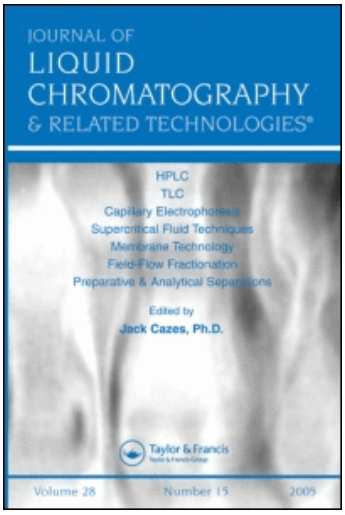

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HPLC DETERMINATION OF SULFADIMETHOXINE AND ITS HYDROXY METABOLITES FOLLOWING SPE OF EDIBLE CHICKEN TISSUES

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ABSTRACT

New cleanup techniques by an anion-exchange SPE column and a centrifugal ultra-filtration unit and a high performance liquid chromatographic (HPLC) determination of residual sulfadimethoxine (SDM) and its hydroxy metabolites (OH-SDMs), 4-sulfanilamido-6-methyl-2-hydroxy-4-pyrimidine (2-OH-SDM), 4-sulfanilamido-2-methyl-6-hydroxy-4-pyrimidine (6-OH-SDM), and 4-sulfanilamido-2,6-dihydroxy-4-pyrimidine (2,6-diOH-SDM), in edible chicken tissues (liver and muscle) were presented. The sample preparation was performed by homogenizing with a mixture of 90% (v/v) acetonitrile solution and n-hexane (5:4, v/v) to minimize the fat amount followed by ISOLUTE® SAX column and Ultrafree™ C3 as a centrifugal ultra-filtration unit. A Mightysil® RP-4 GP column and an isocratic mobile phase of 4% (v/v) acetic acid solution – acetonitrile – N,N-dimethylformamide (83:12:5, v/v/v) with a photo-diode array detector was used. Average recoveries of spiked SDM and OH-SDMs (0.1 and 0.5 ppm), respectively, were > 80.1% with coefficients of variation between 0.5 and 4.8%. The practical limit of detection was 0.05 ppm for all compounds.

INTRODUCTION

Sulfadimethoxine (4-sulfanilamido-2,6-dimethoxy-pyrimidine, SDM) is widely used by veterinarians for prevention and treatment of poultry diseases because of its wide spectrum of anti-bacterial activity and economic advantage gained from its application. As a result, there is concern that residues of this compound may be retained in chicken products.

For metabolites of SDM in chickens, the presence of N_4-acetyl SDM and N_1-glucuronide of SDM in urine of hens[1] and N_4-acetyl SDM and a hydroxy SDM, 4-sulfanilamido-2-methyl-6-hydroxy-4-pyrimidine (6-OH-SDM), in excreta of cocks[2,3] have been found. The tissue residues of 6-OH-SDM and two other kinds of hydroxy SDM, 4-sulfanilamido-6-methyl-2-hydroxy-4-pyrimidine (2-OH-SDM) and 4-sulfanilamido-2,6-dihydroxy-4-pyrimidine (2,6-diOH-SDM), in liver or breast-/thigh- muscles of male chicks[4] have been reported. In these metabolites, it has been found that acetyl metabolites and glucuronides of sulfonamides have no antibacterial activities, but their hydroxy metabolites have antibacterial activities.[4,5] The findings concern the residues of SDM and its hydroxy metabolites in the marketing of chicken liver and muscle since these residues may have toxic effects on consumers. To prevent some health problems with consumers, at present, an analytical method for monitoring residual SDM and its hydroxy SDMs (OH-SDMs) in chicken products is needed. Until now, an effective and practical method is lacking.

The difficulties in drug residue analyses in complex biological matrices, such as animal tissues, are caused by interfering co-extractants when target compounds are isolated. With all, some analytical methods for sulfonamides and its acetyl metabolites in edible meat using high-performance liquid chromatography (HPLC) in the reversed-phase system have been reported.[6-14] These methods are not applicable to the analysis of hydroxy sulfonamides in animal tissues due to the following reason: since the capacity factors of hydroxy sulfonamides are slightly smaller than those of the parent drugs,[15,16] the high polarity hydroxy metabolites are especially difficult to isolate.

Previous papers have presented the reversed-phase HPLC method for determination of SDM and its hydroxy metabolites, 2-OH-SDM, 6-OH-SDM and 2,6-diOH-SDM (OH-SDMs) (Figure 1), in human plasma[17] or edible tissues of male chicks.[4] There were the following problems in these methods: interfering peaks for OH-SDMs in the resulting chromatograms and low recoveries of 2,6-OH-SDM and 6-OH-SDM from the liver.

The aim of this study was to develop a simultaneous HPLC determining method for monitoring residual SDM and OH-SDMs in chicken products. This paper presents: (1) a new technique having a higher efficiency of cleanup and

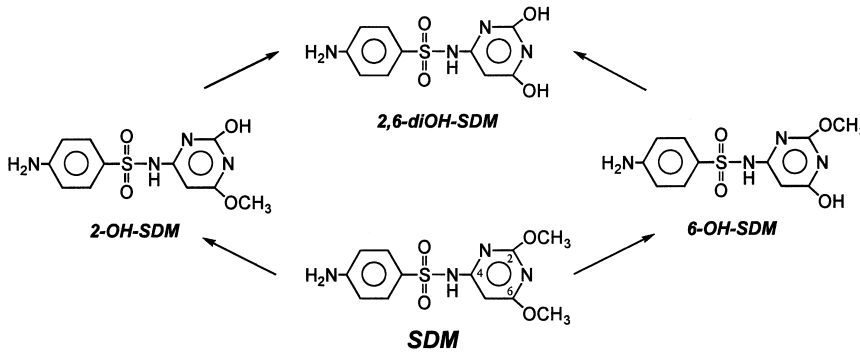

Figure 1. Structures of sulfadimethoxine (SDM) and its hydroxy metabolites.

reproducibility; (2) the simultaneous detection of SDM, 2-OH-, 6-OH- and 2,6-diOH-SDM by an isocratic HPLC system.

EXPERIMENTAL

Materials and Reagents

Edible tissues of chicken, i.e. muscle and liver, served as samples, and were stored in a refrigerator until analysis. SDM standard was obtained from Sigma (St. Louis, MO, USA). Three hydroxy metabolites of SDM (OH-SDMs), i.e. N^1-(6-methyl-2-hydroxy-4-pyrimidinyl) sulfanilamide (2-OH-SDM), N^1-(2-methyl-6-hydroxy-4-pyrimidinyl) sulfanilamide (6-OH-SDM) and N^1-(2,6-dihydroxy-4-pyrimidinyl) sulfanilamide (2,6-diOH-SDM), were the generous gifts from Dr. Miura (Daiichi Seiyaku, Tokyo, Japan). Other chemicals were obtained from Wako Pure Chem. Ltd. (Osaka, Japan). Distilled water and acetonitrile were of HPLC grade.

Each stock standard solution (100 μg/mL) of SDM and its hydroxy metabolites was prepared by accurately weighing 5 mg, dissolving it in 50 mL in distilled water. Working standard solutions were prepared by diluting the stock solution with distilled water. These solutions were stored in a refrigerator and were stable for up to one month.

Apparatus

The following apparatus were used in the sample preparation: Homogenizer, Model Histocolon® NS-50 (Microtec Nichion, Tokyo, Japan);

Two centrifuges, Model H-103N (Kokusan Enshinki Co. Ltd., Tokyo, Japan) and Model CFM-200 (micro-centrifuge, Iwaki Glass, Co. Ltd., Funabashi, Japan).

Five non-polar sorbents, three cation-exchange sorbents, and three anion-exchange sorbent types of ISOLUTE® SPE columns (100mg of the sorbent packed into a 3 mL reservoir) were purchased from International Sorbent Technology Ltd. (UK): non-polar phases were (C_{18}; C_8; CH, cyclohexyl,; PH, phenyl), cation-exchangers (CBA, carboxypropyl; PRS, propylsulphonic acid; SCX, benzenesulphonic acid), and the anion-exchangers (NH2, aminopropyl,; PSA, ethylenediamine-N-propayl,; SAX, quaternary amine).

An Ultrafree™ C3 LBC, disposable ultra-filtration unit, was purchased from Millipore (Milford, MA, USA).

HPLC analyses of five standard and extracted compounds were conducted using an JASCO HPLC (Model PU-980 pump and DG-980-50 degasser) (Jasco Corp., Tokyo, Japan), equipped with an SPD-M10A_{VP} diode array detector (Shimadzu, Kyoto, Japan) interfaced with an Fujitsu FMV-5133D7 personal computer (Fujitsu, Tokyo, Japan). The separation was performed on a Mightysil® RP-4 GP (5 μm) column (4.6 × 250 mm) (Kanto Chem. Co., Inc., Tokyo, Japan) with a guard column (4.6 × 5 mm) (Kanto Chem.) using 4% (v/v) acetic acid solution (in water) – acetonitrile – N,N-dimethylformamide (83:12:5, v/v/v) as the mobile phase at a flow-rate of 1.0 mL/min at ambient temperature. The injection volume was 20 μL.

Application of SDM and OH-SDMs to SPE Columns

The retention abilities of SDM (pKa=6.0) and OH-SDMs (dissolved in water) on the above SPE columns (bed volume = ca. 120 μL) were examined. These columns were pre-conditioned by washing with 1 mL of methanol followed by: 2 mL of water for the non-polar; 2 mL of 0.1 M acid aqueous buffer (pH 5.0) or water for cation-exchangers; 2 mL of 0.1 M basic aqueous buffer (pH 8.5) or water for anion-exchangers. The flow-rate was < 5 mL/min.

A 0.5 mL of a mixture standard solution (in water) containing 1.0 μg of SDM and OH-SDMs, respectively, was applied to the column and the column was then washed with 2 mL of water. Unretained compounds in the passed solution were determined by HPLC.

Extraction and Cleanup Procedures

An accurately weighed 1 g of sample was taken into a centrifuge glass tube (50 mL size) and homogenized with 20 mL of 90% (v/v) acetonitrile (saturated

with n-hexane) solution (in water) and 16 mL of n-hexane[14] for 2 min using a high-speed homogenizer. After centrifugation at 3,500 rpm for 5 min, the acetonitrile layer in supernatant was evaporated to ca. 2 mL. The evaporated solution was poured into an ISOLUTE SAX column, and the column was washed with 3 mL of water. SDM and OH-SDMs were eluted from the column with 1 mL of 0.5 N HCl, and the eluate was then made up to 2 mL precisely with distilled water. A 0.4 mL of the eluate was put into ultrafree™ and centrifuged at 2,000 × g for 10 min. The ultra-filtrate was injected into the HPLC system.

Recovery Test

The recoveries of SDM, 2-OH-SDM, 6-OH-SDM and 2,6-diOH-SDM were determined from blank samples spiked at 0.1 and 0.5 μg/g. These spiked levels were prepared by adding 100 μL of the above standard solutions (1.0 and 5.0 μg/mL), respectively, with separate 1.0 g portions of the sample. These fortified samples were allowed to stand at 4°C for 12 h after the standard additions, followed by mixing.

RESULTS AND DISCUSSION

The separation of OH-SDMs from interfering peaks in the high performance liquid chromatogram of the tissue extract is very difficult as can be seen in the previous papers.[4,17] The cleanup procedure had to be very effective. The advantage of the present study is that SDM and OH-SDMs in chicken tissues can be determined without interferences. The extraction was performed by homogenizing with a mixture of 90% (v/v) acetonitrile and n-hexane (5:4, v/v) to minimize the lipid content, as described previously.[14] The 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 cellar pellet or transfer into the hexane layer. The extract was further purified by a SPE column and a centrifugal ultra-filtration to remove interfering materials.

Cleanup Techniques

Commercial solid-phase extraction (SPE) cartridges, Bond Elut® -C_{18}[10] (non-polar phase), Sep-Pak® Alumina N[14]/Baker 10® amino[11] (polar phases) and Bont-Elut SCX[4] (caion-exchanger), have been applied to the cleanup of sulphonamides in foods of animal origin. Using three types of ISOLUTE SPE columns, non-polar, cation-exchanger, and anion-exchanger, the present study was tested and compared to the retention of SDM and OH-SDMs in the columns. As a result, 2-OH-SDM, 6-OH-SDM and 2,6-OH-SDM (dissolved in water) on the non-polar phase, cation-exchanger, and anion-exchanger columns

other than the SAX column were hardly retained. Therefore, the SAX column was used for the cleanup column and the elution volumes of SDM and OH-SDMs (1 μg each compound) from the column using 0.5 N HCl as the mobile phase was examined. These target compounds in 0.5 mL of each collected fraction were determined by HPLC. All compounds were eluted with an elution volume of 1.5 mL. The average recoveries (n=5) were > 95% when the elution volume was 1.5 mL. To purify the obtained extract further, the extract was cleaned up by an Ultrafree™ C3 as the centrifugal ultra-filtration unit. This disposable device was able to deproteinize the extracted solution easily, in a short period, only with centrifugal processing. A considerable saving of time was achieved.

HPLC Operating Conditions

As for the HPLC analysis of SDM or OH-SDMs the reversed-phase (RP) system used the C_{18} [6,10-13] or C_8 [4,17] column is a mainstream. In the present study, three types of RP columns, i.e. Mightysil columns (- RP-18GP (C_{18}); - RP-8GP (C_8); - RP-4GP (C_4)), with a mixture of acetic acid solution – acetonitrile as the mobile phase were tested and compared with regard to the separation: among 2-OH-SDM, 6-OH-SDM, 2,6-diOH-SDM and SDM; OH-SDMs from the interfering peaks; the their sharp peaks obtained upon injection of equal amounts.

The C_{18} and C_8 columns were difficult to separate between 2,6-diOH-SDM and 2-OH-SDM, and among 2,6-diOH-SDM and the interference, in the resulting extract. The best chromatogram with complete separation of all the target compounds and the interfering peaks and clear/short retention times was obtained with the C_4 column (Mightysil® RP-4GP) and an isocratic mobile phase of 4% acetic acid solution (in water) – acetonitrile – N,N-dimethylformamide (83:12:5, v/v/v). This made it unnecessary to use the gradient system to improve the separation.

The absorption spectra of 2-OH-SDM, 6-OH-SDM, 2,6-diOH-SDM, and SDM standard solutions were measured by the photo-diode array detector. The observed maximum absorptions were: 2-OH-SDM, 203, 264, and 286 nm; 6-OH-SDM, 204 and 278 nm; 2,6-diOH-SDM, 203, 260, and 279 nm; SDM, 203 and 268 nm. The wavelength for the measurement was adjusted to 273 nm, which is an average maximum (except for 203 and 204 nm) for all the compounds. The target compounds were detected within 21.1 min (Figure 2B).

Figure 2 shows examples of typical HPLC traces of blank and spiked samples analyzed by the proposed method. The resulting extracts, free from interference, indicates that satisfactory purification could be achieved by the present method. The clean extracts should extend the lifetime of the HPLC column. No SDM's conjugates, like N_1-glucuronide and N_4-acetyl SDM, showed interference in the peaks of SDM and OH-SDMs.

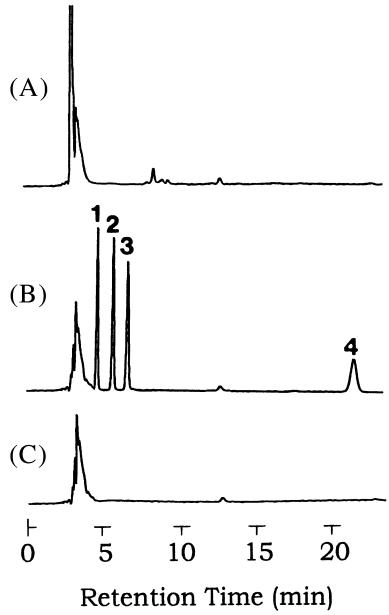

Figure 2. HPLC chromatograms (photo-diode array set at 273 nm). A, blank liver sample; B, spiked (each compound 0.5 μg/g) muscle liver sample; C, blank muscle sample. For HPLC conditions see text. Peaks: 1= 2,6-OH-SDM (Retention time = 4.51 min); 2= 2-OH-SDM (5.55 min); 3= 6-OH-SDM (6.49 min); 4= SDM (21.08 min).

Calibration, Recoveries and Identification

The calibration graphs were linear over ranges 0.1 - 20 ng for 2-OH-SDM, 6-OH-SDM, and 2,6-diOH-SDM and 0.2 - 20 ng for SDM, respectively. The correlation coefficients, >0.9997, for all compounds were highly significant ($P<0.01$). The detection limits were 0.1 ng for 2-OH-SDM, 6-OH-SDM and 2,6-diOH-SDM and 0.2 ng for SDM, respectively. Moreover, the precision of the procedure was obtained from the relative standard deviation of areas calculated for five replicate injections of 1.0 ng of each compound. The values for all compounds were 1.2% or less.

To evaluate the precision and the accuracy of the present method, a series of 1 g tissue samples were spiked with standard SDM and OH-SDMs. Using the method, five replicates were analyzed at each fortification level (0.1 or 0.5 ppm). The recoveries are summarized in Table 1. Overall, good results were obtained. Average recoveries were greater than 80.1% with coefficients of variation (C.V.) between 0.5 and 4.8% (n=5). In a practical analysis, the limits of

Table 1

Recoveries of SDM and OH-SDMs from Chicken Tissues*

Sample	Spiked (ppm)	2,6-diOH-SDM	2-OH-SDM	6-OH-SDM	SDM
Liver	0.1	83.4 (2.5)	80.1 (4.8)	81.0 (2.3)	85.0 (0.9)
	0.5	81.3 (3.1)	82.9 (3.5)	83.4 (3.0)	84.1(1.6)
Muscle	0.1	82.0 (2.0)	81.1 (2.9)	82.8 (1.9)	86.2 (2.1)
	0.5	88.0 (3.2)	82.3 (1.8)	88.5 (2.6)	87.6 (0.5)

* Data are averages. n = 5; coefficients of variation in parentheses (%).

detection (peak-to-noise ratio > 5) were 0.05 ppm for all target compounds. The high recovery, low C.V., and low limits of detection indicate that the present method has good precision and may be accurate. Also, the total time required for the analysis of one sample was below 1 hr.

The HPLC photo-diode array detector chosen allows the separation of target compounds and identification of them by retention time and spectrum. SDM and OH-SDMs could be identified in samples with their retention times and absorption spectra. Their spectra obtained from samples are practically identical with those of the standards. The proposed cleanup technique removed almost all interfering peaks, and allowed a reliable confirmation by plotting of absorption spectrum taken at the peak.

Thirty different samples of market chicken liver and muscle (from Osaka City, Japan) were analyzed by the present method. No SDM and OH-SDMs were detected. The resulting chromatograms were free from interference.

CONCLUSIONS

Cleanup techniques by using an ISOLUTE SAX and an Ultrafree C3 and HPLC determination of residual SDM and OH-SDMs in edible chicken tissues (liver and muscle) have been developed. Since the proposed procedure gives higher efficiency of cleanup and is highly precise, this procedure may be useful for the monitoring residue.

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REFERENCES

1. R. H. Adamson, J. W. Bridges, M. R. Kibby, S. R. Walker, R. T. Williams, Biochem. J., **118**, 41-45 (1970).

2. Y. Takahashi, Jpn. J. Vet. Sci., **48**, 105-109 (1986).

3. Y. Takahashi, Jpn. J. Vet. Sci., **48**, 999-1002 (1986).

4. T. Nakata, Y. Fukuda, J. Pharm. Pharmacol., **46**, 1004-112 (1994).

5. J. M. Nouws, T. M. Vree, Y. A. Hekster, Vet. Q., **7**, 70-72 (1985).

6. G. D. Paulson, A. D. Mitchell, R. G. Zaylskie, J. Assoc. Off. Anal. Chem., **68**, 1000-1006 (1985).

7. N. Haadsma, H. G. J. M. Pluijmakers, M. M. L. Aerts, W. M. L. Beek, Biomed. Chromatogr., **2**, 41-45 (1987).

8. M. J. Mengelers, M. B. Oorsprong, H. A. Kuiper, M. M. Aerts, E. R. Gogh, A. S. Miert, J. Pharm. Biomed. Anal., **7**, 1765-1776 (1989).

9. M. Horie, K. Saito, Y. Hoshino, N. Nose, N. Hamada, H. Nakazawa, J Chromatogr., **502**, 371-378 (1990).

10. S. Horii, C. Momma, K. Miyahara, T. Maruyama, M. Matsumoto, J. Assoc. Off. Anal. Chem., **73**, 990-992 (1990).

11. Y. Ikai, H. Oka, N. Kawamura, J. Hayakawa, M. Yamada, K. Harada, M. Suzuki, H. Nakazawa, J. Chromatogr., **541**, 393-400 (1991).

12. A. R. Long, L. C. Hsieh, M. S. Malbrough, C. R. Short, S. A. Barker, J. Assoc. Off. Anal. Chem., **73**, 868-871 (1991).

13. R. Malisch, B. Bourgeois, R. Lippold, Deutsche Lebensmittel-Rundschau, **88**, 205-216 (1992).

14. N. Furusawa, T. Mukai, J. Chromatogr. A, **677**, 81-85 (1994).

15. T. B. Vree, M. W. Tijhuis, J. F. W. Nouws, Y. A. Hekster, Pharma. Weekbl. Sci. Ed., **6**, 80-87 (1984).

16. T. B. Vree, J. F. M. Hekster, M. W. Tijhuis, M. Baakman, M. J. M. Oosterbaan, E. F. S. Termond, Pharma. Weekbl. Sci. Ed., **6**, 150-156 (1984).

17. T. B. Vree, M. Shimoda, J. Chromatogr., **526**, 119-128 (1990).

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