High-Pressure Liquid Chromatographic Determination of Sulfamethazine Residue in Bovine Tissue

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Abstract □ A sensitive high-pressure liquid chromatographic assay procedure is described for residues of sulfamethazine in bovine kidney, liver, muscle, and fat tissue at a sensitivity of 0.04 ppm. Studies were conducted using sulfamethazine-negative bovine tissue to ensure that the peak eluting at the indicated retention volume was sulfamethazine and that the recoveries from spiked tissues were consistent. Data are presented showing the levels of sulfamethazine in the tissue of 12 treated animals found by this technique. The method is more specific than the frequently used colorimetric methods and does not require the tedious separations involved in TLC assays.

Keyphrases □ Sulfamethazine—residues, high-pressure liquid chromatographic determination, bovine tissue □ High-pressure liquid chromatography—residue analysis, sulfamethazine, bovine tissue

Sulfamethazine is frequently used in intravenous injections, oral solutions, tablets, and bolus forms for the treatment of bovine diseases. These veterinary preparations are utilized for mastitis, metritis, calf pneumonia, calf diphtheria, and calf scours (1) caused by pathogens sensitive to sulfamethazine.

Previous analytical methods relied upon diazotization of the aryl amine followed by either a potentiometric titration (2) or a colorimetric assay (3). These methods suffer from a lack of specificity since they react with all aryl amines and, to some extent, with primary and secondary alkyl amines (4) to yield diazotization products and colored addition compounds which interfere with the assay. More recently, TLC methods (2) have been used to separate sulfonamides and their various degradation products but such methods are tedious to perform.

An alternative approach was sought which would allow for the separation from bovine tissue and the quantitation of residues of sulfamethazine remaining in bovine tissue after cessation of treatment. The method outlined in this paper separates sulfamethazine from other tissue components; therefore, it is more specific than existing colorimetric assays. Furthermore, it is sensitive enough to detect levels as low as 0.04 ppm of sulfamethazine present in the tissue. The technique is more specific than most existing assays but does not suffer from decreased sensitivity.

EXPERIMENTAL

Animals—Twelve calves[1] were divided into four groups of three each and administered sulfamethazine sodium, 214.5 mg/kg po for 1 day followed by 107.2 mg/kg/day for 4 days. Group I was slaughtered at 0–6 hr, Group II at 5 days, Group III at 8 days, and Group IV at 10 days posttreatment. Samples of muscle, fat, liver, and kidney were taken for assay purposes.

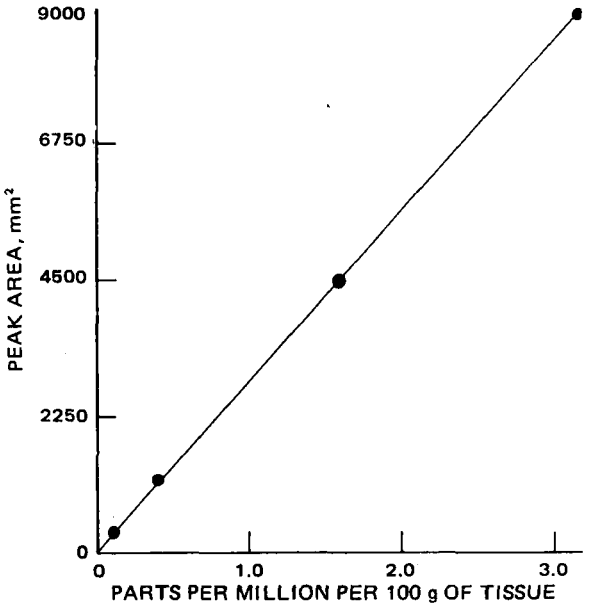

Figure 1—*Calibration curve prepared with sulfamethazine standards (see text).*

Materials—All reagents were ACS or analytical reagent grade, except the *n*-hexane[2] which was technical grade. The *n*-hexane and chloroform were freshly distilled from an all-glass system; the first and last 100 ml from each 2-liter distillation were discarded.

Liquid Chromatography—A 50-μl aliquot of solution was injected by syringe[3] into a high-speed liquid chromatograph[4] equipped with a 254-nm UV detector. The detector signal was fed to a 2-mv strip-chart recorder[5] operating at a chart speed of 0.5 cm/min. A 0.61-m (2-ft) × 2-mm i.d. column, packed with a permanently bonded octadecyl–porous silica[6] material, was purged for at least 2 hr with the mobile phase prior to use. All purging and assay work was done at a constant flow of 0.6 ml/min.

Mobile Phase—The mobile phase, pH 7.70 buffer–2.5% (v/v) isopropyl alcohol, was prepared as follows. Potassium phosphate monobasic crystals, 6.89 g, and 8.79 g of potassium phosphate dibasic (anhydrous) were dissolved in 800 ml of distilled water. The pH was adjusted to 7.70 with 1.0 *M* sodium hydroxide. The solution was transferred to a 1-liter volumetric flask containing 25 ml of isopropyl alcohol and diluted to volume with distilled water.

This solution was poured into a 2-liter round-bottom flask and refluxed for 45 min under a 400-mm Allihn-type water-cooled condenser. The solution was allowed to cool to room temperature with the condenser attached and then was poured gently into the reservoir of the liquid chromatograph. The reservoir was designed to minimize the evaporation of isopropyl alcohol.

Standards—Sulfamethazine USP, 100 mg, was accurately weighed into a 100-ml volumetric flask, dissolved in 0.1 *M* hydrochloric acid, diluted to volume, and mixed. Aliquots of 1.0, 4.0, 16.0, and 32.0 ml were transferred into separate 100-ml volumetric flasks and diluted to volume with distilled water. (These solutions

[1] All animals were located at the Philips Roxane Clinical Research Farm, Cosby, Mo., under identical conditions and were fed antibiotic- and sulfonamide-free rations.

[2] Mallinckrodt Chemical Co., St. Louis, Mo.

[3] Precision Sampling model B-110, Precision Sampling Corp., Baton Rouge, La.

[4] Waters Associates ALC-202, Waters Associates, Inc., Milford, Mass.

[5] Linear Instruments model 252, Linear Instruments Corp., Irvine, Calif.

[6] Porasil B/C_{18} Bondapak, 37–50 μm, Waters Associates, Milford, Mass.

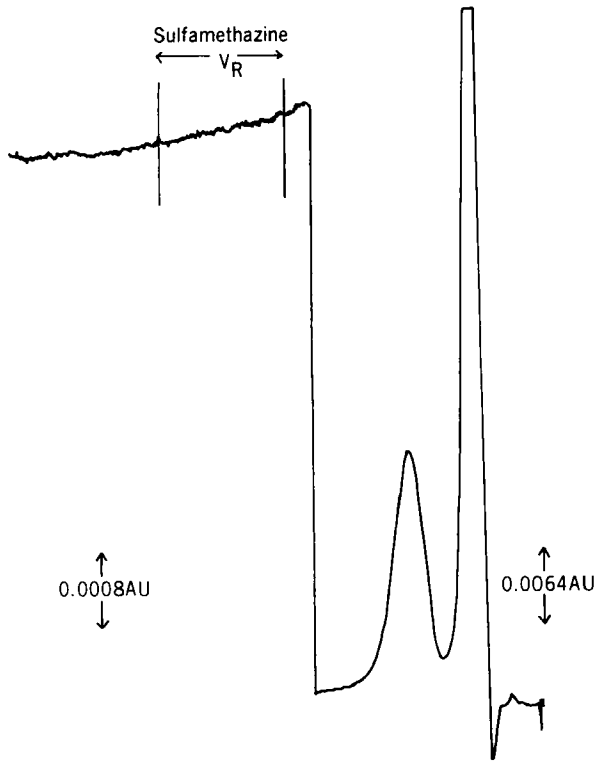

Figure 2—*Chromatogram of control liver extract.*

corresponded to 0.01, 0.04, 0.16, and 0.32 mg/ml of sulfamethazine, respectively, and were retained to prepare recovery samples.)

One-milliliter aliquots of each of the four standards were transferred into 50-ml glass-stoppered graduated cylinders, the volumes were adjusted to 30 ml with 1 *M* hydrochloric acid, and the solutions were mixed. (These solutions contained 10, 40, 160, and 320 μg and corresponded to 0.1, 0.4, 1.6, and 3.2 ppm of sulfamethazine

Table I—Sulfamethazine Residue Results

Animal Number	Slaughter Time Post-treatment	Sulfamethazine Residue, ppm			
		Fat	Muscle	Kidney	Liver
132	0–6 hr	325	54.1	88.6	42.5
177	0–6 hr	150	45.3	62.4	28.0
178	0–6 hr	115	42.1	95.6	33.2
138	5 days	0.06	<0.04	<0.04	0.04
168	5 days	0.27	<0.04	0.08	0.09
169	5 days	0.36	0.17	0.37	0.31
174	8 days	0.04	<0.04	<0.04	<0.04
175	8 days	0.06	<0.04	<0.04	<0.04
176	8 days	0.12	<0.04	<0.04	<0.04
170	10 days	0.06	<0.04	<0.04	<0.04
171	10 days	<0.04	<0.04	<0.04	<0.04
172	10 days	0.06	<0.04	<0.04	<0.04

present, respectively, in a 30-ml extract of 100 g of tissue.) Fifty microliters of each of the last four standards was injected into the liquid chromatograph, and the area of the peak eluting at a retention volume (V_r) of 12 ml was determined.

Since the concentration ranges were so great, the detector attenuation was varied between 0.04 and 0.32 absorbance unit full scale (aufs). The equivalent area at 0.04 aufs was then calculated using:

$$\text{corrected area (0.04 aufs)} = \frac{\text{area sulfamethazine peak} \times \text{aufs used}}{0.04} \quad \text{(Eq. 1)}$$

A calibration curve was prepared (Fig. 1) by plotting the corrected area (0.04 aufs) against the concentration (parts per million) present in 100 g of tissue.

Procedure—The tissue was sliced in squares (2 × 2 × 1 mm thick), and 100 g was weighed into a wide mouth glass jar. Then 150 ml of acetone–chloroform (1:1 v/v) was added, and the mixture was shaken vigorously for 2 min and allowed to set for 15–20 min. The samples were again shaken vigorously for 2 min. The solvent was decanted into a funnel containing an 18.5-cm phase-separating

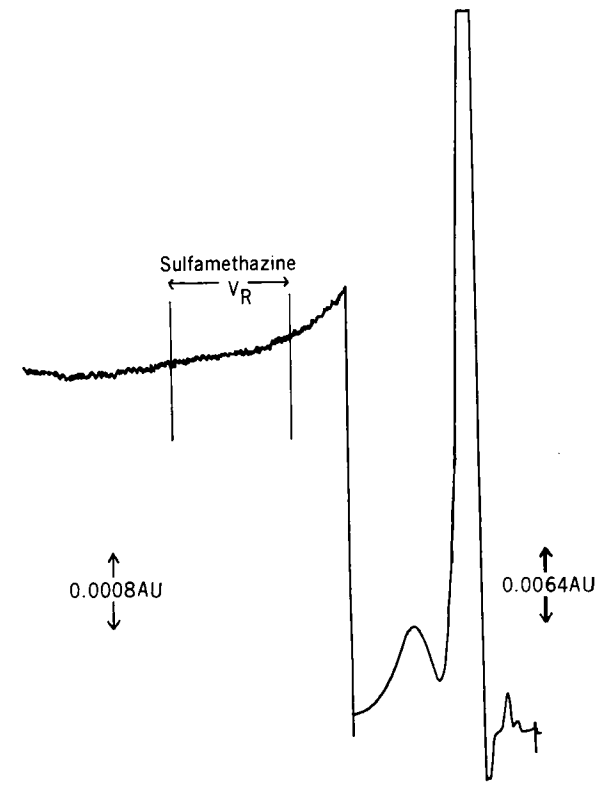

Figure 3—*Chromatogram of control kidney extract.*

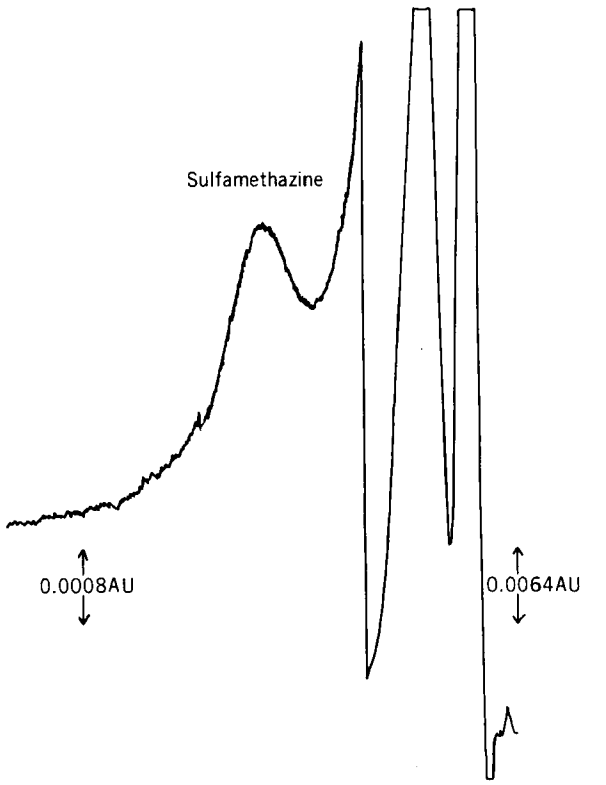

Figure 4—*Chromatogram of bovine liver containing 0.31 ppm of sulfamethazine.*

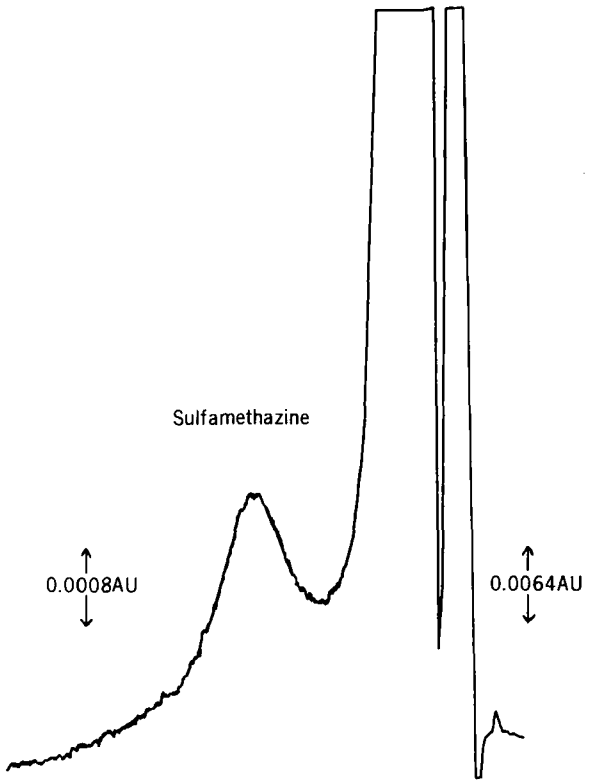

Figure 5—*Chromatogram of bovine kidney containing 0.37 ppm of sulfamethazine.*

paper[7], and the filtrate was collected in a 1-liter round-bottom flask. Two additional 2-min extractions of the tissue were made using 100-ml portions of acetone–chloroform (1:1) and filtering each extract. The filter was rinsed with 100 ml of the acetone–chloroform (1:1). (If water from the muscle tissue seeped through the filter, the filtrate was refiltered through fresh paper.)

The filtrate was evaporated to an oily residue, using a hot water bath at 60° and a rotary thin-film vacuum evaporator[8], and was transferred to a 250-ml separator. The following rinses were used: (*a*) 4 × 25 ml of *n*-hexane, (*b*) 1 × 3 ml of acetone, (*c*) 2 × 10 ml of 3.5 *M* hydrochloric acid (the flask was heated in hot water during this rinse), (*d*) 1 × 3 ml of acetone, and (*e*) 2 × 25 ml of *n*-hexane.

The mixture was shaken gently for 2 min. The emulsion, if present, was allowed to separate. The aqueous layer, including the emulsion, was drained into a 125-ml separator. The organic layer was washed with 3 × 10-ml portions of 1 *M* hydrochloric acid, shaking more vigorously on each successive wash, and the washes were combined in the 125-ml separator. The combined hydrochloric acid extracts were washed gently with 5 ml of chloroform, and the chloroform was discarded. The chloroform wash was repeated twice, shaking more vigorously on each succeeding wash.

The pH of the aqueous phase was adjusted to 8.5–9.0 using 14 *M* ammonium hydroxide. The ammoniacal solution was then washed with 3 × 5-ml portions of chloroform, and the washes were discarded. The aqueous phase was transferred to a 250-ml wide mouth erlenmeyer flask, and the separator was rinsed with a small amount of water. The solution was evaporated on a hot plate until the salts present began to precipitate. Then the mixture was cooled and transferred to a 50-ml glass-stoppered graduated cylinder using 1 *M* hydrochloric acid and diluted to 30 ml with 1 *M* hydrochloric acid. A portion was filtered through a 0.45-μm membrane filter[9] and transferred to a screw-capped test tube. Fifty microliters was injected into the liquid chromatograph under the same conditions as the standard calibration curve.

The corrected area (0.04 aufs) was determined using Eq. 1, and the amount of sulfamethazine present in the tissue was determined from the standard calibration curve. The results obtained from this study are shown in Table I. Representative chromatograms of actual tissue samples are shown in Figs. 2–5.

[7] Whatman PS-1 phase-separating paper.
[8] Rinco Inc.
[9] Nalge 245, Sybron Corp., Rochester, N.Y.

Table II—Sulfamethazine Recovery Data

Sample Number	Parts per Million Added	Peak Area	Corrected Area	Parts per Million Recovered	Percent
		Bovine Fat			
1	0.1	125	127	0.042	42
2	0.4	363	725	0.246	61
3	0.4	296	592	0.200	50
4	0.4	336	672	0.228	57
5	1.6	361	2886	0.978	61
6	1.6	433	3465	1.17	73
				Average recovery	57
		Bovine Muscle			
1	0.1	240	240	0.081	81
2	0.1	228	228	0.077	77
3	0.1	317	317	0.107	107
4	0.4	352	704	0.240	60
5	0.4	975	975	0.330	83
6	0.4	876	876	0.296	74
7	1.6	418	3344	1.14	71
8	1.6	415	3323	1.13	71
9	1.6	535	4282	1.45	91
				Average recovery	79
		Bovine Liver			
1	0.1	172	172	0.058	58
2	0.1	170	170	0.058	58
3	0.4	383	767	0.260	65
4	0.4	320	640	0.217	54
5	0.4	362	724	0.245	61
6	1.6	420	3360	1.14	71
7	1.6	432	3456	1.17	73
8	1.6	378	3024	1.02	64
9	1.6	387	3096	1.05	66
				Average recovery	63
		Bovine Kidney			
1	0.1	149	149	0.050	50
2	0.1	188	188	0.064	64
3	0.4	371	741	0.251	63
4	0.4	564	1128	0.382	96
5	1.6	383	3066	1.04	65
6	1.6	375	3000	1.02	64
7	1.6	337	2700	0.91	57
				Average recovery	66

Recoveries—Sulfamethazine-negative muscle, kidney, fat, and liver tissue were obtained from bovines with a negative antibiotic and sulfonamide treatment history for at least 60 days. Eight control samples (two of each tissue) were then assayed according to the procedure outlined here. Seven were found to be negative; one muscle control was positive but was calculated to be <0.02 ppm.

Six to nine samples of each kidney, fat, muscle, and liver tissue were individually "spiked" with the water dilutions of sulfamethazine prepared in the *Standards* section. Each 1 ml of the 0.01-, 0.04-, 0.16-, and 0.32-mg/ml standard added to 100 g of sample (1–2-mm slices) and mixed well corresponds to 0.1, 0.4, 1.6, and 3.2 ppm of sulfamethazine in the tissue, respectively. These recoveries were assayed according to the procedure, and results are shown in Table II.

DISCUSSION

Several publications (3, 5–7) reported the recoveries of sulfonamides utilizing the colorimetric assays, and all reported values of 75–86%. Only Houston and Umstead (7) reported on sulfamethazine, and these results were a combination of sulfamethazine–sulfathiazole residues in milk.

The major points critical to the sensitivity of this method are the success of the "cleanup" steps and the "noise level" of the liquid chromatograph.

The tissue component eluting just before the sulfamethazine must be reduced to a level so that its tail is not so steep that it ren-

ders quantitation of small sulfamethazine peaks impractical. The chloroform wash of the basic aqueous mixture seems to affect this component most. Overwashing at this point, however, causes a loss of sulfamethazine.

Homogenizing or grinding the tissue prior to extraction tends to improve recovery somewhat but usually generates cleanup difficulties which negate the improved recovery.

Techniques critical to reducing the noise level of the liquid chromatograph include the following:

1. The solvent should be degassed to minimize the effect of small bubbles which are not visible but give detector response. This procedure also helps prevent buildup of air in the pump head, which would result in pulsations.
2. All fittings should be very tight to eliminate even the smallest leaks.
3. Detector electronics and cell windows should be dry, and any air or gas used to purge the reference cell should be scrupulously clean and dry.
4. In some instances, an arrangement to equilibrate the solvent thermally to the temperature of the detector might be helpful.
5. The electrical line to which the chromatograph is connected must be free of interference and large fluctuations. Both the chromatograph and the recorder should be well grounded.
6. The final aqueous solution must be adjusted to pH 8.5–9.0 before evaporation to reduce the volume. If the solution is acidic at this point, the sulfamethazine can be hydrolyzed to sulfanilamide and sulfanilic acid which elute with tissue components.

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Comparative Pharmacokinetics of Coumarin Anticoagulants XIV: Relationship between Protein Binding, Distribution, and Elimination Kinetics of Warfarin in Rats

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Abstract □ The relationships between the protein binding, distribution in the body, and kinetics of elimination of warfarin were studied. Individual rats eliminated warfarin by apparent first-order kinetics, with a biological half-life of 5.9–41 hr and a total plasma clearance of 2.4–22 ml kg^{-1} hr^{-1}. There is a strong positive correlation between the apparent volume of distribution (V_d) and the elimination rate constant (k_{el}). There was no apparent concentration dependence of warfarin binding to serum proteins over a wide concentration range, but there were pronounced intersubject variations in protein binding, with the free fraction of drug (f) in serum ranging from 0.172×10^{-2} to 1.53×10^{-2}. There are strong positive correlations between f and k_{el}, f and V_d, and f and the kidney–serum concentration ratio of warfarin. Consistent with theory, there is an excellent positive linear correlation between f and total plasma clearance of the drug. The intersubject variation in f is not related to variations in serum albumin or total protein concentration. There is a strong correlation between values of f for serum and liver homogenate in individual animals, consistent with the lack of correlation between f in serum and the liver–serum concentration ratio of warfarin. These results show that the pronounced intersubject variation in the elimination of warfarin observed in this investigation was related to interindividual differences in plasma protein binding of the drug. The differences in protein binding cannot be ascribed to differences in plasma protein concentrations and may reflect configurational differences of proteins or the presence of an endogenous displacing agent at different concentrations.

Keyphrases □ Coumarin anticoagulants—relationship between protein binding, distribution, and elimination kinetics of warfarin in rats □ Warfarin—relationship between protein binding, distribution, and elimination kinetics in rats □ Plasma protein binding, warfarin—relationship to distribution and elimination, rats

The anticoagulant warfarin is a highly plasma protein bound drug with pronounced intersubject variability in its elimination kinetics in humans and animals (1–7). In this investigation, the relationships between the protein binding, distribution in the body, and kinetics of elimination of warfarin were studied in individual rats. A theoretical basis for a relationship between plasma protein binding and the total clearance of warfarin by the body was presented in a preliminary report (8).

EXPERIMENTAL

The studies were carried out on adult male Sprague–Dawley rats, weighing 400–450 g. They were selected from a larger number of animals on the basis of the results of a dicumarol screening test (9, 10) in order to obtain a group of animals with a wide difference