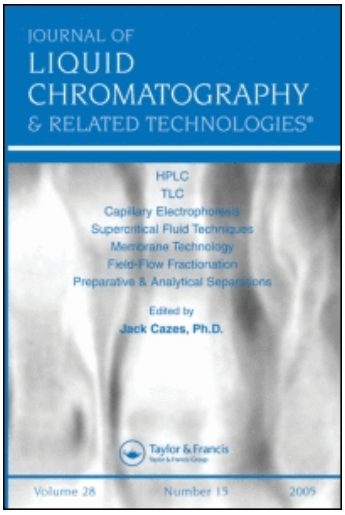

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High Performance Liquid Chromatographic Determination of Sulfisomidine and N4-Acetylsulfisomidine in Swine Tissues

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF SULFISOMIDINE AND N4-ACETYLSULFISOMIDINE IN SWINE TISSUES

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ABSTRACT

A method is described for the determination of the residual sulfisomidine and its metabolite, N4-acetylsulfisomidine, in swine muscle, kidney, liver and plasma by high performance liquid chromatography. Sulfisomidine and N4-acetylsulfisomidine were extracted from the swine tissues with acetonitrile, followed by alumina column clean-up procedure, and analyzed using a reversed-phase C18 column (250x4.6mm id),with UV detection at 270nm and a mobile phase of 10mM phosphate buffer ($_{p}$H 5.6)-acetonitrile (92:8; v/v). The average recoveries of sulfisomidine and N4-acetylsulfisomidine from swine tissues fortified with 0.1µg/g were ranged from 83.2 to 91.3%, 88.6 to 99.5%, respectively. The detection limits were 0.01µg/g for each drug. The applicability of the method was demonstrated by determining concentration of sulfisomidine and metabolite in tissues from swine administrated with sulfisomidine.

INTRODUCTION

Sulfisomidine (SID) is used as an antimicrobial agent to prevent and treat swine deseases in Japan. It has been reported that SID was metabolized by acetylation and became an inert compound, N4-acetylsulfisomidine (ASID) which was reconverted to active antimicrobial SID after their being uptaken in a human body (1). Therefore, in SID residue analysis, it is necessary to determinate its metabolite, ASID too. Recently, residue analysis for sulfonamides has been developed using high performance liquid chromatography (HPLC) (2,3,4). Although several reports of SID used HPLC have been published, SID was qualitated but not quantitated (5), SID was used as an internal standard of sulfonamides analysis (6), or SID was determinated in human plasma (1). Any methods for determination of residual SID and ASID using HPLC have not yet been published.

We had reported a HPLC residue method of some sulfonamides except SID (7). So we have developed a residue method of SID which would become a similar method as possible as the reported method (7), because it is important to develop a universal method that would be applicable to all sulfonamides.

This paper describes a HPLC method for the simultaneous determination of SID and ASID residue in swine muscle, liver, kidney and plasma. Moreover, we acertain applicability of the method to determination of SID and ASID in tissues of swine administrated with SID.

MATERIALS AND METHODS

Reagents

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

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

(c) Alumina - Alumina B Akt.1 (ICN Biomedicals, Eschwege, FRG).

(d) Sulfisomidine (Sigma Chemicals Co.,St. Louis, MO), N4-acetylsulfisomidine was synthesized by the method reported previously (8). Sulfisoxazole (internal standard, Sigma Chemicals).

(e) Standard solution - Stock solutions in concentration of 100μg/ml were prepared in MeCN and stored in the dark at 4 °C.

(f) Sulfisomidine injectable solution - Sulfisomidine was dissolved with sodium hydroxide,followed by adjust at $_{p}$H 9.0 by hydrochloric acid.

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

Apparatus

(a) Homogenizer - Bio-mixer BM-2 (Nition,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 300 x 15 mm id column, 6 g alumina was packed into the column with MeCN-methanol (60:40,v/v), and the column was washed with 30 ml of MeCN-methanol (60:40,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 250 x 4.6 mm id stainless steel column packed with Nucleosil 5C18 (Machery-Nagel, Duren, FRG). The mobile phase was consisted of MeCN - 10 mM phosphate buffer ($_{p}$H 5.6) (8:92, v/v). The injection volume was 20 µl, and flow-rate was 1.0ml/min. The detection wavelength was 270 nm. The column

temperature was 30° C. The chromatograms were recorded with a chart speed of 5 mm/min.

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

Control tissue samples

Three nonmedicated swine (body weight,15-20kg,youkushia) were sacrificed after bleeding, and the muscle, liver, and kidney 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 or kidney, 5ml of plasma was homogenized for 2 min with 25 ml MeCN. The homogenizer and glassware were washed twice with 20 ml MeCN. The mixture was filtrated through a cotton plug, washed with 30 ml n-hexane saturated with MeCN, and 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 30ml 2-propanol was added to the filtrate. The filtrate was evaporated to dryness at

Sample 5g
|
| Homogenize for 2 min with 25 ml MeCN
| Wash for 2 times with 20 ml MeCN
| Filter through a cotton plug
Filtrate
| Wash with 30 ml n-hexane saturated with MeCN
|
|
|——————————————n-hexane layer
|
MeCN layer
| Add 30 g sodium sulfate
| Stand for 30 min
| Filter through a cotton plug
Filtrate
| Add 30 ml 2-propanol
| Evaporate to dryness at 35° C
Residue
| Dissolve with 5 ml MeCN:MeOH (60:40)
| Sonicate for 0.5 min
Alumina column (6 g)
| Column loaded residue from muscle,liver and kidney;
| Wash with 15 ml MeCN:MeOH (60:40)
|
| Column loaded residue from plasma;
| Wash with 10 ml MeCN:MeOH (60:40)
|
| Elute with 40 ml MeOH:H_2O (85:15)
Eluate
| Added 30 ml 2-propanol
| Evaporate to dryness at 40° C
Residue
| Dissolve with 1 ml of mobile phase contained
| 0.5µg/ml sulfisoxazole
| Sonicate for 0.5min
| Filter through Ekicro disk 13 CR
Filtrate
| Inject 20 µl to HPLC
Analyze by HPLC

SCHEME 1. Analytical Procedure.

35° C, and the residue was dissolved with 5ml MeCN-methanol (60:40, v/v), sonicated, and applied to an alumina column. The column was washed with 15 ml MeCN-methanol (60:40, v/v), except the column for plasma. The column loaded plasma was washed with 10 ml MeCN-methanol (60:40, v/v). SID and ASID were eluted with 40 ml methanol-water (85:15, v/v). 30 ml 2-propanol was added to the elute, and followed to evaporate to dryness at 40° C. The residue was dissolved with 1ml mobile phase which contained 0.5μg/ml sulfisoxazole. the solution were filtrated through Ekicrodisk 13 CR (Gelman Sciences Japan, Tokyo, Japan) and subsequently injected with 20 μl into HPLC system.

Recovery

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

Application

Three swine of about 20 Kg body weight were used. They were kept indoors and provided nonmedicated feeds and water at libitum. 3 hours after they were given once intramuscular injection with 30 ,60 or 100mg SID per Kg body weight, they were sacrified after

bleeding, and the muscles,livers and kidneys were removed. Plasma and tissue samples were stored frozen at - 80° C before analysis.

RESULTS AND DISCUSSION

Sample preparation

We previously reported the residue method of sulfonamides(7), in which the sample was extracted with MeCN and the extract was applied to an alumina column , with 15 ml MeCN-methanol (60:40, v/v) washing solution,followed with 12 ml methanol-water (85:15, v/v; fraction 1) elute solution, and with 30 ml methanol-water (50:50, v/v; fraction 2) elute solution. In this condition, sulfonamides (sulfamethoxazole, sulfaquinoxaline, sulfadiadine, sulfadimetoxine, and sulfamonometoxine.) were eluted in fraction 2. When we analyzed samples fortified with SID and ASID according to the method we reported (7), SID and ASID were eluted in fraction 1 and fraction 2, especially, SID and ASID in plasma were eluted in washing solution too. Therefore elute solution was investigated.

Profiles of drugs eluted from the alumina column, which 1 ml working standard (1 ug/ml) was loaded on to, were showed in Fig.1. Increase of amount of water

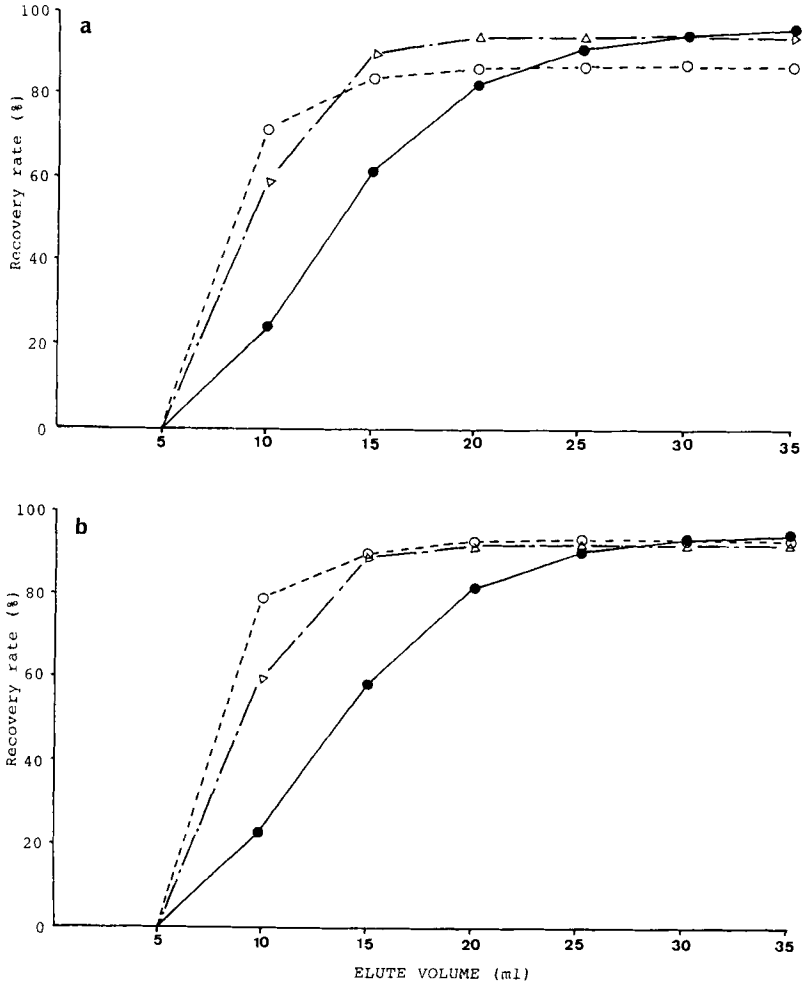

FIGURE 1. Elution Profiles of SID (a) and ASID (b) from an Alumina Column by Elute Solution.
Elute solution; —●—: Methanol-Water (85:15,v/v),
--△--: Methanol-Water (70:30,v/v),
----○----: Methanol-Water (50:50,v/v).

in elute solution caused that the drugs began to elute with small amount of elute solution, but recoveries of the drugs were low (fig.1). With 40 ml methanol-water (85:15,v/v) as elute solution, the recovery rates of SID and ASID were 97.5 and 95.0%, respectively. So, this solution was selected as elute solution.

When a plasma sample fortified with drugs was loaded on to an column and followed by 15 ml washing solution, a small parts of the drugs were eluted in washing solution. So the column was washed with 10ml washing solution. One of the causes might be that the co-extractives from plasma made weak activity of alumina, therefore drugs were eluted in washing solution.

HPLC conditions

When we analyzed a sample according to the HPLC condition we reported (7), we could not separate SID from coextracted components in tissues ,because SID was eluted from HPLC column at retention time of 4.8 min. So HPLC column and mobile phase were investigated.

Column used were ODS-1251-ss (Senshu chemical companey, Tokyo, Japan) , Hibar Lichrosorb RP-18 7um (Kantoukagaku Company,Tokyo,Japan), Capcell Pak C18

TABLE 1

Retention Time of Compounds

Compound	Retention time (min)
Sulfisomidine	13.2
N4-acetylsulfisomidine[a)]	23.0
Sulfisoxazole[b)]	20.2
Sulfachloropyridazine	30.9
Sulfadimidine	37.3
sulfamethoxypyridazine	41.7
Sulfamethoxazole	44.0
Sulfamonomethoxine	47.8
Sulfadoxine	56.0
Sulfadimethoxine	189.5

a) Metabolite of sulfisomidine
b) Internal standard

SG 300, Capcell Pak CN SG 300, Capcell Pack Phenyl SG-120 (SHISEIDO Companey, LTD. Tokyo, Japan) and Nucleosil 5C18 (Machery-Nagel,Duren,FRG). $_{p}$H was varied from 5.4 to 6.0. Mixture rate of phosphate buffer ($_{p}$H5.6) and acetonitrile was varied from 87:13, 90:10, 92:8 to 93:7 , respectively. Based on the results we selected HPLC conditions noted apparatus (e).

Retention time of SID, ASID, internal standard and sulfonamides which is used for swine deseases in Japan,is showed in Table 1.

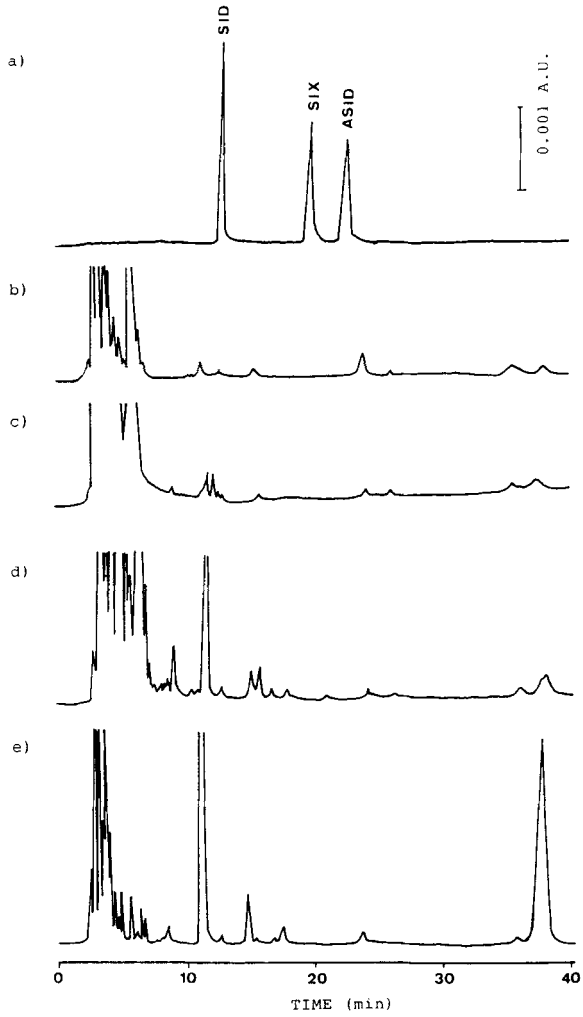

FIGURE 2. Typical Chromatograms of Standards and Control Tissue Extracts.
A standard solution (a) contains each 0.5 ug/ml SID, ASID and SIX. Control tissue extracts were from muscle (b), liver (c), kidney (d) and plasma (e).

Chromatograms

Fig.-2 (a) shows typical chromatogram from a standard solution of SID, ASID and an internal standard, sulfisoxazole (SIX). SID and ASID were well separated from each other.

Fig.-2 (b-e) show typical chromatograms from muscle, liver, kidney and plasma extract of a control swine, respectively. Several peaks derived from a tissue component appeared in the chromatograms,but they did not interfere to determinate SID and ASID.

Calibration curves and detection limits

The calibration curves of SID and ASID were linear and reproducible through the investigated concentration range of 0.05 - 50 μg/ml, which is equivalent to 0.01 -10 $\mu g/_{g \text{ or } ml}$ in tissue,(R=0.999, R=0.999, respectively. n=5).

The detection limits of the method were $0.01 \mu g/_{g \text{ or } ml}$ for both drugs in swine tissues (signal to noise ratio of 3), and satisfactory for residual analysis.

Recovery

recovery studies were conducted by adding 0.1 μg/g of SID or ASID to each 5g of control tissue sample. The extract from each sample was analyzed by

TABLE 2

Recoveries from Swine Tissues Fortified with 0.1ug/g of SID and ASID

Sample	Recovery (C.V.)	
	SID	ASID
Muscle	90.0[a] (2.1)[b]	99.5 (2.1)
Liver	83.2 (8.1)	88.6 (4.4)
Kidney	91.3 (4.5)	91.9 (6.9)
Plasma	85.4 (4.6)	88.8 (1.5)

a);Recovery(%)
b);Coefficient of variation,%,n=5

the present method. Table 2 shows recovery data of SID and ASID. Recoveries ranged from 83.2 to 99.5 % for drugs from swine tissues. Coefficient of variation ranged from 1.5 to 8.1 %. The recoveries were satisfactory for residual analysis.

Application

An attempt was made to comfirm whether components extracted from tissues were parent drug (SID) and major metabolite (ASID), moreover SID and ASID could be determinated by present method.

Fig.3 shows a typical chromatogram of muscle of swine administrated with 30 mg / kg SID.

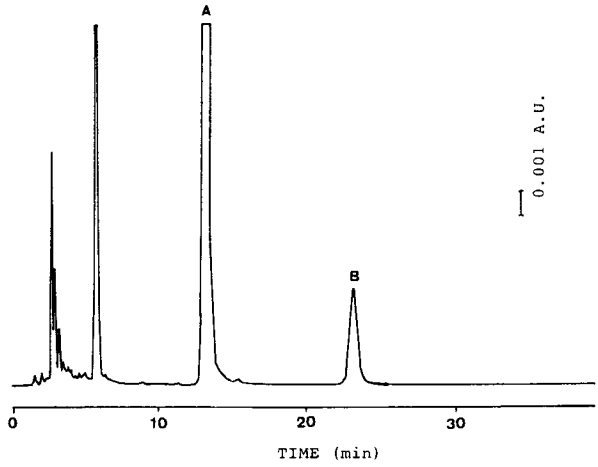

FIGURE 3. Typical Chromatogram of muscle extract from a swine administrated with 30 mg SID/kg

Identification of peaks in the chromatogram (Fig.3.) was done using photodiode array detector. When spectrum of the peaks at retention time 13.2 and 23.0 min (Fig. 3.) were compared with spectrum of the peaks of standard SID and ASID, respectively,they were almost identical, with similarity index of 0.9999 and 0.9995, respectively. Purity of the peaks at retention time 13.2 and 23.0 min (Fig.3.) were almost pure with purity index of 0.9999 and 0.9997,respectively. So, these peak components were confirmed as SID and ASID. No interfering peaks to determinate SID or ASID were there.

TABLE 3

Concentration of SID and ASID in Tissues of Swine Administrated with SID

Sample	SID[a)] (mg)	SID (ppm)	ASID (ppm)	100xASID/SID (%)
Muscle	30	11.6	0.8	6.9
	60	18.6	1.5	8.1
	100	28.9	2.2	7.6
Liver	30	14.2	3.0	21.1
	60	22.5	6.7	29.8
	100	30.4	6.5	21.4
Kidney	30	51.2	14.8	28.9
	60	57.9	34.1	58.9
	100	51.9	44.3	85.4
Plasma	30	25.6	5.9	23.0
	60	27.4	8.3	30.3
	100	47.0	12.0	25.5

a); SID (mg) per Kg swine body weight.

Table 3 shows concentration of SID and ASID in tissues of swine administrated with SID. Major metabolite, ASID was detected in all tested tissues. Rates of ASID to SID in tissues of swine administrated 30, 60, and 100 mg SID were ranged from 6.9 to 28.9 , from 8.1 to 58.9 and from 7.6 to 85.3 %, respectively.

CONCLUSION

A HPLC method with UV detection of SID and ASID in swine muscle, liver, kidney and plasma has been

developed, and this method was shown to be applicable to tissue samples from swine administrated with SID. The detection limits and recoveries were satisfactory to residue analysis.

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