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Solid-phase extraction of sulfamethazine in milk with quantitation at low ppb levels using thin-layer chromatography

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ABSTRACT

A method for the solid-phase extraction and thin-layer chromatographic (TLC) quantitation of sulfamethazine (SMZ) residues in milk is presented. Sulfabromomethazine was added as an internal standard to homogenized milk samples which were then diluted and passed through C_{18} solid-phase extraction columns. The C_{18} columns were eluted with methanol, and interfering components in the methanol were removed by passing eluate over an acidic alumina column. The analyte was then concentrated on a small ion-exchange resin. SMZ was eluted and applied to a silica gel TLC plate. Fluorescence detection was induced with fluorescamine and quantitated with a scanning densitometer. Recoveries were 88.36–103.15% in the analysis range [0.51–15.34 ppb (μg/l)]. The average recovery over the analysis range was 96.07%, with a coefficient of variation of 12.52%.

INTRODUCTION

Sulfamethazine (SMZ) is an effective and conveniently used agricultural drug. The United States of America's Food & Drug Administration (U.S.F.D.A.) permits SMZ use to treat various respiratory diseases, foot rot, acute mastitis, acute metritis and coccidiosis in beef cattle and non-lactating dairy cattle [1]. The U.S.F.D.A. does not permit SMZ to be used in lactating animals, but has set 10 ppb[a] as an unofficial level of concern for SMZ residues in milk [2] and recently has suggested the possibility of lowering the residue limit to 1 ppb or lower [3]. Some recent screening surveys show that SMZ residues are present in many market milk samples [4–8]. Several commercial immunochemical-based test kits are available to visually screen for SMZ in milk at the 10-ppb level [6,7]. Some of these kits have the advantage of being usable on the farm, but none of them appears to distinguish between the free SMZ and any of its N-4-conjugated metabolites [6]. Only one analytical method [9] was located in the literature to specifically analyze milk for SMZ at the 10-ppb level, and the authors confirmed the SMZ residues by adapting gas chromatographic–mass spectrometric (GC–MS) methods [10,11] initially developed for tissues. These methods involve

[a] Throughout the article the American billion (10^9) is meant.

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liquid–liquid extractions, concentration by evaporation and the use of halogenated solvents.

We report here a method to extract and isolate SMZ using a liquid–solid system which is fast, requires no evaporation and uses a minimal amount of non-halogenated solvents. Chromatographic analysis is then carried out using high-performance thin-layer chromatography (HPTLC). The use of TLC to quantitate SMZ further minimizes solvent use and the cost of the analysis.

EXPERIMENTAL

Reagents and equipment

All solvents were HPLC grade. Water was HPLC grade from Modulab Polisher I (Continental Water Systems, San Antonio, TX, U.S.A). All reagent were Baker-analyzed (J. T. Baker, Phillipsburg, NJ, U.S.A.) except fluorescamine, sulfamethazine, N-acetylsulfanilyl chloride and 2-amino-4,5-dimethylpyrimidine, which were obtained from Sigma (St. Louis, MO, U.S.A.). Sulfabromomethazine (SBZ) was synthesized according to the method of English *et al.* [12] and purified by crystallization from acetone (98% purity by TLC). Acidic alumina was purchased as activated, 95 + %, –60 mesh (Alfa Products, Danvers, MA, U.S.A), and used as received. Extraction columns were Bakerbond C_{18}, 3 ml (Baker No. 7020-03). Cation-exchange resin was AG MP-1, 100–200 mesh, chloride form (Bio-Rad Labs., Richmond, CA, U.S.A.). Quik-Snap columns with bottom disc were from Isolab (Akron, OH, U.S.A.). A Lida's twelve-port vacuum manifold equipped with Teflon® needles and stopcocks was purchased from Thomson Instrument (Newark, DE, U.S.A.).

Homogenized/pasteurized milk was obtained from local food markets; raw milk was obtained from Delaware Valley College of Science and Agriculture (Doylestown, PA, U.S.A) or Saul Agricultural High School (Philadelphia, PA, U.S.A.), and raw goat milk was purchased at a local health food store.

Solutions

Stock solutions of SMZ and SBZ (1 mg/ml) were made in acetone and stored at −20°C. Working solutions of 1 μg/ml SBZ and 1.5, 1.2, 1.0, 0.75, 0.60, 0.50, 0.25, 0.10 and 0.05 μg/ml SMZ were made monthly in water, by diluting stock solutions, and they were stored at 0–5°C. A 100-μl volume of SMZ working solution was added to 10 ml of milk to obtain milk fortified at 15, 12, 10, 7.5, 6, 5, 2.5, 1 and 0.5 ppb SMZ. A 100-μl volume of SBZ working solution was added to each 10-ml milk sample to obtain a 10-ppb spike for the internal standard.

Extraction column conditioning

C_{18} columns were attached to the vacuum manifold using Luer stopcocks, washed with two 3-ml volumes of methanol and two 3-ml volumes of water; an additional volume of *ca.* 1.5 ml of water was placed above bed and a 30-ml Luer Lok syringe barrel was attached as a reservoir.

Preparation of anion-exchange resin

A 10-g amount of AG MP-1 was shaken (*ca.* 1 min) with 300 ml of 10% acetic acid in acetone, permitted to settle for 15 min and decanted; then shaken with 300 ml

of water, settled for 15 min and decanted and finally shaken with 300 ml of 2 *M* hydrochloric acid, settled for 5 min and decanted. After rinsing with water in a course-fritted funnel until the water was neutral, it was shaken for 1 h (using a mechanical shaker) with 300 ml of 0.2 *M* K_2HPO_2 (pH 7.9) buffer, filtered through a course-fritted funnel, washed with water until the water was neutral and dried in the funnel (vacuum, 5 min). The 10 g of resin was stored refrigerated in 200 ml of ethanol–water (1:1) and 0.5 ml of suspension was used for column B.

Concentration column (column B)

The end of a 1-ml disposable pipet tip was plugged with a 70-μm porous polypropylene disc (2.5 mm disc punched from 1.59 mm sheet 70-μm Fritware®; BEL-ART, Pequannock, NJ, U.S.A.), then 0.5 ml of the anion-exchange resin suspension was added and permitted to drain to waste.

Clean-up column (column A)

A Quik-Snap column was filled to the reservoir with methanol, then 0.50 ± 0.02 g acidic alumina was slowly poured into the column. A bed of course sand (*ca.* 5 mm) was placed on top of the alumina after it had settled. The bottom closure was then snapped off and the column was placed above the concentration column (column B, see Fig. 1) letting methanol drain through column B to waste.

Sample preparation

Pasteurized/homogenized milk. Milk was permitted to warm to room temperature. Then 10 ml were pipetted into a 50-ml polypropylene centrifuge tube. A 100-μl volume of the internal standard solution was added and the mixture vortex-mixed for 10 s. For fortified samples, 100 μl of appropriate working solution were also added. After the mix was vortex-mixed, the mixture was left at room temperature for at least 15 min before proceeding. After 15 min, 10 ml of 0.2 *M* pH 5.7 phosphate buffer were added and the mixture was again vortex-mixed for 15 s.

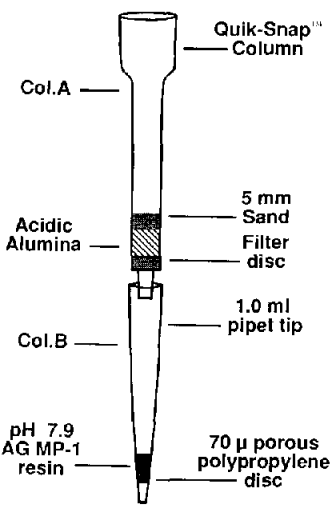

Fig. 1. Tandem column arrangement used in clean-up and isolation of sulfonamides.

Raw milk. Raw milk was heated to 60°C for 5 min, and then passed through a Logeman mulsifier (Thomas Scientific, Swedesboro, NJ, U.S.A.) twice to homogenize. Milk was cooled to room temperature and treated as pasteurized/homogenized milk.

Raw goat milk. Raw goat milk was treated as pasteurized/homogenized milk.

Extraction and isolation

The diluted milk was poured into the reservoir on the extraction column, and a vacuum was applied to obtain a 2–4 ml/min flow-rate. (The stopcock was used to control the flow-rate and to prevent the column bed from drying.) The flow was stopped when the reservoir emptied. The sample tube was rinsed with 10 ml of water, poured into a reservoir and a vacuum was resumed. The reservoir was discarded after it emptied and the extraction column was washed with an additional 3 ml of water. The column was then washed with 3 ml of hexane and dried by vacuum (20–25 in.Hg) for 10 min. The column was eluted with methanol (3 × 0.5 ml + 1 × 1.0 ml) and the eluate was collected in disposable culture tubes (10 × 75 mm).

The eluate was transferred to column. A with a pasteur pipet and permitted to flow by gravity through a tandem column set-up (Fig. 1). After the eluate had passed through Column B, the culture tube and pipet were washed twice with 1.0 ml of methanol and the washes passed through the columns. The walls of column. A were then washed with 1.0 ml of methanol. After draining, column A was discarded and the walls of column B were washed with 1.0 ml of methanol and drained.

Thin-layer chromatography. The sulfa drugs were eluted from column B into 5-ml Reacti-Vials (Pierce, Rockford, IL, U.S.A.) using 0.5 ml of methanol–acetic acid–acetone (1:5:94). The eluate was vortex-mixed for 10 s, and 50 μl were then applied to the plate as described below.

High-performance liquid chromatographic (HPLC) confirmation (optional). The sulfa drugs were eluted from column B into 1.0-ml volumetric flasks with 1 ml of 1 *M* sodium acetate (pH 6.0). The volume was adjusted to mark, mixed, and 20 μl were injected into the system described below.

Thin-layer chromatography

Ascending, one-dimensional development in a twin trough chamber of 10 × 10 cm (Camag, Muttenz, Switzerland) with chamber saturation for 10 min was used. HPTLC plates (10 × 10 cm) precoated with Silica Gel 60 were obtained from Merck (Darmstadt, F.R.G.). The plates were first washed by immersing in methanol for 5 min and then dryed at 80°C for 30 min. Nitrogen was used to spray 50 μl of sample onto the plate in 7-mm bands at a rate of 10 s/μl using a Linomat IV applicator (Camag). Bands were applied 10 mm from the plate bottom, with 3 mm of separation and 10 mm of edge space. The solvent was ethyl acetate–toluene (1:1); 10 ml were split evenly between the troughs. Running time and distance were 11 min and 63.0 ± 0.4 mm from plate bottom, respectively. To allow detection, the dried chromatogram (5 min under a flow of nitrogen at room temperature) was mechanically dipped (Camag Immersion Device II) at low speed for 1 s in 100 ml of a fluorescamine solution (25 mg in 10 ml acetone to which 90 ml hexane were added). The dipped plate was dried with nitrogen for 5 min, then sprayed with 0.2 *M* H_3BO_3 (adjust pH to 8.0 with 1 *M* sodium hydroxide) and scanned. For densitometry lanes were scanned with a 0.025 ×

5 mm band at 0.5 mm/s in the fluorescence mode using a Camag TLC Scanner II. Excitation was at 366 nm (Hg lamp) and emission was measured after a 400-nm cut-off filter. The densitogram was recorded, and peak heights were measured on a Camag SP4290 integrator.

HPLC for confirmation (optional)

Reversed-phase HPLC with isocratic elution was carried out using an LC-18 column (Supelco, Bellefonte, PA, U.S.A.) 25 xm × 4.6 mm I.D., 5-μm packing. The mobile phase was 0.05 *M* K_2HPO_4 (pH 6.0)–methanol (65:35) mixed by a Hewlett Packard Series 1050 pumping system (Hewlett Packard, Avondale, PA, U.S.A.). The flow-rate was 1.0 ml/min. Detection was achieved by measuring the absorbance at 270 nm with a Kratos Spectroflow 773 detector. Chromatograms were recorded by a Hewlett Packard 3396A integrator. The injector was a Rheodyne 7125 (Cotati, CA, U.S.A.) 20-μl sample loop (overfill loop).

RESULTS AND DISCUSSION

Extraction

The solid-phase extraction, clean-up and concentration procedure is a rapid, low-solvent-consumption method. The initial extraction of milk using the C_{18} column successfully eliminates the use of chloroform in the reported HPLC procedure [9]. The retention of the sulfa drugs on the C_{18} column is enhanced by adjusting the pH of the milk samples to *ca.* 5.9 using the pH 5.7 phosphate buffer. The phosphate buffer also acts to dilute the milk, aiding the flow of the sample through the C_{18} column. Raw cow milk does not flow freely through the C_{18} column. We believe this is due mainly to the large size of the fat globules in bovine milk, as there are no flow difficulties encountered when non-homogenized raw goat milk (which has smaller and more uniformly sized fat globules) is analyzed. Raw cow milk must therefore be homogenized prior to analysis. Milk which is homogenized as we have described here is not as stable as industrially processed milk and will separate in one or two days; therefore, we recommend analyzing the milk on the same day it is homogenized.

After the analytes are on the C_{18} SPE column, we found that SMZ and SBZ are best eluted using methanol. However this eluate contains lipids and riboflavin (in addition to other compounds) which interfere with the subsequent chromatographic analysis and fluorescent quantification. Therefore, a further clean-up is necessary. Alumina and AG MP-1 have been used in a tandem column arrangement to clean up and isolate SMZ from organic feed extracts [13,14] and sulfathiazole from honey [15,16]. These methods were optimized to isolate (on the basis of pK_a) only the sulfa drug in question. We modified the clean-up method for SMZ in feeds by eliminating the pH 5.7 AG MP-1 resin from column A in the tandem column set-up [14] (Fig. 1). This leaves the aluminum oxide (column A) which removes the interfering fluorescent riboflavin and any free fatty acids from the extract, but permits any sulfa drugs present to pass through column A so they may be concentrated on the pH 7.9 AG MP-1 resin (column B). The SBZ and SMZ can then be eluted from the AG MP-1 resin (column B) using the volatile acidic acetone solvent mixture and easily applied to the TLC plate without any further steps.

Thin-layer chromatography

Fig. 2 shows examples of fluorescamine-visualized TLC densitograms from the analysis of a control milk and milk fortified with SMZ at 0.5, 5.1 and 10.2 ppb and SBZ at 10 ppb each. The identity of the material at the origin and at R_F 0.18 (contained in both control and fortified samples) is unknown. This pattern has appeared in all milk samples analyzed to date. However, the densitogram of the control milk is free of interferences where both SMZ and SBZ migrate. SBZ was chosen as an internal standard because of SBZ's close structural similarity to SMZ and because SBZ has a low probability for abuse (Merck Sharp & Dohme, SBZ's only U.S.A. sponsor, has not sold nor commercially manufactured SBZ for over ten years). SBZ proved to be suitable as an internal standard because (1) SBZ is completely resolved from SMZ (Fig. 2) and (2) a plot of the ratio of peak heights *vs.* SMZ concentration (ng/ml) is linear ($R^2 = 0.999$, $p < 0.01$) over the desired range of analysis (0.5–15 ppb). These facts qualify SBZ as an acceptable internal standard, which allows correction for both the recovery and its variability.

Table 1 lists the results from the TLC analysis of fortified sulfonamide-free milk samples analyzed each day for six days in the 0.5–15 ppb range. Three separate milk

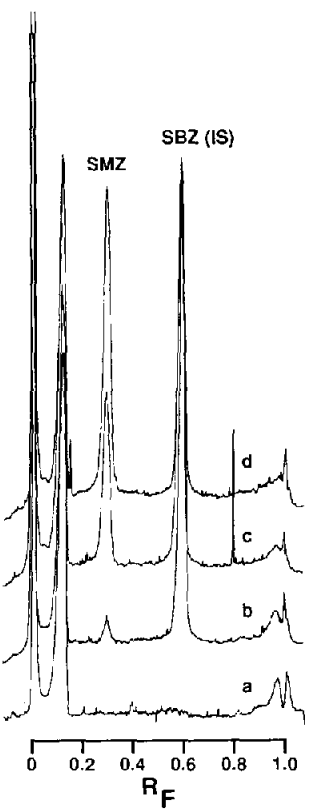

Fig. 2. TLC fluorescence densitograms of SMZ-fortified milk samples. (a) Control milk; (b) 0.5 ppb; (c) 5.1 ppb; (d) 10.2 ppb. Conditions as described in text.

TABLE I

TLC QUANTITATION OF SULFAMETHAZINE IN FORTIFIED MILK SAMPLES

SMZ added (ng/ml)	SMZ found (mean ± S.D.)[a] (ng/ml)	Recovery[b] (%)	Coefficient of variation (%)
0.51	0.46 ± 0.07	90.61	14.95
1.02	0.90 ± 0.12	88.36	13.05
2.56	2.27 ± 0.15	88.73	6.43
5.11	5.02 ± 0.57	98.24	11.39
6.13	6.33 ± 0.54	103.15	8.48
7.67	7.71 ± 1.28	100.47	16.58
10.22	9.95 ± 1.76	97.33	17.69
12.27	11.88 ± 1.48	96.86	12.45
15.34	15.48 ± 1.81	100.91	11.66

[a] One determination at each level for six days.
[b] Relative recovery based on SMZ found.

samples fortified at 0, 7.5 and 15 ppb SMZ were used to calculate the calibration curve. The average correlation coefficient (r) for the calibration lines was 0.999 (n = 12, coefficient of variation = 0.11%). The average recovery of SMZ (SMZ found/SMZ added × 100) was 96.07% showing the high accuracy of the method; the average coefficient of variation was 12.52% showing an acceptable day-to-day precision at this level of analysis.

Table II lists TLC quantitative results from the within-day triplicate analysis on two incurred milk samples obtained from a local market. The two market milk samples were first identified as containing SMZ residues by another group in our laboratory evaluating immunochemical screening assays [7]. Sulfonamide-free milk fortified at 0, 7.5 and 15 ppb was used for calibration. The coefficients of variation (8.08 and 6.46%) for these two samples show the excellent within-day precision of the TLC method even at the low 1.99 ppb level found.

Confirmation by HPLC (optional)

Further confidence in analytical methods is often desired, especially in the method development stages. Mass spectral confirmation would be best. However, mass spectral capabilities are not always available. The method to detect SMZ in

TABLE II

TLC QUANTITATION OF SULFAMETHAZINE IN INCURRED MILK SAMPLES

Homogenized milk was purchased at a local market.

Sample No.	Fat content (%)	SMZ found (mean ± S.D., n = 3)[a] (ng/ml)	Coefficient of variation (%)
1	2	1.99 ± 0.16	8.08
2	4	6.57 ± 0.43	6.46

[a] Same-day analysis.

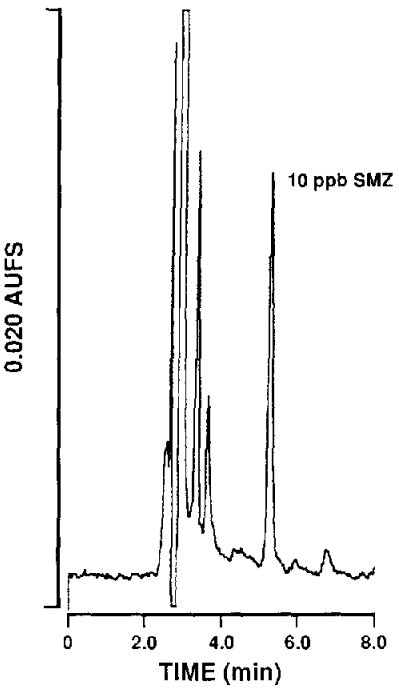

Fig. 3. HPLC (UV detector 270 nm) of a milk sample fortified with 10 ppb SMZ. Conditions as described in text.

feeds suggested a micromethod for making the N^1-methyl derivative followed by TLC [14] as a chemical/chromatographic means of confirmation. But a recent report has determined that the micromethod used results in two products (the N^1-methyl and varying amounts of a pyrimidine ring methylated isomer) [17], thus making N^1-methylation using diazomethane unreliable.

Without mass spectral capabilities and because of the difficulties reported for the micro derivatization we chose reversed-phase HPLC as an alternative means to confirm the presence fo SMZ detected by TLC. Except for the solvent used to elute SMZ and SBZ from the ion-exchange resin (column B), the extraction procedure for HPLC is the same as for TLC. A different eluting solvent is necessary for HPLC in order to eliminate chromatographic peak distortions. Fig. 3 shows an example of a market milk sample which was fortified to 10 ppb SMZ. HPLC analysis of control milks were free of interferences in the area of SMZ and SBZ elution.

CONCLUSION

The use of solid phases to extract, clean up and concentrate SMZ and SBZ results in an easy, low-solvent-consuming isolation procedure. When this isolating procedure is combined with instrumental TLC for identification and quantitation, the result is a method which uses an extremely low amount of solvent (< 20 ml total, of organic solvents per sample) and which is also highly sensitive, accurate and precise.

Using solid phases combined with TLC also results in a rapid method. For this work one analyst easily completed twelve samples: three calibration standards and nine unknowns. This value was dictated by the twelve-port vacuum manifold and the use of 10 × 10 cm HPTLC plates. If a twenty-four-port manifold and 10 × 20 cm plates were used, then one analyst could probably complete sixteen samples in a day: three calibration standards and thirteen unknowns.

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