoxine was 86.5% in muscle and 90.2% in plasma. The limit of detection at a signal-to-noise ratio of 3 was 50 ng/g and 30 ng/ml for ormethoprim in muscle and plasma respectively and 30 ng/g and 15 ng/ml in muscle and plasma respectively for sulphadimethoxine.

1. Introduction

The use of antibacterial agents to treat bacterial infections in aquaculture has a long history commencing in the late 1930s when sulphamerazine was introduced in the USA [1]. In the 1950s oxytetracycline was employed in both the USA and Europe [2] while in the 1970s oxolinic acid gained entry into the fish farming industry in Japan [3,4]. As new drugs were introduced into human and veterinary medicine, suitable representatives were soon evaluated for application to fish farming. However, despite the great expansion of the fish farming industry during the last 30 years, the range of drugs legitimately available to fish farmers is still very limited.

In Norway, oxytetracycline and furazolidone were for many years the agents of first choice. However, in later years oxolinic acid and flumequine, both quinolone derivatives, have found broader use. At present the only sulphonamide approved for the fish farming industry in Norway is the combination of sulphadiazine and trimethoprim (5:1), Tribrissen. However, another potentiated sulphonamide (sulphadimethoxine and ormethoprim [5:1], Romet[30]) is widely used in the aquaculture industry in other countries and was shown to be efficacious in the treatment of infections caused

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by *Yersinia ruckerii* (enteric redmouth disease), *Aeromonas salmonicida* (furunculosis) and *Edwardsiella ictaluri* (enteric septicemia) [5–7].

There is great public concern about residues of antibacterial agents in fish and fish products. However, control for drug residues in farmed fish for human consumption to date is very effective in Norway. Both chemical and microbiological methods have been developed for analysis of residues in fish tissues for all drugs currently used. Routine residue controls are being performed using microbiological methods while chromatographic methods are used for confirmation of positive results obtained with the microbiological methods. Chromatographic methods usually offer the advantage of selectivity and increased sensitivity.

Several high-performance liquid chromatographic (HPLC) methods to determine residues of various sulphonamides in plasma and tissues of fish have been published. In particular, the use of matrix solid-phase dispersion [8,9] and solid-phase extraction [10] for sample clean-up have been described. Radioisotope methods involving liquid scintillation counting have also been used to quantitate sulphadimethoxine residue levels in rainbow trout (*Salmo gardnieri*) [11] and channel catfish (*Ictalurus punctatus*) [12]. However, these methods are not applicable for routine analysis of fish treated with non-radioactively labelled drug.

Walliser et al. [13] described a reversed-phase HPLC method for the simultaneous determination of ormethoprim (OMP) and sulphadimethoxine (SDM) in muscle of chinook salmon (*Oncorhynchus tshawytscha*) applying solid-phase extraction for sample clean-up. Weiss et al. [14] extracted OMP and SDM simultaneously from tissues of catfish using dichloromethane and tetrabuthylammonium hydroxide.

To carry out pharmacokinetic and residue studies of drugs in fish, rapid, simple and selective extraction and analytical methods are important when analysing large series of plasma and tissue samples.

This paper, describes a simple and rapid extraction and clean-up procedure for the simultaneous determination of sulphadimethoxine and ormethoprim in plasma and muscle of Atlantic salmon (*Salmo salar*).

2. Experimental

2.1. Chemicals and reagents

Sulphadimethoxine (SDM) and sulphametoxazole (SMX) were from Sigma (St. Louis, MO, USA), while ormethoprim (OMP) was kindly supplied by Hoffmann-La Roche (Nutley, NJ, USA). 1-Heptanesodiumsulphonate acid was from Fluka Chemie (Buchs, Switzerland). Methanol, acetonitrile (HPLC-grade) and sodium dihydrogen phosphate, triethylamine, trichloroacetic acid, phosphoric acid (p.a. grade) were all from Merck (Darmstadt, Germany).

Stock solutions of SDM, SMX and OMP were prepared at a concentration of 1 mg/ml in methanol and stored in dark stoppered flasks at 4°C. Working standards were prepared by dilution from the stock solutions with methanol.

2.2. Chromatography

The HPLC system used consisted of a Spectra-Physics SP 8800 ternary HPLC pump (Spectra-Physics, San Jose, CA, USA) connected to a Spectra-Physics SP 8780 × R autosampler and a Spectra-Physics SP-8480 × R scanning UV detector operating at 270 nm. The integrator was the Model SP-4270 from Spectra-Physics.

The analytical column (150 × 4.6 mm I.D.) was packed with 3-μm particles of ODS-Hypersil (C_{18}) (Shandon Southern Products, Cheshire, UK) in our laboratory using a Shandon column packing machine. A short precolumn (20 × 4.6 mm I.D.) was packed with ODS-Hypersil 40-μm pellicular material. The column was operated at room temperature.

The mobile phase used was a modified version of that described by Hormazabal and Rogstad [15]. The ion-paring agent 1-heptanesodiumsulphonate was dissolved in phosphate buffer (0.025 *M* NaH_2PO_4) to a concentration of 0.015 *M* and the pH was adjusted to 2.8 with 5 *M* phosphoric acid (solvent A). Solvent B was acetonitrile

containing 0.1% triethylamine. The mobile phase was a mixture of 80% A and 20% B. The solution was filtered through a 0.2-μm Millipore filter and sonicated for 5 min before use. The flow-rate was 1 ml/min and the pressure approximately 14.1 MPa. Aliquots of 50 μl were injected onto the column.

2.3. Sample preparation

Drug-free plasma and muscle were sampled from Atlantic salmon obtained from Matre Research Station (Matredal, Norway).

Plasma

To 200 μl of fish plasma, 90 μl of trichloroacetic acid (24%) in methanol and 10 μl of methanol containing 0.1 μg of SMX as internal standard was added. The mixture was vortex-mixed for ca. 30 s followed by centrifugation at 14 000 *g* in a Biofuge A table-centrifuge (Heraeus Sepatech, Osterode am Harz, Germany) for 5 min. A clear supernatant was obtained, ready for analysis.

Muscle

Samples of fish muscle (1 g) were homogenised in 1.5 ml of a mixture of solvent A and methanol (80:20, v/v). Internal standard, 50 μl of methanol containing 0.5 μg of SMX, was added before blending. Following homogenisation, 0.5 ml of the homogenate was transferred to a polypropylene tube already containing 0.5 ml of methanol. After thorough mixing (1 min) and centrifugation (14 000 *g* for 5 min) the sample was ready for analysis.

2.4. Calibration

Standard calibration curves for OMP and SDM in the range 0.0625–1.0 μg/ml (plasma) and 0.125–1.0 μg/g (muscle) were prepared in three replicates using drug-free plasma or muscle samples and 0.1 μg (plasma) or 0.5 μg (muscle) of SMX as internal standard. Standard curves were drawn by plotting the known drug concentration against the ratio of drug to internal standard peak heights.

2.5. Recovery and precision studies

The extraction recoveries of OMP, SDM and SMX were determined by comparing peak heights from the analysis of drug-free samples spiked with 0.125 and 1.00 μg/ml (plasma) and 0.125 and 1.00 μg/g (muscle) of OMP and SDM and 0.5 μg/ml (plasma) and 0.5 μg/g (muscle) of SMX with peak heights resulting from direct injection of the standards.

To determine the within-run precision, 6 replicates of a plasma and muscle sample spiked with 0.5 μg/ml (plasma) and 0.5 μg/g (muscle) of the three drug were analysed.

3. Results and discussion

No baseline separation of the OMP and SDM peaks was achieved with the concentration of the ion-pairing agent described by Hormazabal and Rogstad [15]. However, reducing the concentration from 0.02 to 0.015 *M* of 1-heptanesodiumsulphonate, the retention time for OMP was shifted by approximately 1 min while the modification had little influence on the retention time of the sulphonamide peaks.

Chromatograms of blank samples of plasma and muscle, samples of plasma and muscle spiked with OMP, SDM and SMX and muscle tissue from fish treated with Romet[30] are shown in Fig. 1. An additional peak occurred between the OMP and SDM peaks in Fig. 1E. This could be the N-acetylated metabolite of SDM. However, since this metabolite is not available it is impossible to do confirmational analysis.

A single protein precipitation step followed by centrifugation was found to give good recovery and chromatograms free from interfering peaks when analysing the plasma samples. The limit of detection was found to be 30 ng/ml for OMP and 15 ng/ml for SDM at a signal-to-noise ratio of 3.

A simple method involving extraction and a subsequent protein precipitation step was developed to simultaneously recover OMP and SDM from salmon muscle tissue. At a signal-to-noise ratio of 3, the limit of detection was found

to be 50 and 30 ng/g for OMP and SDM, respectively.

Both OMP and SDM show good recovery from salmon muscle tissue and plasma. The average recoveries from plasma over the concentration range of the standard curve were 95.7% for OMP and 90.2% for SDM. In muscle the average recovery was 97.2% for OMP and 86.5% for SDM. This is higher [13] or slightly lower [14] than the values reported in previous studies. The results also show that the precision of the method was good. The recovery and precision of OMP and SDM from at the 0.125 and 1.0 μg/ml (plasma) or 0.125 and 1.0 μg/g (muscle) levels are given in Table 1. The precision and recovery of the internal standard (SMX) at the 0.5 μg/ml or 0.5 μg/g level was found to be 94.6% in plasma with a coefficient of variation (C.V.) of 3.7%, and 87.2% (C.V. = 5.5%) in muscle when analysing 6 samples.

The linearity (r) of the standard curves in plasma were 0.993 and 0.999 for OMP and SDM, respectively, and 0.991 for OMP and 0.997 for SDM in muscle when using the internal standard method. The within-run coefficient of variation for the OMP peak height was 0.8%, for the SDM peak height 0.7% and for the SMX peak height 0.7%.

The present study has shown that it is possible to simultaneously determine OMP and SDM in plasma and muscle of fish using a simple sample preparation. The amount of solvent required for the extraction was minimized and pretreatment of the samples by liquid–liquid or solid-phase extractions was avoided. The assay showed good recovery and precision, and the quantitation is linear over a wide concentration range. The method is simple and sensitive and very suitable for pharmacokinetic and residue studies of OMP and SDM in fish plasma and muscle where

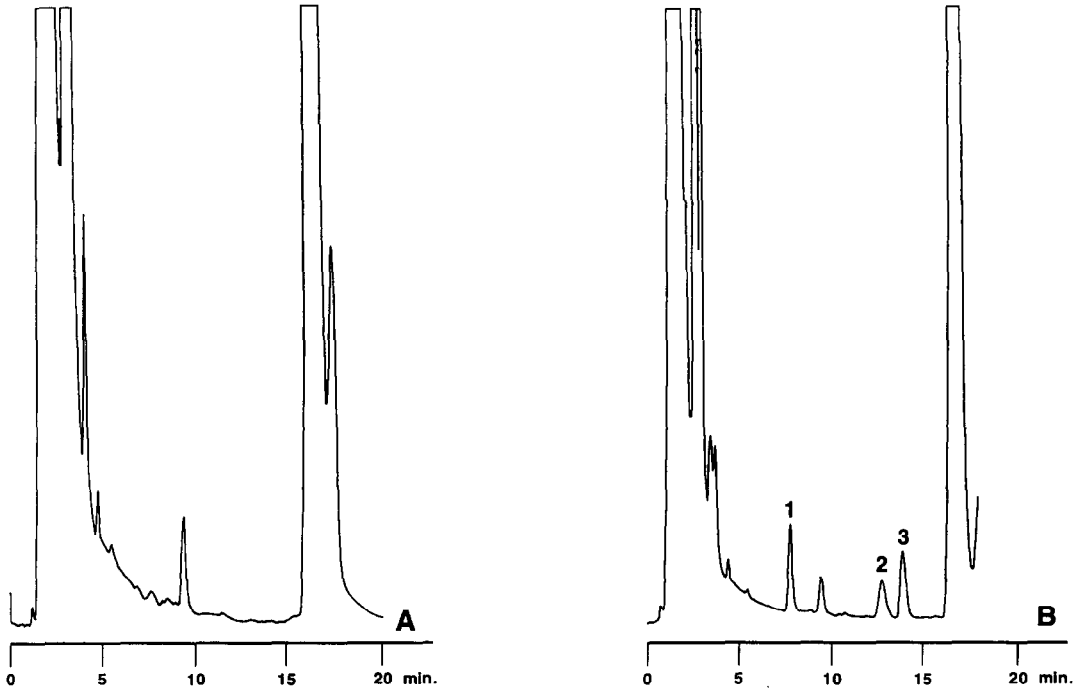

Fig. 1. Chromatograms of extracts from fish plasma and muscle. (A) Drug-free salmon plasma; (B) plasma spiked with 0.5 μg/ml of sulphamethoxazole (1), ormethoprim (2) and sulphadimethoxine (3); (C) drug-free salmon muscle; (D) muscle spiked with 0.5 μg/g of sulphamethoxazole (1) and 0.25 μg/g of ormethoprim (2) and sulphadimethoxine (3); (E) muscle sample from fish treated with a single dose of 25 mg/kg sulphadimethoxine and 5 mg/kg ormethoprim. Integrator sensitivity: range = 0.003, attenuation = 8.

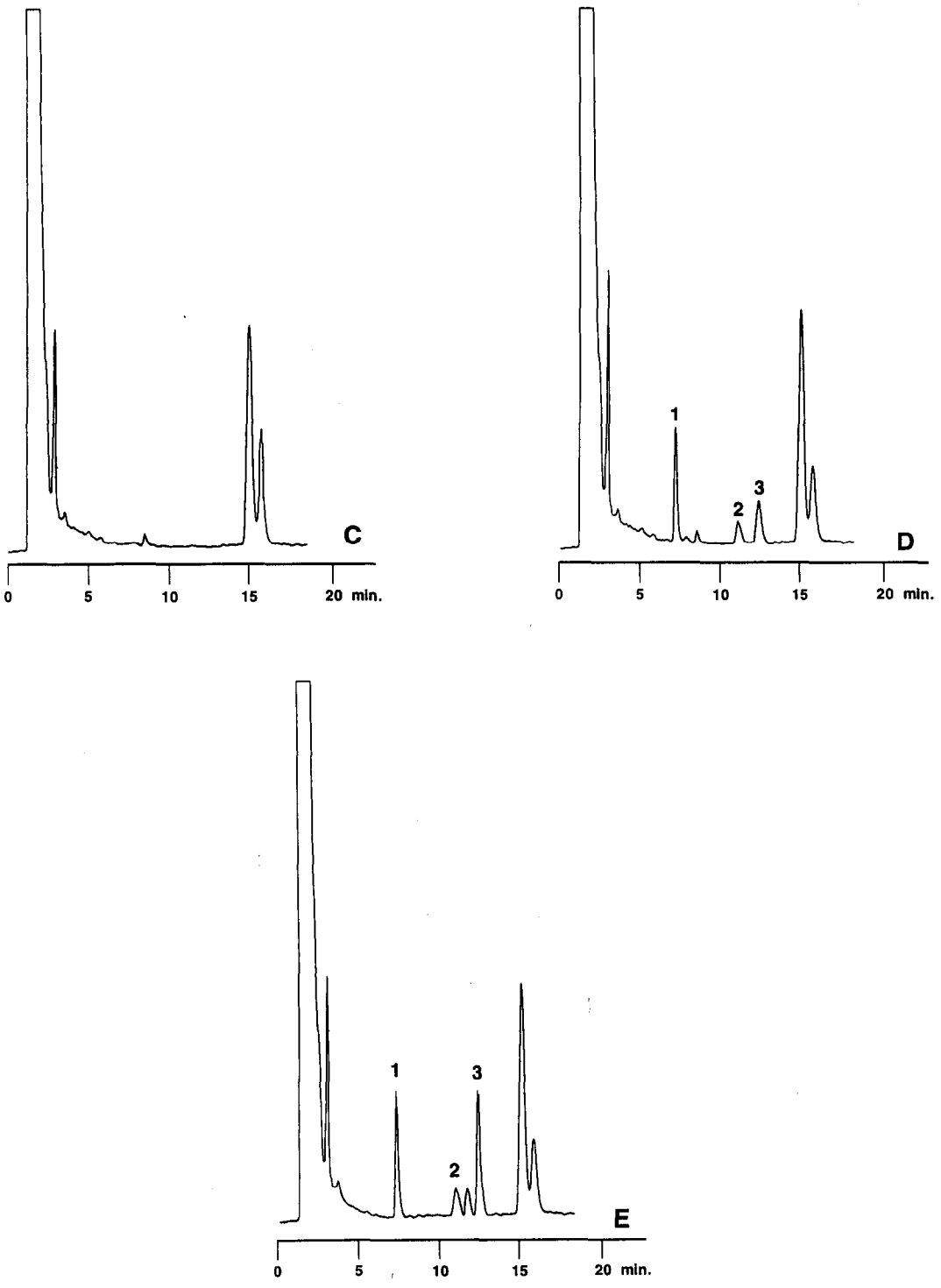

Fig. 1. (*continued*)

Table 1
Recovery and reproducibility of ormethoprim and sulphadimethoxine in fish plasma and tissue

Drug	Tissue	Amount added (μg/ml, plasma) (μg/g, muscle)	Recovery (%)	C.V. (%)	n
OMP	Plasma	0.125	94.6	2.6	6
	Plasma	1.000	96.5	1.95	6
	Muscle	0.125	96.3	7.8	6
	Muscle	1.000	99.1	5.4	6
SDM	Plasma	0.125	89.5	5.7	6
	Plasma	1.000	92.3	4.8	6
	Muscle	0.125	84.5	5.5	6
	Muscle	1.000	88.7	3.1	6

OMP = ormethoprim, SDM = sulphadimethoxine, C.V. = coefficient of variation.

analysis of large series of plasma samples is necessary.

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