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ˣ To whom inquiries should be directed.

Specific TLC Tissue Residue Determination of Sulfadiazine following Fluorescamine Derivatization

CARL W. SIGEL ˣ, JOSEPH L. WOOLLEY, Jr., and CHARLES A. NICHOL

Abstract □ A spectrodensitometric method for the direct determination of sulfadiazine at the tissue residue level (0.1 ppm) is based upon the measurement of the fluorescence of a sulfadiazine–fluorescamine derivative formed directly on a TLC plate by dipping it into a fluorescamine solution. The linear dynamic range for the assay is about 150 from 200 to 0.2 ng, the lower limit of sensitivity. Recoveries from various spiked tissues including milk, eggs, liver, kidneys, muscle, skin, and fat varied with the tissue type but were reproducible. This assay technique has also been used for the assay of sulfamethoxazole and has been explored for use in specifically assaying sulfonamide mixtures.

Keyphrases □ Sulfadiazine—specific TLC tissue residue determination following fluorescamine derivatization □ Fluorescamine—used to prepare sulfadiazine derivative, specific TLC tissue residue determination of sulfadiazine □ TLC—tissue residue determination, sulfadiazine, fluorescamine derivatization

The antibacterial activity of trimethoprim is potentiated by administering it with a sulfonamide (1). Investigation of the use of the combination[1] of trimethoprim with sulfadiazine for treatment of infections in food-producing animals required the development of a method capable of determining sulfadiazine tissue concentrations down to 0.1 ppm. Previous work in this laboratory showed that quantitative TLC could be applied to the direct and specific determination of sulfamethoxazole extracted from plasma by measuring its absorbance on a silica gel plate (2). Although quite satisfactory for kinetic measurement of drug levels in body fluids, this procedure lacked the sensitivity needed for the determination of tissue residue levels of sulfadiazine. Therefore, derivatization on the TLC plate to form a fluorescing compound was considered as an alternative. Fluorescamine, which was first reported as a means of generating a fluorescing derivative of primary amino acids (3), was tested. The reagent was used for detecting amino acids after TLC development, but the fluorescing zones were not quantitated (4).

Sulfonamides and fluorescamine can form fluorescing derivatives directly on silica gel TLC plates, and the resulting zones can be quantitated using a scanning spectrodensitometer. These observations led to a sensitive and specific assay for sulfadiazine, which was applied to the determination of the drug at tissue residue levels. Determination of other sulfonamides, singly and in combination, was explored and will be discussed.

EXPERIMENTAL

Thin-Layer Plates—Silica gel 60 plates without fluorescent indicator (20 × 20 cm, 0.25 mm) were used[2].

Solvents and Solutions—All solvents were reagent grade[3] and all were distilled before use. The tissue homogenization buffer was 0.56 *M* sodium chloride, 0.01 *M* sodium phosphate dibasic, and 0.0013 *M* sodium phosphate monobasic (pH 7.4). Solutions of sulfadiazine (1.0 mg) in 100 ml of ethyl acetate and solutions of triethanolamine (1.25 ml) in 250 ml of chloroform were prepared each week. Fluorescamine[4] (25 mg) was dissolved in 250 ml of acetone. The solution was used for derivatization of not more than 15 plates and was not kept longer than 2 weeks.

Instrumentation—Plates were spotted with an automatic spotter[5] or by hand. Fluorescence was determined by scanning TLC plates with a spectrodensitometer[6], using the reflectance mode and only the sample beam with a secondary cutoff filter at 400 nm. Excitation was at 290 nm for the sulfadiazine–fluorescamine derivative, and total emission (above 400 nm) was determined using a density computer[7]. Peak areas were electronically integrated[8], and standard curves were calculated using the method of least squares.

Sulfadiazine Determination—*Procedure for Milk and Muscle*—In a stainless steel or glass cup kept at 4°, tissue (10 g) was homogenized in saline phosphate buffer (15 ml) using a tissue homogenizer[9]. After adjusting the pH to 6.0 with 1 *M* phosphoric acid, an aliquot equivalent to 1 g of tissue was pipetted into a 15-ml glass-stoppered centrifuge tube. The homogenate was extracted with three 3-ml portions of ethyl acetate. The combined

[1] Tribrissen, Cooper, Inc.

[2] E. M. Laboratories, Inc.
[3] Mallinckrodt.
[4] Fluram, Roche Diagnostics.
[5] Analytical Instrument Specialties Multi-spotter.
[6] Schoeffel model SD 3000 using a reflectance mode assembly.
[7] Schoeffel SDC 30.
[8] Autolab System IV.
[9] Virtis "45."

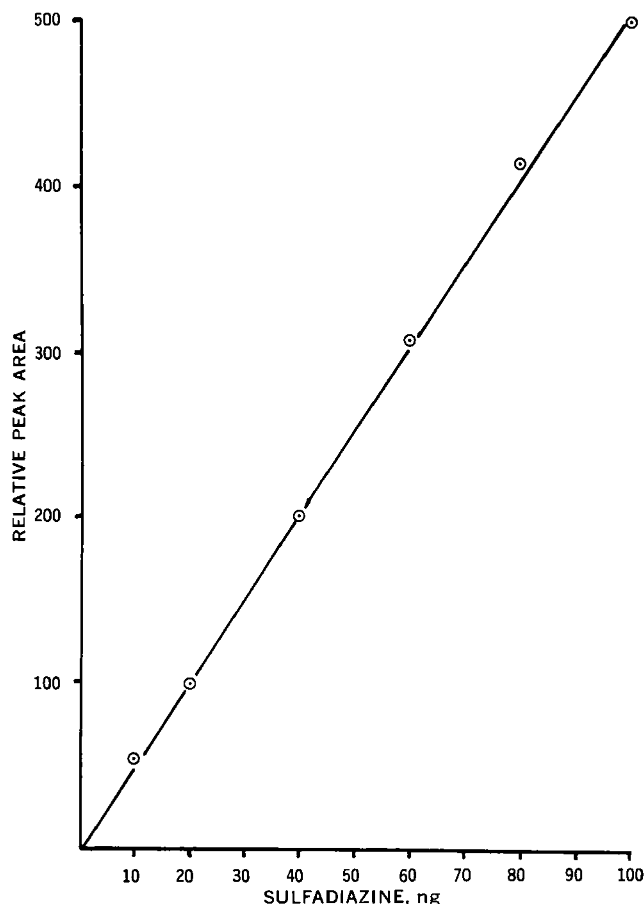


Figure 1—*Standard curve for sulfadiazine determination.*

extracts were dried by transferring them with a pipet into a 1 × 20-cm column containing anhydrous sodium sulfate (2 g).

Following elution, the column was rinsed with additional ethyl acetate (3 ml). Diethylaniline (5 μl) and 26.5% biphenyl in phenyl ether[10] solution (5 μl) were added to the combined eluates, and the sample was evaporated at 30° under a stream of dry nitrogen. The residue was redissolved in acetone (200 μl), and an aliquot (50 μl) was spotted on a scored silica gel TLC plate (20 channels/plate). Standards (0.01–0.1 μg) were spotted on the same plate. The plate was developed to 10–12 cm twice using diethyl ether in a tightly sealed solvent chamber.

The plate was air dried and dipped once quickly in a fluorescamine–acetone solution in a stainless steel tank (21.5 × 21.5 × 0.7 cm) to derivatize the sulfonamide. Then the plate was air dried for about 10 min and dipped once in a 0.5% triethanolamine–chloroform solution to stabilize the derivative (4). A second dipping into each of the solutions, respectively, did not cause either diffusion of the spots or diminution of the fluorescence. After air drying again, the plate was scanned with excitation wavelength at 290 nm and the total emission above 400 nm was measured. Sulfadiazine appeared at about R_f 0.40.

Procedure for Eggs, Liver, Fat, Skin, and Kidneys—The tissue was homogenized and extracted as described for milk and muscle samples. Before drying with sodium sulfate, the combined ethyl acetate extracts were back-extracted with 2 *N* aqueous sodium hydroxide (1 ml). The pH of the aqueous solution was checked and, if less than 9, the organic extract was washed with additional base (1 ml). The combined alkaline washes were adjusted to pH 6.0 with 1 *M* H_3PO_4 and extracted with three 3-ml portions of ethyl acetate. The combined extracts were dried over anhydrous sodium sulfate and assayed as described for milk and muscle samples.

Procedure for Plasma—A modification of the procedure for sulfamethoxazole was used (2). An equal volume of buffer [0.5 *M* citric acid and 1 *M* sodium phosphate dibasic (pH 3.9)] was added to the plasma and extracted with two 3-ml volumes of ethyl acetate. Samples were assayed as described for milk and muscle samples.

Table I—Recovery and Reproducibility of Sulfadiazine from Spiked Rat (R) and Chicken (C) Tissues and Cow's Milk

Tissue	Number of Samples	Amount Added, μg/g	Recovery, %	Deviation, %
Milk[a]	10	0.100	91.1	12.7
Egg (C)[a]	11	0.100	54.0	4.9
Muscle (R)	12	0.100	72.8	9.4
Liver (R)	10	0.100	76.3	15.0
Liver (C)[a]	6	0.100	71.7	10.4
Kidney (R)	11	0.100	59.8	10.3
Fat (C)[a]	10	0.100	59.8	7.7
Skin (C)[a]	5	0.100	81.6	4.2

[a] Obtained from commercial sources.

[10] Dowtherm A, added to prevent decomposition.

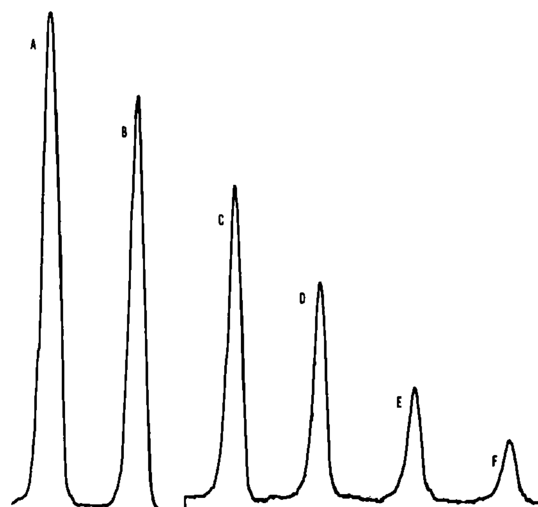


Figure 2—*Typical peaks observed when scanning the fluorescamine derivatives of sulfadiazine standards. Key: A, 0.1 μg; B, 0.08 μg; C, 0.06 μg; D, 0.04 μg; E, 0.02 μg; and F, 0.01 μg.*

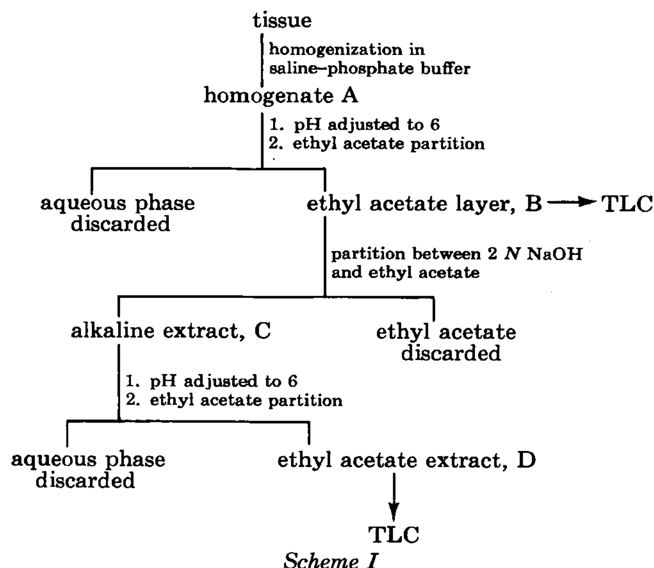
RESULTS

Sulfadiazine can be derivatized directly on a developed silica gel TLC plate with a fluorescamine solution by either spraying the plate or dipping it. The latter technique was more convenient and quicker, the sensitivity fourfold greater, the standard deviation lower, and the background more uniform. The derivative can be stabilized for about 5 hr by posttreating with triethanolamine (4). The standard deviation for the determination of nine samples of 0.05 μg of sulfadiazine was ±0.001 μg or 2%.

A calibration curve for the sulfadiazine assay is shown in Fig. 1. The linear dynamic range was found to be about 150 from 200 to 0.20 ng, the lower limit of sensitivity. Above 200 ng, linearity declined. Typical peaks observed on the recorder chart after scanning derivatized sulfadiazine standards in the 10–100-ng range are

Table II—Tissue Levels of Sulfadiazine in the Rat following a Single Oral Dose of Trimethoprim (4 mg/kg) and Sulfadiazine (20 mg/kg)

Rat	Hours	Plasma, μg/ml	Liver, μg/g	Muscle, μg/g
1	5	53.9	13.6	7.84
2	24	5.8	0.86	0.661
3	48	0.83	0.09	0.129
4	75	0.016	>0.006	>0.006



Scheme I

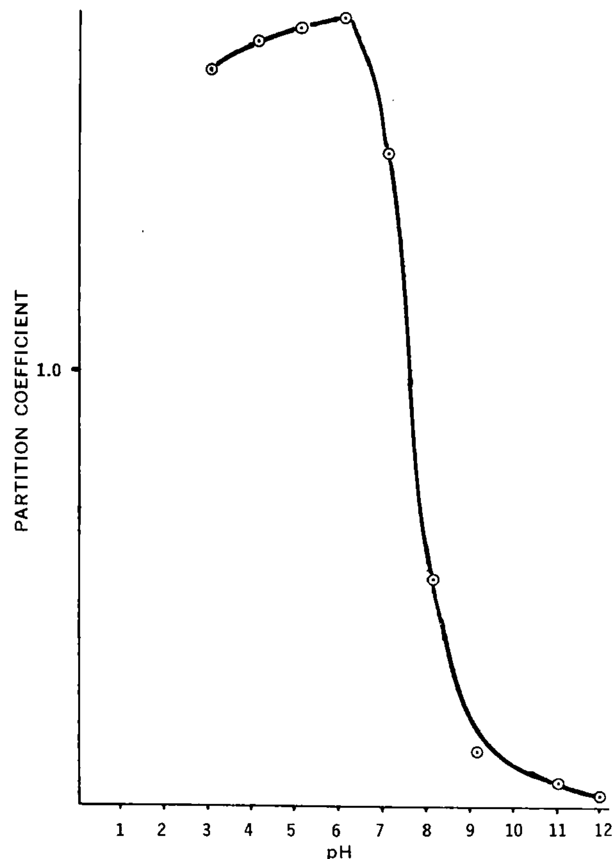


Figure 3—*Relationship between partition coefficient and pH for sulfadiazine.*

shown in Fig. 2. This range was used for assaying residue amounts for 1-g tissue samples.

Recovery data for extraction of sulfadiazine from spiked tissues are shown in Table I. Rat and chicken tissues were used for the recovery experiments. For milk and muscle, a simple "clean-up" procedure involving homogenization and a single extraction (Scheme I) could be used; however, for eggs, liver, kidneys, skin, and fat, a back-extraction into base was required to separate sulfadiazine from endogenous material partitioning into the ethyl acetate extract of the homogenate. The percent recovery of drug depended upon the tissue and whether a back-extraction was required but was considered reproducible for each tissue type.

These results were in the same range as reported by Crisp (5) who used a similar isolation scheme for a tissue residue assay based upon the GC determination of sulfaquinoxaline. In the current work, recoveries were improved by at least 30% by the addition of small aliquots of a solution of 26.5% biphenyl in phenyl ether and diethylaniline to the ethyl acetate extracts (B or D, Scheme I) prior to evaporation[11]. Addition of both components was necessary for recovery improvement. Treatment of the glassware with siliconizing fluid did not improve recoveries.

Careful adjustment of the pH of both the homogenate A and alkaline extract C was an important factor for reproducible recoveries. The relationship between partition coefficient and pH of the aqueous solution for sulfadiazine is shown in Fig. 3.

Four rats were dosed orally with the sulfadiazine–trimethoprim combination (20 and 4 mg/kg, respectively) and were sacrificed sequentially over 4 days. Plasma, muscle, and liver tissue levels of sulfadiazine were determined (Table II). Sulfadiazine appeared as a well-resolved symmetrical peak with no interference due to endogenous compounds, sulfadiazine metabolites, or trimethoprim or its metabolites.

A mixture of five sulfonamides also was chromatographed, derivatized with fluorescamine, and scanned (Fig. 4), thereby illustrating that the basic approach has potential for assaying sulfonamide mixtures.

DISCUSSION

The most widely used method for the determination of sulfonamides is a colorimetric procedure based upon the Bratton–Marshall reaction (6). For tissue concentrations, however, the method has limitations in terms of lack of specificity and background interference. With these considerations in mind, Crisp (5) developed a tissue residue procedure for sulfaquinoxaline based upon electron-capture GLC. The procedure was specific and extremely sensitive but time consuming. This TLC method for sulfadiazine, based upon fluorescence scanning, has the advantages that the sample preparation is simpler and the time required for analysis is shorter. The instrumentation is extremely reliable and easily operated. Very uniform TLC plates are commercially available. Even if there are plate or instrumental variations for different runs, these factors are eliminated by determining a series of standards on the same plate used for the unknown samples. This procedure still allows the analysis of at least 10 unknown samples on each plate. By using this procedure, about 8–10 tissue samples can be assayed by one person in 1 day.

The assay of sulfadiazine has been emphasized in this report. Sulfamethoxazole (following fluorescamine derivatization) has been assayed in vaginal washes[12] and other fluids and tissues down to 1 ng/ml by a modification of the basic TLC procedure previously reported from these laboratories (2). Presumably, the TLC method could be adapted for measurement of specific sulfonamide drug levels in cases where sulfonamide drug mixtures are used. TLC conditions for the separation of up to 15 different sulfonamides on one plate have been reported (7). The potential of this approach was demonstrated by derivatizing and scanning five sulfonamides following separation on a TLC plate. Of course, the method is specific for each of these compounds only insofar as there are no other molecules with primary amino moieties that comigrate with the drugs. Careful selection of the TLC solvent would obviate this problem.

CONCLUSIONS

Quantitative fluorescence scanning is a sensitive analytical technique which has the advantage of moving the drug away from interfering substances to an R_f on the chromatogram characteristic of the compound and the solvent system. Derivatization of sulfonamides with fluorescamine on the developed TLC plate amplifies the sensitivity beyond that attainable by absorbance scanning. A

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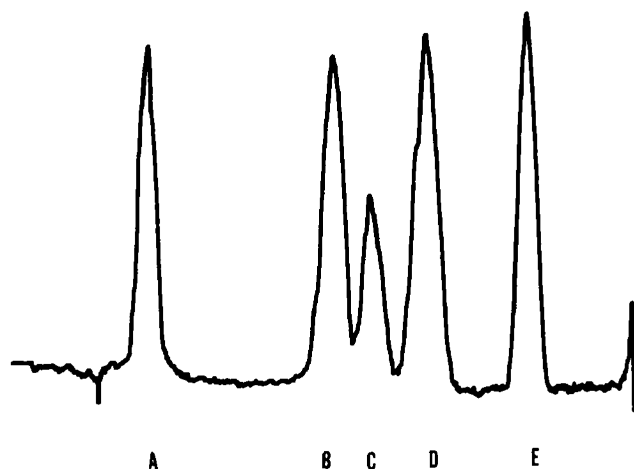

Figure 4—*Scan of derivatized sulfonamides separated on a silica gel TLC plate by development twice in diethyl ether (11 cm). Sulfonamides are: A, sulfathiazole; B, sulfadiazine; C, sulfamerazine; D, sulfadimidine; and E, sulfamethoxazole.*

sensitive and specific method utilizing these principles for the development of a tissue residue assay for sulfadiazine is described.

Fluorescamine was used previously as a means for detection of amino acids on TLC plates, but the quantitative aspects were not pursued (4). This report emphasizes the utility of this reagent as a useful analytical tool, especially for residue level determinations of sulfonamides. Undoubtedly, numerous extensions of this approach to include a broad range of drugs and pesticides can be made.

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[x] To whom inquiries should be directed.

Predicting Subjective Spreadability, Viscosity, and Stickiness

MARIA L. DeMARTINE and E. L. CUSSLER [x]

Abstract □ Subjective spreadability, viscosity, and stickiness perceived with the fingers were predicted from fluid mechanics. The correlation coefficients of these predictions were 0.95 for spreadability, 0.95 for viscosity, and 0.90 for stickiness. The two important assumptions in the predictions were that spreadability and viscosity were perceived as shear stress and that stickiness was perceived as time. When the finger geometry was approximated as two parallel plates, the predictions only required rheological data for the Newtonian and non-Newtonian liquids used. While these liquids covered a range of 10^6 in apparent viscosity, large variations in other fluid properties such as density and surface tension were not studied.

Keyphrases □ Spreadability, subjective—predicted from fluid mechanics, equations □ Viscosity, subjective—predicted from fluid mechanics, equations □ Stickiness, subjective—predicted from fluid mechanics, equations □ Liquid texture—predicting subjective spreadability, viscosity, and stickiness

Previous papers on liquid texture presented interesting empirical relationships between subjective attributes and rheological parameters (1–7). For example, Stevens and Guirao (2) found for Newtonian liquids that:

$$\begin{pmatrix}\text{subjective}\\ \text{viscosity}\end{pmatrix} \propto \begin{pmatrix}\text{objective}\\ \text{viscosity}\end{pmatrix}^{0.44} \qquad \text{(Eq. 1)}$$

where the exponent of 0.44 is found empirically. As a second example, empirical curves were found (4, 6, 7) giving the conditions under which fluids of different rheological properties have the same subjective texture. Why these curves have their peculiar shape remained uncertain.

This paper predicts some attributes of liquid texture without introducing empiricism. It depends on two key assumptions: *(a)* that subjective stickiness is perceived as the time during which the finger is pulled away from the sticky surface, and *(b)* that subjective spreadability and viscosity are perceived as the shear stress on the fingers. That these assumptions are sensible is easily tested. For example, when water and honey are rubbed between the fingers, the