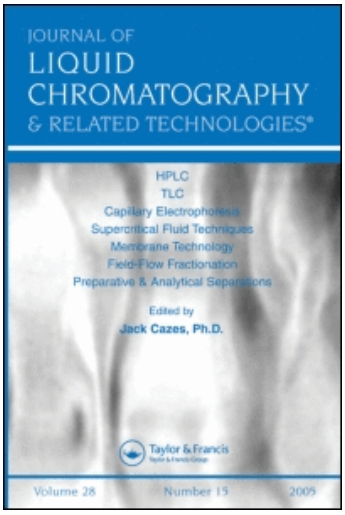

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APPLICATION OF SOLID PHASE EXTRACTION FOR THE ANALYSIS OF SULFONAMIDES IN MILK BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A High Performance Liquid Chromatographic (HPLC) method for the analysis of Sulfadiazine, Sulfathiazole, Sulfapyridine, Sulfamerazine, Sulfamethiazole, Sulfamethazine, Sulfachloropyridazine, Sulfadimethoxine, and Sulfaquinoxaline is described. Milk (5 ml) is diluted with 5 ml potassium phosphate buffer (1 M, pH 4.4) and passed through a Cyclobond-I solid phase extraction (SPE) column. Sulfonamides, retained on the SPE column, are eluted with 4 ml methanol. The eluate is further cleaned using an alumina and AGMP-1 ion exchange column to remove coeluting matrix components. The cleaned extract containing sulfonamides is analyzed by HPLC using a reverse phase column and diode array detector at 265 nm.

INTRODUCTION

Sulfonamides are a class of veterinary drugs which are extensively used on farm animals for the treatment of a variety of bacterial infections. The use of sulfonamides is not limited to therapeutic purpose but are also used for prophylactic purposes. In the past, residues of sulfonamides have been found in milk (1, 2). The concern over the residues of sulfonamides, especially sulfamethazine, has grown after the National Center for Toxicological Research (NCTR) of the Food and Drug Administration (FDA) reported that sulfamethazine may be a carcinogen (3). As a result, The FDA has

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limited the use of sulfonamides in lactating animals to only sulfadimethoxine, and the residues must not exceed 10ppb in milk (4).

High performance liquid chromatography has become the most widely used technique for the analysis of veterinary drug residues in foods of animal origin. A number of HPLC methods have been developed for the analysis of sulfonamides in milk, tissue and eggs (5-13). Most of the HPLC methods use traditional solvent extraction techniques. Unruch et. al. developed a method using solid phase extraction which is applicable only for sulfamethazine (11). In general all HPLC methods which can detect sulfonamides to the 10ppb level require extensive cleanup steps before HPLC analysis.

In this report a HPLC method for the determination of nine sulfonamides at a low level of 10ppb is described. The solvent extraction step is replaced by a convenient solid phase extraction step.

EXPERIMENTAL

Chemicals & Reagents

(a) Sulfadiazine (SDZ), Sulfathiazole (STZ), Sulfapyridine (SPD), Sulfamerazine (SMR), Sulfamethiazole (SMTZ), Sulfamethazine (SMZ), Sulfachloropyridazine (SCP), Sulfadimethoxine (SDM), Sulfaquinoxaline (SQX) (Sigma Chemical Co., St. Louis, MO).

(b) Potassium phosphate buffers (mono and dibasic), ammonium acetate, acetic acid, chloroform, acetone and HPLC grade methanol, neutral alumina (Fisher Chemical Co., Fairlawn, NJ). AGMP-1 ion exchange resin, chloride form (100-200 mesh, Bio-Rad Labs., Richmond, CA).

(c) Potassium phosphate buffer: Dissolve 13.60 g monobasic potassium phosphate buffer in 100 ml distilled water to prepare 1M , pH 4.4 buffer.

(d) Elution solvent for alumina column: Mix 60 ml methanol, 30 ml distilled water, and 2 ml potassium phosphate buffer (c).

(e) Elution solvent for AGMP-1 resin column: Mix 50 ml methanol, 45 ml distilled water, and 5 ml conc. acetic acid.

(f) Ammonium acetate buffers: To prepare buffer 1, dissolve 1.95 g ammonium acetate in 900 ml distilled water, adjust pH to 4.7 with acetic acid and make final volume to 1000 ml. To prepare buffer 2,

dissolve 1.95 g ammonium acetate in 900 ml distilled water, adjust pH to 8.0 with ammonium hydroxide and make final volume to 1000 ml.

(g) Mobile phase for HPLC:

Solvent A: Ammonium acetate buffer 1(pH 4.7):methanol (850:150, V:V).

Solvent B: Ammonium acetate buffer 2(pH 8.0):methanol (700:300, V:V).

Use following gradient at 0.4 ml/minute flow rate.

Time 0 minutes 0% B

Time 5 minutes 0% B

Time 20 minutes 100% B

Time 40 minutes 100% B

Time 45 minutes 0% B

(h) Sulfonamide standard solutions: Dissolve 100 mg of each of the nine sulfonamides in 100 ml methanol. Dilute 1 ml of this solution to 100 ml with distilled water in a volumetric flask (Solution B).

(i) Working standard: Dilute 10 ml of solution B to 100 ml with distilled water to prepare a solution with a concentration of 1000 ng/ml of each sulfonamide.

Apparatus

(a) Pear shaped flask, 125 ml.

(b) Solid Phase Extraction Cartridges: Cyclobond-I, SPE cartridges, 3 ml size (ASTEC, Whippany, NJ).

(c) Liquid Chromatograph: A Perkin Elmer binary LC pump 250 solvent delivery system equipped with a Perkin Elmer ISS 100 autosampler and a Perkin Elmer 235 diode array detector was used at 265 nm. A PE Nelson 1020 data system was used for quantitation by peak heights.

(d) LC Column: LC 18-DB, 250 X 2.1 mm, 5um particle size. (Supelco Inc., Bellefonte, PA).

(e) Preparation of Alumina column: Use glass wool to plug the bottom of a pasteur pipet and then fill neutral alumina to a 1 cm height. Wash with 5 ml methanol.

(f) Preparation of AGMP-1 resin column: Follow the procedure described by Unruch et al (11) to prepare AGMP-1 resin in 0.2M K2HPO4 (pH 7.9) buffer. Use a one ml disposable pipet tip used with Eppendorf

style pipet to prepare the column. Plug the end of the tip with glass wool and load the resin slurry to a 1 cm height.

Fortification of Milk

Fortify milk with sulfonamides at 10 and 20 ppb levels by diluting 100 and 200 ul of working standard to 10 ml with milk.

Extraction and Cleanup Procedure

Take 5 ml of decreamed milk and dilute with 5 ml potassium phosphate buffer (c) (1M, pH 4.4). Condition a cyclobond-I SPE column by washing with 5 ml distilled water followed by 5 ml potassium phosphate buffer (c). Pass 10 ml diluted milk through the SPE column, wash the column with 5 ml potassium phosphate buffer (c), followed by an additional 5 ml mixture of buffer/distilled water (1/4, V/V). Elute sulfonamides, retained on the SPE column, with 4 ml methanol and pass eluate through an alumina column. Collect the eluate from the alumina column in a pear shaped flask. Elute remaining sulfonamides from the alumina column with 4 ml elution solvent (d) prepared by mixing water, buffer, and methanol and collect the eluate in the same pear shaped flask. Evaporate the combined eluate in the pear shaped flask to dryness on a rotary evaporator maintaining temperature approximately 50°C. Add 1 ml methanol to the flask to dissolve sulfonamides and load on an AGMP-1 column. Wash the flask with additional 1 ml methanol and load on the AGMP-1 column. Elute sulfonamides from AGMP-1 column with 4 ml acidic elution solvent (e) prepared with methanol, acetic acid, and distilled water and collect in a pear shaped flask. Concentrate the total volume to approximately 0.5 ml by using a rotary evaporator at 50°C and make final volume to 2 ml. Inject 100 ul in to HPLC.

RESULTS AND DISCUSSION

The solid phase extraction technique is advantageous due to its rapidness as well as low solvent requirements. The extraction of milk with a cyclobond-I SPE column eliminates the use of traditional solvent extraction. The retention of sulfonamides on a cyclobond-I SPE column has been investigated earlier and all the sulfonamides investigated here can be retained on the cyclobond-I SPE column in

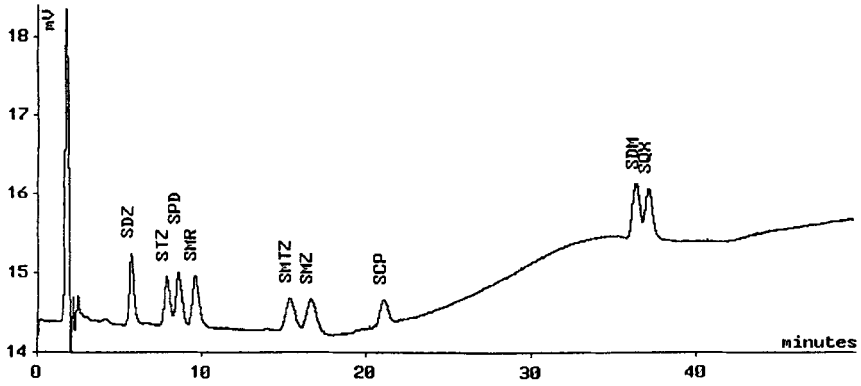

Figure 1. Chromatogram of Standard sulfadiazine (SDZ), Sulfathiazole (STZ), Sulfapyridine (SPD), Sulfamerazine (SMR), Sulfamethiazole (SMTZ), Sulfamethazine (SMZ), Sulfachloropyridazine (SCP), Sulfadimethoxine (SDM), Sulfaquinoxaline (SQX).

aqueous medium within a pH range of 4-5 (14). Therefore, the milk samples were diluted with a pH 4.4, 1 molar potassium phosphate buffer before loading on the cyclobond-I SPE column.

After eluting sulfonamides from the SPE column with methanol, further cleanup was necessary to remove interferring components coeluting with the sulfonamides. An alumina and AGMP-1 resin columns have been used in the past for the analysis of sulfmethazine in milk, and organic feed extracts (11, 15, 16), and sulfathiazole in honey (17, 18). These two columns were also used in this method for further cleanup. Certain modifications however, were necessary as described in the experimental section.

Figure 1 shows a chromatogram of standard sulfonamides. All nine sulfonamides are well resolved. Figure 2 shows a chromatogram of a blank milk sample. Three peaks from the UV absorbing matrix components were present. None of these, however, interferred with the analysis of sulfonamides except the first one which eluted close to the retention time of sulfadiazine (SDZ). Figure 3 shows a chromatogram of a milk sample fortified at 20 ppb level. Under the liquid chromatographic conditions used, all sulfonamides can be quantitated except sulfadiazine. The quantitation of sulfadiazine is difficult

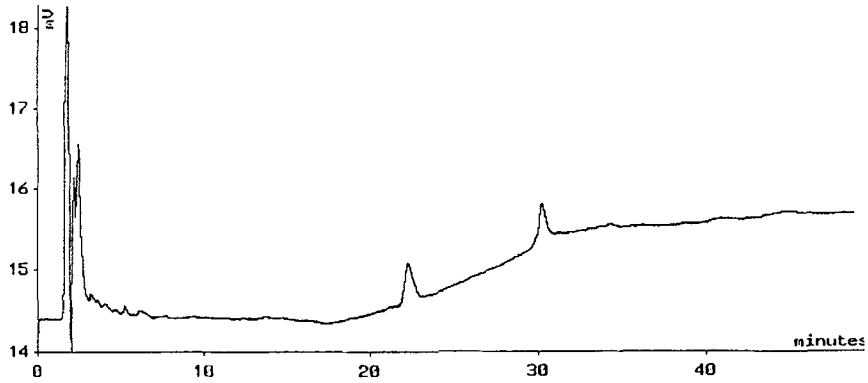

Figure 2. Chromatogram of Blank Milk Sample.

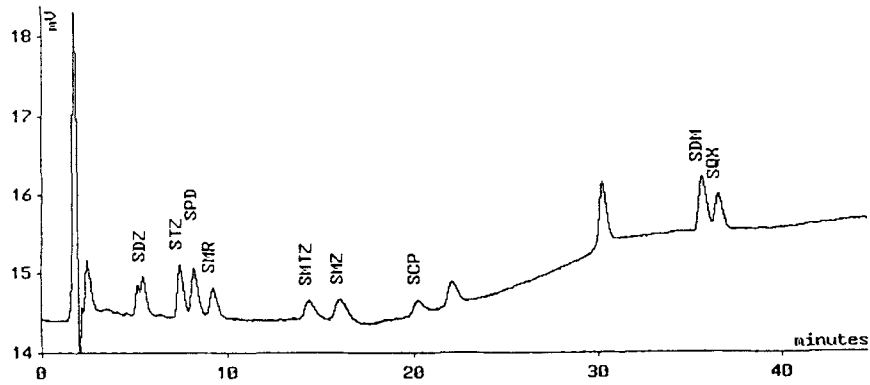

Figure 3. Chromatogram of Milk Sample Spiked with Nine Sulfonamides Each at 20 ppb Level.

because of the presence of an interferring peak close to its retention time. By reducing the concentration of methanol in the mobile phase, however, it is possible to resolve the interferring peak from the sulfadiazine peak, and quantitate sulfadiazine also. The recoveries of all the sulfonamides from milk samples fortified at 10 and 20 ppb levels were greater then 50%.

In conclusion, the present method is a significant improvement over the existing methods for the analysis of sulfonamides in milk. No solvent extraction is required. This method, however, needs further

work to improve the recoveries of sulfonamides and also to be able to remove the interferring matrix peak which elutes near the retention time of sulfadiazine.

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