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Simultaneous determination of sulphadiazine and trimethoprim in plasma and tissues of cultured fish for residual and pharmacokinetic studies

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ABSTRACT

Clean-up and high-performance liquid chromatographic methods for the simultaneous determination of sulphadiazine and trimethoprim in fish plasma and tissues have been developed. The average recovery of sulphadiazine varied from 74% in liver to 92% in plasma, whereas that of trimethoprim varied from 60% in liver to 97% in plasma. The sample pretreatment procedures were simple, selective and robust, having a limit of quantification of 250 ng/ml for trimethoprim and 50 ng/ml for sulphadiazine in plasma, 15 ng/g for sulphadiazine and 80 ng/g for trimethoprim in muscle, and 30 ng/g for sulphadiazine and 160 ng/g for trimethoprim in liver. The assay was tested on plasma from Atlantic salmon treated with Tribrissen.

INTRODUCTION

Sulphonamides and potentiated sulphonamides (combinations of sulphonamides and trimethoprim) are widely used as antibacterials in veterinary medicine for the treatment of diseases in both livestock production and farmed fish production. As early as 1937 sulphanilamide was applied on brook trout (*Salvelinus fontinalis*) infected with *Heamophilus piscum* [1]. Since that time various sulphonamides have been tested for controlling bacterial fish diseases [2,3]. In Norway the only sulphonamide approved for the fish farming industry is the combination of sulphadiazine and trimethoprim (5:1), Tribrissen. Compared with oxytetracycline and oxolinic acid, Tribrissen has been applied for the medication of fish in small quantities only [4] and has been the drug of choice when bacterial resistance has occurred owing to frequent treatment with drugs such as oxytetracycline and oxolinic acid [5]. Oxytetracycline was extensively used in Norwegian fish farming in 1984–1985 and oxolinic acid in 1990–1991. When administering Tribrissen to salmonides, the fish very often show reduced appetite and hesitate to eat the medicated pellets because of the pronounced flavour of the drugs in question. These problems have been overcome by co-extrusion of the drug with pharmaceutical excipients in the cavity of pellets made of a two-layer structure with an outer phase consisting of fish meal, Aqualets [6].

There has for several years been great concern about antibiotic residues in food products of

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treated fish and the potential health risk for humans. In Norway proper withdrawal periods have been established, and the fishery control authorities carry out both a pre-slaughter and a post-slaughter control. Residue control is being performed using microbiological methods, which are rapid to perform, but with the possibility of unspecific inhibition zones and poor sensitivity. Chromatographic methods have been introduced in the control laboratories for confirming positive detection of oxytetracycline, oxolinic acid and flumequine, at the expiry of the withdrawal period [7–9]. These substances are the most important antibacterial drugs in Norwegian fish farming. Chromatographic methods offer the advantages of selectivity and increased sensitivity over many other analytical procedures.

Sulphonamide residues in fish may be monitored by a variety of analytical techniques. Recently, a screening method using thin-layer chromatography was published by Reimer and Suarez [10], whereas Pleasance *et al.* [11] have analysed residues of various sulphonamides in cultured salmon flesh using mass spectrometry. Generally, a control laboratory will be less well equipped and will require rapid clean-up assays for monitoring drugs because of high throughput of samples. Methods for the simultaneous determination of sulphonamides and other antibacterials (quinolones, furazolidones, ormetoprim) in fish tissues have been published by Walisser *et al.* [12] and Horie *et al.* [13].

A variety of methods for the simultaneous determination of sulphonamides and trimethoprim in body fluids from mammals have been published. In particular, the use of solid-phase extraction, both on-line and off-line, for sample pretreatment of these matrices has been described. For assaying sulphadiazine and trimethoprim in more complex matrices Taylor *et al.* [14] applied solid-phase extraction on microbiological cultures. The assays mostly apply liquid chromatography with UV detection at a sensitive wavelength for quantification. Despite selective clean-up procedures the results are still prone to interferences from the matrix, which will reduce confidence in the analyses.

This paper describes simple and rapid extraction and clean-up procedures designed for simultaneous determination of sulphadiazine (SD) and trimethoprim (TM) in fish plasma and tissues, for the purpose of carrying out pharmacokinetic studies and residue analyses of the antibacterial compounds in Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). The methods are reliable and sensitive without interferences from the matrix in the chromatographic analysis. Because TM concentrations in both plasma and tissues are much lower than concurrent SD concentrations, the chromatographic system and the extraction procedures were optimized for TM.

EXPERIMENTAL

Chemicals and reagents

All chemicals were of analytical grade. SD and TM were purchased from Sigma (St. Louis, MO, USA). Sulphadimidine (SDM) was applied as internal standard for the plasma analyses and sulphamethoxazole (SM) for the tissue analyses. These compounds were supplied by Serva (New York, NY, USA) and Sigma, respectively.

Solvents were of analytical and high-performance liquid chromatography (HPLC) grade. Stock solutions (1 mg/ml) of SDM and SM were prepared by dissolving the compounds in a small amount of acetone and diluting to volume with ethanol. Stock solutions (1 mg/ml) of TM and SD were prepared in a mixture of 0.02 *M* phosphoric acid and ethanol (1:1) and a mixture of 0.03 *M* sodium hydroxide and ethanol (1:1), respectively. The solutions were stored in the refrigerator in dark, stoppered flasks. Working standards were prepared by diluting with ethanol or water for analysis of plasma and tissues, respectively.

Spin-X centrifuge filter units from Costar (Cambridge, MA, USA) were also applied.

Samples of fish and medication

Drug-free plasma and tissues were sampled from Atlantic salmon and rainbow trout obtained from the Norwegian Institute of Water

Research, Biological Research Centre at Solbergstrand (Norway).

An absorption study of SD and TM in plasma of Atlantic salmon [15] after a dose of Tribrissen was also carried out at Solbergstrand. Healthy fish were kept in fibreglass tanks of 1.8 m^3. The sea temperature was 8°C and salinity 29‰. The fish were force fed a single dose of 100 mg per kg body weight of fish, corresponding to 0.5%. Blood was sampled from the caudal vein of five fish at each sampling time, being 8, 12, 24, 48, 72, 96, 120 h, 7 and 14 days after administration of the dose. Plasma was isolated by centrifugation of the blood at 13 840 *g* for 10 min, immediately frozen and stored at −20°C until the analyses were performed.

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 cooler (14°C) Lauda RMT6 from Messgeräte Werk Lauda (Lauda-Königshafen, Germany) and an LC 95 UV detector (Perkin Elmer, Norwalk, CT, USA). The integration was carried out using of the software program Omega-2 (Perkin Elmer), which was operated on an Olivetti M300 personal computer connected to a Star LC24-10 printer. The detector was operated at 270 nm.

The analytical column (stainless steel, 25 cm × 4.6 mm I.D.) and guard column (stainless steel, 2 cm × 4.6 mm I.D.) were packed with 5-μm particles of the reversed-phase material Supelcosil LC-18 DB (Supelco, Gland, Switzerland).

For LC analysis of plasma samples the mobile phase was 0.025*M* sodium phosphate with hexane sulphonate (pH 2.8, adjusted with 5 *M* phosphoric acid)–acetonitrile with 0.1% triethylamine added (77:23, v/v). The ion-pairing agent sodium 1-hexanesulphonate was dissolved in the phosphate buffer to a concentration of 0.02 *M*. For analysis of tissue samples the mobile phase was a mixture of the same solvents at a ratio 80% aqueous to 20% organic phase. The flow-rate was 0.9 ml/min. Aliquots of 10 μl were injected into the column.

Sample pretreatment

Plasma. The pretreatment of plasma samples was as follows. To 0.5 ml of plasma were added 100 μl of internal standard solution (SDM, 30 μg/ml), 150 μl of 3% trichloroacetic acid (TCA) in ethanol and 100 μl of ethanol. The mixture was vortex-mixed and left in the freezer (−20°C) for 5 min. Following centrifugation the sample mixture was left for another 10 min at −20°C and then centrifuged through a Spin-X filter tube. This supernatant was injected into the chromatograph.

Tissue. The sample pretreatment of tissues is shown in Fig. 1. Spiked samples (1–3 g) of muscle and liver were mixed with 0.7% TCA in acetone (3 ml to muscle and 2 ml to liver) in the Whirlimixer and then ultrasonicated for 10 min at 40°C. Internal standard SM (3 μg to muscle and 6 μg to liver) was added before blending. Following addition of 2 ml of 0.01 *M* disodium hydrogenphosphate (pH 6) and ultrasonication (5 min), 100 μl of 0.5 *M* sodium hydroxide and 9 ml of dichloromethane (10 ml to liver) were added. After thorough mixing (1 min) and centrifugation (2240 *g* for 5 min), 6 ml of the organic layer were evaporated to dryness at 40°C under a stream of nitrogen gas. The dry residue was dissolved in 400 μl of 0.01 *M* phosphate buffer (pH 2.8)–acetonitrile (80:20, v/v) (800 μl for liver), ultrasonicated and extracted with 1 ml of hexane. The aqueous phase was ultrasonicated (1 min) and centrifuged through a Spin-X filter tube before injection into the chromatograph.

Validation of the assays

The within-day precision, the accuracy, the recovery of the sample clean-up procedures relative to standard solutions and the linearity of the calibration curve of the assays were determined by analyses of spiked samples of plasma, muscle and liver in the concentration range 100–3000 ng/ml (plasma) and 25–1000 ng/g (tissue) for SD and in the concentration range 300–3000 ng/ml (plasma) and 100–1000 ng/g (tissue) for TM. Each concentration range was assayed in triplicate. Both the internal standard method and the external standard method were validated.

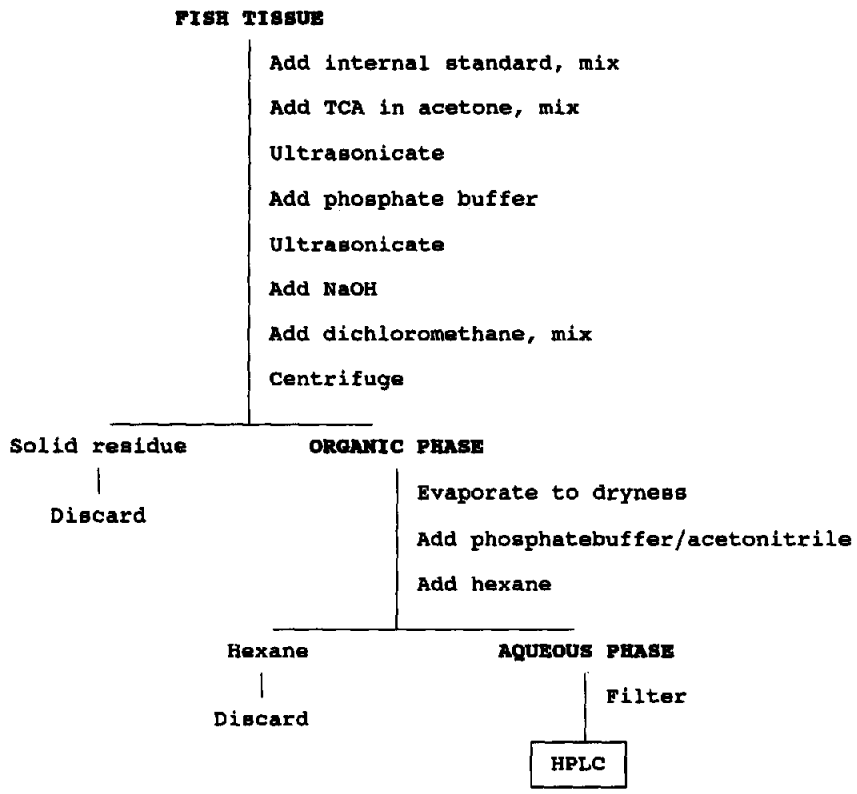

Fig. 1. Extraction and clean-up procedure for sulphadiazine and trimethoprim in fish tissue.

RESULTS AND DISCUSSION

Chromatographic system

Reversed-phase liquid chromatography was used and deactivated C_{18} phase on a silica support was chosen for its good separation and low asymmetry factor of the sulphonamides. Both SD and TM eluted at retention times shorter than SDM and SM using a mobile phase of phosphate buffer–acetonitrile (70:30). However, it appeared that both TM and the internal standards either coeluted or eluted very close to residues of endogenous compounds in the extracts of fish tissue. The TM peak was highly asymmetric. The tailing was suppressed by addition of triethylamine, which had little effect on the sulphonamide peaks [14,16].

Minor modifications of the aqueous phase/organic phase ratio of the mobile phase did not improve the separation of the peaks of the drugs from those of the endogenous compounds. However, the problem was overcome by adding an anion-pairing agent to the mobile phase. This modification had little effect on the retention times of the impurity peaks, whereas that of TM was shifted from 7 to 14 min in the chromatograms of tissue samples and from 6 to 9 min in plasma samples. The shift of the sulphonamides was less dramatic, from 5 to 5.5 min for SD and from 15 to 17 min for SM in the chromatograms of tissue samples. The long retention time of the internal standard (SM) was considered acceptable for the quantitative analyses.

Chromatograms of extracts of blank samples of plasma and muscle and samples of plasma and muscle from fish treated with Tribrissen are shown in Fig. 2.

Sample pretreatment

Both solid-phase extraction columns [17,18]

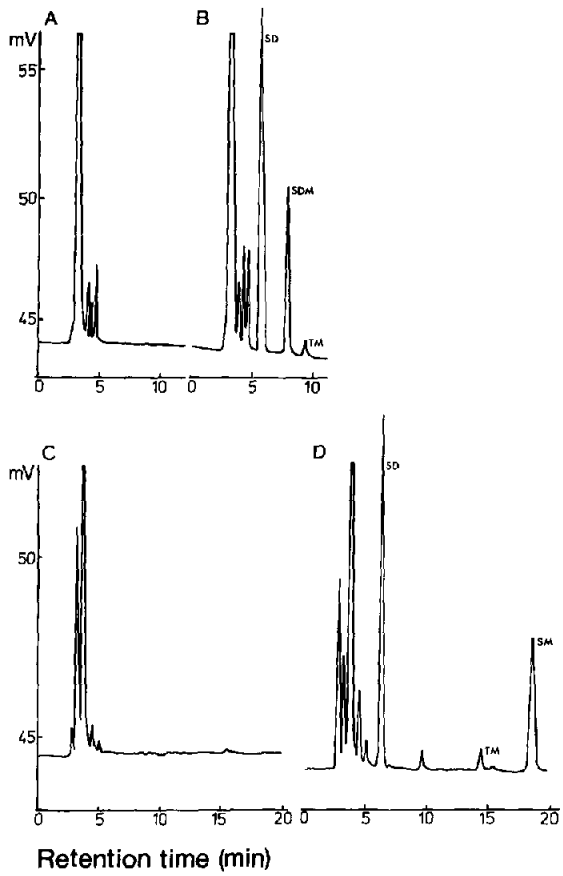

Fig. 2. Chromatograms of extracts from fish plasma and liver. (A) Drug-free salmon plasma; (B) plasma from treated salmon, 11.8 μg/ml sulphadiazine (SD), 1.75 μg/ml trimethoprim (TM), 6.0 μg/ml sulphadimidine (SDM); (C) Drug-free salmon muscle; (D) muscle from treated salmon, 1.87 μg/g sulphadiazine, 0.43 μg/g trimethoprim, 2.0 μg/g sulphamethoxazole (SM). Detection: 270 nm. Column: Supelcosil LC-18DB. Mobile phase: 0.025 *M* sodium phosphate with hexane sulphonate (pH 2.8)–acetonitrile with triethylamine (0.1%) at an aqueous/organic phase ratio of 77:23 for A and B, at ratio 80:20 for C and D.

and a single protein precipitation step were tested on plasma. Protein precipitation followed by centrifugation through Spin-X filters was found to give good recovery and was less time-consuming than application of extraction columns. The limit of quantification was 50 ng/ml SD and 250 ng/ml TM in plasma, which could be improved by application of a larger sample amount or by a preconcentration step. No interfering peaks were observed in the plasma extracts.

When pretreating tissue samples it appeared to be difficult to get rid of minor residues of endogenous compounds in the extracts by either solid-phase extraction or liquid–liquid extraction. This problem was more pronounced for liver than muscle. Therefore the liver samples were diluted to 800 μl with the consequence of somewhat reduced sensitivity. The critical step of the clean-up procedure was the extraction of the drugs from the aqueous homogenate to the dichloromethane phase without transfer of tissue impurities. Changing the internal standard from SDM to SM appeared to be necessary to obtain reliable quantification. The limit of quantification was 15 ng/g for SD and 80 ng/g for TM in muscle and 30 ng/g for SD and 160 ng/g for TM in liver. However, the sensitivity may be enhanced by using a larger sample amount.

The extraction procedures were validated, and the results are presented in Table I, showing good recovery of both TM and SD. The average recoveries from plasma over the concentration range of the standard curve were 90% for SD and 96.5% for TM. For SD in muscle the average recovery was 77.5% and for TM 71.5%, whereas for liver the average recovery achieved was 76.5% for SD and 62.5% for TM.

The results also show that the precision and accuracy of the quantification of TM and SD are good. When adding SD to blank plasma or tissues about 101–96% was recovered, whereas for TM the recovery was 102–95% when using internal standard. The precision of these recovery studies varied from 1.5 to 4.0% for SD and from 2.8 to 9.0% for TM. The calculations were also performed without internal standard, and the results are quoted in Table I. The precision and recovery of the internal standards were calculated, and the average recovery of SDM in plasma was found to be 94% [coefficient of variation (C.V.) = 4.5%], 79% for SM in muscle (C.V. = 4.6%) and 73% in liver (C.V. = 4.3%).

The linearity of the standard curve was ≥ 0.989 for both SD and TM when using the internal standard method. The external standard method of calculation gave a linearity coefficient of more than 0.984 for both compounds. The extraction procedure appeared applicable to tissues of both Atlantic salmon and rainbow trout.

TABLE I

VALIDATION OF ANALYTICAL METHOD FOR QUANTIFICATION OF SULPHADIAZINE AND TRIMETHOPRIM IN FISH PLASMA AND TISSUES

Sample	n	Amount of drug added[a]	Sulphadiazine			Trimethoprim		
			C.V.[b] (%)	Recovery (%)	Linearity (I.S. or E.S.)[c]	C.V. (%)	Recovery (%)	Linearity (I.S. or E.S.)
Plasma (0.5 ml)	8	500	2.6	99	0.996 (I.S.)	4.1	101	0.989 (I.S.)
			5.9	92	0.996 (E.S.)	11.3	97	0.984 (E.S.)
	8	3000	2.7	96	(I.S.)	5.0	99	(I.S.)
			7.8	88	(E.S.)	7.0	96	(E.S.)
Muscle (3 g)	10	100	1.5	98	0.999 (I.S.)			
			2.3	80	0.998 (E.S.)			
	10	150				7.0	101	0.996 (I.S.)
						8.2	73	0.995 (E.S.)
	10	1000	1.8	96	(I.S.)	2.8	96	(I.S.)
			2.6	75	(E.S.)	3.5	70	(E.S.)
Liver (3 g)	10	200	4.0	101	0.998 (I.S.)			
			6.6	79	0.990 (E.S.)			
	8	300				9.0	102	0.990 (I.S.)
						9.9	60	0.987 (E.S.)
	10	1000	3.0	97	(I.S.)	5.5	95	(I.S.)
			5.9	74	(E.S.)	6.1	65	(E.S.)

[a] Concentration: ng/ml for plasma, ng/g for tissue.
[b] C.V. = coefficient of variation
[c] I.S. = internal standard; E.S. = external standard.

Absorption study

An *in vivo* experiment with Atlantic salmon in seawater was performed to test the extraction procedure and HPLC assay for monitoring SD and TM simultaneously. Fig. 3 shows the absorption and distribution profile of SD and TM in plasma of salmon. Large variations were observed in the antibiotic concentration in individual fish sampled at the same time after administration, as the error bars included in Fig. 3 demonstrate. TM appeared to be absorbed and distributed rapidly, and the peak concentration was 3.25 μg/ml 12 h after medication, whereas the absorption peak of SD appeared 24 h post administration, and the concentration was 20.3 μg/ml. The decline was rapid to a concentration of 1.27 μg/ml SD and 0.61 μg/ml TM 120 h after administration. SD was not detected two weeks post administration, whereas an average of 0.36 μg/ml TM was determined in four of the five fish sampled.

CONCLUSIONS

This study has shown that SD and TM may be determined simultaneously in plasma and tissues of fish using minimal sample manipulation. An experienced technician can carry out sample clean-up of about 60–80 plasma samples or 20–30 tissue samples per day. The assays show good precision using both the internal standard and external standard methods. The quantification is linear over a wide concentration range. The methods are robust, sensitive and specific with good recovery of both substances. The amount of solvents is minimized, and pretreatment of tissues by liquid–liquid extractions combined with centrifugation filters is preferable to solid-phase ex-

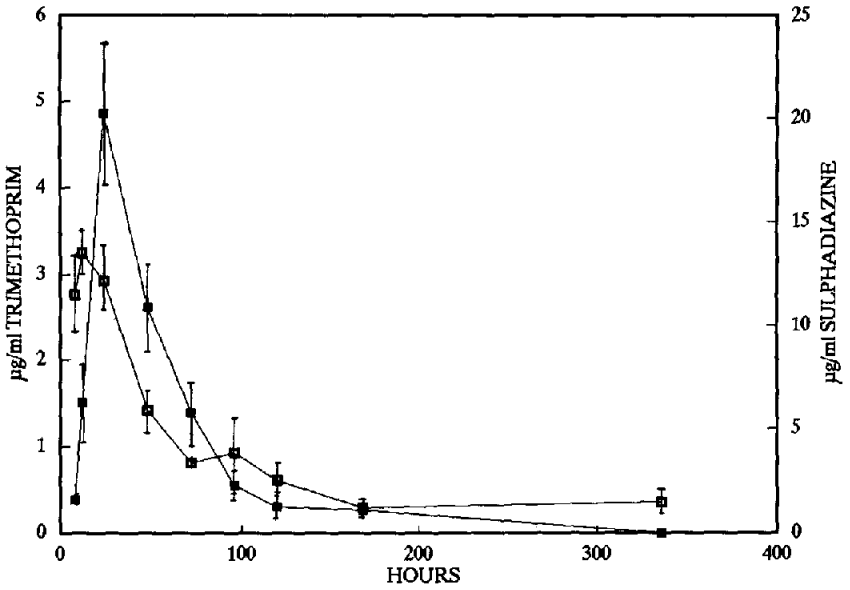

Fig. 3. Concentration of sulphadiazine (■) and trimethoprim (□) in plasma from Atlantic salmon (*Salmo salar*) following oral administration of pellets coated with Tribrissen (sulphadiazine/trimethoprim = 5:1). Sea temperature: 8°C. Dose: 100 mg of antibiotic per kg body weight of fish.

traction columns when performing the pretreatment manually. The chromatographic system was specific with regard to sulphadiazine and trimethoprim.

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