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High-performance liquid chromatographic determination of sulphadiazine and trimethoprim in Chinook salmon muscle tissue

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ABSTRACT

A sensitive high-performance liquid chromatographic (HPLC) analytical procedure was developed for the quantitative determination of sulphadiazine (SDZ) and trimethoprim (TMP) in Chinook salmon muscle tissue. SDZ and TMP were extracted from salmon muscle tissue using solid-phase extraction, and the extract was subsequently subjected to separate HPLC assay for each of the drugs. An Ultrasphere octadecylsilyl ion-pair column (250 × 4.6 mm I.D.) was used for both assays. A mobile phase of methanol–0.05 *M* phosphate buffer pH 2.5 (17:83), with ultraviolet detection at 280 nm was used for the SDZ assay. A mobile phase of acetonitrile–0.05 *M* phosphate buffer pH 2.5 (5:95), with ultraviolet detection at 224 nm was used for the TMP assay. The calibration curves for both assays in Chinook salmon tissue were linear over the concentration range of 0.1 to 10 μg/g for SDZ (r^2 = 0.9990) and 0.1–15 μg/g for TMP (r^2 = 0.9996). The minimum detectable quantities in Chinook salmon muscle tissue for both SDZ and TMP were 0.1 μg/g at signal-to-noise ratios of 10:1. The average recoveries for the drugs from Chinook salmon muscle tissue were 63% for SDZ and 42% for TMP.

INTRODUCTION

The ocean net-pen culture of Chinook salmon (*Oncorhynchus tshawtschya*) represents an important component of the finned-fish aquaculture industry. As with terrestrial animal husbandry, control of infectious disease is an important requirement for successful growing of stock to marketable size. Administration of antibacterial drugs to fish, either prophylactically or therapeutically, is commonly used to control infection. The choice of the antibacterial agent depends on the identity of the pathogen, however, most such xenobiotics exhibit a broad spectrum of antibacterial activities. The drug product Tribrissen (Burroughs Wellcome, Kirkland, Canada) classified therapeutically as a potentiated sulphonamide, is a commonly used broad-spectrum antibacterial agent. Tribrissen is a mixture of sulphadiazine (SDZ) and trimethoprim (TMP) in a fixed-dose combination of 5:1. Both SDZ and TMP interfere with bacterial growth by inhibiting the folic acid biosynthetic pathway synergistically. In order to ensure that acceptable levels of SDZ and TMP occur in Chinook salmon muscle

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following adequate depletion times, selective and sensitive assays for SDZ and TMP are required.

Both SDZ and TMP have been previously determined in fish tissue. McCarthy *et al.* [1] measured SDZ in rainbow trout muscle using the colorimetric Bratton–Marshall reaction and TMP in the same samples utilizing a microbiological bioassay procedure. These techniques gave sensitivities of approximately 1 ppm for both drugs which exceeded the acceptable residue level for most antibacterial agents in farmed fish muscle. Both techniques were also non-selective, since non-analytes commonly present in the fish extracts contributed to the values measured. Bergsjo and Sognen [2], analyzed TMP in muscle tissue of rainbow trout using a microbiological bioassay procedure that had a sensitivity of approximately 0.5 μg/g, also considered inadequate for residue depletion studies. Tissue concentrations of SDZ have been determined in rat muscle, liver and kidney at a sensitivity of greater than 0.1 μg/g using radioisotope-labeled SDZ and liquid scintillation spectrophotometry by Woolley and Sigel [3]. Concentrations of SDZ have also been determined in rat liver and chicken liver by an assay incorporating extraction into ethyl acetate followed by thin-layer chromatography of the extract [4]. Quantitation was achieved by fluorescamine derivatization of SDZ followed by densitometric scanning. The sensitivity of this assay was approximately 0.1 μg/g, however there was substantial variability in SDZ measurements. Jacobsen [5], using a sensitive and selective HPLC assay reported a sensitivity of 0.01 ppm for TMP extracted from rainbow trout muscle tissue that was suitable for residue depletion studies in rainbow trout. These studies can not be completely extrapolated to salmon since cultivated salmon muscle tissue has a higher lipid content than rainbow trout muscle tissue [6]. Lipids can be a limiting factor in the efficiency of TMP extraction by reported liquid–liquid techniques [7,8], due to the large amount of co-extracted endogenous material associated with the tissue matrix. Solid-phase extraction methods offer an attractive alternative for sample preparation since drugs can be preferentially bound to a solid matrix and subsequently selectively eluted prior to chromatographic analysis [4]. The aim of this research was to utilize solid-phase extraction in the development of sensitive and specific HPLC assays for SDZ and for TMP in Chinook salmon muscle tissue.

EXPERIMENTAL

Chemicals and reagents

SDZ, TMP and salbutamol (SAL) were obtained from Sigma (St. Louis, MO, USA), *p*-toluenesulphonamide (PTS) was obtained from Matheson Coleman & Bell (Los Angeles, CA, USA). Phosphoric acid (H_3PO_4) 85% was obtained from Fisher (Fair Lawn, NJ, USA). HPLC-grade solvents were obtained from BDH Chemicals (Vancouver, Canada). Purified water was produced using a Milli-Q water purification system from Millipore (Mississauga, Canada).

Apparatus

The HPLC system consisted of a Beckman Model 100A solvent metering system (Palo Alto, CA, USA), a Rheodyne Model 7125 sample injection valve (Berkeley, CA, USA) equipped with a 20-μl loop, a Gilson Model HM Holochrome variable wave-length detector (Villiers, FRA) attached to a Hewlett-Packard Model 3390A integrator (Avondale, PA, USA). Ultraviolet detection was at 224 nm for the TMP assay and at 280 nm for the SDZ assay. The HPLC column for both assays was an Ultrasphere octadecylsilyl ion-pair 5-μm column (250 × 4.6 mm I.D.) from Beckman. A NewGuard holder equipped with an RP-18 cartridge (15 × 3.2 mm I.D.) (Brownlee, Santa Clara, CA, USA) was used as a guard column. The mobile phase for the SDZ assay consisted of acetonitrile–0.05 *M* phosphate buffer pH 2.5 (5:95) delivered isocratically at 1.0 ml/min. The mobile phase for the TMP assay consisted of methanol–0.05 *M* phosphate buffer pH 2.5 (17:83) delivered isocratically at 1.0 ml/min. Filtration of the mobile phase prior to use was accomplished using an HPLC solvent filtration apparatus (Kontes, Vineland, NJ, USA) and FP Vericel 47 mm, 0.45-μm membrane filters (Gelman, Ann Arbor, MI, USA).

Preparation of standard solutions and reagents

All standard solutions were prepared with HPLC-grade solvents, and all solutions were stored at 4°C until required for use.

A stock solution of TMP was prepared by dissolving 50 mg of TMP in 100 ml of acetonitrile to yield a concentration of 500 μg/ml. This solution was diluted with acetonitrile to produce solutions

with final TMP concentrations of 1, 5, 10, 50 and 100 μg/ml.

A stock solution of SDZ was prepared by dissolving 50 mg of SDZ in 100 ml of methanol to yield a concentration of 500 μg/ml. This solution was diluted with acetonitrile to produce solutions with final SDZ concentrations of 1, 5, 10, 50 and 100 μg/ml.

A stock solution of PTS was prepared by dissolving 10 mg of PTS in 100 ml of acetonitrile to yield a concentration of 100 μg/ml. A stock solution of SAL was prepared by dissolving 10 mg of SAL in 50 ml of methanol to yield a concentration of 200 μg/ml. The internal standard solution was prepared by adding 100 ml of the PTS stock solution to 50 ml of the SAL stock solution and diluting the resulting solution to 100 ml with acetonitrile to yield final concentrations of PTS 10 μg/ml and SAL 100 μg/ml.

Chinook salmon muscle tissue

Farmed Chinook salmon of approximately 1.5 to 2 kg total body weight were obtained from an ocean net-pen farm site where they had been grown out on a standard salmon aquaculture diet. They were maintained free of drugs for at least 120 days prior to being obtained for analysis. Fish were killed by cranial fracture and edible muscle tissue samples were removed and stored frozen at −20°C until required for analysis.

Sample preparation and extraction procedure

Chinook salmon muscle tissue samples (5 g) were minced with a scalpel to form cubes approximately 1 mm on each side. The samples were transferred quantitatively to 150 × 18 mm test tubes and 9 ml acetonitrile, 0.5 ml internal standard solution and 0.5 ml drug solution were added. The samples were homogenized for 1 min at medium speed using a Brinkmann (Rexdale, Canada) Polytron Model PT 10/35 homogenizer. The samples were centrifuged for 10 min at 1500 *g* in a Beckman GP centrifuge equipped with a GH-3.7 swing-head rotor. The supernatants were decanted to 125 × 16 mm test tubes. Acetonitrile (10 ml) was added to each of the tissue pellets which were then homogenized and centrifuged as before. The supernatants from the first and second extraction of each sample were combined and the tissue pellet was discarded. The organic extracts were evaporated under a stream of dry nitrogen in a 37°C water bath to a residual volume of 0.5 to 1 ml. At this stage the extracts comprised an aqueous phase and a semi-solid phase which consisted primarily of lipids and lipid-soluble orange pigments. The volumes were adjusted to 2 ml with purified water and the extracts were vortex-mixed for 1 min. The samples were then centrifuged for 10 min as before, to cause the semi-solid phase to coalesce. Following centrifugation, the aqueous portions of the extracts were passed through activated C_{18} Sep-Pak solid-phase extraction cartridge assemblies (Waters, Mississauga, Canada). The cartridge assemblies consisted of two 400-mg cartridges connected in series. The cartridges were wetted by passing through 3 ml of methanol, and then equilibrated to the aqueous phase by passing through 5 ml of water. The eluate from loading the muscle tissue extracts to the cartridges was discarded. The drugs were subsequently eluted from the cartridges with 5 ml of methanol and the eluate was collected and evaporated to dryness under a stream of dry nitrogen in a 37°C water bath. The dried extract was dissolved in 0.5 ml of methanol and vortex-mixed prior to HPLC analysis.

Calibration curves, assay precision, and recovery

Calibration curves were prepared by taking 5-g samples of Chinook salmon muscle tissue and adding 0.5 ml of the prepared solutions of either SDZ or TMP to yield final drug concentrations of 0.1, 0.5, 1.0, 5.0 and 10.0 μg/g tissue. In addition, 0.5 ml of the internal standard solution was added to each of these samples to yield final concentrations of SAL 10 μg/g of tissue or PTS 1 μg/g of tissue. The samples were then prepared for analysis as described in the extraction procedure.

Intra-assay variability was determined in control Chinook salmon muscle tissue by preparing and analyzing 6 extracts containing SDZ at a concentration of 0.1 μg/g of tissue and TMP at a concentration of 0.4 μg/g tissue in one day.

Inter-assay variability was determined in control Chinook salmon muscle tissue by preparing and analyzing extracts containing SDZ at 0.1, 5 and 10 μg/g (n = 5 at each concentration) and extracts containing TMP at 0.2, 2 and 10 μg/g (n = 5 at each concentration). These extracts were prepared and analyzed over a period of several days to test for

day-to-day precision and variability in the assays.

Recoveries of SDZ and TMP from Chinook salmon muscle tissue were determined by adding 0.5, 2.5, 5, 25 and 50 μg of SDZ and TMP to 5-g samples of muscle tissue (0.1, 0.5, 1, 5 and 10 μg/g, respectively) and carrying out the extraction and analysis as described. Identical quantities of SDZ and TMP in solution were also analyzed directly using the HPLC methods described. The recoveries were determined by comparison of the peak areas (average of 3 injections) of the tissue extracts with those (average of 3 injections) of the corresponding drug solution (*i.e.* 10 μg/ml drug solution corresponds to 1 μg/g tissue extract).

RESULTS AND DISCUSSION

Representative chromatograms of a control salmon muscle tissue extract and extract containing SDZ with the internal standard, SAL, are presented in Fig. 1. The calibration curve for SDZ extracted from Chinook salmon muscle gave a linear relationship over the concentration range of 0.1–10 μg/g of SDZ ($y = 1.994x + 0.085$; $r^2 = 0.9990$).

Representative chromatograms of a control salmon muscle tissue extract and extract containing TMP with the internal standard, PTS, are presented in Fig. 2. The calibration curve for TMP extracted from Chinook salmon muscle tissue gave a linear relationship over the concentration range of 0.1–15 μg/g ($y = 0.702x + 0.0015$; $r^2 = 0.9996$).

The intra-assay relative standard deviation (R.S.D.) was 9.1% for SDZ and 4.7% for TMP, as shown in Table I. The precision of the extraction method for SDZ was determined at three different concentrations over a period of several days and the resulting inter-assay R.S.D.s are presented in Table II. This procedure was also performed for TMP and the resulting data are presented in Table II.

Both assays employed an initial solvent extraction of the drugs from muscle tissue, followed by a purification of the primary extract by retention and subsequent elution of the drugs from a solid-phase extraction cartridge. The average recoveries were 63% for SDZ and 42% for TMP (Table III).

Initial experiments were undertaken to develop a single extraction procedure and chromatographic method for the simultaneous recovery and analysis of SDZ and TMP, using PTS as an internal standard. However, SDZ with a UV absorption maximum at approximately 280 nm, and TMP with a UV absorption maximum at approximately 224 nm, did not allow for detection at a common wave-

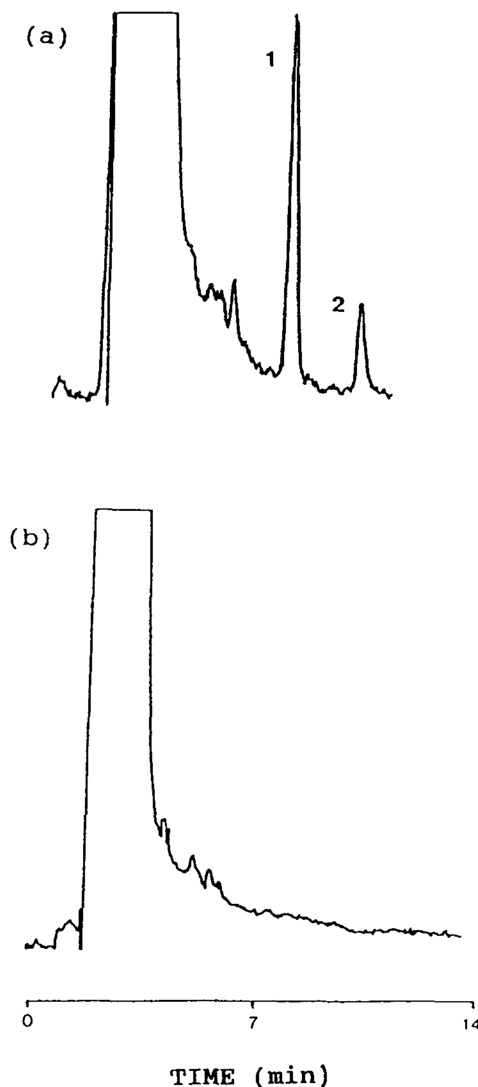

Fig. 1. Chromatogram of (a) a Chinook salmon muscle tissue extract spiked with 0.1 μg/g sulphadiazine and 10 μg/g salbutamol and (b) a control Chinook salmon muscle tissue extract. Chromatographic conditions: column, Ultrasphere I.P. 5 μm (25 cm × 4.6 mm I.D.); mobile phase, acetonitrile–0.05 *M* phosphate buffer pH 2.5 (5:95); HPLC flow-rate, 1.0 ml/min; ultraviolet detection wavelength, 280 nm. Peaks: 1 = salbutamol; 2 = sulphadiazine.

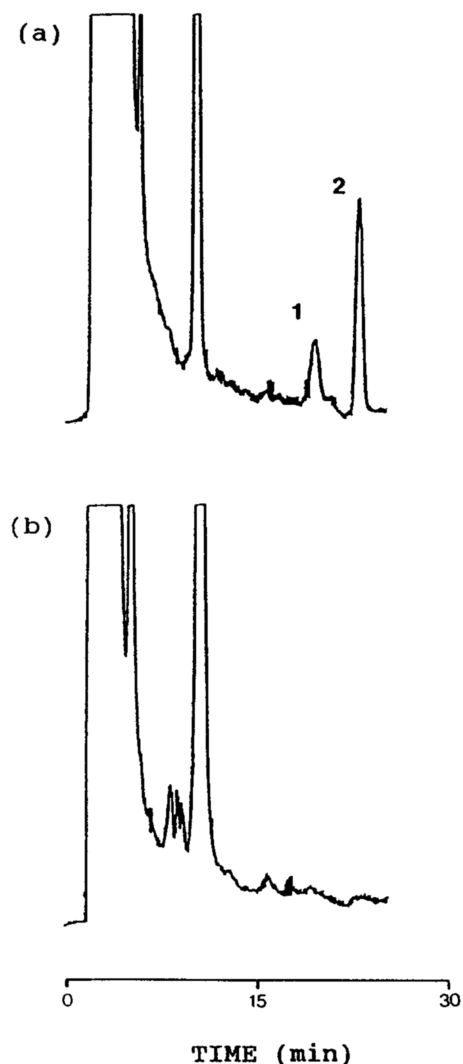

Fig. 2. Chromatogram of (a) a Chinook salmon muscle tissue extract spiked with 0.1 μg/g trimethoprim and 1 μg/g *p*-toluenesulphonamide and (b) a control Chinook salmon muscle tissue extract. Chromatographic conditions: column, Ultrasphere I.P. 5 μm (25 cm × 4.6 mm I.D.); mobile phase, methanol–0.05 *M* phosphate buffer pH 2.5 (17:83); HPLC flow-rate, 1.0 ml/min; ultraviolet detection wavelength, 224 nm. Peaks: 1 = trimethoprim; 2 = *p*-toluenesulphonamide.

length with adequate sensitivities for both drugs. Accordingly, two separate chromatographic procedures were developed. TMP was analyzed at 224 nm using PTS as the internal standard. SDZ was analyzed at 280 nm, however since PTS exhibited

TABLE I

INTRA-ASSAY VARIABILITY OF SULPHADIAZINE AND TRIMETHOPRIM IN CHINOOK SALMON MUSCLE TISSUE

Extract No.	SDZ/I.S. area ratio	TMP/I.S. area ratio
1	0.126	0.292
2	0.120	0.291
3	0.114	0.292
4	0.111	0.317
5	0.105	0.296
6	0.134	0.324
Mean area ratio	0.118	0.302
S.D.	0.011	0.014
R.S.D. (%)	9.1	4.7

TABLE II

INTER-ASSAY VARIABILITY OF SULPHADIAZINE AND TRIMETHOPRIM IN CHINOOK SALMON MUSCLE TISSUE

Sulphadiazine		Trimethoprim	
Sample concentration (μg/g)	R.S.D. (%)	Sample concentration (μg/g)	R.S.D. (%)
0.1	5.9	0.2	10.4
5.0	3.3	2.0	7.8
10.0	4.9	10.0	4.7

TABLE III

RECOVERY OF SULPHADIAZINE AND TRIMETHOPRIM FROM CHINOOK SALMON MUSCLE TISSUE

Sample concentration (μg/g)	Recovery (%)	
	Sulphadiazine	Trimethoprim
0.1	73	39
0.5	61	44
1.0	69	43
5.0	56	43
10.0	57	47
Mean	63	42

poor absorbance at this wavelength, SAL was selected as internal standard.

Preliminary extraction methods based on liquid–liquid extraction procedures outlined in the literature [4,5,7,8] resulted in low recoveries and substantial chromatographic interferences from co-extracted endogenous substances. Since cultivated salmon muscle tissue contains substantial quantities of lipid material, the low recoveries and chromatographic interferences were felt to be at least partially due to lipid constituents in the salmon muscle. The extraction procedure was modified to reduce the final extract to a small volume, with drugs predominantly isolated in the aqueous phase. The extracts at this stage still exhibited significant interference with the chromatographic analysis due to co-extracted endogenous materials. Incorporation of a solid-phase extraction cartridge protocol [9] improved the chromatographic appearance of the extracts, and permitted concentration of the extracts which enhanced the sensitivity of detection.

The method outlined in this study incorporates a single extraction procedure using two internal standards to satisfy detection requirements and provides analytical control of extraction variations from sample to sample. While the recoveries of SDZ and TMP were 63% and 42%, respectively, the sensitivity of the assay allows for quantitation of SDZ and TMP at 0.1 μg/g. While the recoveries are not as efficient as those previously reported in rainbow trout [1,2,5], attempts to increase the recovery led to increased co-extraction of endogenous materials which interfered with the chromatography. Similar observations with extraction of the combination of sulphadimethoxine and ormetoprim from Chinook salmon muscle tissue have been reported [9].

In summary, the methods reported in this study enable the quantitation of trimethoprim and sulphadiazine to 0.1 μg/g in Chinook salmon muscle tissue. Further research to incorporate these methods for Tribrissen analysis in pharmacokinetic studies in Chinook salmon are certainly warranted.

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