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ACKNOWLEDGMENTS

The authors thank the Medical Research Council of Canada for providing a Studentship to W. A. Turner and the Government of Saskatchewan for providing funds under the Students Temporary Employment Programme (1971) for D. K. Kowal. Dr. Harry B. Wood, Jr., Drug Research and Development Division, National Cancer Institute, Bethesda, Md., kindly arranged for two compounds to be screened against the L-1210 and P-388 tumor systems.

Simple High-Pressure Liquid Chromatographic Determination of Trisulfapyrimidines in Human Serum

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Received April 19, 1977, from the **Departments of Medicinal Chemistry and* ‡*Pharmaceutics, School of Pharmacy, University of Pittsburgh, Pittsburgh, PA 15261,* and the §*Division of Biopharmaceutics, Food and Drug Administration, Washington, DC 20204.* Accepted for publication June 30, 1977.

Abstract □ A simple and rapid high-pressure liquid chromatographic method was developed for the determination of sulfadiazine, sulfamerazine, and sulfamethazine in human serum. After the trichloroacetic acid precipitation of the serum proteins, an aliquot of the supernate is injected into a high-pressure liquid chromatograph equipped with a reversed-phase microparticulate column and a fixed wavelength UV detector. For each of the three components of trisulfapyrimidines, a linear calibration curve was observed in the 1-30-μg/ml range, with the precision of the assay estimated to be ±2% (*RSD*). Preliminary pharmacokinetic data are also presented.

Keyphrases □ Sulfadiazine—high-pressure liquid chromatographic analysis in human serum □ Sulfamethazine—high-pressure liquid chromatographic analysis in human serum □ Sulfamerazine—high-pressure liquid chromatographic analysis in human serum □ High-pressure liquid chromatography—analyses, sulfadiazine, sulfamethazine, and sulfamerazine in human serum □ Antibacterials—sulfadiazine, sulfamethazine, and sulfamerazine, high-pressure liquid chromatographic analyses in human serum □ Trisulfapyrimidines—high-pressure liquid chromatographic analyses in human serum

The antibacterial properties of the sulfonamides were first recognized with the discovery of sulfamidochrysoidine (1), and these drugs are still frequently used in the treatment of urinary tract infections. Unfortunately, kidney damage has been associated with sulfonamide therapy, resulting from the crystallization in the renal tubules of the N^4-acetylated metabolites of many sulfonamides. To overcome this problem, combinations of sulfa drugs are commonly utilized. One such combination is the trisulfapyrimidines: sulfadiazine, sulfamerazine, and sulfamethazine.

A colorimetric method based on a diazotization reaction, as proposed by Bratton and Marshall (2), is commonly employed for quantitating sulfonamides. However, when applied to biological fluids, interferences from metabolites possessing a free amino group was observed. To overcome this lack of specificity, Rieder (3) introduced an extraction step.

Many other spectrophotometric and TLC methods subsequently were proposed for the determination of sulfonamides in biological fluids (4–12). GLC of the sulfa drugs also was attempted (13–15). However, derivatization must be performed to eliminate adsorption of these compounds to the chromatographic support. The separation and quantitation of sulfa drugs, alone (16–18) and in pharmaceutical preparations (19, 20), by high-pressure liquid chromatography (HPLC) were described. Recently, methods were proposed for sulfamethazine residues in bovine tissue (21) and for sulfamethazine, sulfamerazine, sulfathiazole, and their metabolites in cattle urine (22).

A study relating dissolution and bioavailability profiles of triple sulfa suspensions required a specific and sensitive method for the individual trisulfapyrimidine components. This report describes a simple, rapid, specific, and sensitive HPLC method devised for this purpose.

EXPERIMENTAL

Apparatus—A modular high-pressure liquid chromatograph consisted of a constant-flow pump[1], a valve-type injector[2], a fixed wavelength (254 nm) UV detector[3], and a strip-chart recorder[4]. A stainless steel column (3.9 mm × 30 cm) packed with fully porous 10-μm silica particles, to which is chemically bonded a monomolecular layer of octadecylsilane[5], was obtained commercially.

Chromatographic Conditions—The mobile phase consisted of acetonitrile–1% acetic acid (13:87). A flow rate of 1.5 ml/min was established (1100 psi). The column was maintained at 29.6° by inserting it into a glass sleeve which was then immersed in a constant-temperature water bath[6].

[1] Chromatography pump model M-6000A, Serial No. SDS-5235, Waters Associates, Milford, Mass.
[2] Universal injector model U6K, Serial No. U6K-6065, large capacity, Waters Associates, Milford, Mass.
[3] Model 440 absorbance detector, Serial No. 440-01249, Waters Associates, Milford, Mass.
[4] A-25 dual channel, Varian Associates, Walnut Creek, Calif.
[5] Waters Associates prepacked μBondapak C_{18} column.
[6] B. Braun Thermomix II (No. 26394), Bronwill Scientific Co., Rochester, N.Y.

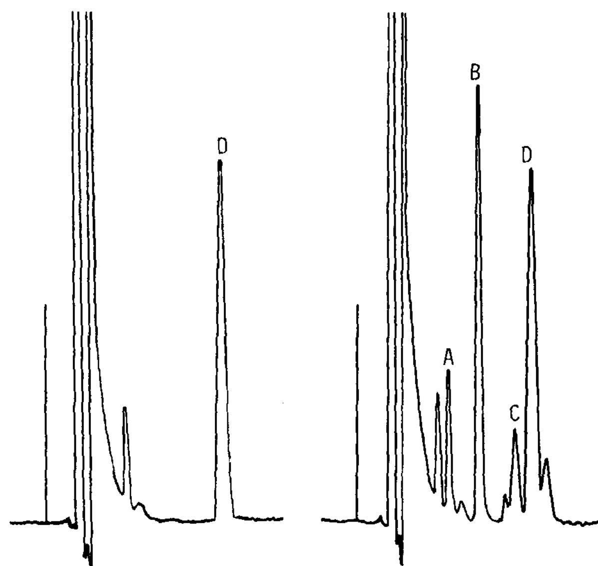

Figure 1—*Chromatogram obtained from HPLC assay of control human serum spiked with internal standard (left) and serum from a volunteer who had received a single dose of a trisulfapyrimidines suspension (right). Key: A, sulfadiazine (4.3 μg/ml); B, sulfamerazine (18.6 μg/ml); C, sulfamethazine (5.2 μg/ml); and D, sulfamethizole (25 μg/ml).*

Reagents and Materials—Samples of sulfadiazine[7], sulfamerazine[8], sulfamethazine[9], and sulfamethizole[10] were used as received. All other materials were reagent grade. A stock solution of trisulfapyrimidines was prepared by dissolving 100 mg each of sulfadiazine, sulfamerazine, and sulfamethazine in 0.1 *N* NaOH and adjusting the volume to 50 ml in a volumetric flask. The internal standard stock solution was prepared by dissolving 50 mg of sulfamethizole in 1 *N* NaOH and adjusting to volume in a 100-ml volumetric flask. Plasma standards were prepared by taking appropriate small volumes of the stock solutions and adding these to control plasma[11].

HPLC Assay—To 0.2 ml of serum or citrated plasma in a 1-ml capacity vial[12] was added 10 μl of the internal standard solution, followed by 0.1 ml of a 14% solution of trichloroacetic acid. The samples were mixed[13] and then centrifuged[14] at 2500 rpm for 15 min. A 5-μl aliquot of the clear supernate was then injected onto the column.

Calculations—Peak height ratios were calculated by dividing the peak height corresponding to the sulfa drug by the peak height corresponding to the internal standard. Calibration curves were constructed daily from results obtained from spiked control plasma samples containing equal amounts of each trisulfapyrimidine in the 1–30-μg/ml range by plotting peak height ratios *versus* concentration of the individual sulfa drug.

Colorimetric Assay—A modification of the Bratton–Marshall method (2) was used. To 0.5 ml of serum was added 0.5 ml of a 14% trichloroacetic acid solution. The samples were vortexed and centrifuged, and 300 μl of the supernate was transferred to a 12-ml centrifuge tube containing 3.0 ml of 0.1 *N* HCl. To each tube were added 0.5 ml of 0.1% sodium nitrate, 0.5 ml of 0.5% ammonium sulfamate, and 0.5 ml of 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride. The absorbance was then determined at 544 nm. Calibration curves were constructed for each batch of samples from spiked control plasma containing equal amounts of each trisulfapyrimidine in the 1–30-μg/ml range.

Human Study Protocol—To establish the usefulness of the HPLC method for bioavailability studies, a 10-ml dose of a trisulfapyrimidines oral suspension (providing 0.33 g of each trisulfapyrimidine) was administered to a normal human volunteer; 15 blood samples of approximately 5 ml each were collected. Each sample was assayed in duplicate by both HPLC and the conventional Bratton–Marshall method.

[7] Lot R02114, Eli Lilly and Co., Indianapolis, Ind.
[8] Lot M07059, Eli Lilly and Co., Indianapolis, Ind.
[9] Lot R02011, Eli Lilly and Co., Indianapolis, Ind.
[10] Lot R159178, Ayerst Laboratories, New York, N.Y.
[11] Citrated plasma obtained from the Central Blood Bank, Pittsburgh, Pa.
[12] Reacti-Vials No. 13261, Pierce, Inc., Rockford, Ill.
[13] Vortex-Genie, Catalog No. 12-812-VI, Fisher Scientific Industries, Springfield, Mass.
[14] IEC EXD centrifuge 460G, Damon/IEC Division, Needham Heights, Mass.

Table I—Precision and Accuracy of the HPLC Method for the Estimation of the Trisulfapyrimidines in Serum

Concentration, Theoretical, μg/ml	Concentration, Experimental, Mean[a] (Range)	CV	Relative Error, %
	Sulfadiazine		
1	1.0 (0.9–1.0)	3.0	4.0
2	2.0 (1.9–2.1)	3.9	3.0
5	5.1 (5.0–5.1)	1.5	1.7
10	9.7 (9.4–10.0)	2.6	2.7
15	15.3 (15.1–15.4)	0.8	2.0
20	20.2 (19.9–20.3)	0.9	1.1
25	24.7 (24.5–25.0)	1.0	1.4
30	30.1 (29.4–30.9)	2.1	1.6
	Sulfamerazine		
1	1.0 (0.9–1.1)	5.3	4.3
2	2.0 (1.9–2.1)	3.6	2.8
5	5.0 (5.0–5.1)	1.1	1.0
10	9.7 (9.4–9.9)	2.0	3.5
15	15.3 (15.3–15.4)	0.4	2.1
20	20.3 (20.3–20.4)	0.2	1.7
25	24.6 (24.4–25.0)	1.0	1.5
30	30.0 (29.4–30.8)	2.1	1.5
	Sulfamethazine		
1	1.0 (0.9–1.0)	5.6	4.3
2	2.0 (2.0–2.2)	2.8	3.3
5	5.0 (5.0–5.1)	0.5	0.6
10	9.6 (9.3–9.9)	2.4	3.8
15	15.2 (15.1–15.3)	0.6	1.4
20	20.3 (20.2–20.4)	0.3	1.5
25	24.8 (24.6–25.0)	0.7	1.0
30	30.0 (29.5–30.9)	2.0	1.4

[a] $n = 4$.

RESULTS AND DISCUSSION

A method that was capable of distinguishing among the trisulfapyrimidines and their metabolites in serum at suitably low concentrations was desired. Because of the large number of blood samples expected in a planned bioavailability-dissolution study, the total time required for each analysis also posed a critical limitation. HPLC seemed to offer the best approach for solving these problems because of its great versatility. To separate the various sulfa drugs, several column packings have been tried (16–22). In this present study, a reversed-phase microparticulate column was chosen. This type of column offered the possibility of minimum sample preparation prior to actual injection into the chromatograph.

Initial work with pure solutions of the trisulfapyrimidines showed good separation on the reversed-phase column using an acetonitrile–dilute acetic acid mobile phase. This result suggested the possibility of precipitating the serum proteins with acetonitrile and the subsequent direct injection of the supernate onto the column. At first, this approach appeared satisfactory since no interfering peaks were noted in the chromatogram. Within a short time, however, column back-pressure increased; on inspection of the column inlet frit, it seemed obvious that precipitation was not complete. Trichloroacetic acid precipitation was tried next and proved satisfactory.

The chromatogram obtained from the analysis of serum from a volunteer who had ingested a single dose of a triple sulfa suspension showed the peaks for the trisulfapyrimidines plus other peaks that were probably metabolites. These additional peaks were not resolved from the parent drug peaks. Adjustment of both the polarity of the mobile phase and the column temperature was again required to resolve the drug peaks.

Heparin, when used as an anticoagulant, interfered, whereas citrate did not. A peak for caffeine also interfered, requiring further refinement of the polarity of the mobile phase and the column temperature.

For the determination of the stability of sulfadiazine, sulfamerazine, and sulfamethazine in plasma, citrated plasma was spiked with a solution of the trisulfapyrimidines to produce concentrations of 5, 10, and 20 μg of each sulfa drug/ml. These samples were divided into 15 smaller portions, and then the vials were sealed and frozen. The samples were assayed immediately and then at weekly intervals. No appreciable decomposition was noted over at least 3 weeks. Repeated analysis of selected clinical samples at weekly intervals showed similar results.

Chromatograms obtained on the analysis of control human serum and human serum from a volunteer who had received a single dose of a triple sulfa suspension are shown in Fig. 1. The chromatogram of control serum showed no interfering peaks. In the other chromatogram, peaks corre-

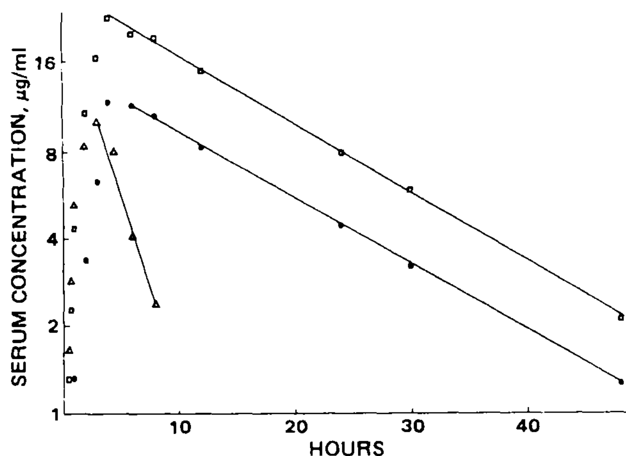

Figure 2—*Plot of serum concentrations of sulfadiazine* (●), *sulfamerazine* (□), *and sulfamethazine* (△) versus *time obtained after a dose of a trisulfapyrimidines suspension.*

sponding to sulfadiazine (retention time of 4.85 min), sulfamerazine (retention time of 6.43 min), sulfamethazine (retention time of 8.37 min), and the internal standard (retention time of 9.23 min) were observed. In addition, two peaks corresponding to metabolites (under investigation) were seen.

Verification that these peaks corresponded to the three unchanged drugs was obtained by comparing their retention times and peak width at half-height with standards and by using the standard addition technique. These methods confirmed that all three drugs were present in the clinical serum samples examined and that no hidden peaks were interfering in the assay.

On administration of sulfadiazine alone, at a dose three times higher than that given in the triple sulfa suspension study, a metabolite was found that had a retention time similar to sulfamerazine. The peak, however, was small even at this higher dose. Thus, this size would account for the inability to detect the peak in the blood samples obtained in the triple sulfa study.

The standard curves for the assay of all three trisulfapyrimidines were linear in the 1–30-μg/ml range, and the precision and accuracy of the method were studied. To control human plasma were added known amounts of the three compounds, and then these samples were assayed as described. The mean percent relative standard deviation (*CV*) and the mean relative error for each compound are reported in Table I. Virtually complete recovery was found with this method.

The utility of this new HPLC method was shown by assaying serum samples obtained from a human volunteer who had ingested a 10-ml dose of a trisulfapyrimidines suspension. In addition, these same samples were assayed by the conventional Bratton–Marshall method.

Since it was possible to separate unchanged drug from metabolites using HPLC, the concentrations of the individual components in these serum samples were determined. Serum concentrations for sulfadiazine, sulfamerazine, and sulfamethazine *versus* time are shown in Fig. 2. Elimination half-lives were 13.0, 12.7, and 2.3 hr, respectively. However, the spectrophotometric analysis of trisulfapyrimidines by the Bratton–Marshall method measures total sulfa concentrations and other primary amines. Mean total sulfa concentrations obtained from duplicate analysis of the volunteer's serum samples by both the Bratton–Marshall and HPLC methods are shown in Table II. For the HPLC method, total sulfa concentrations were obtained by summing the mean values for each component at each time period. The two methods showed differences in concentrations probably because of interference by metabolites in the Bratton–Marshall method. These differences became more marked at later sampling times, as indicated by a plot of the ratio of drug concentrations obtained by the Bratton–Marshall method to concentrations determined by HPLC (Fig. 3).

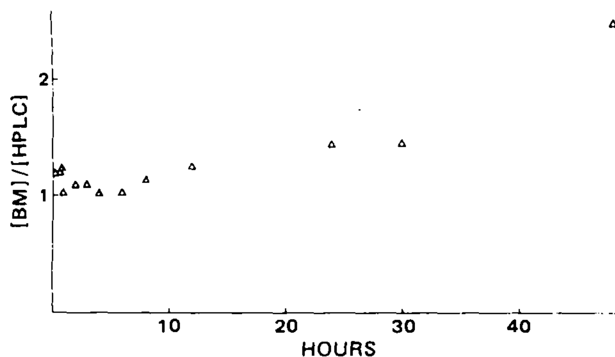

Figure 3—*Plot of ratio of total drug concentrations obtained by the Bratton–Marshall method to that determined by the HPLC method* versus *time (see text).*

Table II—Mean Total Sulfa Serum Concentrations Determined by HPLC and Bratton-Marshall Methods in a Single Subject after Administration of a Single Dose of a Trisulfapyrimidines Suspension

Time	Total Sulfa Drug Concentration, μg/ml HPLC Method	Bratton–Marshall Method
10 min	<1	1.0
20 min	1.2	1.5
30 min	3.4	4.1
45 min	5.9	7.3
60 min	10.6	10.8
2 hr	22.7	24.6
3 hr	33.3	36.1
4 hr	43.0	43.8
6 hr	35.8	36.8
8 hr	32.6	36.9
12 hr	24.1	30.2
24 hr	12.4	17.8
30 hr	9.1	13.2
48 hr	3.4	8.3

In conclusion, a simple, specific, and sensitive HPLC method has been described; it can be used for bioavailability determination of the trisulfapyrimidines.

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ACKNOWLEDGMENTS

Supported by Food and Drug Administration Contract 223-76-3010.

The authors thank Mr. Chan-Loi Yong and Mrs. Kathy Weyant for technical assistance, and Mr. Jack Korpi, Waters Associates, Inc., Millford, Mass., for helpful suggestions.