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Restricted-access media liquid chromatography for determination of sulfamonomethoxine, sulfadimethoxine, and their N^4-acetyl metabolites in eggs

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Abstract

Simultaneous determination of sulfamonomethoxine, sulfadimethoxine, and their N^4-acetyl metabolites in eggs using restricted-access media high performance liquid chromatography (RAM-HPLC) with a photo-diode array detector is developed. The target compounds are extracted by a handheld ultrasonic homogenizer with saturated ammonium sulfate solution followed by centrifugation. The separation is performed by a Hisep shielded hydrophobic phase column, isocratic elution with 0.3% acetic acid solution (pH 2.9, in water)–ethanol (75:25, v/v). Average recoveries from samples spiked at 0.1–1.0 ppm for each drug were >91% with relative standard deviations within 4%. The limits of quantitation were ≦0.08 ppm. No hazardous-chemicals were used in all the processes.

Keywords: Restricted-access media high performance liquid chromatography; Environmental-friendly analysis; Sulfonamide; Eggs

1. Introduction

Sulfamonomethoxine (SMM) and sulfadimethoxine (SDM) are frequently used for prevention or treatment of diseases in poultry in Japan and all the countries of the world, respectively. There is a risk of drugs remaining in poultry products because of illegal use such as an excessive administration and an inappropriate withdrawal period. Drug residues may cause allergic or toxic reaction to consumers and promote occurrence of antibiotic-resistant bacteria.

Sulfonamides, including SMM and SDM, can be acetylated at the N_4-position. N^4-acetyl (Ac) metabolite (AcSMM or AcSDM), one of the main metabolites of SMM or SDM in food-producing animals (Vree, Hekster, & Tijhuis, 1985, 1987), possess the following chemical and pharmacokinetic properties:

- lower solubility (pH 7.0), which may cause the renal toxicity as a result of precipitation in the kidney (Vree & Hekster, 1985);
- they are deacetylated to the parent compound in vivo and vitro (Furusawa, 1998, 2000a, 2000b, 2001; Shimoda, Kokue, Shimizu, Muraoka, & Hayama, 1988; Shimoda, Vree, Beneken, & Arts, 1990; Vree et al., 1985);
- plasma protein binding is higher than the parent compound, which may affect their excretion rates (Vree, Hekster, Nouws, & Dorrestein, 1987).

Since eggs are nutritious "perfect foods" and readily available, it is important to detect both the parent drugs and the Ac metabolites (Fig. 1) to guarantee the food safety. The acceptable method for residue monitoring should be easy to perform and economical in cost and time because a large number of samples are analyzed.

Previous analytical methods (Balizs, Benesch-Girke, Börner, & Hewitt, 1994; Furusawa, 2000a, 2000b; Furusawa & Mukai, 1994; Shaikh, Rummel, & Donoghue, 1999; Uno

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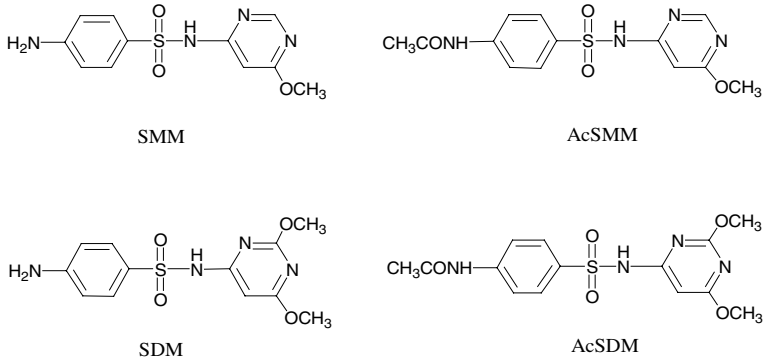

Fig. 1. The structures of sulfamonomethoxine (SMM), sulfadimethoxine (SDM), and their N^4-acetyl metabolites.

& Maeda, 1995) have described that sulfonamides and Ac metabolites residues in animal products could be acceptably determined by high performance liquid chromatography (HPLC). However, these methods have a crucial disadvantage. Procedures require the use of hazardous solvents, like acetonitrile (Balizs et al., 1994; Furusawa, 2000a, 2000b; Furusawa & Mukai, 1994; Shaikh et al., 1999; Uno & Maeda, 1995), dichloromethane (Balizs et al., 1994), and hexane (Balizs et al., 1994; Furusawa & Mukai, 1994), as the extraction solvents or the HPLC mobile phase. Although these substances are widely used and highly valued because of their excellent solvency in a wide range of analytes, they are harmful to the environment and to humans. Waste hazardous solvents is a severe world-wide problem. Risks associated with these chemicals extend beyond their direct effect on the health of humans and wildlife in the environment and ecosystem in which we all reside, and the cost of treatment and disposal of analytical chemicals has increased steadily over the past ten years. Analytical methods should therefore avoid the use of hazardous solvents and reagents (Anastas & Warner, 1998; Dost, Jones, & Davidson, 2000).

In order to simplify analytical procedure, restricted-access media (RAM)-HPLC column has been created to determine plasma drug concentrations with direct injection. RAM-HPLC has been successfully applied to determination of analytes in plasma and serum. However, only a few applications to another matrix like muscle or milk have been reported (Agarwal, 1990; Kishida & Furusawa, 2004; Ueno & Aoki, 1996). As far as we aware, there is no application to analysis of drugs in eggs.

This paper presents a first application of RAM-HPLC determination of SMM, SDM, and their Ac metabolites in eggs without the use of hazardous-chemicals.

2. Experimental

2.1. Materials and Reagents

Eggs were obtained from laying hens kept in individual cages and given drug-free basal diet. Whole eggs (= mixture of egg yolk and albumin) were used as samples.

SMM, SDM, ethanol, distilled water, (HPLC grade), and acetic acid (analytical chemical grade) were obtained from Wako Pure Chem. (Osaka, Japan). AcSMM and AcSDM were generous gifts from Dr. Miura (Daiichi Seiyaku, Tokyo, Japan).

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 are stable for up to one month.

2.2. Procedure

A 0.3 g sample was placed into a microcentrifuge tube together with 0.6 ml of with saturated ammonium sulfate solution (4 mol/l) and homogenized for 30 s with a handheld ultrasonic homogenizer (Model HOM-100, 2 mm i.d. chip, Iwaki Glass Co. Ltd., Funabashi, Japan). The tube was centrifuged at 10,000*g* for 5 min at 4 °C with a microcentrifuge (Biofuge Fresco, Kendo Laboratory Products, Hanau, Germany). The supernatant was injected directly into the RAM-HPLC. 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 FMV-6667CL6c computer (Fujitsu, Tokyo, Japan).

The RAM-HPLC column was a Hisep shielded hydrophobic phase column (250 × 4.6 mm i.d., 5 μm) (Supelco, Bellefonte, PA, USA). The separation was performed using 0.3% (v/v) acetic acid solution (pH 2.9, in water)–ethanol (75:25, v/v) as the mobile phase at a flow rate of 1.2 ml/min at 40 °C. The injection volume was 20 μl.

2.3. Recovery test

Recovery of SMM, SDM, AcSMM, and AcSDM from egg spiked at (0.1, 0.5, and 1.0 ppm) was determined. Each fortified sample was mixed and left to stand at 4 °C for 12 h before analysis.

3. Results and discussion

The main objective of this work was to develop a RAM-HPLC method for determining SMM, SDM, and their Ac metabolites in eggs without the use of hazardous-chemicals.

Ammonium sulfate, a protein precipitant popularly used for biological matrices, acetic acid solution, and ethanol, used here as the extraction/deproteinizing solution or HPLC mobile phase are not hazardous to the environment and human beings.

3.1. Sample preparation

The advantage of the sample preparative operation is using a handheld ultrasonic homogenizer, which is easy-to-use, portable, and yields rapid and easy extraction of target compounds in samples. This operation was able to extract all compounds from a small egg sample (0.3 g) effectively with a small volume of the saturated ammonium sulfate solution (0.6 ml) in a microcentrifuge tube (capacity 1.5 ml) and did not cause recovery loss by "flying off" of the tube content. In addition, the extract did not form an emulsion that was often the cause of low recoveries especially in extraction from eggs. After centrifugation, the target compounds were recovered completely in the clear aqueous layer. The time required for sample preparation was less than 10 min.

3.2. RAM-HPLC optimal conditions

The RAM-HPLC column used in this study, Hisep shielded hydrophobic phase column, was originally created to determine plasma drug concentrations with direct injection. This column has the advantage of enabling rapid separation of the target compounds (low molecular weight compounds) and the extractable proteins (high molecular weight compounds). The sample preparation has finished with rapid single step like the present procedure.

The HPLC conditions were optimized based on our previous report (Kishida & Furusawa, 2004). Since the same conditions were not suitable for the present analyses, mobile phases containing from 0.1% to 0.5% (v/v) acetic acid solution were tested. The best chromatogram, with complete separation of all the analytes as symmetrical sharp peaks in a short analysis time, was obtained using 0.3% (v/v) acetic acid solution (pH 2.9, in water)–ethanol (75:25, v/v) as the mobile phase at a flow rate of 1.2 ml/min at 40 °C. The photodiode-array detector was operated at 267 nm, which was a compromise among the wavelengths of maximum absorption of SMM, SDM, AcSMM, and AcSDM. Fig. 2A and B show the chromatograms obtained from blank and spiked chicken egg samples, respectively. The resulting extracts were free from compounds interfering with detection and identification in all HPLC chromatograms, indicating that no additional sample clean-up was necessary. The procedure thus accomplished rapid and environmental-friendly analysis of SMM, SDM, and their Ac metabolites in chicken eggs. The total time required for analysis of one sample was less than 35 min.

The author's previous paper for determination of SMM, SDM, AcSMM, and AcSDM in chicken plasma using saturated ammonium sulfate solution (Kishida, Nishinari, & Furusawa, 2005) offered relatively lower recoveries (78–84%) than the proposed method (91–96%). The previous sample preparation needed filtration of the sample extracts by 0.20 μm (hydrophilic PTFE) filters before HPLC analyses. The present method did not need filtrating before the RAM-HPLC analyses. It might avoid recovery losses and achieve the saving of analytical time and cost.

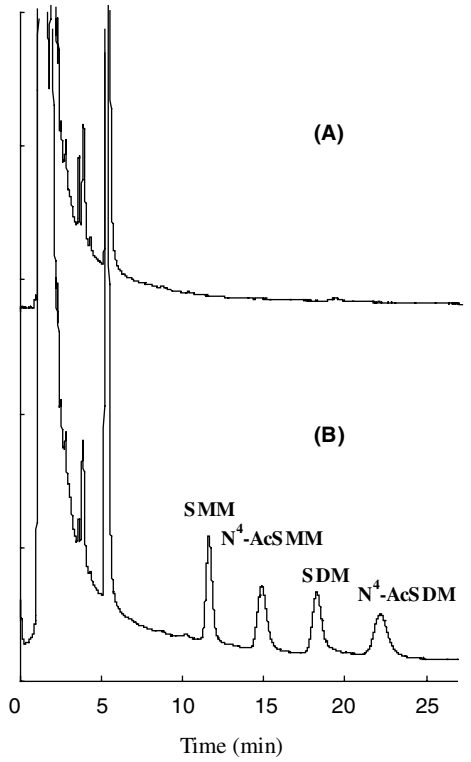

Fig. 2. RAM-HPLC chromatograms obtained from: (A) blank and (B) fortified (0.1 ppm for each drug) egg samples.

3.3. HPLC repeatability

Chromatographic repeatability was determined as the relative standard deviations (RSD) of peak areas and retention times calculated for ten replicate injections of a spiked sample (0.1 ppm, for each drug). The values for all the target compounds were estimated to be ≦0.07% for peak area and ≦0.54% for retention times.

3.4. Method validation and application

Analytical performance data are summarized in Table 1. Average recoveries were >91% with RSD <4%. Inter- and intra-assay variability, expressed as RSD, was ≦2.8%. These values are well within the criteria of the Codex for residue analysis (average recovery 70–110% and RSD <20% for an analyte concentration of ≦0.1 ppm) (Codex Alimentarius Commission, 1993). The limits of detection (LODs) and quantitation (LOQs) were calculated by measuring the analytical background

Table 1
Analytical performance data of the present method

Fortification level (ppm)	Recovery (%) (mean, $n = 5$)			
	SMM	AcSMM	SDM	AcSDM
0.1	91 (2.1)	92 (2.5)	94 (2.6)	91 (2.6)
0.5	94 (2.2)	95 (3.4)	95 (3.0)	92 (3.1)
1.0	92 (1.9)	96 (2.2)	94 (2.1)	93 (2.0)
Interassay variability (% ± SD)	2.0 ± 0.3	2.7 ± 0.6	2.6 ± 0.7	2.8 ± 0.6
Intraassay variability (%, $n = 5$)	1.5	2.2	2.1	2.4
Correlation coefficient[a]	0.999	0.999	0.998	0.998
LOD (ppm)	0.01	0.02	0.02	0.03
LOQ (ppm)	0.02	0.03	0.06	0.08

Values in parentheses are RSDs.
[a] Constructed from four points and each point represented the mean of five injections.

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, LOD and LOQ were defined as the average background of samples (= fluctuations of the baseline) plus 3 and 10 times the standard deviation (SD), respectively. The LOQs for the target compounds ranged from 0.02 to 0.08 ppm. These values were well below the maximum residue limit (MRL = 0.1 ppm) established for SMM or SDM in animal products by European Union (Commission of the European Communities, 1991). No MRL for Ac metabolites in animal products has been fixed up to now. Calibration lines were generated by plotting peak areas obtained from sample extracts fortified at levels ranging from 0.1 to 2.0 ppm. The correlation coefficients (r) for each target compound was >0.998 ($P < 0.01$).

As an application, 20 samples of commercial eggs purchased in Fukuoka Prefecture were analyzed using the present method. No samples contained detectable concentrations of SMM, SDM, and their Ac metabolites. All chromatograms were free from interferences. In order to clarify the pharmacokinetic profiles of SMM, SDM, AcSMM, and AcSDM in eggs, the author is planning to undertake the administration study in laying hens using the proposed method as a next study. In the preliminary experiment with egg samples after application of the drugs, the target compounds were detected completely.

4. Conclusions

A novel method has been developed for determination of SMM, SDM, and their Ac metabolites in eggs. The method requires no hazardous-chemicals and is, therefore, safer both for humans and for the environment. The RAM-HPLC enabled the simple and rapid analysis which avoided analyte losses and resulted in high reproducibility and reliability. This method might, therefore, be useful for practical residue monitoring and pharmacokinetic studies with eggs.

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