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Simultaneous determination of sulfamonomethoxine, sulfadimethoxine, and their hydroxy/N^4-acetyl metabolites with gradient liquid chromatography in chicken plasma, tissues, and eggs

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Abstract

A simultaneous determination of sulfamonomethoxine, sulfadimethoxine, and their hydroxy/N^4-acetyl metabolites in chicken plasma, muscle, liver, and eggs using gradient high-performance liquid chromatography (HPLC) with a photo-diode array detector is developed. All the compounds are extracted by a handheld ultrasonic homogenizer with ethanol followed by centrifugation. The separation is performed by a reversed-phase C4 column with a gradient elution (ethanol:1% (v/v) acetic acid, v/v; 10:90 → 20:80). Average recoveries from samples spiked at 0.1–1.0 $\mu g\,g^{-1}$ or $\mu g\,ml^{-1}$ for each drug were >90% with relative standard deviations within 4%. The limits of quantitation were <30 $ng\,g^{-1}$ or $ng\,ml^{-1}$.

Keywords: Sulfamonomethoxine; Sulfadimethoxine; Hydroxy/N^4-acetyl metabolites; Gradient liquid chromatography; Toxic/harmful solvents-free

1. Introduction

Sulfamonomethoxine (SMM) and sulfadimethoxine (SDM) are frequently used for prevention or treatment of diseases to poultry in Japan and all the countries of the world, respectively. In order to elucidate the effect of the drugs and administer them in such a way as to produce chicken products free from drug residues, it is necessary to clarify the pharmacokinetics of SMM and SDM in chickens.

SMM and SDM can be mainly metabolized by hydroxylation and acetylation in chicken [1,2] (Fig. 1). For SDM, previous study had found hydroxy (OH) metabolites in three ways, at the 2-, 6-, and both 2- and 6-positions of the pyrimidine ring (2-OH-SDM, 6-OH-SDM, and 2,6-diOH SDM), respectively, in chickens in vivo [3]. As well as SDM, three kinds of OH metabolites of SMM (2-OH-SMM, 6-OH-SMM, and 2,6-diOH SMM) have been found in turtles [4]. OH-Metabolites still possess a free *para*-aminophenyl group which interferes with the *para*-aminobenzonic acid synthesis in bacteria, that is, antibacterial [5]. Although N^4-acetyl (Ac) metabolite (Ac-SMM or AcSDM) has no antibacterial activity, it possess the following chemical/pharmacokinetic properties: (1) a lower solubility (pH 7.0), which may lead the renal toxicity by precipitation in kidney [6]; (2) it is de-acetylated to the parent drug in vivo/vitro [1,6–11]; (3) higher plasma protein binding than the parent drug, which may slow down the excretion pace [2]. A determination of the pharmacokinetic profiles for OH/Ac-metabolites of SMM and SDM in chickens is therefore an important means to create the eligible prescription and to guarantee the food safety. In addition, it is necessary to develop an analytical method for the simultaneous determination of SMM, SDM, and their OH/Ac-metabolites. As far as we are aware, there are no acceptable methods.

Previous analytical methods [12–20] have described that sulfonamides and/or OH/Ac-metabolites residues in animal plasma or foods of animal origin could be acceptably determined by high-performance liquid chromatography (HPLC). However, all of these methods could not detect all OH/Ac-metabolites of SMM and SDM, and require the

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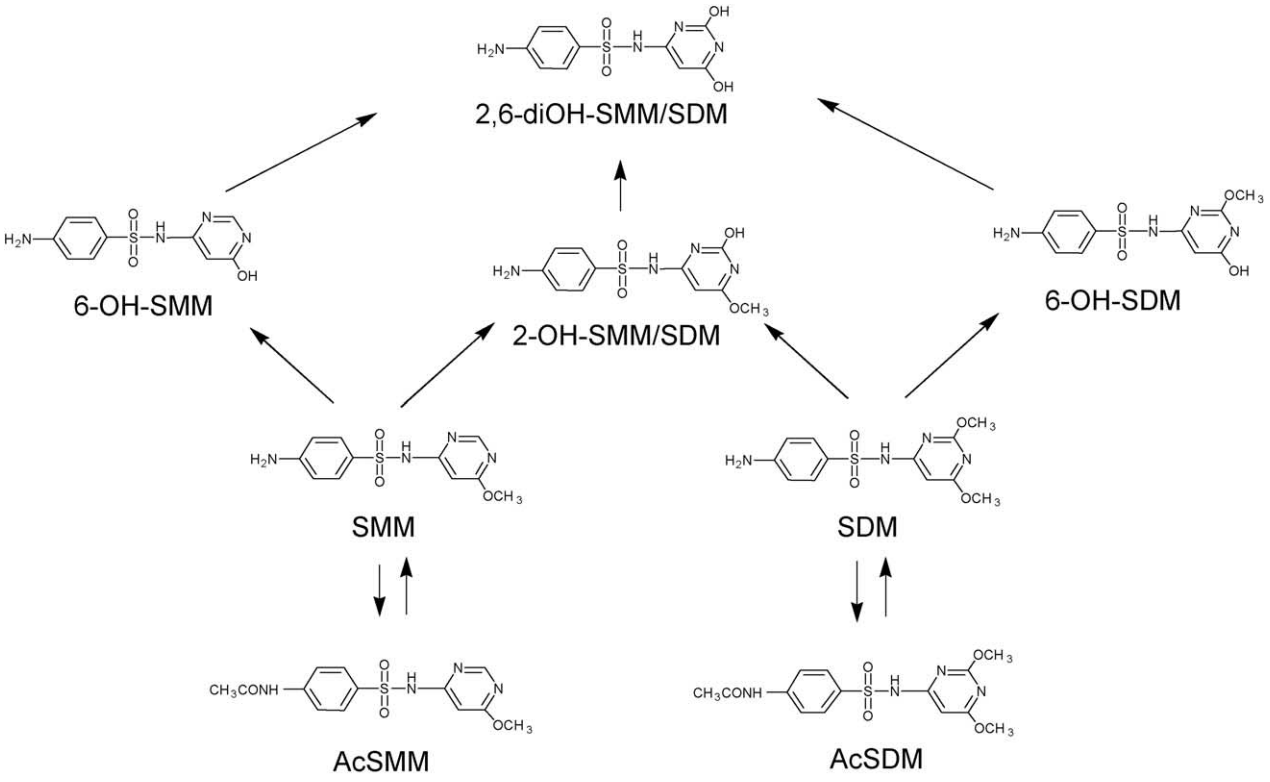

Fig. 1. Structures of sulfamonomethoxine, sulfadimethoxine, and their hydroxy/N^4-acetyl metabolites.

use of toxic or harmful organic solvents, like acetonitrile, dichloromethane, hexane, and ethyl acetate as the extracting solvent or the HPLC mobile phase and some hazardous acids like, trichloroacetic acid and perchloric acid for extraction and deproteinization in sample preparation. These chemicals are harmful to the environments and the analysts [21]. Their disposal is costly and must be performed with ecological responsibility. Because discharging toxic solvent waste is a big problem, analytical methods should avoid their use [22–24].

In this paper, we have developed a green and rapid method for simultaneous determination of SMM, SDM, and their OH/Ac-metabolites in chicken muscle, liver, eggs, and plasma without use of toxic/harmful solvents and reagents.

2. Experimental

2.1. Materials and reagents

Laying hens on a drug-free basal diet were purchased from a poultry farm in Osaka, Japan. Plasma, muscle, liver, and eggs obtained from hens, served as blank samples, and were stored in a refrigerator until analysis.

Ethanol, distilled water, (HPLC grade), and acetic acid (analytical chemical grade) were obtained from Wako Pure Chem. Ltd. (Osaka, Japan).

SMM and SDM standards were obtained from Wako Pure Chem. Ltd. Four hydroxy metabolites of SMM or SDM, i.e., 2-OH-SMM/SDM, 6-OH-SMM, 2,6-diOH-SMM/SDM, 6-OH-SDM, and AcSMM, AcSDM were generous gifts from Dr. Miura (Daiichi Seiyaku, Tokyo).

Each stock standard solution was prepared by accurately weighing (10 mg) and dissolving it in ethanol (100 ml). Working mixed standard solutions were prepared by diluting the stock solutions with distilled water. These solutions can be kept at 4 °C and were stable for up to 1 month.

2.2. Apparatus

The following apparatus were used for the sample preparation: an ultrasonic homogenizer Model HOM-100 (2 mm i.d. chip) (Iwaki Glass Co. Ltd., Funabashi, Japan); a microcentrifuge (Biofuge fresco, Kendo Lab. Products, Hanau, Germany); rotary evaporator Model EYELA N-N (Tokyo Rikakiki Co., Tokyo, Japan); 0.20 μm disposable syringe filter unit, DISMIC-13_{HP} (hydrophilic PTFE) (ADVANTEC, Tokyo, Japan); centrifugal ultrafilter unit, Ultrafree® MC/PL (regenerated cellulose ultrafiltration membrane, fractionating molecular weight = 5000, capacity ≤ 0.5 ml) (Millipore, Bedford, MA, USA).

HPLC analyses were carried out using a LC-10ADvp system equipped with an SPD-M10Avp photo-diode array detector (Shimadzu, Kyoto, Japan) interfaced with a Fujitsu FMV-6667CL6C personal computer (Fujitsu, Tokyo, Japan).

The separation was performed on Mightysil RP-4 GP (150 mm × 4.6 mm i.d.) with a guard column (5 mm ×

4.6 mm i.d.) (Kanto Chemical Co., Tokyo, Japan) using gradient mobile phase at a flow rate of 1.0 ml/min at 30 °C. The gradient mobile phase consisted of an initial combination of 10% ethanol in 1% acetic acid solution (in water) at 0 min, gradient to 20% ethanol in 6 min, held for 20 min. After completion of the HPLC run, the pump was programmed to regain its initial conditions within 3 min. Injection volume was a 20 μl. The obtained absorption maxima ranged from 267 to 272 nm. The wavelength selected for the target compounds was 270 nm (giving an average maximum absorbance for all the compounds).

2.3. Procedure

2.3.1. Chicken plasma and eggs

A 0.2 ml sample was placed into a microcentrifuge tube together with 0.6 ml of ethanol and homogenized with a handheld ultrasonic homogenizer for 30 s. The tube was centrifuged at 10,000 g for 5 min and the supernatant was filtered through a 0.20 μm disposable syringe filter unit. A 20 μl of the filtrate was injected into the HPLC system.

2.3.2. Chicken muscle and liver

A 0.2 g sample was placed into a microcentrifuge tube together with 0.8 ml of ethanol and homogenized with a handheld ultrasonic homogenizer for 30 s. The tube was centrifuged at 10,000 g for 5 min and the supernatant was filtered through a 0.20 μm disposable syringe filter unit. The filtrate was evaporated to dryness, and the residue was dissolved in 0.8 ml of 50% (v/v) ethanol solution (in water). A 0.3 ml portion of the resulting solution was put into an Ultrafree® MC/PL and centrifuged at 5000 g for 5 min. The Ultrafree® easily deproteinized the extraction solution in a short period (5 min), with only centrifuging. A 20 μl of the ultrafiltrate was injected into the HPLC system.

2.4. Recovery test

Recoveries of eight target compounds (2-OH-SMM/SDM, 6-OH-SMM, 2,6-diOH-SMM/SDM, 6-OH-SDM, SMM, SDM, AcSMM, and AcSDM) from blank samples spiked at three levels (0.1, 0.5, and 1.0 $\mu g\,g^{-1}$ or $\mu g\,ml^{-1}$ for each drug) were determined. Fortified samples were left to stand at 4 °C for 12 h after the addition followed by mixing. Recovery tests were done in quintuplicate.

3. Results and discussion

3.1. Sample preparation

The extracting operation using a handheld ultrasonic homogenizer enabled satisfactory extraction of the eight target compounds in chicken plasma, eggs, liver, and muscle samples with ethanol. SMM, SDM, and their OH/Ac-metabolites were extracted from a small sample (0.2 g or 0.2 ml) with a small volume of ethanol (0.6 or 0.8 ml) in a microcentrifuge tube (capacity 1.5 ml). The liver and muscle extracts were

Table 1
Recoveries, R.S.D.s, and limits of quantitation (LOQ) for the target compounds from fortified chicken eggs, muscle, liver, and plasma

Fortification level	Recovery (%) (mean, $n = 5$)							
	2-OH SMM/SDM	6-OH SMM	2,6-diOH SMM/SDM	6-OH SDM	SMM	AcSMM	SDM	AcSDM
Egg ($\mu g\,ml^{-1}$)								
0.1	96 (2.2)	96 (1.9)	95 (2.6)	97(2.2)	97 (2.0)	96 (2.4)	96 (2.3)	96 (2.5)
0.5	95 (2.3)	96 (2.1)	96 (2.2)	95 (1.9)	96 (2.0)	96 (2.1)	96 (2.4)	95 (2.6)
1.0	96 (2.0)	95 (2.5)	95 (1.8)	96 (2.3)	95 (2.1)	96 (2.3)	94 (2.6)	95 (2.0)
LOQ ($ng\,ml^{-1}$)	12.1	14.6	11.8	7.2	11.0	11.5	19.2	27.8
Muscle ($\mu g\,g^{-1}$)								
0.1	91 (3.0)	90 (2.7)	91 (2.7)	90 (2.5)	91 (2.4)	92 (3.0)	93 (2.4)	91 (2.2)
0.5	91 (2.3)	91 (2.2)	91 (2.1)	91 (2.2)	92 (2.4)	91 (2.2)	92 (2.1)	92 (2.6)
1.0	92 (2.0)	91 (2.5)	91 (2.4)	91 (2.0)	92 (2.3)	92 (2.1)	91 (2.2)	91 (2.3)
LOQ ($ng\,g^{-1}$)	12.4	15.3	12.2	7.8	11.5	12.3	20.0	28.4
Liver ($\mu g\,g^{-1}$)								
0.1	90 (1.9)	91 (2.5)	91 (2.3)	91 (2.4)	91 (2.6)	91 (2.0)	91 (2.8)	91 (3.0)
0.5	90 (2.3)	92 (2.0)	91 (2.5)	91 (1.7)	91 (2.1)	91 (2.6)	91 (2.5)	91 (2.2)
1.0	91 (2.3)	92 (2.3)	91 (2.3)	92 (2.1)	90 (2.3)	91 (2.5)	91 (2.4)	91 (2.2)
LOQ ($ng\,g^{-1}$)	12.6	15.7	12.9	7.7	11.7	12.8	20.6	29.8
Plasma ($\mu g\,ml^{-1}$)								
0.1	93 (2.7)	92 (3.5)	93 (3.1)	93 (3.2)	92 (2.6)	93 (2.4)	92 (2.2)	92 (1.5)
0.5	95 (3.0)	94 (3.2)	94 (3.3)	94 (3.4)	95 (3.8)	94 (3.1)	94 (2.0)	93 (3.3)
1.0	96 (2.0)	95 (2.8)	95 (3.1)	96 (2.0)	96 (2.3)	96 (2.1)	94 (2.2)	95 (2.5)
LOQ ($ng\,ml^{-1}$)	12.5	15.6	12.8	7.8	11.6	12.5	20.2	29.8

Values in parentheses are R.S.D.s.

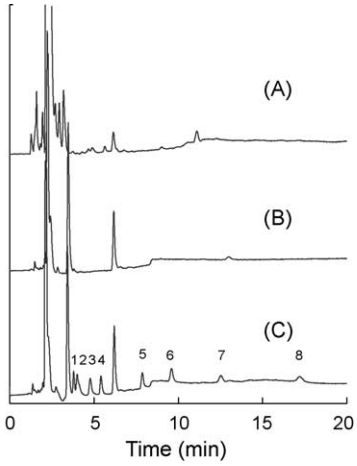

Fig. 2. HPLC chromatograms obtained from (A) liver, (B) egg, and (C) egg (spiked at 0.1 μg ml^{-1} for each drug) samples. Peaks: (1) 6-OH SMM (t_R: 3.8 min); (2) 2-OH SMM/SDM (t_R: 4.0 min); (3) 2,6-diOH SMM/SDM (t_R: 4.8 min); (4) 6-OH SDM (t_R: 5.4 min); (5) SMM (t_R: 7.9 min); (6) AcSMM (t_R: 9.6 min); (7) SDM (t_R: 12.5 min); (8) AcSDM (t_R: 17.2 min).

further cleaned up by Ultrafree® MC/PL as a centrifugal ultrafiltration unit.

Advantages of the present sample preparation method include low harmless solvent consumption, no use of toxic/harmful solvents and reagents, and remarkably low capital equipment costs. The procedure is simple in operation and offers rapid sample turnaround. Ten plasma (or egg) and liver (or muscle) samples can be cleaned up in 0.5 and 1 h, respectively. The simple procedure allowed for high reproducibility of the target compounds (Table 1).

3.2. HPLC optimal conditions

Using the HPLC conditions described, SMM, SDM, and their OH/Ac-metabolites were all well resolved. When a higher percentage of ethanol was used, OH-SMM/SDMs were inadequately resolved from the interference of the resulting sample extract. Optimal resolutions for a batch of OH-SMM/SDM resulted in unacceptably long retention times for SDM and AcSDM. In order to resolve all target compounds in short retention times, gradient conditions were examined. The gradient mobile phase ranged from 10 to 20% ethanol in 1% acetic acid solution. On the proposed HPLC conditions, the retention times of 2-OH-SMM/SDM, 6-OH-SMM, 2,6-diOH-SMM/SDM, 6-OH-SDM, SMM, SDM, AcSMM, and AcSDM were 3.8, 4.0, 4.8, 5.4, 7.9, 9.6, 12.5, and 17.2 min, respectively, yielding capacity factors (k') of 1.9, 2.1, 2.7, 3.2, 5.1, 6.4, 8.6, and 12.2, respectively (Fig. 2). The present HPLC analysis accomplished optimum resolution less than 18 min (giving symmetrical sharp peaks) and was also the possibility of multiple sequential injections, without the risks of interfering late-eluting peaks.

Fig. 2 displays the chromatograms of chicken liver and egg samples. There are no interfering peaks for quantitation and identification. Similar clean chromatograms were obtained from chicken plasma and muscle extracts with no interfering peaks after a simple and rapid sample preparation method.

3.3. HPLC repeatability

The chromatographic repeatability was obtained as the relative standard deviations (R.S.D.s) of peak areas and retention times calculated for 10 replicate injections of a spiked (0.1 μg^{-1} or μg ml^{-1} for each drug) sample. The values for all the target compounds were estimated to be 0.08% for peak areas and 0.62% for retention times.

3.4. Method validation

Table 1 shows the average recoveries of SMM, SDM, and their metabolites from chicken plasma, muscle, liver, and egg samples at three different spiking levels (0.1, 0.5, and 1.0 μg g^{-1} or μg ml^{-1} for each drug). The average recoveries (≥90%) with their R.S.D.s (1.7–3.8%) were well within the acceptable criteria for the residue analysis that Codex sets up (recovery 70–110% and R.S.D. < 20%, for MRL < 100 ng g^{-1} or ng ml^{-1}) [25]. The limits of quantitation (LOQs) were calculated by measuring the analytical background response in accordance with the CCMAS 1993 (Codex Committee for Methods Analyses and Sampling). Based on the peak areas in HPLC chromatograms obtained from blank and fortified samples, LOQ was defined as the average background of samples (=fluctuations of the baseline) plus 10 times the standard deviation (S.D.). The LOQs for the eight target compounds ranged from 7.2 to 29.8 ng g^{-1} or ng ml^{-1}. The LOQs were well below the maximum residue limit (MRL = 100 ng g^{-1} or ng ml^{-1}) established for SMM or SDM in animal products by European Union [26]. No MRL for the OH/Ac-metabolites in animal products has been fixed up to now. The authors generated the spiked recovery graph as the practical calibration line by plotting peak areas of fortified sample extracts ranging from 0.1 to 2.0 μg g^{-1} or μg ml^{-1}. The resulting correlation coefficient for each target compound was >0.998 ($P < 0.01$).

4. Conclusions

The present study has succeeded in making a simple method without the use of toxic/harmful solvents and reagents at all for simultaneous determination of SMM, SDM, and their OH/Ac-metabolites in chicken plasma, eggs, liver, and muscle. This method has been developed to study the pharmacokinetic profiles in chickens and monitor the drug residues of SMM, SDM, and their OH/Ac-metabolites in chicken products. The complete procedure, which harms neither the environment nor humans, is economical and provides reproducible recoveries.

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