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Improvement of chemical analysis of antibiotics

XVII.[a] Application of an amino cartridge to the determination of residual sulphonamide antibacterials in meat, fish and egg

YOSHITOMO IKAI*, HISAO OKA, NORIHISA KAWAMURA, JUNKO HAYAKAWA and MASUO YAMADA
Aichi Prefectural Institute of Public Health, Nagare 7–6, Tsuji-machi, Kita-ku, Nagoya 462 (Japan)
KEN-ICHI HARADA and MAKOTO SUZUKI
Faculty of Pharmacy, Meijo University, Tempaku-ku, Nagoya 468 (Japan)
and
HIROYUKI NAKAZAWA
National Institute of Public Health, 4–6–1, Shirokanedai, Minato-ku, Tokyo 108 (Japan)
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ABSTRACT

A simple, rapid and reliable method for the determination of residual sulphonamide antibacterials (SAs) (sulphathiazole, sulphisozole, sulphamethoxazole, sulphadiazine, sulphamerazine, sulphadimidine, sulphamonomethoxine, sulphadimethoxine, sulphamethoxypyridazine and sulphaquinoxaline) in meat, fish and egg was developed using a combination of high-performance liquid chromatography (HPLC) and clean-up with an amino-type prepacked cartridge. SAs were extracted with ethyl acetate and applied to a Baker 10 amino cartridge. After elution from the cartridge, SAs were determined by HPLC. The recoveries at the level of 0.5 ppm were 73.7–99.1% and the detection limits were 0.05 ppm. The analysis time per sample was about 45 min.

INTRODUCTION

Sulphonamide antibacterials (SAs) are widely used for the treatment and prevention of diseases of animal and fish, and more than ten kinds of SAs are applied to domestic animals and cultured fish in Japan. Several methods have been reported [1–20] for the determination of residual SAs in livestock products such as meat, fish and egg, but most of them are not suitable for routine analysis because of the long analysis time and complicated clean-up procedure. Thus, a simple, rapid and reliable method for the simultaneous determination of SAs is required.

[a] For Part XVI, see ref. 21.

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In order to simplify the clean-up procedure for SAs, prepacked cartridges [4–7,20] have been utilized, among which the reversed-phase type [4–6,20] are the most frequently used. However, we considered that normal-phase and ion-exchange types are more suitable than the reversed-phase type, because SAs have been successfully extracted from various samples with organic solvents [1–4,6–17]. For the clean-up of extracted SAs, reversed-phase cartridges need some time-consuming pretreatments such as evaporation of the organic solvents and elimination of fat from the extract, whereas normal-phase and ion-exchange cartridges do not require such pretreatments. For these reasons, normal-phase and ion-exchange cartridges may be more effective for carrying out the clean-up simply and rapidly.

On the basis of the above considerations, we tried to establish a simple, rapid and reliable clean-up system for the determination of residual SAs in livestock products using a normal-phase or an ion-exchange type of cartridge. This paper describes techniques for the clean-up of residual sulphathiazole (STZ), sulphisozole (SIZ), sulphamethoxazole (SMX), sulphadiazine SDZ), sulphamerazine (SMR), sulphadimidine (SDD), sulphamonomethoxine (SMMX), sulphadimethoxine (SDMX), sulphamethoxypyridazine (SMPD) and sulphaquinoxaline (SQ) in meat, fish and egg using an amino cartridge, and for the simultaneous determination of SAs using high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Materials

Acetonitrile, anhydrous sodium sulphate, ethyl acetate, *n*-hexane, methanol, phosphoric acid and sodium hydroxide were of analytical reagent grade.

SMX, SDZ, SMR, SDD, SDMX and SMPD were obtained from Sigma (St. Louis, MO, U.S.A.), and STZ, SMMX, SQ and SIZ from Wako (Osaka, Japan), Daiichi Pharmaceutical (Tokyo, Japan), Dainippon Pharmaceutical (Osaka, Japan) and Takeda Chemical Industries (Osaka, Japan), respectively.

Baker 10 amino (catalogue No. 7088-3), Baker 10 cyano (7021-3), Baker 10 diol (7094-3), Baker 10 quaternary amine (7091-3), Baker 10 carboxylic acid (7211-3) and Baker 10 aromatic sulphonic acid (7090-3) cartridges were purchased from J. T. Baker (Phillipsburgh, NJ, U.S.A.).

Preparation of standard solution

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

Clean-up procedure

A 5-g amount of sample and 10 g of anhydrous sodium sulphate were weighed into a 50-ml centrifuge tube, blended with 20 ml of ethyl acetate for 30 s using a high-speed blender (Ultra-Turrax T25; IKA Werk, Staufen, Germany) and centrifuged (1500 rpm, 300 *g*) for 2 min and the supernatant was decanted. The above extraction procedure with ethyl acetate was repeated once more and the combined extracts were applied to a Baker 10 amino cartridge pre-washed with 10 ml of methanol. The cartridge was washed with 5 ml of *n*-hexane and air-dried by aspiration for 1 min. SAs were eluted from the cartridge with 5 ml of acetonitrile–0.02 *M* aqueous

phosphoric acid solution (24:76) and 20 μl of the eluate were injected into the HPLC system for the routine determination of SAs.

High-performance liquid chromatography

A high-performance liquid chromatograph equipped with a constant-flow pump (LC-6A; Shimadzu, Kyoto, Japan) was used with a UV detector (Shimadzu SPD-6AV) operated at 272 nm. The separation was performed on Wakosil $5C_{18}$ (5 μm, 250 × 4.6 mm I.D.) (Wako) with acetonitrile–0.02 *M* aqueous phosphoric acid solution (24:76) as mobile phase at a flow-rate of 1.0 ml/min at room temperature.

RESULTS AND DISCUSSION

Establishment of HPLC system

In order to determine SAs, thin-layer chromatography (TLC) [14–15,17], gas chromatography (GC) [1–2,16] and HPLC [3–13,18–20] have mainly been used. However, TLC is unsuitable for precise determinations and GC requires complicated treatment to derivatize SAs. On the other hand, HPLC enables SAs to be determined rapidly and with high sensitivity without any additional treatment such as GC, so we tried to separate ten kinds of SAs using a C_{18} HPLC column and a combination of acetonitrile and aqueous phosphoric acid solution as the mobile phase. After optimization of the mobile phase parameters, concentration of phosphoric acid, pH of the aqueous solution and ratio of the aqueous solution and the organic solvent, satisfactory separations of SAs could be obtained using acetonitrile–0.02 *M* aqueous phosphoric acid solution (pH unadjusted) (24:76). The flow-rate was 1.0 ml/min and the monitoring wavelength was adjusted to 272 nm, which is a common maximum absorption wavelength for all SAs. Under these conditions, the ten kinds of SAs were successfully separated in 26 min, as shown in Fig. 1, the calibration graphs were linear

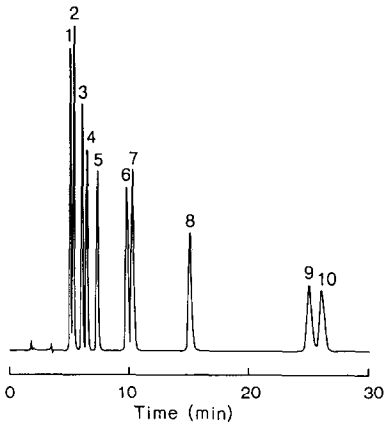

Fig. 1. Typical high-performance liquid chromatogram of SAs under the optimum conditions. Peaks: 1 = STZ ; 2 = SDZ; 3 = SMR; 4 = SDD; 5 = SMPD; 6 = SMMX; 7 = SIZ; 8 = SMX; 9 = SDMX; 10 = SQ (50 ng each). Column, Wakosil $5C_{18}$ (5 μm) (250 × 4.6 mm I.D.); mobile phase, acetonitrile–0.02 *M* aqueous phosphoric acid solution (24:76); flow-rate, 1.0 ml/min; detection, 272 nm.

between 0.5 and 100 ng and the detection limits of SAs on the chromatograms were 0.5 ng (signal-to-noise ratio >5).

Establishment of clean-up system

In a previous study [21], the following clean-up procedure was successfully applied to the determination of residual pyridonecarboxylic acid antibacterials in fish. The analyte is extracted from the sample by blending with organic solvent, the resulting extract is centrifuged and the supernatant is decanted and applied to a prepacked cartridge. After elution from the cartridge with a suitable eluent, the analyte is determined by HPLC. Because this procedure is very simple and rapid, we wished to apply it to the determination of SAs. To apply this procedure effectively, various conditions were optimized as described below and a satisfactory clean-up system as given under Experimental was established.

Comparison of prepacked cartridges. The cartridge to be used in this method is required to possess both the ability to retain SAs with an organic solvent and then to release SAs with a suitable eluent. Several commercially available normal-phase and ion-exchange types (Baker 10 amino, cyano, diol, quaternary amine, carboxylic acid and aromatic sulphonic acid) prepacked cartridges were compared. SAs (5 μg each) were dissolved in 40 ml of ethyl acetate and applied to the cartridges. The ethyl acetate solution which passed through the cartridges was collected and evaporated to dryness. SAs in the residue were determined by HPLC and the amounts of SAs retained in the cartridge were calculated. Although SAs were perfectly retained on the amino and aromatic sulphonic acid cartridges, the proportions of SAs retained on the quaternary amino cartridge were 90–100% and those on the cyano, diol and carboxylic acid cartridges were less than 20%.

Because the amino and aromatic sulphonic acid cartridges gave satisfactory retention of SAs, they were successively compared with respect to the elution behaviour of SAs. After the application of SAs to the cartridges in the same manner as described above, SAs were eluted from the cartridges with the mobile phase, acetonitrile–0.02 *M* aqueous phosphoric acid solution (24:76), and then determined by HPLC. Whereas more than 95% of SAs could be recovered from the amino cartridge using 20 ml of the eluent, only SIZ and SMR were eluted from the aromatic sulphonic acid cartridge. We concluded that the amino cartridge is the most suitable for our objective.

Elution of SAs from the cartridge. In order to determine analytes in the eluent from the cartridge by HPLC, the eluent is usually evaporated and the residue is dissolved in a suitable solution before injection into the HPLC system. However, these treatments are not desirable for a simple and rapid determination, so we wished to elute the SAs with a small volume of the mobile phase, because the eluent can be injected into the HPLC system without any treatment. The elution patterns of SAs from the cartridge were investigated using the mobile phase as the eluent. Usually, the retention power of the cartridge is weakened by the influence of the sample matrix. SAs were applied to the cartridge with an extract of chicken meat. The sample (chicken meat, 5 g) was extracted by blending with 10 g of anhydrous sodium sulphate and 40 ml of ethyl acetate and the resulting extract was centrifuged. After addition of SAs (2.5 μg each) to the supernatant, the mixture was applied to the cartridge. The cartridge was washed with 5 ml of *n*-hexane and then aspirated to remove the *n*-hexane. The SAs

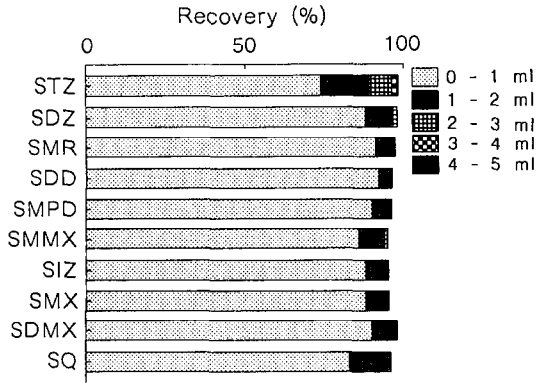

Fig. 2. Elution profiles of SAs from the amino cartridge. Integration of recoveries of SAs in each 1-ml fraction of eluent.

were then eluted from the cartridge with the eluent and the SAs in each collected fraction (1 ml) were determined. As SAs were sufficiently recovered in 4 ml of the eluate, as shown in Fig. 2, we used 5 ml of the mobile phase for elution of SAs from the cartridge.

Application of SAs to the cartridge. Because SAs must be applied to the cartridge together with a sample extract, the retention of SAs on the cartridge would be influenced by the volume of the extract. The influence on the recoveries of SAs was investigated. After the extraction of sample (chicken meat, 5 g) with 20, 40, 60, 80 or 100 ml of ethyl acetate, SAs (2.5 μg each) were added to each extract and the resulting solution was applied to the cartridge in the same manner as described above. SAs were eluted with 5 ml of the eluent and determined. As shown in Fig. 3, all the SAs were satisfactorily retained on the cartridge when 20 or 40 ml of ethyl acetate were used, whereas the retention of SDD became weak when over 60 ml was used, indicating that 40 ml of the extract is the volume limit.

In order to remove completely remaining matrix such as fat from the cartridge, the cartridge was washed with the extraction solvent used in a previous study [21].

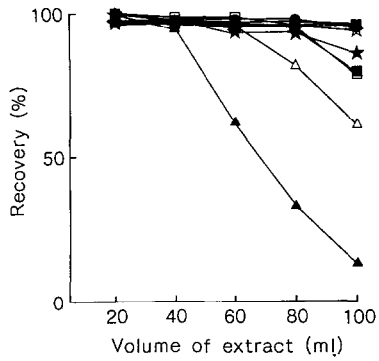

Fig. 3. Influence of volume of the extract on the retention of SAs on the amino cartridge. Recoveries of SAs which were applied to the cartridge with various volumes of extract. ○, STZ, ●, SDZ; △, SMR; ▲, SDD; □, SMPD; ◪, SMMX; ■, SIZ; ☆, SMX; ★, SDMX; ⁎, SQ.

However, the recoveries of SAs were decreased when ethyl acetate was used as a washing solvent. Various organic solvents were examined and *n*-hexane gave the most satisfactory result. We therefore used 5 ml of *n*-hexane as the washing solvent.

Extraction of SAs from the sample. Organic solvents such as methanol [5,9,20], acetone [3–4,8,16], acetonitrile [1,2,11], dichloromethane [12,15], chloroform [4,13–15] and ethyl acetate [14,15,17] have frequently been used for the extraction of SAs from various biological samples because of their excellent deproteinization effect. However, methanol, acetone and acetonitrile were unsuitable for the retention of SAs on the cartridge, and dichloromethane and chloroform also were not advantageous for decantation of the supernatant after centrifugation because of their gravity. Ethyl acetate was used as the solvent for the extraction of SAs from samples.

Because the use of less than 40 ml of ethyl acetate was favourable for the extraction, it was required to extract SAs from samples with at most 40 ml of ethyl acetate or to concentrate the extract to less than 40 ml. It was desirable to avoid the concentration of the extract, for the sake of simple and rapid clean-up. Therefore, extraction of SAs was carefully investigated. SAs (2.5 μg each) were added to the sample (chicken meat, 5 g) and were extracted five times using 20 ml of ethyl acetate. SAs in each extract were determined by HPLC after the treatment with the cartridge. It was found that 83–84% of SAs were recovered in the first extract, 12–13% in the second and 2–3% in the third. As satisfactory recoveries were given by the first and second extractions SAs were extracted twice using 20 ml of ethyl acetate.

We always used anhydrous sodium sulphate in extraction step throughout any optimization process. Probably it is effective in reducing the amount of water in the sample, so that a satisfactory extraction efficiency was obtained. In order to find a suitable amount of anhydrous sodium sulphate to be added, recoveries of SAs (2.5 μg each) from the sample (chicken meat, 5 g) were investigated using various amounts (0, 5, 10, 15 and 20 g). The recoveries increased with increasing amount of anhydrous sodium sulphate but remained almost constant with amounts above 10 g. Therefore, 10 g of anhydrous sodium sulphate were added to the sample before blending with ethyl acetate.

Application of various samples

Using the present method, the recoveries of SAs from chicken, beef, pork, eel, sweet fish, rainbow trout and egg were investigated at the addition level of 0.5 ppm. Although satisfactory recoveries (74.7–99.1%) and relative standard deviations (0.9–4.8%) were obtained, as shown in Table I, egg did not show satisfactory recoveries because of insufficient elution of SAs from the cartridge. However, adjustment of the pH of the aqueous solution to 2.5–5.0 gave satisfactory results. We therefore used acetonitrile–0.02 *M* aqueous phosphoric acid solution (pH adjusted to 3.0 with sodium hydroxide) (24:76) as the eluent for egg samples.

The detection limit for routine analysis was 0.05 ppm, which could be decreased to 0.01 ppm by injecting 100 μl of the eluent into the HPLC system. The time required for the analysis of one sample was about 45 min.

Typical chromatograms of fortified chicken and commercially available meats (chicken, pork and beef), fish (eel) and egg are shown in Fig. 4. Satisfactory clean-up could be achieved, because no interfering peaks appeared on the chromatograms and the peaks near the solvent front on the chromatograms were very small.

TABLE I

RECOVERIES OF SULPHONAMIDES FROM VARIOUS SAMPLES

Recoveries of SAs from 5 g of commercially available meats, fishes and egg at the level of 0.5 ppm according to the present method. Results are averages of six replicate determinations.

Sample	Recovery (%)[a]									
	STZ	SDZ	SMR	SDD	SMPD	SMMX	SIZ	SMX	SDMX	SQ
Chicken	92.0	97.6	95.7	90.0	96.7	95.7	95.7	94.9	94.1	94.8
	(3.4)	(1.2)	(3.3)	(4.8)	(1.4)	(2.3)	(1.6)	(1.9)	(4.5)	(3.0)
Pork	87.4	94.6	96.4	91.2	93.2	94.0	94.5	94.1	91.9	89.1
	(1.9)	(2.0)	(2.1)	(2.3)	(1.6)	(1.4)	(1.8)	(1.2)	(2.5)	(1.6)
Beef	86.3	93.4	95.6	87.7	92.8	93.0	93.8	94.6	91.0	85.2
	(2.9)	(2.5)	(1.6)	(4.0)	(1.7)	(3.4)	(3.0)	(1.5)	(2.1)	(4.4)
Eel	90.9	97.1	99.1	90.3	96.9	98.4	97.8	96.9	98.2	96.8
	(2.1)	(1.1)	(1.0)	(4.2)	(1.0)	(0.9)	(1.4)	(1.4)	(1.4)	(1.8)
Sweet fish	85.6	92.2	95.4	79.8	89.5	93.5	92.7	91.8	90.2	87.1
	(4.4)	(2.5)	(2.7)	(3.8)	(3.6)	(4.6)	(2.4)	(3.2)	(4.0)	(3.6)
Rainbow trout	89.2	95.6	97.6	74.7	91.5	93.7	95.1	95.0	92.8	91.1
	(0.9)	(0.9)	(1.1)	(4.5)	(1.5)	(4.5)	(1.6)	(0.9)	(0.9)	(1.6)
Egg[b]	73.7	86.2	93.0	81.6	91.7	88.9	83.2	88.2	92.2	89.2
	(3.9)	(3.9)	(1.7)	(5.6)	(1.7)	(1.6)	(2.8)	(2.0)	(0.9)	(2.2)

[a] Relative standard deviations (%) in parentheses.

[b] Acetonitrile–0.02 *M* aqueous phosphoric acid solution (pH 3.0) (24:76) was used as the eluent.

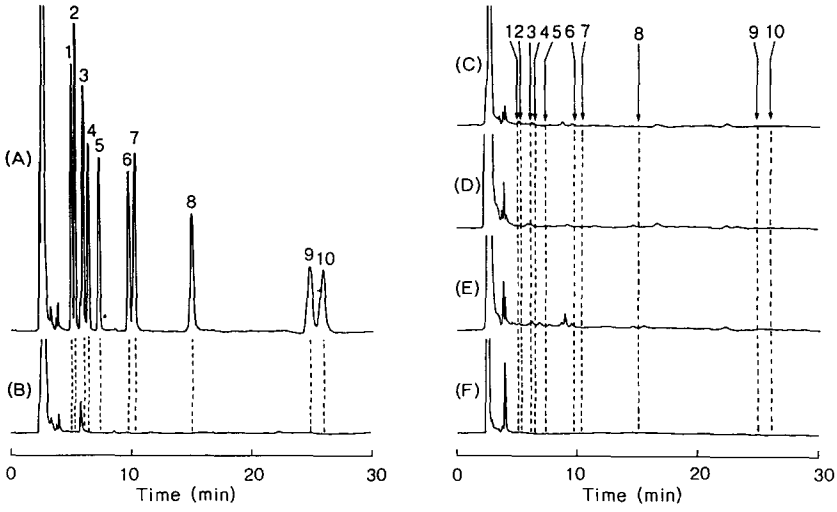

Fig. 4. Typical high-performance liquid chromatograms of commercially available meats, eel and egg. (A) Fortified chicken (0.5 ppm); (B) chicken; (C) pork; (D) beef; (E) eel; (F) egg. Peaks and HPLC conditions as in Fig. 1.

In conclusion, a method for the determination of residual SAs in meat, fish and egg was established using a combination of HPLC and clean-up with Baker 10 amino cartridge. The method is not only simple, rapid and reliable, but also permits the simultaneous determination of ten kinds of SAs with good accuracy, precision and reproducibility.

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