High-Pressure Liquid Chromatographic Separation, Identification, and Determination of Sulfa Drugs and Their Metabolites in Urine

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Abstract □ A high-pressure liquid chromatographic method for the separation and quantitative determination of sulfamethazine, sulfamerazine, sulfathiazole, and their N^4-acetylated metabolites on an amino-bonded reversed-phase column was developed. The method is suitable for the analysis of these compounds in pure solutions as well as in cattle urine. Retention times were reproducible. Injection volumes containing 0.2 μg of individual sulfonamides or their acetyl derivatives were successfully quantitated; coefficients of variation ranged from 0 to 0.073 for individual sulfonamides.

Keyphrases □ Sulfamethazine—and metabolites, high-pressure liquid chromatographic analysis, pure solutions and cattle urine □ Sulfamerazine—and metabolites, high-pressure liquid chromatographic analysis, pure solutions and cattle urine □ Sulfathiazole—and metabolites, high-pressure liquid chromatographic analysis, pure solutions and cattle urine □ High-pressure liquid chromatography—analysis, sulfamethazine, sulfamerazine, sulfathiazole, and acetyl derivatives, pure solutions and cattle urine □ Antibacterial agents—sulfamethazine, sulfamerazine, and sulfathiazole, high-pressure liquid chromatographic analysis, pure solutions and cattle urine

Solutions containing a mixture of three sulfonamides are commonly administered to food-producing animals for the treatment of bacterial diseases. In some instances, treated animals may be slaughtered before the concentrations of sulfonamides have declined to predetermined tolerance limits (≤0.1 ppm) in edible tissues. Animal carcasses containing sulfonamide concentrations in excess of 0.1 ppm are destroyed (1).

The disappearance of a drug from edible animal tissues is a function of time (2). Carcass destruction can be avoided if animals containing excessive tissue concentrations of sulfonamides can be identified and withheld from slaughter. Preslaughter sampling and analysis of animal plasma and urine could be used to identify these animals if a meaningful correlation exists between the concentrations of drug in plasma, urine, and tissues.

A rapid, accurate method for the separation and quantitation of sulfonamides and their metabolites in animal urine was requisite to a projected urine–plasma–tissue study. Previous work (3–5) indicated that the parent sulfonamides and the N^4-acetylated derivatives were major urinary excretion products of sulfonamides.

Previously reported quantitative methods based on TLC or colorimetry (6–12) did not distinguish between parent drugs and/or metabolites. These methods were intended for the analysis of the parent drug in pure solutions and extracts of pharmaceutical preparations and animal feeds. No provisions were included to eliminate possible interference due to biological components in urine.

GLC methods (13–15) were unsuited for this purpose because the derivatization, extraction, and isolation procedures were too exhaustive for routine sample analysis.

High-pressure liquid chromatography (HPLC) can be used to separate nonpolar, polar, and ionized materials. Kram (16) surveyed the conditions required for the separation of pure sulfonamide samples on an anion-exchange column. Poet and Pu (17) described the separation and determination of trisulfapyrimidines in pharmaceutical dosage forms by HPLC. No reports concerning the separation and determination of sulfonamides and their metabolites in pure solutions or biological fluids have appeared.

This paper describes an HPLC method for the separation and quantitative determination of sulfamethazine, sulfamerazine, sulfathiazole (a common triple sulfa preparation), and their N^4-acetylated metabolites in pure solutions and urine.

EXPERIMENTAL

Apparatus—A liquid chromatograph[1] equipped with a digitally controlled dual pumping system, having a 3000-psi pressure limit, was used. Prepacked columns (2.6 mm i.d. and 0.5 m in length) containing amino-bonded phase material[2] were used to effect separations.

A single-beam spectrophotometer[3] equipped with an 8-μl flowcell and digital readout was used for the UV detection (254 nm) of separated components. The detector output was recorded on a variable-span potentiometric recorder[4].

All separations were effected at ambient temperature.

Reagents and Materials—The mobile phase was methanol, spectroscopic grade[5]. A 0.1 *N* sodium hydroxide solution in distilled water was prepared. USP sulfamethazine, sulfamerazine[6], and sulfathiazole[7] were used as received.

Acetyl derivatives of sulfonamides were prepared by acetylation with acetic anhydride, precipitation with water, and recrystallization in dioxane–water (1:1 v/v). Purity of dried samples was checked by TLC and HPLC.

Sulfonamide Stock Solutions—From 200 to 250 mg of each of the three sulfonamides and their acetylated products was weighed and transferred to a clean 100-ml volumetric flask, and distilled water (5 ml) was added. The sulfonamides were solubilized by dropwise addition of 0.1 *N* sodium hydroxide and constant shaking, followed by dilution to volume with distilled water.

Working Standards—Aliquots (1–7 ml) of the stock solution were transferred to 25-ml volumetric flasks and diluted to volume with distilled water. Five microliters of each solution was injected onto the column.

Urine Standards—Six aliquots (2–12 ml) of the stock solution were transferred to 25-ml volumetric flasks and diluted to volume with cattle urine. Aliquots (2 ml) of each dilution were pipetted into 20-ml screw-capped glass tubes containing 8 ml of methanol. Each tube was centrifuged for 5 min at 2000 rpm to remove the gelatinous precipitate

[1] Model 1220, Perkin-Elmer Corp., Norwalk, Conn.
[2] Amino-Sil-X-I, Perkin-Elmer Corp., Norwalk, Conn.
[3] Model LC-55, Perkin-Elmer Corp., Norwalk, Conn.
[4] Linear Instruments Corp., Irvine, Calif.
[5] Burdick and Jackson Laboratories, Muskegan, Mich.
[6] American Cyanamid Co., Pearl River, N.Y.
[7] Merck and Co., Rahway, N.J.

Table I—Retention Times and Standard Curve Parameters for Sulfa Drugs and Their Metabolites in Pure Sample and Urine

Compound	Solution Injected	Retention Time, min	Slope (Response Factor), Absorbance/μg	Intercept, Absorbance Unit	Correlation Coefficient of Standard Curves	*SE* of Estimate	Range of Sample Sizes Used, μg
Sulfamethazine	Sample	2.3	0.0462	−0.0001	0.999	0.0004	0.20–1.65
	Urine	2.6	0.0537	−0.0015	0.998	0.0008	0.14–0.86
Sulfamerazine	Sample	3.6	0.0319	+0.0005	0.999	0.0006	0.2–2.13
	Urine	3.8	0.0310	+0.00002	0.999	0.0002	0.14–0.88
Sulfamethazine acetyl derivative	Sample	5.7	0.0311	−0.0001	0.999	0.0006	0.33–1.67
	Urine	5.8	0.0280	−0.00005	0.999	0.0003	0.18–1.09
Sulfathiazole	Sample	8.1	0.0188	+0.0007	0.999	0.0003	0.40–2.02
	Urine	9.0	0.0163	−0.0001	0.999	0.0002	0.19–1.16
Sulfamerazine acetyl derivative	Sample	11.0	0.0166	+0.0015	0.998	0.0007	0.53–2.14
	Urine	11.0	0.0216	−0.0007	0.999	0.0003	0.16–0.99
Sulfathiazole acetyl derivative	Sample	13.8	0.0153	+0.0002	0.999	0.0004	0.39–1.94
	Urine	15.6	0.0108	+0.0006	0.993	0.0004	0.14–1.15

which formed. The clear supernate (5 μl) was injected onto the column. Unspiked control urine was treated in an identical manner, except that no stock sulfonamide solution was added.

Chromatographic Procedures—Columns[2] were preconditioned with methanol until a stable recorder output was obtained. A flow rate of 1.5 ml of methanol/min was established at a pressure of about 700 psi. Sample aliquots were injected onto the column under stop-flow conditions. Column eluants were monitored at 254 nm. As the peak maxima emerged, the peak absorbance of each component was recorded from the digital spectrophotometer readout as well as on a potentiometric recorder.

RESULTS AND DISCUSSION

Solutions of pure sulfonamides were completely separated with a reversed-phase column packing[2] (Fig. 1).

Raw urine containing sulfonamides and their acetyl derivatives could not be injected directly onto the column packing, since operating pressures increased with each successive injection. This result may be explained by precipitation of urine components onto the column packing in the presence of methanol and other organic solvents. The problem was circumvented by pretreating urine samples with methanol. Following the addition of methanol to urine samples, a gelatinous precipitate was formed; it was subsequently removed by centrifugation. Separation of all sulfonamides in the supernate was then obtained without the attendant problems resulting from the injection of samples containing raw urine.

The separations obtained after the injection of methanol-pretreated urine containing sulfonamides and metabolites as well as a pretreated urine blank are included in Fig. 2. The effect of various flow rates between 1.0 and 1.5 ml/min was investigated to improve the resolution. Although slight improvements in peak resolution were noted at lower flow rates, a rate of 1.5 ml/min was selected to obtain maximum resolution in the shortest possible time.

The separation patterns of sulfonamides following their injection in distilled water or urine were similar but not identical. Although the relative order of elution was maintained, the retention times of certain

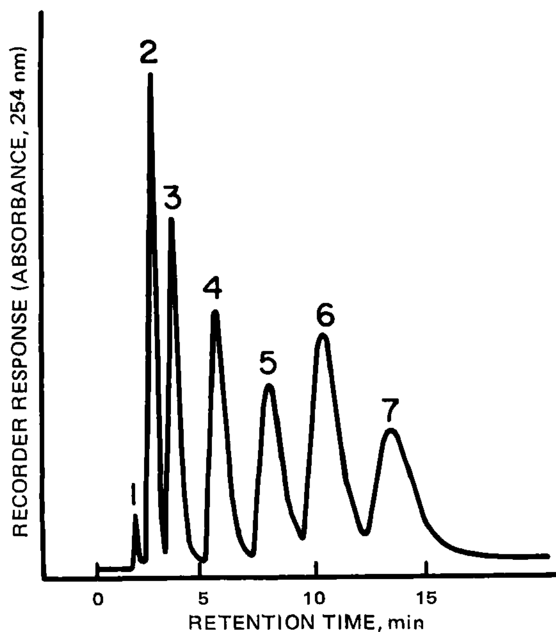

Figure 1—*Separation of sulfa drugs and their acetylated derivatives. Key: 1, solvent front; 2, sulfamethazine; 3, sulfamerazine; 4, sulfamethazine acetyl derivative; 5, sulfathiazole; 6, sulfamerazine acetyl derivative; and 7, sulfathiazole acetyl derivative (7.5 μg total sample).*

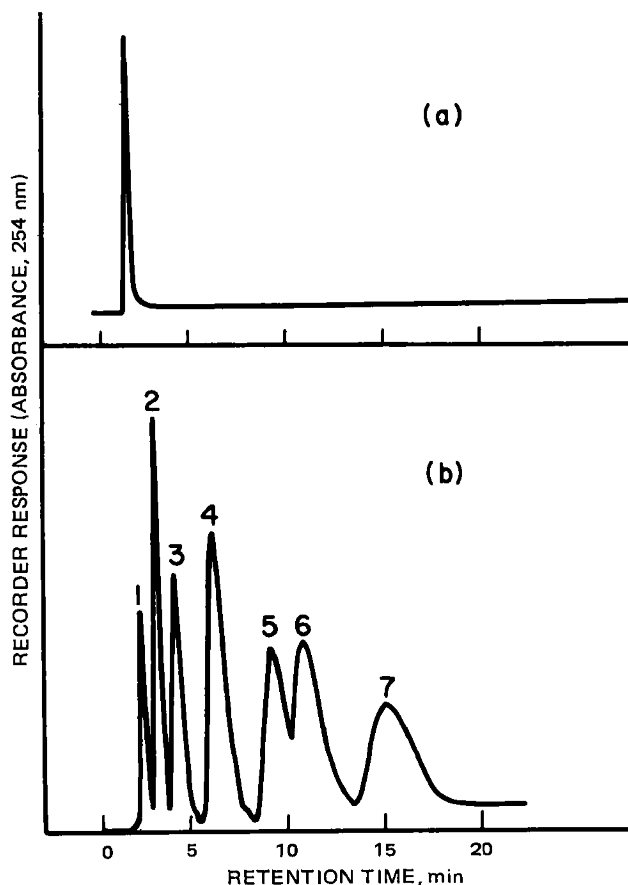

Figure 2—*Separation of sulfa drugs and their metabolites in urine. (a) Blank, urine treated with methanol showing solvent front. (b) Key: 1, solvent front; 2, sulfamethazine; 3, sulfamerazine; 4, sulfamethazine acetyl derivative; 5, sulfathiazole; 6, sulfamerazine acetyl derivative; and 7, sulfathiazone acetyl derivative (3.6 μg total sample).*

Table II—Determination of Sulfa Drugs and Their Metabolites in Pure Samples and Urine

Compound	Amount in Pure Samples, μg			Amount in Urine, μg		
	Added[a]	Recovered[a,b]	SD	Added[a]	Recovered[a,b]	SD
Sulfamethazine	0.83	0.86	±0.012	0.29	0.29	±0.011
	1.65	1.65	±0.012	0.58	0.55	±0.000
Sulfamerazine	0.85	0.87	±0.018	0.30	0.32	±0.000
	1.71	1.72	±0.018	0.59	0.63	±0.019
Sulfamethazine acetyl derivative	0.67	0.67	±0.019	0.37	0.38	±0.021
	1.34	1.31	±0.018	0.75	0.81	±0.021
Sulfathiazole	0.81	0.80	±0.031	0.39	0.41	±0.035
	1.62	1.63	±0.031	0.78	0.82	±0.035
Sulfamerazine acetyl derivative	1.07	1.13	±0.035	0.33	0.37	±0.000
	2.14	2.10	±0.035	0.67	0.71	±0.027
Sulfathiazole acetyl derivative	0.78	0.79	±0.038	0.38	0.43	±0.054
	1.55	1.53	±0.038	0.77	0.75	±0.055

[a] Values reported are correct up to second decimal place. [b] Recovered amounts are averages of triplicate injections of the same amount.

peaks were altered. When urine supernates were injected, the sulfathiazole and acetyl derivative of sulfamerazine peaks merged to some degree and the overall elution time increased slightly (2 min) when compared with that obtained after the injection of sulfonamides in distilled water. Differences in the separation patterns of the sulfonamides in water and urine may have been due to the presence of nonabsorbing inorganic ions in urine and their influence on column characteristics. However, the peak maxima of all components remained well separated.

Mean retention times (T_R) obtained following the injection of identical volumes (5 μl) of sulfonamide mixtures in water and urine are presented in Table I. The retention times were highly reproducible under the experimental conditions previously described.

Slight changes in retention times (and hence peak heights) were observed when the sample volume injected was gradually increased. These changes may be due to the gradual accumulation of inorganic ions on the columns. This problem was minimized by the injection of small sample volumes.

Column regeneration was required after 15–30 urine samples were injected and was effected by flushing the column for 1 hr with methanol–water (98:2 v/v) at a flow of 1.5 ml/min. However, water itself produced changes in column characteristics, as denoted by a complete merging of some peaks when samples were injected immediately after the flushing step. To reduce the water content of the column and provide complete column regeneration, pure methanol was pumped through the column for 2 hr at a rate of 1.5 ml/min.

Peak heights were proportional to the concentration of individual components and were used for their quantitative determination. Peak heights were measured in terms of the absorbance value obtained at the peak maxima of each component. A recent report (18) indicated that the use of peak heights for quantitative determinations gives the same degree of precision and accuracy as may be obtained with electronically measured peak areas.

Standard curves were calculated by linear regression analysis of the peak heights obtained for each component following the injection of sulfonamide mixtures in water and urine. The parameters for these curves are presented in Table I. Ideally, the straight lines should have a zero intercept; in most cases, the intercept values listed in Table I approached zero.

The high correlation of individual results to the line of best fit and the small standard deviation among data points were indicative of the accuracy and precision of the method.

The recovery of individual components following their injection in water and urine is reported in Table II. Recoveries were calculated from the equation for a straight line using slopes (response factors) and intercepts presented in Table I.

CONCLUSION

The present work demonstrates the capability of an amino-bonded reversed-phase packing to separate sulfonamides and their metabolites. The present procedure is suitable for routine analysis of cattle urine for the compounds mentioned and also may serve as a method for screening urine samples for sulfonamides and their metabolites.

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