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Simultaneous high-performance liquid chromatographic determination of residual sulphamonomethoxine, sulphadimethoxine and their N^4-acetyl metabolites in foods of animal origin

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Abstract

A rapid and sensitive method for the determination of residual sulphamonomethoxine, sulphadimethoxine and their N^4-acetyl metabolites in beef, pork, chicken and eggs by high-performance liquid chromatography (HPLC) was developed. The extraction of these compounds was performed using a mixture of 90% (v/v) acetonitrile solution and hexane (5:4, v/v) to minimize the fat content followed by purification by alumina column chromatography. These extracts contained sulphonamide analytes which were free from interfering compounds when examined by HPLC using a LiChrosorb RP-18 column. The average recoveries from spiked meat and egg were in excess of 80% with relative standard deviations between 0.4 and 5.0%. The practical limits of detection were 0.01 ppm for all samples.

1. Introduction

In recent years, the application of veterinary drugs and feed supplements to livestock has increased to prevent diseases of animals and poultry. As a result, there is concern that residues of these compounds may be retained in foods derived from treated animals. In Japanese Food Sanitation Law, no food should contain any antibiotics or synthetic antibacterial substances. To produce animal products such as meat and eggs free from drug residues, it is necessary to set an adequate withdrawal period after administration of the drug, based on data on the residue depletion obtained from pharmacokinetic studies, and to monitor drug residues in animal products.

Sulphamonomethoxine [SMM; 4-amino-N-(2-methoxypyrimidinyl)benzenesulphonamide] and sulphadimethoxine [SDM; 4-amino-N-(2,6-methoxy-4-pyrimidinyl)benzenesulphonamide] are widely used in Japanese farming. Pharmacokinetic studies of SMM and SDM have been performed in cows, hens and pigs, and one of their major metabolites is elucidated to be the N^4-acetyl metabolite, which is acetylated at the N^4-position by N-acetyltransferase [1–5]. According to Japanese Food Sanitation Law, the

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parent compounds such as SDM, and major metabolites such as N^4-acetyl-SDM (N^4-AcSDM), must be measured in food of animal origin. Although rapid clean-up procedures have been developed for the determination of SMM and SDM using high-performance liquid chromatography (HPLC) [6–10], simultaneous methods for these compounds and their major metabolites in various samples such as beef, pork, chicken and eggs have not been published. In this study, we developed a rapid and reliable method for the simultaneous determination of SMM, SDM and their N^4-acetyl metabolites by HPLC in various foods of animal origin.

2. Experimental

2.1. Materials and reagents

Edible muscle tissues of swine, cattle and chicken and eggs served as samples, and were stored in a refrigerator until analysis. SMM and SDM were obtained from Wako (Osaka, Japan) and Sigma (St. Louis, MO, USA), respectively. N^4-AcSMM and N^4-AcSDM were generous gifts from Daiichi Seiyaku (Tokyo, Japan). Each stock standard solution (100 μg/ml) was prepared by accurately weighing 10 mg, dissolving it in 100 ml of HPLC-grade acetonitrile and diluting to the desired volume with the HPLC mobile phase. A 6-g portion of aluminium oxide 90 active basic (activity I, 60–200 μm) (Merck, Darmstadt, Germany) was placed in a column (300 × 15 mm I.D.) and sequentially washed with 30 ml of 90% (v/v) acetonitrile solution and acetonitrile. Other chemicals were obtained from Merck and were of the highest purity available.

2.2. Extraction and clean-up procedure

An accurately weighed 5-g amount of sample was homogenized in 25 ml of 90% (v/v) acetonitrile solution and 20 ml of hexane with a homogenizer (AM-1; Nippon Seiki, Tokyo). After centrifugation at 2100 *g* for 10 min, the supernatant was poured into a separating funnel through a No. 2 filter-paper (Toyo Roshi, Tokyo, Japan). The extraction was repeated twice and the combined supernatant was left until completely separated into two layers. The acetonitrile layer was collected and then dried by adding anhydrous sodium sulphate followed by filtration. The filtrate was applied to an alumina column. After the column had been washed with 30 ml of acetonitrile, sulphonamides and their N^4-acetyl metabolites were eluted with 20 ml of 90% (v/v) acetonitrile solution. The eluate was evaporated to dryness and the residue was dissolved in 1 ml of HPLC mobile phase. A 20-μl volume of the solution was injected into the HPLC system.

2.3. HPLC conditions

Analyses of standard and extracted sulphonamides were conducted using an LC-9A pump (Shimadzu, Kyoto, Japan) equipped with a 20-μl Rheodyne Model 2791 injector and an SPD-6A UV–Vis spectrophotometric detector (Shimadzu) operator at 270 nm. The separation was performed on a LiChrosorb RP-18 (7 μm) column (250 × 4 mm I.D.) (Merck) with a guard column (4 × 4 mm I.D.) (Merck) using acetonitrile–0.05 *M* phosphate buffer (pH 5.0) (25:75, v/v) as the mobile phase at a flow-rate of 1.0 ml/min at ambient temperature.

2.4. Calibration

Working standard solutions of concentrations 0.01, 0.02, 0.05, 0.10, 0.20 and 0.40 μg/ml of SMM, SDM, N^4-AcSMM and N^4-AcSDM were prepared from the stock standard solutions. Volumes of 20 μl of these solutions were injected into the column. Calibration graphs were obtained by measurement of peak heights.

3. Results and discussion

3.1. HPLC conditions

In order to determine sulphonamides, the use of HPLC appears to be much more advantageous than TLC and GC because of the high

sensitivity and speed without the complicated treatment of samples such as is needed in GC analyses [11,12]. We reported previously that six sulphonamides could be determined by HPLC using an ODS column and a mixture of acetonitrile, acetic acid and water as the mobile phase [10]. In preliminary experiments, when the same or similar HPLC conditions were used, SDM and N^4-AcSDM could be separated but not SMM and N^4-AcSMM. We therefore tried to separate these compounds using a combination of acetonitrile and phosphate buffer as the mobile phase. Based on several parameters such as pH and concentration of phosphate buffer, flow-rate and detection wavelength, the optimum HPLC conditions were determined.

The retention of all sulphonamides decreased on raising the pH of the buffer and with increasing concentration of acetonitrile in the mobile phase. The pH of phosphate buffer, rather than its molar concentration, affected the separation of target compounds. The flow-rate was 1.0 ml/min and the monitoring wavelength was adjusted to 270 nm because the maximum adsorption of all sulphonamides occurred at 270 nm [6,7,13]. The best separation of the compounds was obtained using acetonitrile–0.05 *M* phosphate buffer (pH 5.0) (25:75, v/v) as the mobile phase. Fig. 1 shows the chromatogram of N^4-AcSMM, SMM, N^4-AcSDM and SDM obtained under the established conditions. The target compounds were successfully separated within 12.4 min.

3.2. Calibration

The calibration graphs for SMM, SDM, N^4-AcSMM and N^4-AcSDM were linear between 0.02 and 0.4 μg/ml and fitted the following equations, where y is peak height (mm) and x is concentration (μg/ml):

SMM: $y = 376.09x$ ($r = 0.9999$) (1)

SDM: $y = 197.75x$ ($r = 0.9996$) (2)

N^4-AcSMM: $y = 562.08x$ ($r = 0.9999$) (3)

N^4-AcSDM: $y = 247.77x$ ($r = 0.9994$) (4)

All of the correlation coefficients (r) were highly significant ($p < 0.01$) and all the calibration graphs were linear and passed through the origin. The detection limits of all the compounds were determined according to Ref. [9], and were 0.4 ng in each instance.

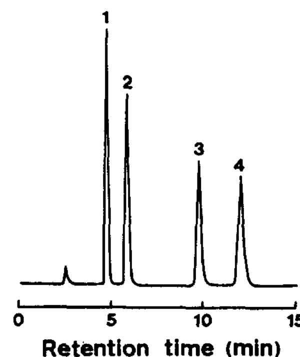

Fig. 1. Typical chromatogram of standard mixture. Peaks: 1 = N^4-acetylsulphamonomethoxide (t_R = 4.6 min); 2 = sulphamonomethoxine (t_R = 5.9 min); 3 = N^4-acetylsulphadimethoxine (t_R = 9.9 min); 4 = sulphadimethoxine (t_R = 12.4 min) (5 ng each). Column, LiChrosorb RP-18 (250 × 4 mm I.D.); mobile phase, acetonitrile–0.05 *M* phosphate buffer (pH 5.0) (25:75, v/v); flow-rate, 1.0 ml/min; detection, 270 nm; absorbance range setting, 0.01 AUFS.

3.3 Extraction and clean-up

The difficulties in drug residue analyses in complex biological matrices such as animal tissues are generally caused by interfering co-extractants when target compounds are isolated [6–8,14,15]. The advantage of the present method is that SMM, SDM and their metabolites in various foods of animal origin can be determined using the same procedure without interferences. The extraction of the target compounds from meat and eggs was performed by homogenizing with 45 ml of 90% (v/v) acetonitrile–hexane (5:4, v/v) to minimize the fat content. Also, with the egg samples, this extract did not form an emulsion that would hinder the recovery of the target compounds. After centrifugation, they were completely recovered into the acetonitrile solution layer without leading to residue losses on the cellular pellet or transfer into the hexane layer (data not shown).

The extract is further purified to remove

interfering materials. Our previous work showed that basic alumina column chromatography was suitable for the clean-up of residual SMM, SDM and sulphaquinoxaline in various chicken tissues [10]. Therefore, basic alumina was used as the packing material for the column chromatography in this study. Fig. 2 shows the elution volumes of the target compounds from the alumina column using 90% (v/v) acetonitrile solution as the mobile phase. The target compounds in 5 ml of each collected fraction were determined by HPLC. All compounds were eluted with 20 ml of the mobile phase. The average recoveries ($n = 3$) of SMM, SDM, N^4-AcSMM and N^4-AcSDM were 98.3, 98.8, 100.1 and 99.9%, respectively, when the elution volume was 20 ml.

The resulting extracts were free from interferences, as can be seen in HPLC traces of blank pork (Fig. 3A), blank eggs (Fig. 3B), spiked pork (Fig. 3C) and spiked eggs (Fig. 3D). Similar chromatograms were obtained from beef and chicken samples (data not shown).

The use of commercial cartridge columns in order to extract and purify residual drugs in various foods of animal origin has been reported [8,9]. However, our preliminary experiments showed that sulphonamide-related compounds

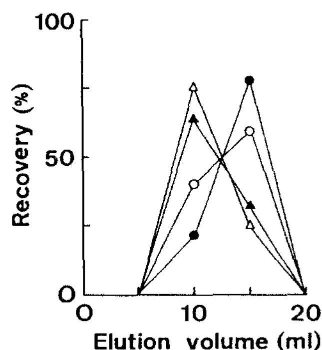

Fig. 2. Elution pattern of sulphamonomethoxine, sulphadimethoxine and their N^4-acetyl metabolites from an alumina B column using 90% (v/v) acetonitrile solution as the mobile phase. A mixture of standard solutions of four compounds was prepared (2.5 μg per 20 ml) and the solution was applied to the column. Results of three replicates. ● = Sulphamonomethoxine; ○ = N^4-acetylsulphamonomethoxine; ▲ = sulphadimethoxine; △ = N^4-aceylsulphadimethoxine.

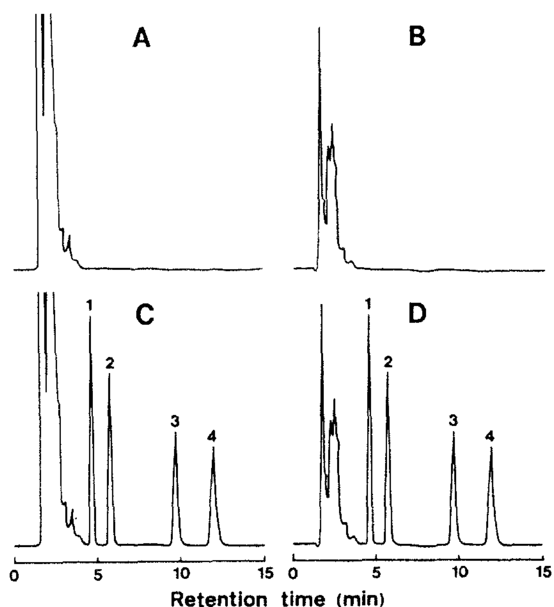

Fig. 3. Typical chromatograms of blank and spiked (0.05 ppm) pork and egg samples. (A) blank pork; (B) blank egg; (C) spiked pork; (D) spiked egg. Peaks and HPLC conditions as in Fig. 1.

including their metabolites from 5-g samples could not be determined simultaneously using the cartridges because of interfering co-extractants. Although the sequence of extraction and clean-up steps described here may be considered more or less classical, they can be evaluated in terms of the minimal steps and amounts of organic solvents which are required without interference. Moreover, packing materials such as alumina are much more economical than commercial cartridges.

3.4. Recovery studies

Using the present method, studies of the recovery of SMM, SDM, N^4-AcSMM and N^4-AcSDM from 5 g of beef, pork, chicken and eggs were carried out at spiking levels of 0.1, 0.5 and 1.0 ppm. As shown in Table 1, satisfactory results were obtained and the average recovery of these compounds from all samples were greater than 80% with relative standard deviations (R.S.D.) between 0.4 and 5.0%. In a practical analysis, the limits of detection from 5 g of

Table 1
Recoveries of sulphamonomethoxine, sulphadimethoxine and their N^4-acetyl metabolites from meat and eggs

Sample	Spiked level (ppm)	Recovery (%)			
		SMM	N^4-AcSMM	SDM	N^4-AcSDM
Beef	0.1	98.0 (3.6)	87.5 (1.1)	96.2 (3.7)	96.0 (0.6)
	0.5	88.7 (4.4)	90.5 (2.9)	86.9 (4.4)	91.7 (0.9)
	1.0	91.3 (3.4)	85.7 (2.4)	94.3 (1.3)	90.0 (3.4)
Pork	0.1	97.6 (0.5)	89.3 (2.6)	95.7 (4.2)	95.0 (3.0)
	0.5	95.9 (1.4)	91.3 (0.6)	94.5 (0.9)	90.9 (4.6)
	1.0	95.1 (0.4)	93.4 (1.8)	95.8 (2.2)	89.3 (1.4)
Chicken	0.1	91.9 (2.9)	82.1 (1.7)	92.3 (2.5)	89.3 (2.8)
	0.5	90.4 (1.6)	88.8 (3.4)	91.3 (2.6)	91.0 (0.5)
	1.0	94.9 (2.6)	86.7 (4.7)	86.2 (2.1)	92.1 (3.3)
Eggs	0.1	90.5 (2.0)	85.7 (2.6)	93.8 (2.5)	90.9 (3.3)
	0.5	88.3 (0.7)	88.0 (5.0)	91.7 (2.6)	94.8 (2.8)
	1.0	93.8 (1.2)	90.3 (2.0)	87.0 (2.1)	89.5 (0.5)

Recoveries of four compounds from 5 g of beef, pork, chicken and eggs at the indicated levels according to the proposed method. Results are averages of five replicates. Figures in parentheses are relative standard deviations (%).

samples were 0.01 ppm for all compounds. The high recovery and low R.S.D. together with the low limits of detection indicate that this method has a good precision and may be accurate.

The method presented here has several minor technical improvements over previously published procedures. The purpose of this study was to develop a method for the simultaneous assay of SMM, SDM and their N^4-acetyl metabolites in various foods of animal origin. Characteristics of this procedure are that it is rapid, sensitive, precise and economical. Therefore, this procedure may be useful for monitoring residual drugs in various foods of animal origin and studying pharmacokinetics.

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