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Short Communication

Simultaneous determination of residual synthetic antibacterials in fish by high-performance liquid chromatography

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ABSTRACT

A simple and rapid high-performance liquid chromatographic (HPLC) method for the simultaneous determination of sulphamonomethoxine (SMMX), sulphadimethoxine (SDMX), sulphisozole (SIZ), nalidixic acid (NA), oxolinic acid (OXA), piromidic acid (PMA), furazolidone (FZ) and sodium nifurstyrenate (NFSA) in cultured fish was developed. The drugs were extracted with 0.2% metaphosphoric acid–methanol (6:4), followed by a Bond Elut C_{18} clean-up procedure. The HPLC separation was carried out on an Inertsil ODS column (150 × 4.6 mm I.D.) using 5 m*M* aqueous oxalic acid–acetonitrile (55:45) as the mobile phase with detection at 265 nm (0.04 a.u.f.s.). The calibration graphs were rectilinear from 1 to 20 ng for OXA, from 2 to 50 ng for SMMX, SDMX, SIZ, NA, PMA and FZ and from 5 to 100 ng for NFSA. The recoveries of each drug added to fish were 65.0–89.5%. The detection limits were 0.02 μg/g for OXA, 0.05 μg/g for SMMX, SDMX, SIZ, NA, PMA and FZ and 0.1 μg/g for NFSA.

INTRODUCTION

Various antibiotics and synthetic antibacterials are widely used for the prevention and treatment of infectious diseases in cultured fish. According to the Japanese Food Sanitation Law, no food should contain antibiotics and, in addition, meat, poultry, eggs, fish and shellfish should not contain any synthetic antibacterial substances.

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In a previous paper [1], we reported a method for the simultaneous determination of nalidixic acid (NA), oxolinic acid (OXA) and piromidic acid (PMA) in cultured fish by high-performance liquid chromatography (HPLC). In addition to NA, OXA and PMA, sulphamonomethoxine (SMMX), sulphadimethoxine (SDMX), sulphisozole (SIZ), furazolidone (FZ) and sodium nifurstyrenate (NFSA) are also used in culture fisheries in Japan. HPLC analysis of these antibacterials is being performed individually [2–7] or with specified groups [8–12], because their physico-chemical properties are different from one another. Although individual methods are useful for the accurate detemination of each drug, it is very labour- and time-consuming to inspect one by one every possible drug that might remain in fish. Consequently, it is necessary to establish a reliable method by which many antibacterials can be determined simultaneously and simply and rapidly.

This paper describes a simple and rapid HPLC method for the simultaneous determination of eight kinds of synthetic antibacterials which were considered to be possible residues in fish using Bond-Elut C_{18} cartridges in a clean-up step.

EXPERIMENTAL

Materials and reagents

The edible muscle tissues of yellowtail, eel, sweet fish, rainbow trout and red sea bream served as samples.

SMMX, SDMX and NA were obtained from Daiichi (Tokyo, Japan), FZ and NFSA from Ueno (Osaka, Japan) and OXA, PMA and SIZ from Tanabe (Osaka, Japan), Dainihon (Osaka, Japan) and Takeda (Osaka, Japan) Pharmaceutical, respectively.

Bond Elut C_{18} (500 mg), Baker C_{18} (500 mg) and Sep-Pak C_{18} were purchased from Analytichem (Harbor City, CA, U.S.A.), J. T. Baker (Phillipsburg, NJ, U.S.A.) and Millipore (Milford, MA, U.S.A.), respectively. The cartridges were washed with 5 ml of methanol and then 10 ml of distilled water before use. Hyflo Super-Cel was purchased from Johns-Manville (Denver, CO, U.S.A.). Other chemicals were of analytical-reagent or HPLC grade.

Preparation of standard solutions

Each standard (10 mg) was weighed accurately into a 100-ml volumetric flask and diluted to volume with acetonitrile. Subsequent dilutions were made with the HPLC mobile phase.

Apparatus

The HPLC system consisted of an LC-6A solvent-delivery system, an SPD-6A UV detector operated at 265 nm and a Chromatopack C-R3A data system, all from Shimadzu (Kyoto, Japan). The separation was performed on an Inertsil ODS (5 μm) column (150 × 4.6 mm I.D.) (Gasukuro Kogyo, Tokyo, Japan) with 5 m*M* aqueous oxalic acid–acetonitrile (55:45) as the mobile phase at a flow-rate of 0.5 ml/min at room temperature. An RP-8 Newguard guard column (15 × 3.2 mm I.D.) (Brown Labs., Santa Clara, CA, U.S.A.) was fitted in front of the analytical column.

The other instruments used were a Model 330 spectrophotometer (Hitachi, Tokyo, Japan) and a Model NS-50 Physcotron homogenizer (Niti-on, Chiba, Japan).

Sample preparation

A 5-g amount of sample was homogenized with 100 ml of 0.2% metaphosphoric acid (MPA)–methanol (6:4) as a deproteinizing extractant at high speed for 2 min. The homogenate was filtered through *ca.* 1 mm Hyflo Super-Cel coated on a suction funnel. The filtrate was evaporated under reduced pressure at 40°C. Evaporation was interrupted when *ca.* 10 ml of solution remained in the flask. The flask contents were applied to a Bond Elut C_{18} cartridge. After washing with 10 ml of distilled water, the cartridge was eluted with 10 ml of methanol. The eluate was evaporated to dryness under reduced pressure and the residue dissolved in 1 ml of HPLC mobile phase; 10 μl of the solution were injected for HPLC.

Calibration graphs

Working standard solutions with concentrations of 0.1, 0.2, 0.5, 1.0 and 2.0 μg/ml of OXA, 0.2 0.4, 1.0, 2.0 and 5.0 μg/ml of SMMX, SDMX, SIZ, NA, PMA and FZ and 0.5, 1.0, 2.0, 5.0 and 10.0 μg/ml of NFSA were prepared from stock standard solutions. A 10-μl volume of these solutions was injected into the column. Calibration graphs were obtained by the measurement of peak heights.

RESULTS AND DISCUSSION

Chromatographic conditions

Each of the eight synthetic antibacterials was dissolved in the HPLC mobile phase and their UV spectra were measured. The maximum UV absorptions of each of SMMX, SDMX, SIZ, FZ, NA and OXA were found to be 260–270 nm and those of PMA and NFSA were *ca.* 280 and 290 nm, respectively. On the basis of these results, a wavelength of 265 nm was chosen for the measurement.

It is known generally that NA, OXA and PMA show peak tailing in reversed-phase chromatography [1,12]. In order to prevent such a phenomenon, methylation of samples [6] or addition of counter ions to the mobile phase [7,11] is recommended. However, the methylation method requires a complex procedure and the addition of counter ions cannot prevent the tailing sufficiently. Previously, we reported that such tailing could be prevented by the use of wide-pore ODS column [1]. Therefore, in this study the effect of a wide-pore ODS column on the mutal separation of the eight drugs was evaluated. These drugs, however, could not be separated efficiently with this column because of its weak retention capacity compared with that of the ODS column.

Ikai *et al.* [12] reported that the tailing of NA, OXA and PMA could be prevented by the addition of oxalic acid to mobile phase. Although Ikai *et al.* used a three-component mobile phase (acetonitrile–methanol–aqueous oxalic acid), operation would be easier if a two-component system could be applied. Consequently, the separation conditions for the drugs were examined by applying acetonitrile–aqueous oxalic acid as the mobile phase and by using end-capped Inertsil ODS, Nucleosil $5C_{18}$ (Macherey-Nagel, Düren, F.R.G.) and LiChrosphere RP-18e (E. Merck, Darmstadt, F.R.G.) in the separation column, because it seemed that residual silanol groups on the surface of the stationary phase could cause the tailing of NA, OXA and PMA [1,12]. Inertsil ODS was chosen for subsequent experiment because it gave the best separations and peak shapes.

Next, optimum separation conditions were evaluated by changing the mixing ratio, oxalic acid concentration and the pH of the mobile phase (acetonitrile–aqueous oxalic acid). As the result, 5 m*M* aqueous oxalic acid–acetonitrile (55:45) was chosen as the mobile phase. Fig. 1A shows a typical chromatogram of the eight antibacterials obtained under the chosen conditions.

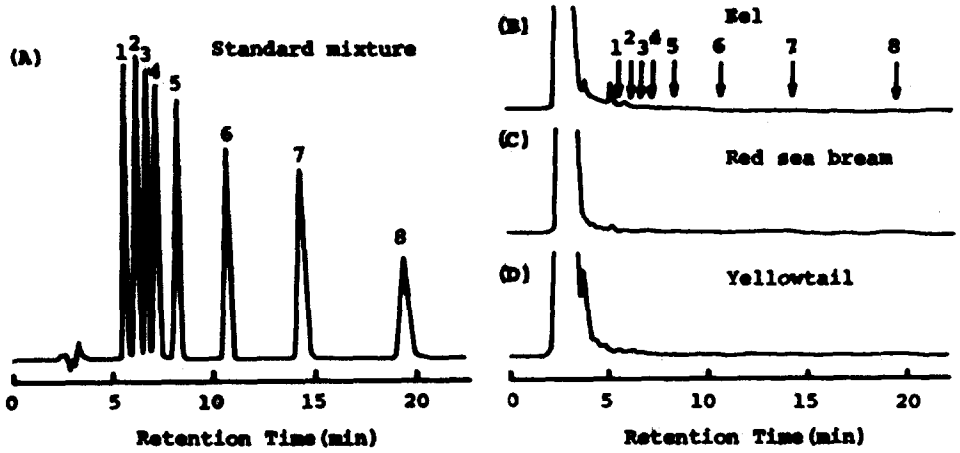

Fig. 1. Typical chromatograms of extracts of cultured fish. (A) Standard mixture. Peaks: 1 = SMMX (10 ng); 2 = SIZ (10 ng); 3 = FZ (10 ng); 4 = OXA (4 ng); 5 = SDMX (10 ng); 6 = NA (10 ng); 7 = PMA (10 ng); 8 = NFSA (20 ng). (B) Eel extract. (C) Red sea bream extract. (D) Yellowtail extract. For chromatographic details, see Experimental.

Clean-up

Various methods have been applied to the extraction and clean-up processes for the HPLC analysis of residual antibacterials in livestock and aquatic products, because their physico-chemical properties are different from one another. In a previous paper [1], we reported the simultaneous determination of NA, OXA and PMA in fish in which samples were extracted with MPA–methanol and cleaned-up using Sep-Pak C_{18} cartridges. Therefore, an evaluation was performed in order to establish whether such a method could be applied to the simultaneous determination of the eight drugs.

The drugs (5 μg each, except for 2 μg of OXA) were added to a yellowtail extract which has been prepared according to the method described under Experimental, and the retention capacity for each drug between the C_{18} cartridges was compared. As shown in Table I, Sep-Pak C_{18} showed a weak SIZ-retaining capacity, resulting in the loss of about 20% of the SIZ. In contrast, the retention capacity of Baker C_{18} for NA, OXA and PMA was so strong that about 20% of each drug could not be eluted. On the basis of these results, it was decided that Bond-Elut C_{18} be used as the cartridge for clean-up.

We have previously reported that the content of methanol in the MPA–methanol extraction solvent affected the recovery of veterinary drugs [1,13]. Here, the effect of methanol on the recovery of the eight drugs was evaluated by changing the content of methanol in the extraction solvent. As shown in Table II, the recovery of these drugs, excluding SIZ, was improved as the content of methanol increased; the recovery of SIZ decreased when the content of methanol exceeded 40%.

TABLE I

COMPARISON OF DISPOSABLE REVERSED-PHASE C_{18} CARTRIDGES

Values are means ± S.D. ($n = 5$). Recoveries of synthetic antibacterials from 10 ml of yellowtail extract. To the samples were added 0.2 μg/ml of oxolinic acid and 0.5 μg/ml of other drugs.

Drug	Recovery (%)		
	Bond Elut C_{18}	Baker C_{18}	Sep-Pak C_{18}
SDMX	91.7 ± 3.1	92.1 ± 2.6	90.5 ± 3.7
SIZ	88.7 ± 3.7	90.6 ± 3.5	73.9 ± 7.7
SMMX	93.2 ± 2.9	92.9 ± 3.7	94.1 ± 4.0
NA	97.0 ± 1.6	74.0 ± 3.1	87.7 ± 2.7
OXA	97.7 ± 1.6	80.5 ± 2.7	91.3 ± 3.3
PMA	97.8 ± 2.2	76.3 ± 2.0	91.9 ± 4.1
FZ	95.8 ± 0.7	93.2 ± 2.2	94.6 ± 1.4
NFSA	91.6 ± 2.8	90.3 ± 3.0	88.7 ± 1.2

The pH of the yellowtail extract prepared according to the method described under Experimental was varied from 2.5 to 7.0 and 5-μg portions of SMMX, SDMX and SIZ were added. As shown in Fig. 2, the recovery of SIZ decreased considerably when the pH exceeded 5.5, probably owing to a weakened retention capacity of the C_{18} cartridge due to the ionization of SIZ at > 5.5. When the content of methanol in the extraction solvent exceeds 50%, the pH of the extract increases to $\geqslant 5$ (Table II). This seems to be a cause of the reduction in SIZ recovery. The pH of the extract and the change in the recovery of SIZ accompaning an increase in methanol concentration are similar for yellowtail as for other kinds of fish (eel, sweet fish, red sea bream and rainbow trout), so that they are independent of the kind of fish. In addition, the peaks on the chromatogram were affected by the coexisting agents when the content of methanol was increased. As a consequence, the content of methanol was fixed at 40%.

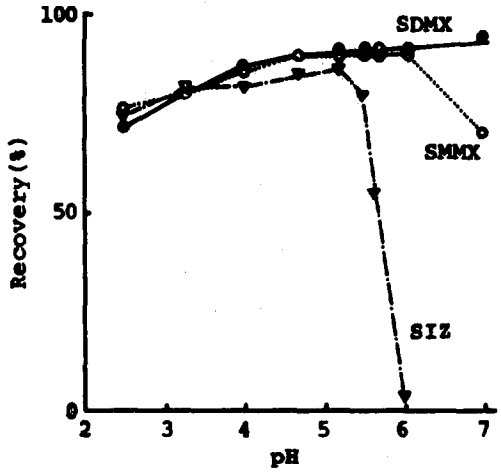

Fig. 2. Influence of pH of yellowtail extract on the recovery of SMMX, SDMX and SIZ.

TABLE II

EFFECT OF METHANOL CONTENT IN EXTRACTING SOLVENT ON THE RECOVERY OF SYNTHETIC ANTIBACTERIALS FROM YELLOWTAIL

Samples were spiked with 0.4 μg/g of oxolinic acid and 1.0 μg/g of other drugs. Mean results of five replicate determinations with relative standard deviations (%) in parentheses.

Extraction solvent	pH[a] of extract	Recovery (%)							
		SDMX	SIZ	SMMX	NA	OXA	PMA	FZ	NFSA
0.2% MPA–methanol(8:2)	3.9	65.3(3.3)	64.5(4.5)	71.1(3.7)	81.5(2.6)	78.0(3.2)	66.5(2.6)	82.4(3.0)	21.7(3.3)
0.2% MPA–methanol(7:3)	4.3	76.1(2.9)	74.0(1.6)	80.0(3.2)	84.2(1.4)	83.7(1.4)	75.6(1.7)	87.5(1.3)	45.7(0.8)
0.2% MPA–methanol(6:4)	4.7	83.8(2.6)	80.1(3.4)	85.8(3.2)	87.3(2.3)	86.4(1.5)	85.4(3.2)	86.1(0.4)	65.3(3.5)
0.2% MPA–methanol(5:5)	5.1	83.7(1.8)	73.6(6.3)	86.1(2.0)	87.8(2.0)	87.3(2.5)	87.3(1.6)	87.3(2.0)	70.9(2.8)
0.2% MPA–methanol(4:6)	5.5	86.9(2.7)	63.4(9.7)	86.9(2.5)	88.6(2.6)	88.5(1.6)	88.8(2.7)	88.6(1.6)	77.9(0.8)

[a] pH of a typical concentrated extract.

TABLE III

EFFECT OF THE CONCENTRATION OF METAPHOSPHORIC ACID IN THE EXTRACTION SOLVENT ON THE RECOVERY OF SYNTHETIC ANTIBACTERIALS FROM YELLOWTAIL

Samples were spiked with 0.4 μg/g of oxolinic acid and 1.0 μg/g of other drugs. Results of five replicate determinations with relative standard deviations (%) in parentheses.

Extraction solvent	Recovery (%)							
	SDMX	SIZ	SMMX	NA	OXA	PMA	FZ	NFSA
0.1% MPA–methanol(6:4)	86.5(1.2)	84.4(4.2)	88.2(1.7)	85.0(1.9)	85.0(1.4)	82.9(2.4)	86.4(2.2)	70.5(4.3)
0.2% MPA–methanol(6:4)	83.8(2.6)	80.1(2.6)	85.8(3.2)	87.3(2.3)	87.9(3.1)	85.4(3.2)	87.5(2.0)	65.3(3.5)
0.5% MPA–methanol(6:4)	76.5(4.5)	76.9(3.4)	80.1(3.0)	89.0(2.7)	86.4(1.5)	85.4(3.6)	85.4(0.4)	53.8(4.9)
1.0% MPA–methanol(6:4)	63.8(5.0)	64.1(3.7)	67.1(3.9)	88.3(1.3)	84.4(1.6)	81.0(4.7)	83.4(1.6)	46.0(3.5)

TABLE IV

RECOVERIES OF SYNTHETIC ANTIBACTERIALS FROM CULTURED FISH

Samples were spiked with 0.4 μg/g of oxolinic acid and 1.0 μg/g of other drugs. Results of five replicate determinations with relative standard deviations (%) in parentheses.

Sample	Recovery (%)							
	SDMX	SIZ	SMMX	NA	OXA	PMA	FZ	NFSA
Yellowtail	83.8(2.6)	80.1(2.6)	85.8(3.2)	87.3(2.3)	87.9(3.1)	85.4(3.2)	87.5(2.0)	65.3(3.5)
Eel	81.7(1.0)	80.5(2.3)	82.9(1.9)	87.9(1.9)	90.2(3.4)	86.4(3.9)	89.0(2.1)	67.8(3.7)
Sweet fish	86.6(1.7)	86.0(4.8)	88.5(2.1)	85.9(2.9)	88.1(1.4)	84.9(1.7)	89.5(3.1)	66.3(1.9)
Red sea bream	85.2(2.2)	82.6(2.7)	86.2(3.0)	87.4(2.7)	89.1(2.0)	87.3(1.8)	89.2(0.7)	66.1(2.7)
Rainbow trout	82.7(2.1)	83.2(2.0)	84.2(2.0)	87.0(3.4)	87.1(2.5)	84.5(2.9)	87.0(2.7)	65.0(1.3)

Table III shows the effect of the MPA concentration on the recovery of the eight drugs. A lower concentration of MPA gave higher recoveries of SMMX, SDMX, SIZ and NFSA, and gave less interfering paks. An extractant of 0.1% MPA–methanol (6:4) was not effective enough with regard to deproteinization. Based on the above experiments, 0.2% MPA–methanol (6:4) was chosen as a deproteinization extractant. Fig. 1B, C and D show typical chromatograms of eel, red sea bream and yellowtail extracts, respectively. Similar chromatograms were obtained from sweet fish and rainbow trout samples.

Recovery

Linear calibration graphs were obtained from 1 to 20 ng for OXA, 2 to 50 ng for SMMX, SDMX, SIZ, FZ, NA and PMA and 5 to 100 ng for NFSA. Table IV summarizes the recoveries of the drugs from commercial samples of yellowtail, eel, sweet fish, red sea bream and rainbow trout fortified with 0.4 μg/g of OXA and 1.0 μg/g of other drugs. Although the recovery of NFSA was low (65.0–67.8%), those of other drugs were higher than 80%, with standard deviations within 5%. The detection limits of the method were 0.02 μg/g for OXA, 0.05 μg/g for SMMX, SDMX, SIZ, FZ, NA and PMA and 0.1 μg/g for NFSA.

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