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Identification and determination of sulphamethazine and N^4-acetylsulphamethazine in meat by high-performance liquid chromatography with photodiode-array detection

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SUMMARY

A simple and selective method for the determination of sulphamethazine (SMT) and its metabolite, N^4-acetylsulphamethazine (N^4-AcSMT), in meat by high-performance liquid chromatography (HPLC) with photodiode-array detection was developed. The drugs were extracted from meat 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 a Supersphere RP-18e column (125 × 4.0 mm I.D.) using 0.05 *M* sodium dihydrogenphosphate (pH 4.5)–acetonitrile (8:2) as the mobile phase at a flow-rate of 0.5 ml/min, and monitored with a photodiode-array detector. The recoveries of SMT and N^4-AcSMT from meat fortified at 0.5 μg/g were 90.1–93.3 and 93.0–94.4%, respectively, with coefficients of variation of 1.9–3.2 and 1.5–2.7%. The limits of detection were 0.02 μg/g for each drug. SMT was found in ten samples of imported meat (12.5%) at levels ranging from 0.05 to 1.05 μg/g.

INTRODUCTION

Various antibiotics and synthetic antibacterials are widely used for the prevention and treatment of infectious diseases in livestock animals. 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. Therefore, a simple and reliable method is need to monitor drug residues in edible tissues of swine, cattle and chicken.

Sulphamethazine [SMT; 4-amino-N-(4,6-dimethyl-2-pyrimidinyl)benzenesulphonamide; sulphadimidine] is widely used in the rearing of food-producing animals

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to prevent and treat diseases and to promote their growth. A number of methods have been developed for the assay of SMT using spectrophotometric procedures[1], thin-layer chromatography[2–4], gas chromatography (GC)[5–7], GC–mass spectrometry (GC–MS)[7–11], enzyme immunoasay[12] and high-performance liquid chromatography (HPLC)[11,13–17].

A conventional HPLC method using a UV detector used for the determination of SMT residues in meat lacks qualitative ability. A few GC–MS methods developed for confirmation of SMT and its metabolites in animal tissues[7,11] are complicated and time consuming.

Haagsma *et al.*[18] reported an HPLC method for the simultaneous determination of SMT and its metabolites in swine tissue. However, they only measured the minor metabolite, desamino-SMT, and did not measure SMT and its major metabolite, N^4-acetyl-SMT (N^4-AcSMT), using a photodiode-array detector. According to Japanese Food Sanitation Law, the parent compound such as SMT, and its major metabolite such as N^4-AcSMT, must be measured in tissue. Hence it is important to identify the parent compound and its major metabolite.

This paper describes a simple and rapid HPLC method for the identification and determination of SMT and N^4-AcSMT using photodiode-array detection and Bond-Elut C_{18} cartridges in a clean-up step.

EXPERIMENTAL

Materials and reagents

Edible muscle tissues of swine, cattle and chicken served as samples. SMT was obtained from ICN Pharmaceuticals (Cleveland, OH, U.S.A.). Bond-Elut C_{18} cartridges (500 mg) (Waters Assoc., Milford, MA, U.S.A.) were washed with 5 ml of methanol and then 10 ml of distilled water before use. Hyflo Super-Cel was obtained from Johns-Manville (Denver, CO, U.S.A.). Other chemicals were of analytical-reagent or HPLC grade.

Synthesis of N^4-acetylsulphamethazine

N^4-AcSMT was prepared by heating SMT and acetic anhydride in glacial acetic acid according to the procedure described by Uno and Ueda[19]. The synthesized compounds were characterized by mass spectrometry (MS) and infrared (IR) spectrophotometry.

Preparation of standard solutions

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

Apparatus

The HPLC system consisted of a Model LC-6A solvent-delivery pump (Shimadzu, Kyoto, Japan), a Model 7125 injector (Rheodyne, Berkeley, CA, U.S.A.) and a Model SPD-M6A photodiode array detector (Shimadzu) interfaced with an NEC PC-9801 VX-4 personal computer (Tokyo, Japan). The chromatograms were recorded on a plotter. The separation was performed on a Supersphere RP-18e column

(125 × 4.0 mm I.D.) (E. Merck, Darmstadt, F.R.G.) with 0.05 *M* sodium dihydrogenphosphate (pH 4.5)–acetonitrile (8:2) as the mobile phase at a flow-rate of 0.5 ml/min.

The other instruments used were a Model GCMS-QP 1000 mass spectometer (Shimadzu), a Model IR-435 infrared spectrophotometer (Shimadzu) and a Hiscotolon homogenizer (Nichion Irika Kikai, Tokyo, Japan).

Sample preparation

A 5-g amount of sample was homogenized with 100 ml of 0.2% metaphosphoric acid–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 20 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 2 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

Standards at concentrations of 0.2, 0.5, 1.0, 2.0 and 4.0 μg/ml of SMT and N^4-AcSMT were prepared from stock standard solutions. A 10-μl volume of these solutions was injected into the column. Calibration graphs were obtained by measurement of peak height.

RESULTS AND DISCUSSION

Chromatographic conditions

Some metabolites of drugs show biological activity and therefore, for the determination of residual drugs in animal tissues, it is desirable to establish a method that takes into consideration their major metabolites. Although several metabolites of SMT are known, the major one is N^4-AcSMT, in which the N^4 position of SMT has been acetylated[20,21].

In the determination of SMT by HPLC, unlike GC, no derivatization is necessary and SMT can be determined directly, and many studies[11,13–17] have been reported. However, there have been few reports[11] of the simultaneous determination of SMT and N^4-AcSMT in animal tissues. Many of the HPLC methods for SMT employ a reversed-phase ODS column[11,13,15,16] which utilizes. Therefore, a study was made of the separation conditions using Supersphere RP-18e, LiChrospher RP-18e, Inertsil ODS, Nucleosil 5C 18 and Capcell Pak C_{18} (SG) columns and phosphate buffer–acetonitrile as the mobile phase. Although the samples were separated well with all the columns tested, the sharp peaks of SMT and N^4-AcSMT were obtained using a Supersphere RP-18e column. Therefore we have chosen the Supersphere RP-18e column in this paper.

Based on a study with different mixing ratios of phosphate buffer and acetonitrile at various salt concentrations and pH, 0.05 *M* sodium dihydrogenphosphate (pH 4.5)–acetonitrile (8:2) without pH conditioning was chosen as the mobile phase. Fig. 1A shows the chromatogram of SMT and N^4-AcSMT obtained under the established conditions.

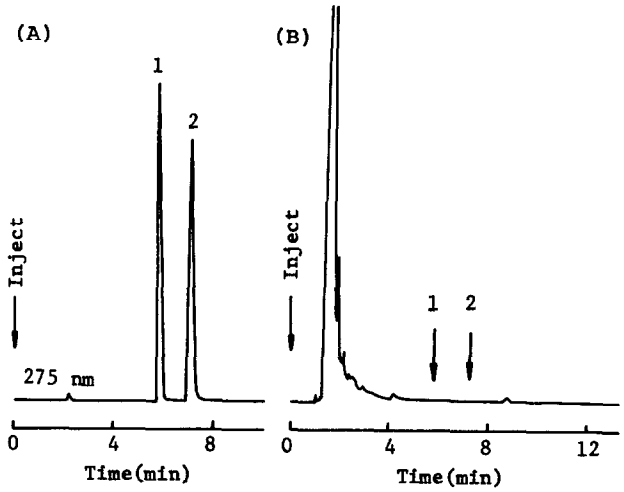

Fig. 1. Typical chromatograms of (A) standard mixture and (B) pork extract. Peaks: 1 = N^4-acetylsulphamethazine (10 ng); 2 = sulphamethazine (10 ng).

Clean-up

Liquid–liquid partition using acetone[5], acetone–chloroform[1,2,6–10,13], ethyl acetate[3,4] and ethyl acetate–chloroform[15] has been reported as a means of cleaning up meat in order to remove fatty compounds. However, these methods are complicated and time consuming, and involve problems such as emulsion formation. Haagsma *et al.*[18] used a silica cartridge for sample treatment. We used a C_{18} instead of a silica cartridge for sample clean-up, because it is important to develop a universal method that would be applicable to other antibacterials. The present method is applicable to other such compounds[22,23].

First, we tested the capacity of a commercial C_{18} cartridge to retain SMT and N^4-AcSMT. As shown in Table I, a recovery of about 90% was obtained for either

TABLE I

INFLUENCE OF EXTRACTION CARTRIDGES ON THE RECOVERY OF SULPHAMETHAZINE AND N^4-ACETYLSULPHAMETHAZINE FROM PORK MEAT

Values are means ± S.D. ($n=5$). Samples were spiked with 0.5 μg/g each of sulphamethazine and N^4-acetylsulphamethazine.

Cartridge	*Recovery (%)*	
	SMT	*N^4-AcSMT*
Bond-Elut C_{18} (200 mg)	67.9 ± 7.0	84.9 ± 3.0
Baker C_{18} (200 mg)	82.5 ± 1.7	88.9 ± 2.3
Bond-Elut C_{18} (500 mg)	93.3 ± 2.6	94.4 ± 2.7
Baker C_{18} (500 mg)	94.1 ± 2.7	94.2 ± 3.3
Easy Chromato C_{18} (500 mg)	89.2 ± 4.1	90.1 ± 3.2
Sep-Pak C_{18} (400 mg)	87.2 ± 3.9	89.1 ± 4.1

sample when 400–500 mg were packed in the column, whereas part of the SMT was not retained and leaked with a packing amount of 200 mg. Bond-Elut C_{18} (500 mg) was used as the clean-up cartridge because it gave the fewest peaks of contaminants.

An unified extraction clean-up procedure is desirable for establishing a rapid and widely applicable method for the determination of antibiotics and synthetic antibacterials in animal and fish samples. When we used 0.2% metaphosphoric acid–methanol (6:4) as the extraction solvent, which we had used previously for the determination of oxolinic acid, nalidixic acid and piromidic acid in fish culture[22]; a good recovery was obtained without any interference from coexisting substances, as shown in Fig. 1B. Similar chromatograms were obtained from chicken and beef samples.

Recovery

Linear calibration graphs were obtained from 1 to 40 ng of SMT and N⁴-AcSMT. Table II summarizes the recoveries of the drugs from commercial samples of pork, beef and chicken fortified with 0.5 μg/g of SMT and N⁴-AcSMT. Greater than 90% overall mean recoveries and 5% standard deviations were obtained with every meat sample. The detection limits were 0.02 μg/g for both SMT and N⁴-AcSMT.

TABLE II

RECOVERIES OF SULPHAMETHAZINE AND N⁴-ACETYLSULPHAMETHAZINE FROM MEATS

Values are means ± S.D. ($n=5$).

Sample	*Added (μg/g)*	*Recovery (%)*	
		SMT	*N⁴-AcSMT*
Pork	0.5	93.3 ± 2.6	94.4 ± 2.7
Beef	0.5	91.8 ± 1.9	93.0 ± 1.7
Chicken	0.5	90.1 ± 3.2	94.1 ± 1.5

Analysis of commercial samples

Using the above method, we analysed commercial samples of pork, chicken and beef for residual SMT. As shown in Table III, SMT was found in ten imported pork samples (12.5%) at levels ranging from 0.05 to 1.05 μg/g. Three samples contained about 1 μg/g of SMT. Judging from a study reported by Cox and Krzeminski[16], the pigs from which these samples were obtained are surmised to have been slaughtered almost without any interruption of the drug treatment. In all pork samples in which SMT was detected, N⁴-AcSMT was also detected at low concentrations.

HPLC using a UV detector, which is generally employed for the determination of sulpha drugs, has poor specificity, and qualitative information obtainable from this technique is limited to the retention time of the sample. Accordingly, for the analysis of meat samples that contain residual sulpha drugs, the samples are further analysed by GC–MS[7–11] to confirm the target substance. However, for GC–MS, sulpha drugs

TABLE III

DETERMINATION OF SULPHAMETHAZINE IN COMMERCIAL MEATS

Sample	*Type*[a]	*No. of samples*	*Samples with positive detection*	*Range (μg/g)*		
				Min.	*Max.*	*Av.*
Pork	I	80	10 (12.5%)	0.05	1.05	0.47
	D	40	N.D.[b]	–	–	
Beef	I	40	N.D.	–	–	
	D	15	N.D.	–	–	
Chicken	I	17	N.D.	–	–	
	D	20	N.D.	–	–	

[a] I = Imported; D = domestic.
[b] N.D. = not detected (<0.05 μg/g).

must be methylated to give suitable volatile derivatives, and thus the process is complicated and time consuming.

A photodiode-array detector, as used here, can measure both retention times and absorption spectra, and peak components can therefore be identified by comparison of the peaks with those of standards.

Figs. 2 and 3 show chromatograms of pork samples in which SMT was detected at 1.05 and 0.10 μg/g. The peak component with a retention time of 7.1 min was compared with a standard sample of SMT. The two spectra were almost identical, confirming that the peak component eluting at of 7.1 min was SMT. The similarity index given in Figs. 2, 3 and 4 represents the similarity of two spectra in terms of numbers.

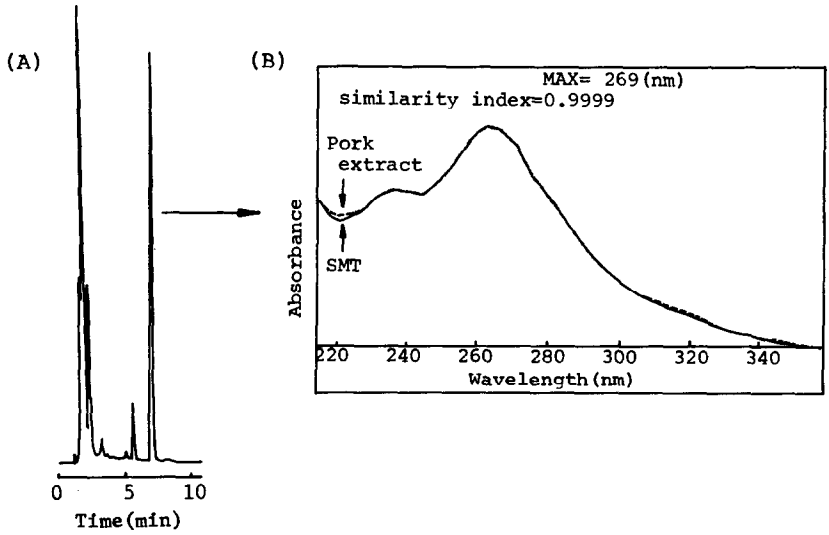

Fig. 2. (A) Chromatogram of pork sample in which sulphamethazine was detected at 1.05 μg/g, plotted at 275 nm. (B) Normalized spectra of the peak (at 7.11 min) obtained from pork extract (dashed line) and standard sulphamethazine (solid line).

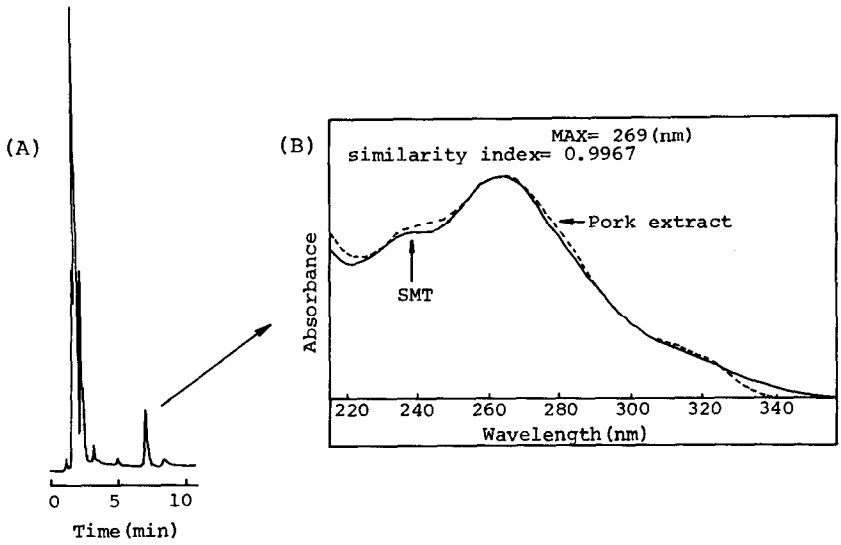

Fig. 3. (A) Chromatogram of pork sample in which sulphamethazine was detected at 0.10 μg/g, plotted at 275 nm. (B) Normalized spectra of the peak (at 7.13 min) obtained from pork extract (dashed line) and standard sulphamethazine (solid line).

In the USA, a tolerance of 0.1 μg/g for SMT[24] in edible animal tissues has been set. Consequently, our proposed method is considered to be useful as a method for the determination of residual SMT down to this level.

In general, when SMT is administered to animals, N^4-AcSMT is also detected in tissues. The retention time of 5.8 min of the peak component in Fig. 2, for pork meat in which 1.05 μg/g of SMT was detected, is in agreement with the retention time of standard N^4-AcSMT. The spectra of this peak and standard N^4-AcSMT were almost identical, with a similarity index of 0.9989 (Fig. 4). Hence this peak component was confirmed as N^4-AcSMT.

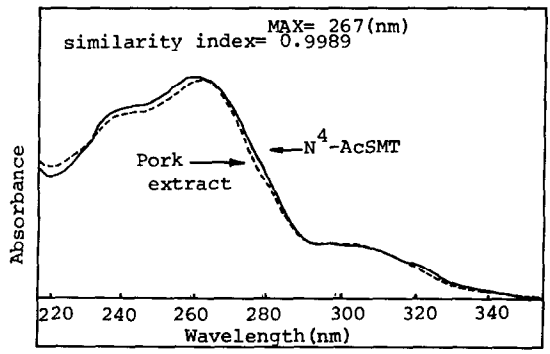

Fig. 4. Normalized spectra of the peak (at 5.80 min) obtained from pork extract (dashed line) in Fig. 3 and standard N^4-acetylsulphamethazine (solid line).

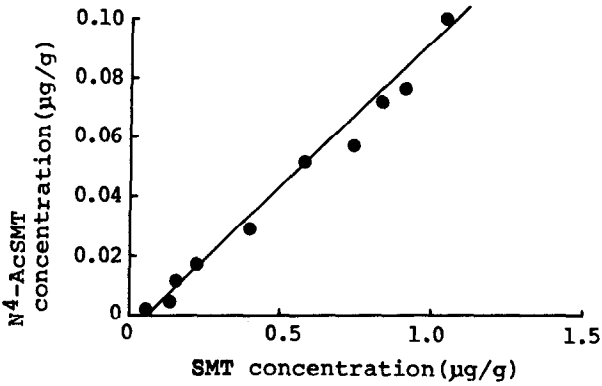

Fig. 5. Correlation between residual sulphamethazine and residual N^4-acetylsulphamethazine in pork meat. $y = 0.10x - 0.00$; $r = 0.989$; $n = 10$.

Correlation between residual SMT and residual N^4-AcSMT

Detection of SMT in meat has already been reported in the USA[15]. However, no detailed studies have been reported on the relationship between the residual concentration of SMT and that of N^4-AcSMT. We studied this relationship employing ten samples. Although this number of samples is small, a high correlation was detected between the concentrations of these two compounds, as shown in Fig. 5.

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