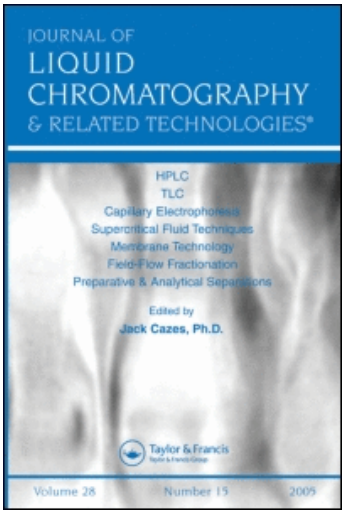

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HPLC DETERMINATION OF SULFONAMIDES, THEIR N4-ACETYL METABOLITES AND DIAMINOPYRIMIDINE COCCIDIOSTATS IN CHICKEN TISSUES

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ABSTRACT

A reversed-phase HPLC method is described for the quantitative determination of 5 sulfonamides, their N4-acetyl metabolites and 3 diaminopyrimidine coccidiostats in chicken muscle, liver, kidney, skin and plasma. Average recoveries from chicken tissues fortified with 0.05 and 0.2 μg/g of the 13 compounds were ranged from 53.9 to 106.3 % for individual compounds from individual tissues, with only 2 compounds from 1 tissue below 70 %. Coefficients of variation (C. V.) ranged from 0.6 to 21.3 %, with only 3 compounds from 1 tissue above 15 %. Detection limits were 0.02-0.05 μg/g for each compounds. The applicability of the method was demonstrated by determining concentrations of the 13 compounds in tissues from chickens administered with sulfonamides and diaminopyrimidines.

INTRODUCTION

Sulfonamides are widely used as antimicrobial agents and coccidiostats to prevent and treat chicken deseases sometimes in combination with diaminopyrimidine coccidiostats.

Recentry Takahashi et al. reported that one sulfonamide has remained as a residue in chicken skin for a longer time than in other tissues (1). So, we have been interested to establish a useful determination method with several sulfonamides residues particularly in chicken skin. It should be also important to determine N4-acetyl sulfonamides, the major metabolites, in chicken tissues because they have been reported to be also remained as residues in chicken meat (2), and they can be reconverted to active antimicrovial sulfonamides after their being uptaken in a human body, although they themselves are inert compounds (3).

Although several methods have been developed to quantitate several sulfonamide residues in animal tissues by thin-layer chromatography (4), gas chromatography (5), HPLC (6), and gas chromatography-mass spectrometry (7), no methods have been available to quantitate several sulfonamides and their N4-acetyl metabolites in most of edible tissues including skin.

One purpose of the present work is to develop a multiple determination method with HPLC capable of quantifying all sulfonamides used for chicken in Japan, their N4-acetyl metabolites, and 3 diaminopyrimidine coccidiostats, which are often administered with sulfonamides, in chicken tissues simultaneusly. The sulfonamides are sulfadiazine (SDZ), Sulfamonomethoxine (SMM), sulfamethoxazole (SMX), sulfaquinoxaline (SQ) and sulfadimethoxine (SDM). The diaminopyrimidines are diaveridine (DIV), trimethoprim (TMP) and ormethoprim (OMP). Moreover, we acertain applicability of the method to determination of these compounds in tissues from the compounds-administered chickens.

MATERIALS & METHODS

Reagents

(a) Solvents - Acetonitrile, methanol, n-hexane and 2-propanol (Wako Chemicals, Osaka, Japan).

(b) Anhydrous sodium sulfate, disodium hydrogenphosphate 12-water, and potassium dihydrogenphosphate (Wako Chemicals).

(c) Alumina - Alumina B Akt. I (ICN Biomedicals, Eschwege, FRG).

(d) Sulfonamides - SDZ, SMX and SDM (Sigma Chemical Co., St. Louis, MO), SMM (Fuji Yakuhin Kogyo, Takaoka, Japan) and SQ (Shionogi & Co., Ltd., Osaka, Japan).

(e) Diaminopyrimidines - DIV (Sigma Chemical Co.), TMP (Tanabe Seiyaku Co., Ltd., Osaka, Japan) and OMP (Fuji Yakuhin Kogyo).

(f) N4-acetyl sulfonamides - N4 acetyl SDZ (ASDZ), N4-acetyl SMM (ASMM), N4-acetyl SMX (ASMX), N4-acetyl SQ (ASQ) and N4-acetyl SDM (ASDM) were synthesized by the method reported previously (8).

(g) Standard solutions - Stock solutions in concentrations of 25-100 µg/ml were prepared in acetonitrile, and stored in the dark at 4°C.

(h) Internal standards - Acetanilide (AA) (E. Merck, Darmstadt, FRG) and Chloramphenicol (CP) (Sigma Chemicals).

(i) Quartz wool - Fine (Nihon Chromato Works, Ltd., Tokyo, Japan).

Apparatus

(a) Homogenizer - Bio-mixer BM-2 (Niti-on, Tokyo, Japan).

(b) Evaporator - Rotary evaporator MINI Model RE-21 (Yamato Scientific Co., Tokyo, Japan).

(c) Centrifuge - Model 90 -3 (Sakuma Seisakusho, Tokyo, Japan).

(d) Cleanup column - A small quartz wool plug was placed at the bottom of a 30 cm x 15 mm id column, 6 g alumina was packed into the column with methanol-acetonitrile (40:60, v/v), and the column was washed with 30 ml of methanol-acetonitrile (40:60,v/v) before use.

(e) HPLC system and conditions - The HPLC system comprised a Model 635A pump (Hitachi, Tokyo, Japan), a Model SIL-6A autoinjector (Shimadzu, Kyoto, Japan), a Model 875-UV detector (Japan Spectroscopic Co., Tokyo, Japan), and a Model C-R5A integrator (Shimadzu). The column was a 25 cm x 4.6 mm id stainless steel column packed with Nucleosil 5C18 (Machery-Nagel, Düren, FRG). The mobile phase-1 and the mobile phase-2 were consisted of acetonitrile-10 mM phosphate buffer (pH 5.0) (18:82,v/v) and acetonitrile-10 mM phosphate buffer (pH 5.9) (15:85,v/v), respectively. The injection volume was 20 ul, and the flow-rate was 1.0 ml/min. The detection wavelength was 240 and 270 nm. The chromatograms were recorded with a chart speed of 5 mm/min.

Control tissue samples

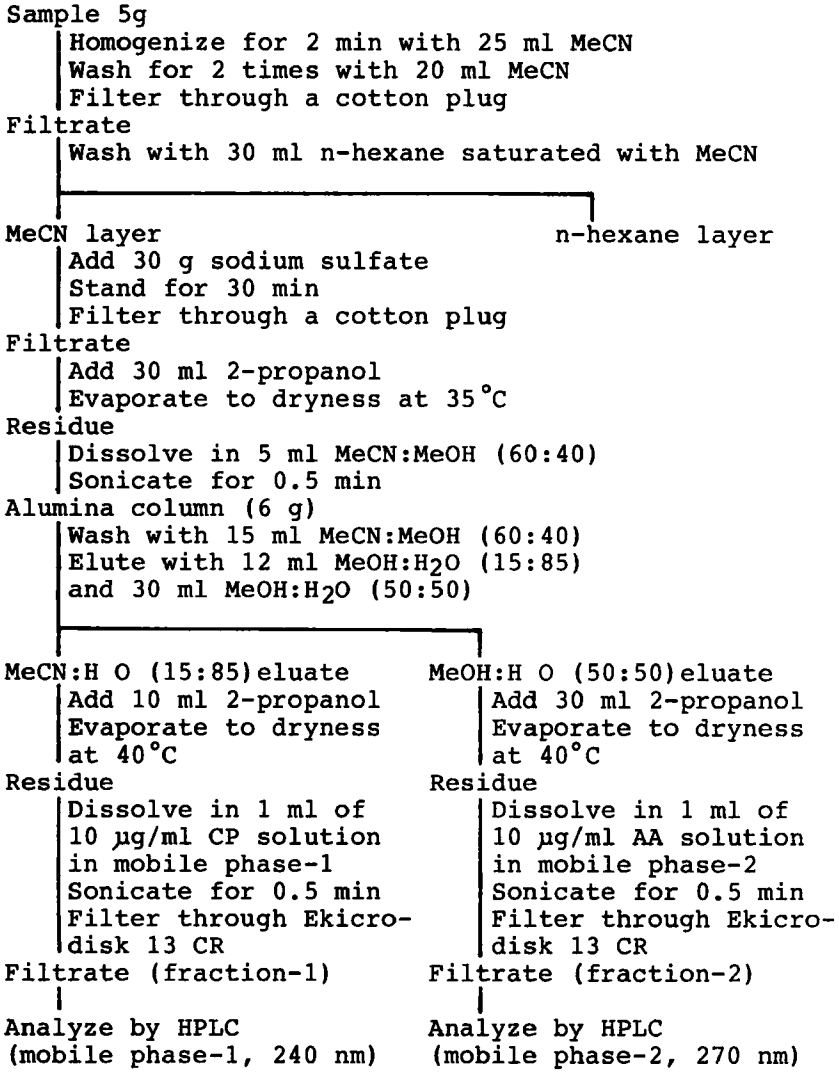
Thirty nonmedicated White Leghorn (Nisseiken Co. Ltd., Tokyo, Japan) were sacrificed after bleeding,

and the muscles, liver, kidney, and skin of trunk were removed. Plasma and Tissue samples were stored frozen at -80°C.

Sample preparation

Sample preparation procedure was shown in Scheme 1.

Five gram of chopped muscle, liver, kidney or skin was homogenized for 2 min with 25 ml acetonitrile, or 5 ml plasma was shaken by hand for 2 min. The homogenizer and glassware were washed twice with 20 ml acetonitrile. The mixture was filtered through a cotton plug, wased with 30 ml n-hexane saturated with acetonitrile ,and 30 g anhydrous sodium sulfate was added to the filtrate. The mixture was allowed to stand for 30 min at room temperature, filtered through a cotton plug, and 30 ml 2-propanol was added to the filtrate. The filtrate was evaporated to dryness at 35°C, and the residue was dissolved in acetonitrile-methanol (60:40,v/v), sonicated, and applied to an alumina column. The column was washed with 15 ml acetonitrile-methanol (60:40,v/v), diaminopyrimidines were eluted with 12 ml methanol-water (15:85,v/v) which is named as fraction-1, then sulfonamides and their N4-acetyl metabolites were eluted with 30 ml



SCHEME 1. Analytical Procedure.

methanol-water (50:50,v/v) which is named as fraction-2. 10 ml and 30 ml 2-propanol was added to fractions-1 and 2, respectively, and both fractions were evaporated to dryness at 40°C. The residues of fractions-1 and 2 were dissolved in 10 μg/ml CP solution in mobile phase-1 and 10 μg/ml AA solution in mobile phase-2, respectively. The solutions were filtered through Ekicrodisk 13 CR (Gelman Sciences Japan, Tokyo, Japan) and subsequently injected into HPLC system. The solution of fraction-1 was analyzed by using mobile phase-1 and detection wavelength of 240 nm, and that of fraction-2 was analyzed by using mobile phase-2 and 270 nm.

Recovery

Recovery values were evaluated by comparing peak-height ratios of each compound extracted from fortified tissue samples with peak-height ratios of standard solutions.

Application

Two White Leghorn (Nisseiken Co. Ltd.) of 5 weeks old were used. They were kept indoors and provided nonmedicated feeds and water ad libitum. They were administered twice at an interval of 24 hours with 20 mg/kg of SDZ, SMM, SMX, SQ, SDM, DIV, TMP and OMP

mixture orally. 5 hours after the second dosing, they were sacrificed after bleeding, and the muscles, liver, kidneys, and skin of trunk were removed. Plasma and tissue samples were stored frozen at -80°C before analysis.

RESULTS AND DISCUSSION

Sample preparation

We selected acetonitrile for extraction of the 13 compounds from tissues and alumina column chromatography for purification of the extract according to previous papers (9,10). We separated diaminopyrimidines from sulfonamides and their N4-acetyl metabolites prior to HPLC analysis, because we could not separate the former from the latter completely by HPLC. The former compounds were well separated from the latter ones completely by alumina column chromatography described in Materials & Methods in the case of muscle, liver, kidney or skin extracts. Although SQ remained in fraction-1 even after alumina column chromatography only in the case of plasma extracts, it was separated completely from other compounds in fraction-1 through a subsequent HPLC procedure with a condition as described in Materials & Methods.

HPLC conditions

Reversed-phase HPLC was used for determination of the 13 compounds on the basis of previous studies (9,10). The ODS (Nucleosil 5 C18) column gave better separation of the 13 compounds from each other and from tissue components than did the octyl silane (Nucleosil 5 C8) column. The optimal HPLC conditions described in Materials & Methods were selected after many trials. Although we could not manage to have a single isocratic HPLC condition which made it possible to analyze the 13 compounds separately from each other and various tissue components, we got two isocratic HPLC conditions to separate them. Thus, we used mobile phase-1 with detection wavelength of 240 nm for fraction-1, and mobile phase-2 with detection wavelength of 270 nm for fraction-2. Only ASMM and ASMX in fraction-2 could not be separated from each other by using mobile phase-2, and we used mobile phase-1 instead of mobile phase-2 in the case of the sample which contained both of them. The retention time of 13 compounds, internal standards and other sulfonamindes and diaminopyrimidine by using these two mobile phases was shown in Table 1. When unknown peak appeared chromatograms of tissue extract, we could confirm the peak by analyzing the sample with these two conditions.

TABLE 1

Retention Time of Compounds

Compound	Retention time (min) Mobile phase-1	Mobile phase-2
SULFONAMIDES		
SDZ	6.4	6.4
SMM	12.4	11.2
SMX	16.1	12.6
SQ	34.7	28.5
SDM	34.7	34.2
sulfisomidine	4.8	5.7
sulfathiazole	6.3	7.8
sulfamerazine	8.0	9.7
sulfisoxazole	10.1	5.9
sulfamethoxypyridazine	10.0	13.6
sulfamethazine	10.9	14.0
sulfadoxine	16.2	15.2
sulfaphenazole	33.3	30.1
sulfachlorpyridazine	NE[a)]	NE
N4-ACETYL SULFONAMIDES		
ASDZ	6.2	5.7
ASMM	10.7	8.5
ASMX	14.6	8.5
ASQ	28.9	19.0
ASDM	31.3	21.3
DIAMINOPYRYMIDINES		
DIV	8.6	17.4
TMP	10.1	21.3
OMP	12.7	28.4
pyrimethamine	NE	NE
INTERNAL STANDARDS		
AA	12.4	15.6
CP	26.1	38.1

a)NE; Not eluted within 40 min.

Chromatograms

Fig. 1-(a) and Fig. 2-(a) show typical chromatograms from a standard solution of diaminopyrimidines and from that of sulfonamides and their N4-acetyl metabolites, respectively. Fig.1-(b-f) and Fig. 2-(b-f) show typical chromatograms from fraction-1 solutions and fraction-2 solutions of five tissue extracts of a control chicken, respectively. All compounds tested were well separated from each other with the exception of ASMM and ASMX in Fig. 2-(a). Although several small peaks derived from a tissue component appeared in the chromatograms of fraction-2 of tissue extracts (Fig. 2-(b-f)), all peaks except one did not interfere with detrmination of the 13 compounds at 0.05 μg/g concentration. The interfering peak came out at the retention time of 18.4 min in the case of fraction-2 of a kidney extract (Fig. 2-(d)), which interfered with ASQ determination. We could not get rid of the peak, so we exceptionally used mobile phase-1 with detection wavelength of 270 nm for determination of ASQ in kidney.

Calibration curves and detection limits

The calibration curves of the 13 compounds were linear and reproducible through the investigated

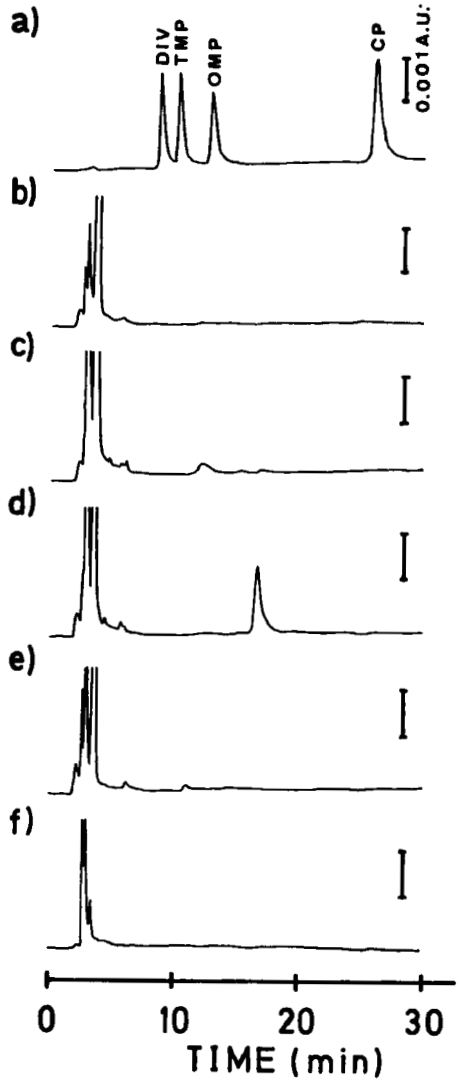


FIGURE 1. Typical chromatograms of (a) standard solution which contains 1.0 µg/ml DIV, TMP and OMP, and fraction-1 of blank (b) muscle, (c) liver, (d) kidney, (e) skin and (f) plasma extracts.

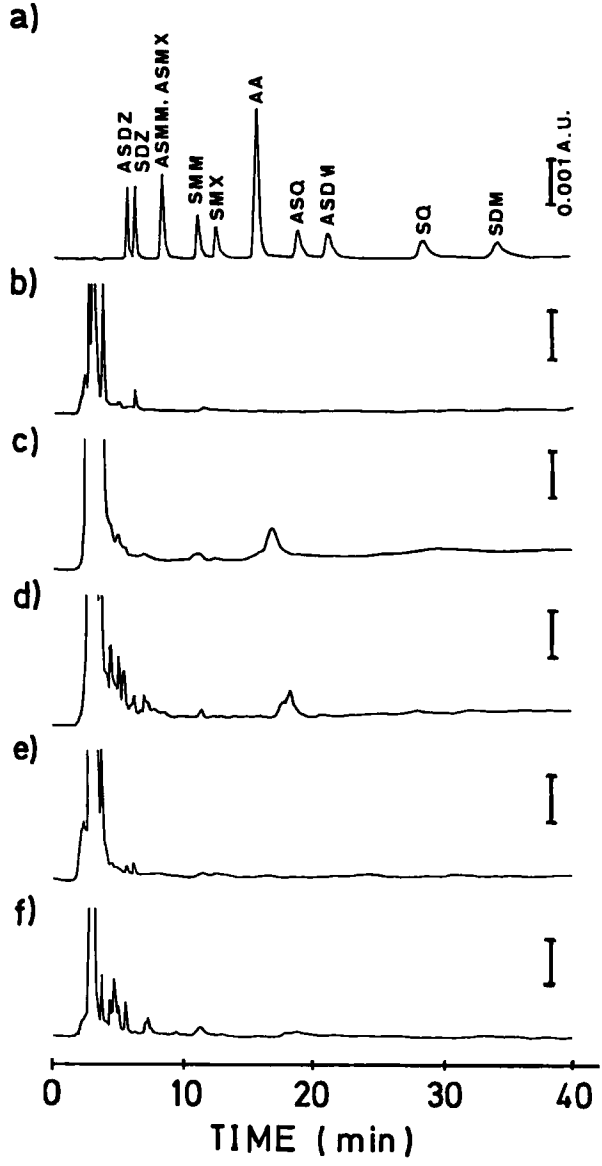


FIGURE 2. Typical chromatograms of (a) standard solution which contains 0.25 μg/ml SDZ, SMM, SMX, SQ, SDM, ASDZ, ASMM, ASMX, ASQ and ASDM, and fraction-2 of blank (b) muscle, (c) liver, (d) kidney, (e) skin and (f) plasma extracts.

TABLE 2

Detection Limit of Compounds in Chicken Tissues.

Compound	Detection limit (μg/ml)				
	Muscle	Liver	Kidney	Skin	Plasma
DIV	0.02	0.02	0.02	0.02	0.02
TMP	0.02	0.02	0.02	0.02	0.02
OMP	0.02	0.02	0.02	0.02	0.02
SDZ	0.03	0.02	0.05	0.02	0.02
SMM	0.03	0.03	0.03	0.03	0.03
SMX	0.02	0.02	0.02	0.03	0.02
SQ	0.02	0.02	0.02	0.02	0.02
SDM	0.02	0.02	0.02	0.02	0.02
ASDZ	0.02	0.03	0.05	0.03	0.05
ASMM	0.02	0.02	0.02	0.02	0.02
ASMX	0.02	0.02	0.02	0.02	0.02
ASQ	0.02	0.02	0.02 a)	0.02	0.02
ASDM	0.02	0.02	0.02	0.02	0.02

a) Determined by HPLC using mobile phase-1 and wavelength of 270 nm.

concentration range of 0.1-50 μg/ml, which is equivalent to 0.02-10 μg/g in tissue (R=0.999, n=5).

The detection limits were shown in Table 2 (signal-to-noise ratio of 3), and satisfactory for residue analysis.

Recoveries

Recovery studies were conducted by adding 0.05 and 0.2 μg/g of the 13 compounds to each 5 g of control tissue sample. The extract from each sample was analyzed by the present method. Tables 3 and 4 show

TABLE 3

Recovery from Chicken Tissues Fortified with 0.05 μg/g of the 13 Compounds.

Compound	Recovery (%) (C.V. (%)[a]) (n=5)				
	Muscle	Liver	Kidney	Skin	Plasma
DIV	93.0 (3.2)	101.3 (9.7)	95.2 (8.1)	91.4 (10.2)	97.1 (1.8)
TMP	94.5 (3.6)	94.6 (10.5)	96.6 (8.1)	87.4 (8.5)	91.0 (3.5)
OMP	62.7 (5.3)	97.4 (11.6)	98.3 (8.1)	90.0 (8.8)	92.0 (1.9)
SDZ	90.4 (6.7)	84.7 (10.7)	103.4 (11.8)	84.6 (6.4)	95.9 (2.0)
SMM	80.1 (7.0)	83.5 (8.4)	91.2 (3.5)	84.6 (5.2)	86.6 (4.0)
SMX	91.9 (4.3)	81.0 (5.6)	96.1 (4.6)	81.6 (4.8)	86.2 (3.9)
SQ	96.1 (4.6)	85.6 (9.7)	90.4 (8.8)	88.8 (5.5)	53.9 (16.0)
SDM	96.8 (6.3)	92.3 (21.3)	90.8 (5.7)	80.7 (5.4)	72.3 (4.7)
ASDZ	95.0 (4.5)	83.1 (4.8)	87.3 (13.6)	87.0 (6.1)	100.5 (3.9)
ASMM	86.2 (4.0)	73.6 (11.8)	86.0 (20.9)	102.4 (11.2)	95.4 (5.1)
ASMX	97.6 (2.9)	99.3 (2.8)	99.6 (3.0)	85.8 (7.6)	80.7 (11.2)
ASQ	102.0 (2.0)	94.7 (2.4)	97.4 [b] (6.6)	90.4 (5.4)	81.0 (7.0)
ASDM	102.9 (1.5)	94.6 (3.0)	90.1 (3.0)	92.7 (5.5)	104.4 (2.2)

a) C.V.; Coefficient of variation
b) Determined by HPLC using mobile phase-1 and wavelength of 270 nm.

TABLE 4

Recovery from Chicken Tissues Fortified with 0.2 µg/g of the 13 Compounds.

Compound	Recovery (%) (C.V. (%)[a]) (n=5)				
	Muscle	Liver	Kidney	Skin	Plasma
DIV	94.9 (3.6)	95.6 (3.2)	105.6 (3.8)	96.5 (4.0)	94.9 (1.3)
TMP	96.9 (4.9)	98.3 (2.4)	106.3 (3.7)	98.7 (3.8)	90.5 (2.6)
OMP	88.4 (4.0)	95.6 (3.3)	105.6 (3.3)	96.6 (4.2)	95.8 (1.9)
SDZ	91.4 (5.7)	85.9 (3.7)	87.6 (2.4)	80.9 (5.2)	87.7 (3.7)
SMM	86.7 (6.5)	83.5 (3.9)	87.2 (4.1)	79.9 (4.3)	89.1 (5.9)
SMX	89.8 (4.5)	84.7 (3.0)	88.9 (3.3)	78.8 (3.5)	89.9 (4.8)
SQ	95.6 (4.0)	85.1 (3.0)	87.2 (2.6)	85.1 (3.4)	55.9 (16.5)
SDM	98.3 (3.0)	88.4 (7.4)	90.3 (3.2)	87.7 (5.4)	81.6 (4.0)
ASDZ	98.7 (2.3)	86.1 (3.8)	84.1 (3.1)	86.6 (3.6)	89.6 (7.2)
ASMM	88.2 (2.1)	89.4 (4.8)	83.5 (3.5)	90.2 (5.7)	91.1 (3.2)
ASMX	90.5 (1.8)	83.5 (2.6)	94.7 (0.6)	85.9 (4.4)	89.3 (2.0)
ASQ	100.9 (0.9)	86.4 (3.5)	97.0[b] (1.6)	90.1 (1.8)	82.0 (5.5)
ASDM	103.1 (1.8)	88.0 (3.5)	91.1 (2.6)	92.9 (1.7)	96.8 (2.8)

a) C.V.; Coefficient of variation
b) Determined by HPLC using mobile phase-1 and wavelength of 270 nm.

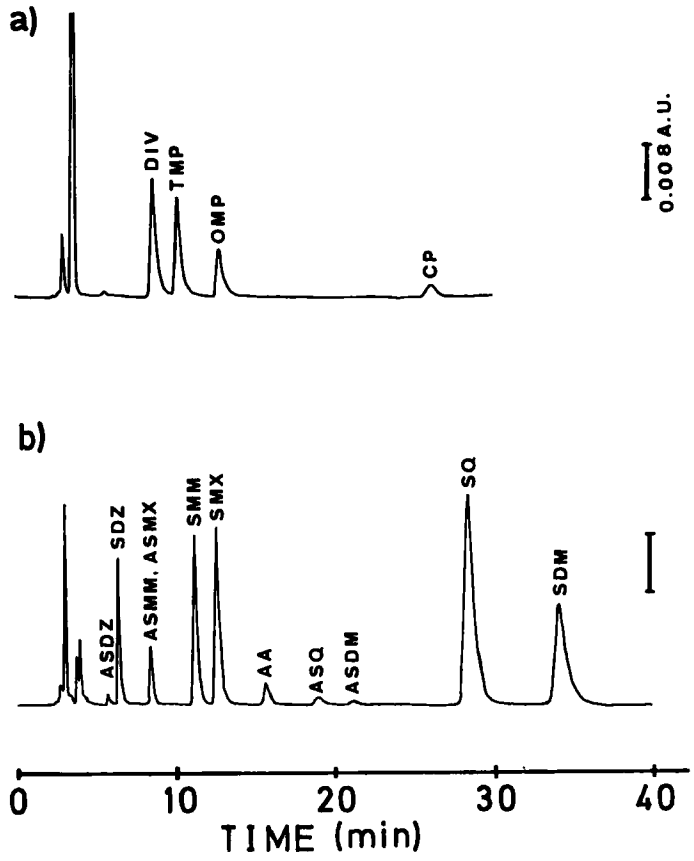


FIGURE 3. Typical chromatograms of (a) fraction-1 and fraction-2 of a muscle extract from a chicken 5 hours after the second administration orally with 20 mg/kg of SDZ, SMM, SMX, SQ, SDM, DIV, TMP and OMP.

recovery data of the 13 compounds. Recoveries ranged from 53.9 to 106.3 % for individual compounds from individual tissues, with only OMP from muscle at 0.05 µg/g and SQ from plasma below 70 %. SQ was partially eluted in fraction-1, and the contaminated SQ in

fraction-1 was less than 30% of fortified amount. Coefficients of variation (C. V.) ranged from 0.6 to 21.3 %, with only SDM from liver at 0.05 μg/g, ASMM from kidney at 0.05 μg/g and SQ from plasma above 15 %. The recoveries were satisfactory for residue analysis.

Application

An attempt was made to determine whether the present method is applicable to quantification of the 13 compounds in tissues from chicken which had been given the 5 sulfonamides and the 3 diaminopyrimidines. Fig. 3 shows typical chromatograms of fraction-1 and fraction-2 from a muscle extract. The 13 compounds from 5 tissues were well separated not only each other but also from tissue components with the exception of ASMM and ASMX in fraction-2 by using mobile phase-2, but the two compounds were separated from each other by using mobile phase-1 (data not shown).

CONCLUSION

A HPLC method with UV detection of sulfonamides, their N4-acetyl metabolites and diaminopyrimidine coccidiostats in chicken muscle, liver, kidney, skin and plasma has been developed, and this method was shown to be applicable to tissue samples from a drugs

administered chicken. The detection limits and recoveries were satisfactory to residue analysis.

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