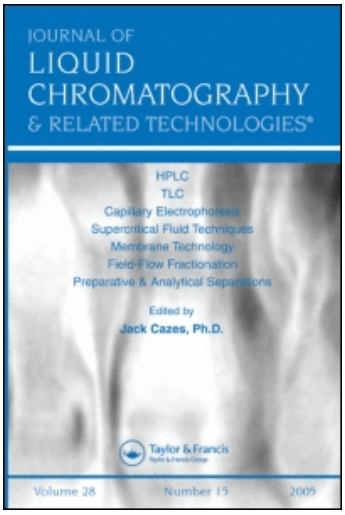

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A Sensitive High-Performance Liquid Chromatographic Method for Detecting Sulfonamide Residues in Swine Serum and Tissues After Fluorescamine Derivatization

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A SENSITIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR DETECTING SULFONAMIDE RESIDUES IN SWINE SERUM AND TISSUES AFTER FLUORESCAMINE DERIVATIZATION

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ABSTRACT

A highly sensitive and rapid high-performance liquid chromatographic method for determining sulfonamides (sulfadiazine, sulfamethazine, sulfamonomethoxine, sulfamethoxazole and sulfadimethoxine) in swine serum and tissues is described. The sulfonamides were extracted from the samples, derivatized with fluorescamine, chromatographed on a Nova-Pak C_{18} column using acetonitrile-10 mM potassium phosphate (30:70, v/v) as the mobile phase and detected spectrofluororimetrically (excitation 390 nm, emission 475 nm). The retention times were 7.1 to 18.2 min and there was no interference from any co-extractives. The detection limit for each standard sulfonamide solution was 0.1 ng/ml and their calibration curves were linear between 1 and 100 ng/ml. In the presence of sulfadiazine as an internal standard, sulfonamide recovery from spiked serum, muscle, liver and kidney samples (10 ng/ml) was 94.0 ± 4.7 to 97.3 ± 5.9%, 58.5 ± 3.1 to 73.9 ± 5.7 %, 65.9 ± 7.1 to 86.9 ± 10.6% and 86.2 ± 4.0 to 92.8 ± 6.4% respectively.

INTRODUCTION

Antimicrobial agents are given to animals in subtherapeutic concentrations for three reasons: (1) to prevent infectious diseases caused by bacteria or protozoa; (2) to decrease the amount of feed needed and (3) to increase the rate of weight gain [1]. Sulfonamides were the first chemotherapeutic agents used for the systematic control of bacterial diseases in livestock [2], as they had a broad spectrum of activity and were cheap [3]. However, the use of antimicrobial agents in animals that end up as food for human consumption results in the presence of illegal residues in meat (especially the liver and kidneys) [1]. A study by the National Center for Toxicological Research indicated that sulfamethazine may be a thyroid carcinogen [4].

Various authors have published procedures for determining different sulfonamides in animal fluids and tissues, most of which involve reversed-phase high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection [2]. However, these methods require elaborate and time-consuming clean-up procedures or have low detection sensitivities. Japanese food safety laws have established a zero residual level for all antimicrobial agents in edible animal tissues [5]. Therefore, improved analytical procedures are needed to monitor them for sulfonamide, and other antibiotic, residues.

Fluorescamine was first reported to be a means of generating fluorescent derivatives of primary amino acids [6]. Sigel *et al.* [7] detected sulfadiazine after derivatization with fluorescamine solution using a thin-layer chromatographic (TLC) method and recently, several TLC methods [3, 8, 9] for analyzing sulfonamides at ppb levels in animal tissues using a fluorescamine solution for derivatization have been described. Although van Haaster *et al.* [10] developed a highly sensitive HPLC method for determining histamine and 3-methylhistamine in biological samples using fluorescamine as the derivatizing agent. So far, to our knowledge, no reports of methods for identifying sulfonamide residues in animal tissues using HPLC with fluorescamine derivatization have been published. The objective of this study was to develop a rapid and sensitive screening method for sulfonamide residues in swine serum and tissues using HPLC with fluorescamine as the derivatizing agent.

EXPERIMENTAL

Materials

Sulfadiazine (SDZ), sulfamethazine (SMT), sulfamonomethoxine (SMM) and sulfadimethoxine (SDM) were purchased from Sigma (St. Louis, MO, USA) and sulfamethoxazole (SMX) was obtained from Shionogi Pharmaceutical, Osaka, Japan. Acetonitrile, potassium dihydrogen phosphate (PDP) and N,N'-dimethylformamide were obtained from Wako Pure Chemical Industries, Tokyo, trichloroacetic acid (TCA, analytical reagent grade) was from Yoneyama Yakuhin Kogyo, Osaka, Japan, fluorescamine was obtained from Sigma and HPLC-grade water was produced in a Milli-Q purification system (Millipore, Milford, MA, USA). Stock sulfonamide solutions (1 mg/ml) were prepared by dissolving 10 mg each standard compound with 1 ml N,N'-dimethylformamide and diluting them to 10 ml with distilled water. The stock solutions were diluted to the desired concentrations (1, 5, 10, 50 and 100 ng/ml) with 0.01% TCA solution for calibration curve study. Sulfadiazine (10 ng/ml) was added to the assay solution as an internal standard.

Extraction procedure

Serum

A 0.1-ml aliquot of each sulfonamide solution (100 ng/ml) was added to 0.9 ml swine serum (spiked 10 ng/ml), then kept in the refrigerator (4°C) overnight to allow drug incorporation into the serum, after which 4 ml acetonitrile was added for extraction and deproteinization, the mixture was stirred with a vortex stirrer and centrifuged for 15 min at 1,000 g. The supernatant was evaporated to dryness under a stream of nitrogen gas using a 40°C water bath, the residue was dissolved in 0.05 ml water and mixed vigorously, 1 ml acetonitrile was added to the mixture, which was for centrifuged 15 min at 1,000 g and the resulting upper layer was evaporated to dryness. The residue was dissolved in 1.0 ml 0.01% (w/v) TCA solution containing 10 ng/ml sulfadiazine (internal standard) and shaken, 0.1 ml hexane was added to the solution, which was shaken again

and centrifuged at 1,000 g for 15 min, after which, a 0.5-ml aliquot of the clear layer was collected carefully with a Pasteur pippet and used for derivatization.

Tissues

Swine kidney, muscle and liver tissues (100-200 g) were cut into small pieces and homogenized in a blender. The ground tissues were stored at -30°C until analyzed, when 1.0 g was placed in a 10-ml centrifuge tube, 0.1 ml sulfonamide mixture (100 ng/ml) was added to produce 10 ng/ml spiked samples, which were kept in the refrigerator (4°C) overnight to allow drug incorporation into the tissues, after which, the extraction procedure was carried out as described for serum.

Derivatization

A 0.1-ml aliquot of freshly prepared fluorescamine solution in acetonitrile (1 mg/ml) was added to each tube containing 0.5-ml purified samples, which were shaken by hand 1 min, and a 50-µl aliquot of each derivatized sample was injected into the HPLC column.

HPLC analysis

The HPLC system comprised a Model 600E multisolvent delivery pump connected to a U6K injector (Waters Associates, Milford, MA) and a Hitachi F-1050 fluorescence spectrophotometric detector (Hitachi, Tokyo, Japan) operating at excitation (Ex) and emission (Em) wavelengths of 390 and 475 nm respectively. The separation procedure was performed using a Nova-Pack C_{18} column (prepacked, 10-µm particle size, 300 mm x 3.9 mm ID, Waters Associates), the chromatographic data system used was Chromatopac C-R6A (Shimadzu Seisaku, Kyoto, Japan), the mobile phase comprised acetonitrile-10 mM potassium dihydrogen phosphate (30:70, v/v) at room temperature, which was degassed using an ultrasonic bath and the flow-rate was 1.0 ml min^{-1}.

Calculation

A standard calibration curve (four replicates each) for each of the four sulfonamides of their peak-height (h) to that of the internal standard (IS) ratios against their concentrations (1, 5, 10, 50, 100 ng/ml) was plotted using the following equations: Y = aX + b and Y = h (of each sulfonamide)/h (IS); X = concentrations; a = slope; b = intercept. The recovery of each sulfonamide from each spiked sample was calculated by comparing its peak-height ratio with those of the standard control solutions under identical HPLC analytical conditions.

RESULTS and DISCUSSION

Linearity and stability

The linearity of thefluorescence intensity of the sulfonamide mixture solution was evaluated by analyzing a concentration range of 1 to 100 ng/ml of the mixture of four sulfonamides. The chromatogram of 0.5 ml standard sulfonamide mixture solution (1 ng/ml) containing 10 ng/ml sulfadiazine (internal standard) derivatized with 0.1 ml fluorescamine (1 mg/ml) is shown in Fig. 1. Each peak was symmetrical and the retention times of SDZ, SMT, SMM, SMX and SDM respectively were 7.1, 7,9, 9.1. 14.1 and 18.2 min.

The standard calibration curves (four replicates) for the four sulfonamides were linear with correlation coefficients in excess of 0.99 as follows: SMT: $Y=(0.1383 \pm 0.0033)X + (0.1315 \pm 0.0617)$, $r=0.9993 \pm 0.0005$; SMM: $Y=(0.1033 \pm 0.0037)X + (0.1202 \pm 0.0671)$, $r=0.9977 \pm 0.0025$; SMX: $Y=(0.0544 \pm 0.0032)X + (0.079 \pm 0.0546)$, $r=0.9987 \pm 0.0014$; SDM: $Y=(0.0458 \pm 0.0032)X + (0.0895 \pm 0.0504)$, $r=0.9974 \pm 0.0029$. In a standard solution, with a 50-μl injection sample, 0.1 ng/ml sulfonamides was the lowest concentration that could be detected. van Haaster *et al.* [10] detected 20 pg histamine and 3-methylhistamine on their column at a signal-to-noise ratio of 3:1, which was about the same sensitivity as our method. They also demonstrated that only 10% of the fluorescence intensity was lost over a period of 7 days. In this study, we

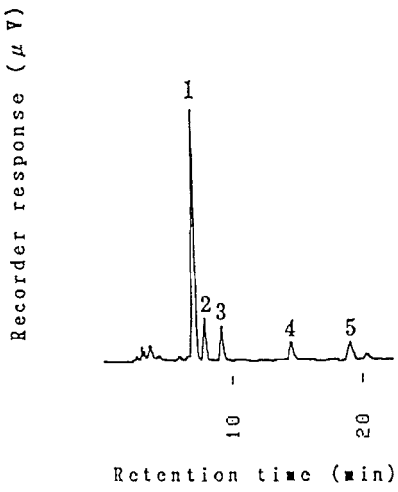

Figure 1. Chromatogram of the four standard sulfonamides (1: SDZ at 10 ng/ml, the internal standard, 2: SMT, 3: SMM, 4: SMX and 5: SDM with respective retention times of 7.1, 7.9, 9.1, 14.1 and 18.2 min) at 1 ng/ml derivatized with 0.1 ml fluorescamine solution (1 mg/ml).

analyzed 0.5 ml mixture of standard sulfonamides (10 ng/ml) solution on day 5 after derivatization with 0.1 ml fluorescamine (1 mg/ml), on day 5 and found the peak height was only about 10% (corresponding to about 1 ng/ml) of that obtained after derivatization for 1 min. Most of fluorophore activity appeared to have declined, which agrees with the results of Lai [11], who reported that the fluorescence intensity stayed constant for about 1 h, then diminished slowly thereafter van Poucke *et al.* [3] reported that after spraying with fluorescamine solution, the HPTLC plate should be scanned within 30 min. However, the fluorophore was found to be stable for up to 3 h in this study and its fluorescence intensity had halved 24 h after derivatization of a 10 ng/ml standard solution (0.5 ml) with 0.1 ml fluorescamine (1 mg/ml).

Recovery

The sulfonamide recoveries from swine 10 ng/ml spiked serum and tissue samples were determined using six replicates. The chromatograms of blank and spiked serum samples after acetonitrile extraction and fluorescamine derivatization are shown in Fig. 2. Those of the muscle, liver and kidney samples are shown in Figs. 3, 4 and 5 respectively. The recovery results for the four sulfonamides from swine spiked serum and tissue samples are presented in Table 1. The recoveries from muscle and liver were lower than those from serum and kidney. This may be due to an unknown substance that affected fluorophore formation or the sulfonamides may have bound to these tissues. Reimer and Suarez [8] demonstrated that low sulfonamide recovery from salmon muscle tissue appeared to be related to its relatively high cholesterol level.

Derivatization

Usually, o-phthalaldehyde is used as the derivatizing agent for determining sulfonamides with fluorescence detection. Morita *et al.* [12] analyzed sulfonamide residues in livestock products using HPLC with spectrofluorometric detection (Ex 285 nm, Em 445 nm). They used the o-phthalaldehyde as the derivatizing agent and the sample extracts had to be reacted with it for 30 min at 60°C. In this study, derivatization was quickly and easily carried out at room temperature for 1 min before injection into the HPLC column. Furthermore, Lai demonstrated that o-phthalaldehyde yielded background fluorescence several fold higher than fluorescamine [11]. Fluorescamine reacts with peptide primary amino groups almost instantaneously at room temperature in aqueous solution at pH 7.5-9 to form a fluorescent compound [11]. Initially, we used a solution of this pH for the derivatization reaction, but obtained a large interference peak that overlapped with the sulfonamides peaks. Eventually, we used 0.01% (w/v) TCA solution (pH 3.6) to dissolve the residues after drying under nitrogen gas and then derivatized them with fluorescamine for 1 min. The derivatized samples were eluted with a mobile phase (pH 5.3) of acetonitrile-10 mM PDP (30:70, v/v). These were found to be

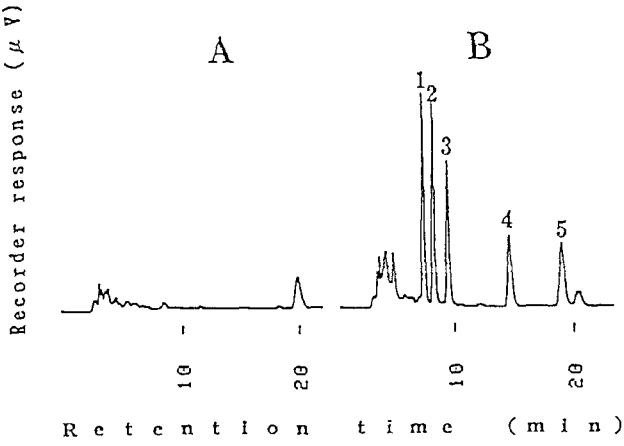

Figure 2. Chromatogram of fluorescamine-derivatized acetonitrile extracts from (A) blank serum and (B) a serum sample spiked with standard sulfonamides (10 ng/ml) and the internal standard (10 ng/ml) sulfadiazine. The elution order is (1) SDZ, (2) SMT, (3) SMM, (4) SMX and (5) SDM with respective retention times of 7.1, 7.9, 9.1, 14.1 and 18.2 min.

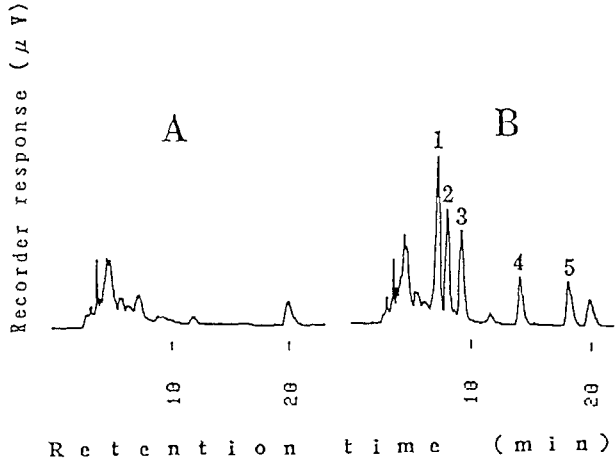

Figure 3. Chromatogram of fluorescamine-derivatized acetonitrile extracts from (A) blank and (B) spiked muscle samples. Spiking data and retention times as Figure 2.

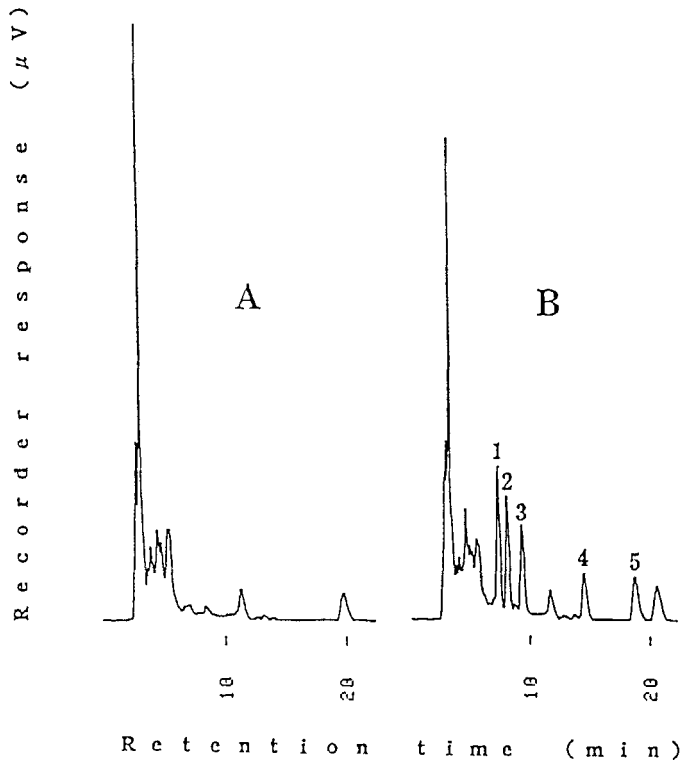

Figure 4. Chromatogram of fluorescamine-derivatized acetonitrile extracts from (A) blank and (B) spiked liver samples. Spiking data and retention times as Figure 2.

the best conditions for fluorophore formation and HPLC elution. Maybe owing to the HPLC system could change the circumstance for fluorophore formation.

Extraction

Usually, sulfonamides are extracted from solid samples, such as muscle and some tissues, by homogenizing the sample in an extraction solvent [3, 5, 8, 9, 12] and liquid samples, for example serum, milk and urine, are treated similarly by multiple extraction with organic solvents [2, 3, 12-14]. Both types of extract require additional clean-up

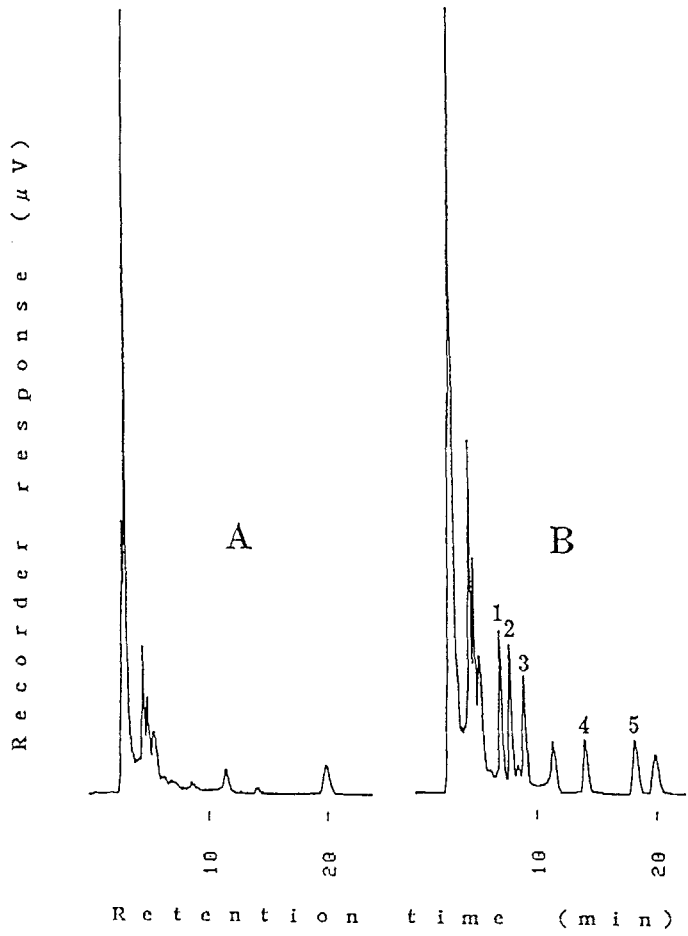

Figure 5. Chromatogram of fluorescamine-derivatized acetonitrile extracts from (A) blank and (B) spiked kidney samples. Spiking data and retention times as Figure 2.

steps and concentration using C_{18} packing material or another cartridge before they can be assayed [3, 5, 13] and these procedures are time-consuming. In this study, we developed a highly sensitive method for detecting sulfonamide residues at ppb levels. Only a small sample (1 ml serum or 1 g ground tissue) needs to be treated to monitor any residual sulfonamides and the extraction method is easy, using only 5 ml acetonitrile.

TABLE 1. RECOVERY RESULTS FOR THE FOUR SULFONAMIDES FROM SPIKED (10 ng/ml) SWINE SERUM AND TISSUES (n=6)

Drugs	serum	muscle	liver	kidney
SMT	97.0± 4.9	58.5± 3.1	65.9± 7.1	86.5± 4.3
SMM	94.1± 2.4	64.1± 2.7	73.5± 9.8	86.2± 4.0
SMX	97.3± 5.9	69.6± 3.2	83.1± 8.2	90.4± 5.0
SDM	94.0± 4.7	73.9± 5.7	86.9±10.6	92.8± 6.4

CONCLUSION

A simple, sensitive and rapid HPLC analytical method for determining sulfonamide residues in swine serum and tissues using fluorescamine as the derivatizing agent has been described. The advantages of this method over the others currently available are that a small sample only is needed, sulfonamide extraction is easy, derivatization with fluorescamine takes only 1 min at room temperature and at least four sulfonamides in a sample can be detected simultaneously. Our method may be useful for regulatory purposes for routine screening for some residual sulfonamides in animal edible tissues. However, further studies are necessary to evaluate the effectiveness of this system in vivo.

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