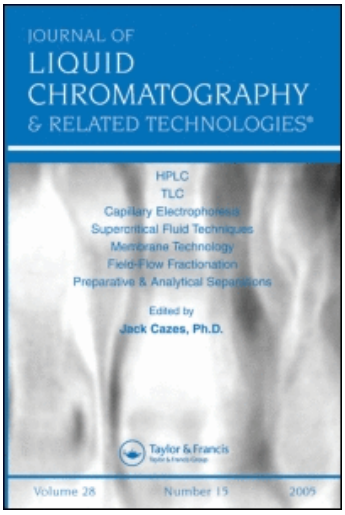

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Simultaneous High-Performance Liquid Chromatographic Determination of Amprolium, Ethopabate, Sulfaquinoxaline and N4-Acetylsulfaquinoxaline in Chicken Tissues

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To cite this Article Takahashi, Y. , Sekiya, T. , Nishikawa, M. and Endoh, Y. S.(1994) 'Simultaneous High-Performance Liquid Chromatographic Determination of Amprolium, Ethopabate, Sulfaquinoxaline and N4-Acetylsulfaquinoxaline in Chicken Tissues', Journal of Liquid Chromatography & Related Technologies, 17: 20, 4489 — 4512
To link to this Article: DOI: 10.1080/10826079408013633
URL: http://dx.doi.org/10.1080/10826079408013633

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(20), 4489-4512 (1994)

SIMULTANEOUS HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF AMPROLIUM, ETHOPABATE, SULFAQUINOXALINE AND N4-ACETYLSULFAQUINOXALINE IN CHICKEN TISSUES

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ABSTRACT

A reversed-phase high-performance liquid chromatographic method is described for the quantitative simultaneous residue determination of amprolium with fluorometric detection using post-column reaction, and ethopabate, sulfaquinoxaline and its major metabolite, N4-acetylsulfaquinoxaline with UV detection, in chicken muscle, liver, kidney, skin and plasma. Average recoveries from chicken tissues fortified with 0.1 μg/g of the four compounds tested were ranged from 81.0 to 103.8 % for individual compounds from individual tissues. Coefficients of variation were ranged from 1.1 to 8.6 %. Detection limits were 0.002-0.004 μg/g for each compound. The applicability of this method was demonstrated by determining concentrations of the four compounds in tissues from chickens administered with the three parent compounds.

INTRODUCTION

Amprolium (AMP), Ethopabate (EB) and Sulfaquinoxaline (SQ) are widely used to prevent coccidiosis and leukocytozoonosis in chickens. Since they are usually used as a combination of AMP + EB, AMP + SQ or AMP + EB + SQ, it is very useful and important to establish a simultaneous determination method with them in chicken tissues. Determination of N^4-acetyl SQ (ASQ) residue in chicken tissues should be also important, because ASQ, a major metabolite of SQ, can be detected in most of edible tissues from chickens administered SQ (1) and will be reconverted to SQ after their being uptaken in human body (2).

Several analytical methods involving gas chromatography (GC;3,4) and high-performance liquid chromatography (HPLC) with ultraviolet (UV; 5,6,7,8), fluorescence (9,10) detection have been reported for detecting AMP, EB and SQ individually, or the combination of EB and SQ in chicken muscle and liver. Nose et al.(11) have been reported to detect AMP, EB, SQ and other seven compounds simultaneously in chicken muscle with GC, though the GC conditions were separated in each compound and detection limits were

not enough for residue analysis. However, any methods to determine AMP, EB, SQ and ASQ simultaneously in most of edible chicken tissues including skin using HPLC with a low sensitivity limit have not yet been reported.

The purpose of the present study was to develop a simultaneous quantitative determination method with HPLC for AMP, EB, SQ and ASQ from chicken muscle, liver, kidney, plasma and skin. Further, applicability of this method was ascertained to determine the four tested compounds in tissues from chickens administered a commercial preparation containing AMP, EB and SQ.

MATERIAL AND METHODS

Reagents

(a) Solvents - Acetonitrile (MeCN), methanol (MeOH), n-hexane and 2-propanol (Wako Pure Chemical Industry Ltd., Osaka, Japan).

(b) Anhydrous sodium sulfate, disodium hydrogenphosphate 12-water, potassium dihydrogenphosphate, sodium 1- hexanesulfonate, sodium hydroxide and potassium ferricyanide (Wako Pure Chemical Industry Ltd.).

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

(d) Coccidiostats - AMP-HCl and SQ (Sigma Chemical Co., St. Louis, MO) and EB (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan).

(e) Metabolite - ASQ was synthesized by the method reported previously (12).

(f) Internal standard - Chloramphenicol (CP, Sigma Chemical).

(g) Standard solutions - Stock solutions in concentration of 25 - 100 μg/ml were prepared in MeCN for EB,SQ and ASQ and in 2% water-MeCN for AMP, and stored in dark at 4°C. A working solutions of lower concentrations were prepared from this solution by dilution with MeCN.

(h) Quartz wool - Fine (Nippon Chromato Works, Ltd., Tokyo, Japan).

(i) Reaction solution - Dissolve 50 g sodium hydroxide and in water, add 0.8 g potassium ferricyanide, and dilute to 1 liter with water.

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 8800 (Kubota Co., 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 MeCN - MeOH (6:4, V/V), and the column was washed with 30 ml of the same solution before use.

(e) HPLC system and conditions - The HPLC system comprised a Model PU-980 pump (Japan Spectroscopic Co., Tokyo, Japan), a Model LC-9A pump (Shimadzu Co., Kyoto, Japan), a Model SIL-6A autoinjector (Shimadzu Co.), a Model 860-CO column oven (Japan Spectroscopic Co.), a Model 875-UV detector (Japan Spectroscopic Co.) placed between the column and the reactor coil, a Model RF-535 spectrofluorometer (Shimadzu Co.) and Model C-R5A integrators (Shimadzu Co.). The column was a 25 cm x 4.6 mm id stainless steel ODS column (L-column ODS, Chemicals Inspection and Testing Institute, Tokyo, Japan). The reactor coil placed in the column oven was 10 m x 0.25 mm id stainless steel tube. The mobile phase-1 and the mobile phase-2 were consisted of 0.2 M potassium dihydrogenphosphate - MeCN (85:15, V/V) containing 5 mM sodium 1-hexanesulfonate and 10 mM phosphate buffer (pH 5.0) - MeCN (79:21, V/V), respectively. The injection volume was 20 µl. The flow-rates of the mobile

phase-1 and reaction solution were both 0.7 ml/min, and the flow-rate of the mobile phase-2 was 1.0 ml/min. The fluorescence of AMP derivative converted by oxidation with ferricyanide in alkaline solution were detected at 367 nm excitation and 470 nm emission in spectrofluorometer by using mobile phase-1, and EB, SQ and ASQ were detected at 270 nm with spectrophotometer by using mobile phase-2. The column and the reactor coil temperature was 40°C. The chromatograms were recorded with a chart speed of 5 mm/min.

(f) Photodiode-array system - The detector was a Model SPD-M6A (Shimadzu Co.) interfaced with a PC-9801 VX personal computer (NEC Corporation, Tokyo, Japan). The recorder was a Model UP-2000 (Shimadzu Co.).

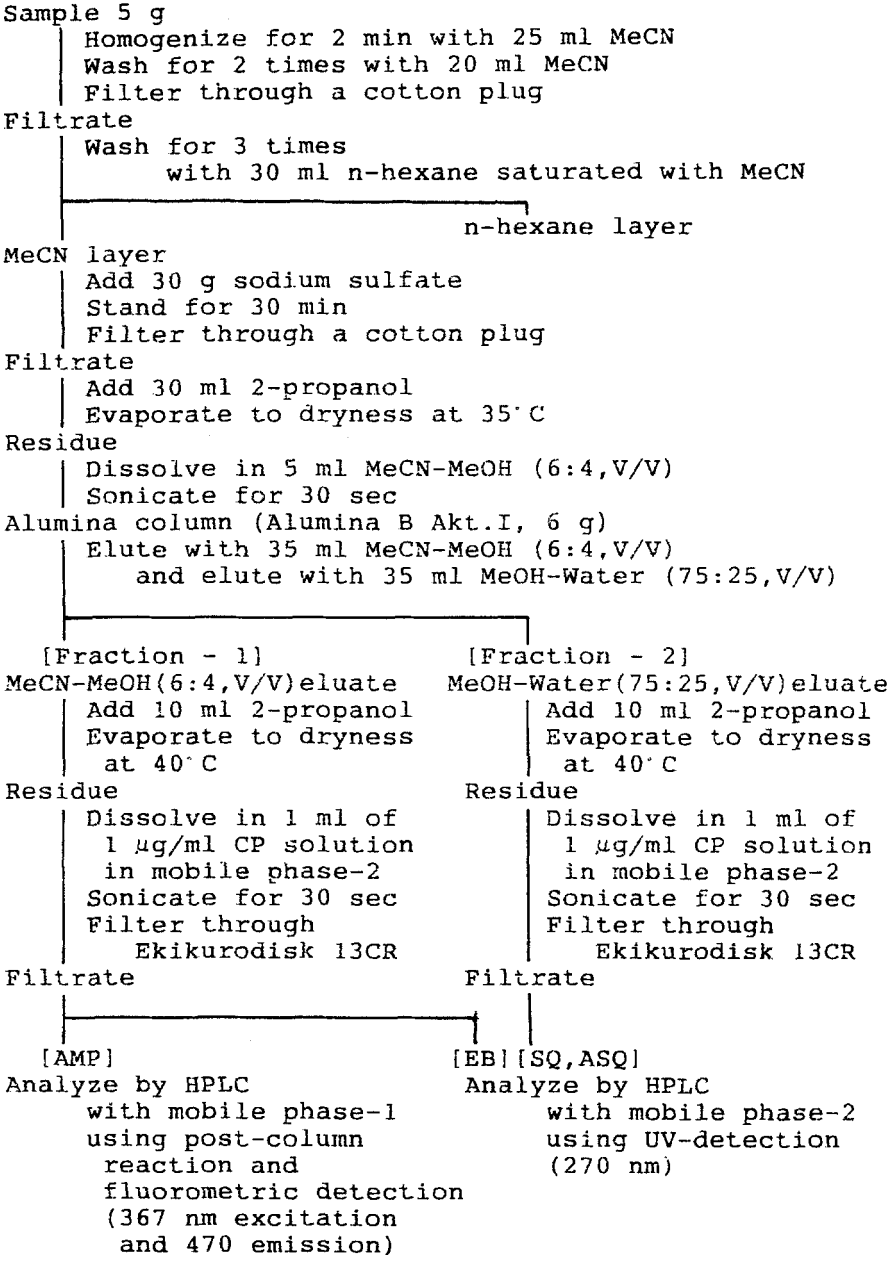
Control tissue samples

Ten non-medicated White Leghorn chickens (Nisseiken Co., Ltd., Tokyo, Japan) were sacrificed after bleeding, and the muscle, liver, kidney and skin were removed. The plasma after centrifuged at 3,000 rpm for 5 min and tissue samples were stored frozen at - 80°C until analysis.

Sample preparation

Sample preparation procedure was shown in Scheme 1.

5 g of chopped muscle, liver, kidney, skin or plasma was homogenized for 2 min with 25 ml MeCN. The homogenizer and glassware were washed twice with 20 ml MeCN. The mixture was filtered through a cotton plug, washed with 30 ml n-hexane saturated with MeCN for three times, 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 5 ml MeCN-MeOH (6:4, V/V), sonicated, and applied to an alumina column. AMP and EB were eluted with 35 ml MeCN-MeOH (6:4, V/V) which was named as fraction-1, then SQ and ASQ were eluted with MeOH-water (75:25, V/V) which was named as fraction-2. The both fractions were added 10 ml 2-propanol and evaporated to dryness at 40°C. These residues were dissolved in the mobile phase-1 containing with 1 μg/ml CP. The solutions were filtered through Ekikurodisk 13 CR (Gelman Sciences Japan, Tokyo, Japan) and subsequently injected into HPLC system. The resulting solution of fraction-1



SCHEMA 1. Analytical Procedure.

was analyzed by using the mobile phase-1, and AMP was detected by using spectrofluorometer at 367 nm excitation and 470 nm emission after mixing with the reaction solution in reactor coil to convert of AMP to a fluorescent derivative by oxidation with ferricyanide in alkaline solution. The resulting solution of fraction-1 (EB) and fraction-2 (SQ and ASQ) were analyzed by using the mobile phase-2 and detected by using spectrophotometer with detection wavelength of 270 nm.

Recovery

Recovery values of AMP were evaluated by comparing peak-areas of AMP extracted from fortified tissue samples with peak-areas of the standard solutions. Recovery values of EB, SQ and ASQ were evaluated by comparing peak-area ratios of each compound extracted from fortified tissue samples with peak-area ratios of the standard solutions.

Application

Two White Leghorn chickens (Nisseiken Co.) of 7 weeks old were used. They were kept in cages individually and provided non-medicated feeds and water ad libitum. They were administered 0.4 g/kg PANCOXIN (Dainippon Pharmaceutical Co.) containing

AMP (200 mg/g), EB (10 mg/g) and SQ (120 mg/g) orally with catheter. 6 and 24 hours after the administration they were sacrificed after bleeding, and the muscle, liver, kidney and skin of trunk were removed. Plasma and tissue samples were stored frozen at -80°C until analysis.

RESULTS AND DISCUSSION

Sample preparation

In the present study the sample preparation method developed was based on our previous reports concerning residue analytical methods of some coccidiostats (1,13). It is very useful and convenient to develop a universal method that would be applicable to determine all residual coccidiostats in animal tissues. In our previous report samples applied to an alumina column were firstly washed with 15 ml MeCN-MeOH (6:4, V/V) to remove lipo-soluble tissue components from samples. Since AMP and EB were eluted with this solution, we could not use the solution for this purpose. We selected the procedure of washing with n-hexane saturated with MeCN was repeated three times before applying to alumina column for this purpose.

Profiles of compounds eluted from the alumina column, which the sample solution from a control kidney added 1 μg of each compounds (1 μg/ml working standard solutions) was loaded on to, were shown in Fig.1.

EB was eluted by MeCN-MeOH (6:4, V/V) from the first eluate fraction (5 ml) and AMP was eluted in succession. 30 ml MeCN-MeOH (6:4, V/V) was necessary to elute AMP completely. In the case of plasma, only AMP was delayed one fraction (5 ml) to be eluted, but 35 ml MeCN-MeOH (6:4, V/V) was enough to be eluted. Recoveries of EB and AMP were 99.5 and 96.1 % , respectively. Then, 35 ml MeCN-MeOH (6:4,V/V) was selected as the first elution solution. After eluted EB and AMP, three different MeOH-water solutions, 85:15, 75:25 and 50:50 (V/V), were applied to elute SQ and ASQ from the alumina column. Increasing water content of elution solution, the total volume of solution was decreased, but recoveries of SQ and ASQ were 95 - 96, 98 - 100 and 76 - 79 % in 85:15, 75:25 and 50:50 (V/V) MeOH-water, respectively. Then, 35 ml MeOH-water (75:25, V/V) was selected as the second elution solution.

HPLC conditions

HPLC condition with UV detection was used for

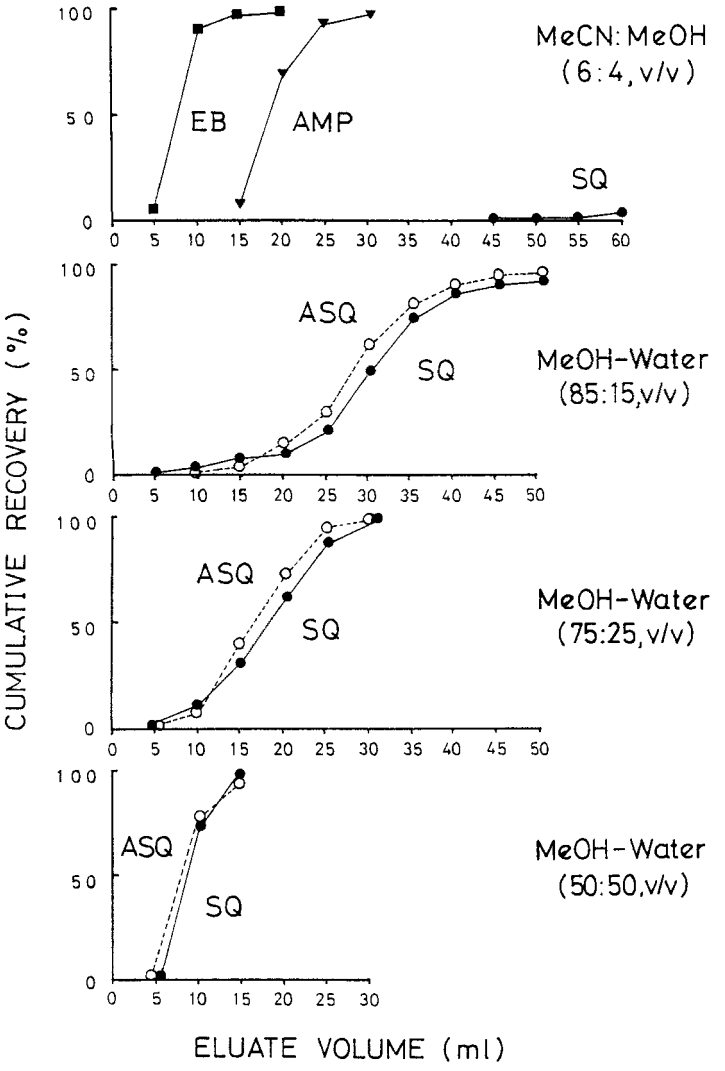


FIGURE 1. Elution Profiles of AMP(—▼—), EB(—■—), SQ(—●—) and ASQ(---○---) from an Alumina Column with the First Elution of MeCN-MeOH (6:4, V/V) and the Second Elution Using Three Kinds of Solvents, MeOH-Water (85:15, 75:25, 50:50, V/V).

determination of sulfonamides on the basis of our previous studies (1). But, we could not use a single HPLC condition to determine four compounds tested, because AMP was eluted at a very short retention time in the condition, and could not be separated from tissue components (tR=3.1 min in mobile phase-2). Then, fluorometric detection using post-column reaction was selected for AMP detection on the basis of previous study (10).

HPLC condition of AMP was changed a little from the previous study (10). For the purpose of ensuring conversion of AMP to a fluorescent derivative more, volume of reactor coil was increased about two fold, column temperature was raised from 30 to 40°C, and the content of MeCN in mobile phase-1 was decreased.

Optimal HPLC condition with UV detection was determined using tissue sample solutions described in sample preparation. Column was selected after some trials using 5 kinds of ODS columns, Capcell Pak C18 (Shiseido Company Ltd.,Tokyo, Japan), TSKgel-80Ts (Tosoh Co. Ltd., Tokyo, Japan), Chemco Pak-Nucleosil 5C18 (CHEMCO Scientific Co. Ltd., Osaka, Japan), Senshu Pak-ODS-1251-SS (Senshu Scientific Co. Ltd., Tokyo, Japan) and L-column ODS. Optimal mobile phase for each tissue was selected after trials of varying

pH from 5.0 to 5.8, mixture rate of MeCN in phosphate buffer from 15 to 25 %.

The retention time of tested compounds and other sulfonamides, their N^4-acetyl metabolites, diaminopyrimidines and other drugs which are used for poultry diseases by using mobile phase-2 with UV-detection are shown in table 1. All tested compounds except sulfadimethoxine were not interfered for determination by the compounds tested in the present study.

Chromatograms

Fig.2-(a), Fig.3-(a) and Fig.4-(a) show typical chromatograms of standard solutions of AMP, EB, SQ, ASQ and internal standard, CP. Fig.2-(b-f) shows typical chromatograms of fraction-1 of five tissue extracts from a control chicken using fluorometric detection. Fig.3-(b-f) and Fig.4-(b-f) show typical chromatograms of fraction-1 and fraction-2 of five tissue extracts from a control chicken using UV detection, respectively. Several peaks derived from tissue components appeared in the chromatograms, but all compounds tested were not interfered by them. AMP and EB in fraction-1 were not interfered each other, because AMP was detected at very short

TABLE 1

Retention Time of Compounds in UV-detection Method.

Compounds	Retention Time (min)
Sulfonamides	
SQ	20.7
sulfadiazine	4.8
sulfamethazine	6.2
sulfachloropyridazine	3.3
sulfamonomethoxine	8.5
sulfamethoxazole	11.2
sulfadimethoxine	20.8
sulfadoxine	11.1
N4-acetyl sulfonamides	
ASQ	16.7
N4-acetyl sulfamonomethoxine	7.2
N4-acetyl sulfadiazine	4.8
N4-acetyl sulfamethazine	6.2
N4-acetyl sulfamethoxazole	10.0
N4-acetyl sulfadimethoxine	18.1
Diaminopyrimidines	
trimethoprim	5.1
ormethoprim	5.8
pyrimethamine	21.4
diaveridine	4.5
Others	
EB	20.0
CP	18.8
AMP	3.1
oxolinic acid	16.1
nalidixic acid	33.8
nitrofurazone	6.1
furazolidone	8.7
thiamphenicol	6.1

retention time with UV detection, and EB was not detected with fluorometric detection.

Calibration curves and detection limits

The calibration curves of four compounds were

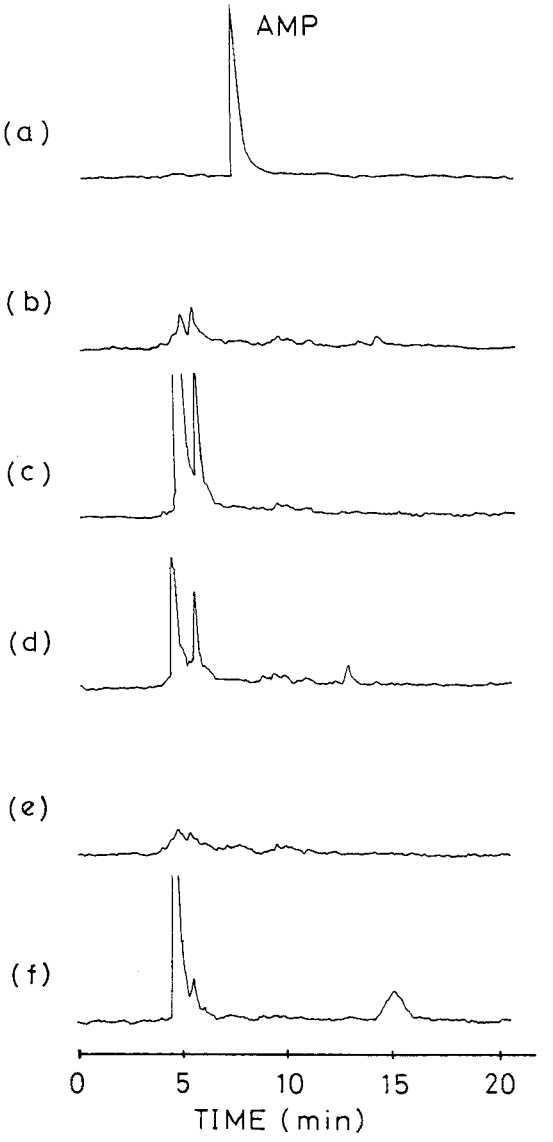


FIGURE 2. Typical Chromatograms of Standards containing 0.1 g/ml AMP (a) and Fraction 1 of Control Tissue Extracts, muscle (b), Liver (c), Kidney (d), Skin (e) and Plasma (f).

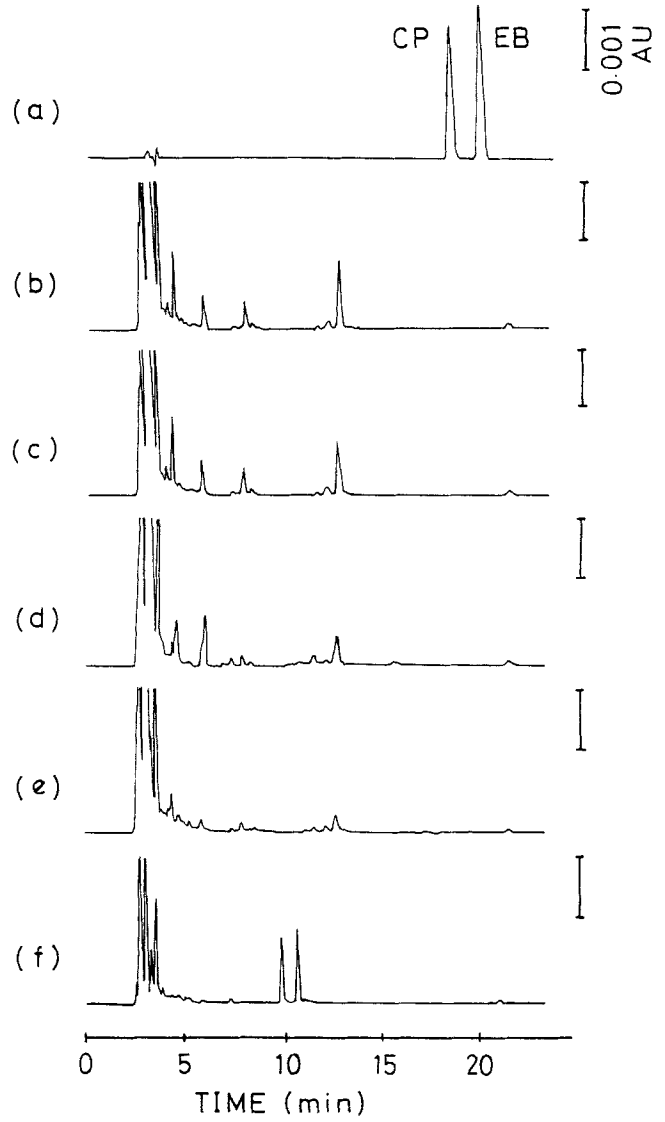


FIGURE 3. Typical Chromatograms of Standards containing 1.0 g/ml EB (a) and Fraction - I of Control Tissue Extracts, muscle (b), Liver (c), Kidney (d), Skin (e) and Plasma (f).

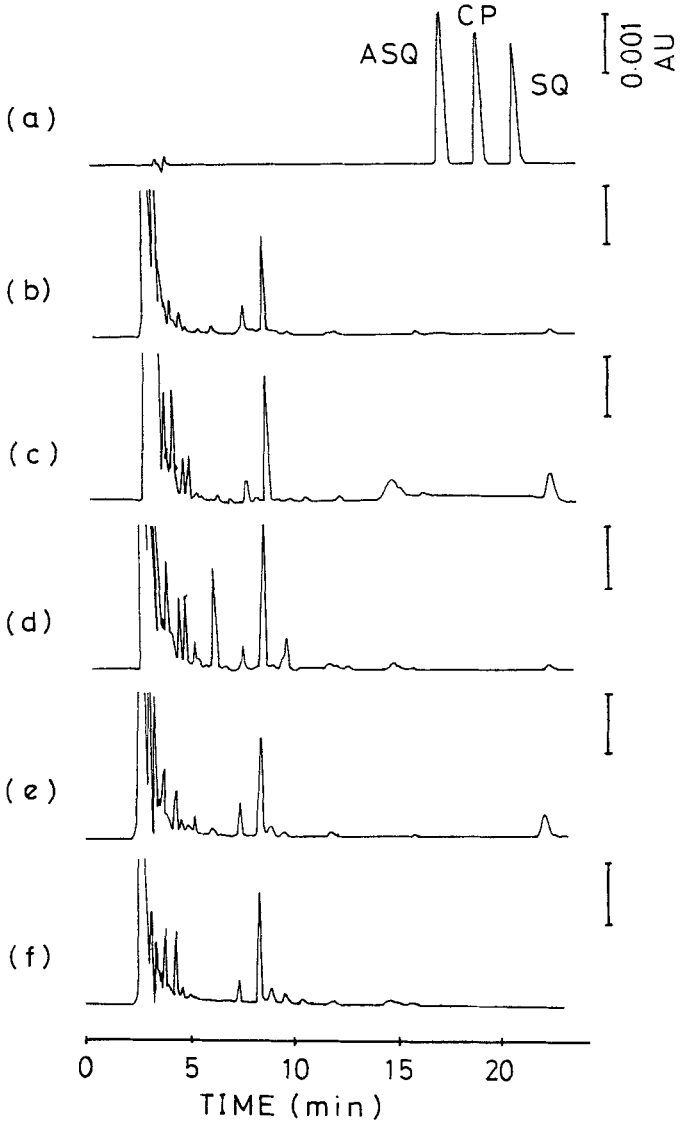


FIGURE 4. Typical Chromatograms of Standards containing 1.0 g/ml SQ and ASQ (a) and Fraction - 2 of Control Tissue Extracts, muscle (b), Liver (c), Kidney (d), Skin (e) and Plasma (f).

linear and reproducible through the investigated concentration range of 0.05 - 50 μg/ml, which is equivalent to 0.01 - 10 μg/g in tissue (R=0.999, n=5).

The detection limits shown in Table 2 (signal-to-noise ratio of 3) were satisfactory for residue analysis. These detection limits of SQ and ASQ were more sensitive than those in our previous report (1). This improvement might be caused by using L-column ODS for HPLC analysis and sample pretreatment of washing with n-hexane for three times.

Recovery

Recovery studies were conducted by adding 0.1 μg/g of AMP, EB, SQ and ASQ to each 5 g of control tissue sample. The extract from each sample was analyzed by the present method. Table 3 shows recovery data of the four compounds from tissues. Recoveries ranged from 83.8 to 103.8 % for individual compounds from individual tissues. Recoveries of SQ and ASQ from plasma were a little lower than those of other tissues, and this tendency was similar to that of our previous report (1). This tendency might be caused by delaying of SQ and ASQ elution from the alumina column. Coefficient of variation (C.V.)

TABLE 2

Detection Limits of AMP, EB, ASQ and SQ in Chicken Tissues.

Tissue	Detection Limit (μg/g)			
	AMP	EB	ASQ	SQ
Muscle	0.003	0.002	0.003	0.003
Liver	0.002	0.003	0.003	0.003
Kidney	0.004	0.003	0.003	0.003
Skin	0.002	0.003	0.003	0.003
Plasma	0.003	0.003	0.003	0.003

TABLE 3

Recoveries from Chicken Tissues Fortified with 0.1 μg/g of AMP, EB, ASQ and SQ.

Tissue	Recovery (%) (Coefficient of variation (%))			
	AMP	EB	ASQ	SQ
Muscle	90.2 (5.1)	99.7 (1.7)	99.7 (1.3)	103.8 (3.4)
Liver	85.8 (5.5)	97.3 (1.8)	89.4 (4.8)	97.4 (5.3)
Kidney	93.5 (4.0)	99.3 (1.1)	92.8 (3.1)	99.1 (3.3)
Skin	94.6 (8.6)	95.6 (3.8)	95.0 (4.6)	100.1 (4.2)
Plasma	98.1 (5.3)	90.8 (6.8)	83.8 (2.6)	81.0 (6.5)

n=5

ranged from 1.1 to 8.6 %. The recoveries were satisfactory for residue analysis.

Application

The application study was made to confirm whether the present method is applicable to quantitative assay of AMP, EB, SQ and ASQ in tissues from chickens administered AMP, EB and SQ.

Typical chromatograms of a liver extract are shown in Fig.5. The four compounds from five tissues were well separated not only from each other but also from tissue components. Further, the purity of SQ and ASQ peaks from individual tissues were determined by using the photodiode-array detector. Purity indices were 0.9999 for SQ and ranged from 0.9991 to 0.9999 for ASQ.

Table 4 shows concentration of four compounds in tissues. Though EB could not been detected in all samples because of a small dosage amount in the commercial preparation, AMP, SQ and ASQ were detected in all samples. AMP concentration was low in spite of high dosing, but SQ concentration was very high, especially in plasma which was three-fold greater than in muscle. ASQ concentration was low, and ratios of ASQ to SQ ranged from 0.9 to 4.3 % and was highest in liver.

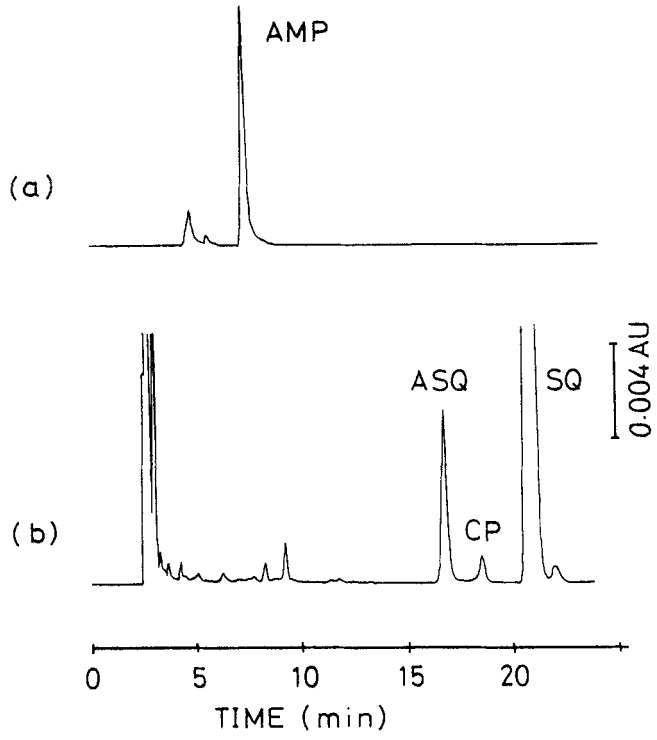


FIGURE 5. Typical Chromatograms of Ffraction - 1 with Fluorometric Ddetection (a) and Fraction - 2 with UV-detection for SQ and ASQ (b) of a Liver Extract from a Chicken 24 Hours after Orally Administration of AMP, EB and SQ.

CONCLUSION

A simultaneous HPLC residue analytical method with fluorometric detection using post-column reaction of AMP and with UV-detection of EB, SQ and ASQ in chicken muscle, liver, kidney, skin and plasma has been developed. This method was shown to be

TABLE 4

Concentration of AMP, EB, ASQ and SQ in Tissues from Chicken Administered with Commercial Drug Containing AMP 200 mg/g, EB 10 mg/g and SQ 120 mg/g.

Tissue	chicken[1)]	Concentration in Tissues (μg/g)			
		AMP	EB	ASQ	SQ
Muscle	1	0.45	-[2)]	1.51	53.58
	2	0.37	-	1.67	52.68
Liver	1	3.76	-	4.33	101.61
	2	1.41	-	3.98	111.70
Kidney	1	3.29	-	2.36	167.02
	2	0.59	-	2.06	163.66
Skin	1	0.59	-	2.08	80.29
	2	0.28	-	1.77	72.96
Plasma	1	0.31	-	1.54	171.49
	2	0.21	-	1.63	172.20

1) Chicken 1 and 2 were sacrificed 6 and 24 hours after single oral administration of 0.4 g/kg commercial drug, respectively.
2) Not detected.

applicable to tissue samples from a drug administered chicken. The detection limits and recoveries were satisfactory to residue analysis.

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Received: May 5, 1994
Accepted: May 24, 1994