In addition, the new method requires only one solvent–column system for all catecholamines studied and phenylephrine.

The developed method was used for the quantitative determinations of II–V in dosage forms, and the results were in agreement with the results obtained using literature methods (Table I). The presence of methylparaben and propylparaben in the commercial dosage (phenylephrine hydrochloride nose drops) did not interfere (Table I). In all catecholamines studied and phenylephrine, the areas of the peaks were directly related to concentration (range 0.5–1.5 μg). The sensitivity of the method can be further improved by reducing the absorbance unit for full-scale deflection. The standard deviations based on five injections of the standard solution were estimated to be 0.53, 0.84, 0.76, 0.83, and 0.98% for I, II, III, IV, and V, respectively.

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High-Performance Liquid Chromatographic Assay of Sulfapyridine and Acetylsulfapyridine in Biological Fluids

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Abstract □ A high-pressure liquid chromatographic method for the sensitive, rapid, and specific determination of sulfapyridine and its *N*-acetyl derivative in plasma and saliva was developed. A cyano-bonded, reversed-phase, high efficiency column was used. The system detected these sulfonamides in serum to 0.25 mg/liter and within only 6 min. Sulfapyridine was separated from its acetyl derivative with little interference from other drugs. The assay reproducibility was within 3%. The assay was highly useful for routine monitoring of patients receiving sulfasalazine for inflammatory bowel disease.

Keyphrases □ Sulfapyridine and acetyl metabolite—high-performance liquid chromatographic analyses in biological fluids □ High-performance liquid chromatography—analyses, sulfapyridine and acetyl metabolite in biological fluids □ Dermatitis suppressants—sulfapyridine and acetyl metabolite, high-performance liquid chromatographic analyses in biological fluids

The measurement of sulfasalazine[1] metabolites in serum is useful for monitoring patients with inflammatory bowel disease (1–4). As a combination drug, sulfasalazine (I) is composed of sulfapyridine (II) azo linked to 5-aminosalicylic acid (IV). The azo linkage is cleaved by bacteria in the colon, releasing II and IV. Compound II metabolizes to acetylsulfapyridine (III), sulfapyridine *O*-glucuronide (VI), and acetylsulfapyridine *O*-glucuronide (VII).

[1] Azulfidine, Pharmacia; SAS-500, Rowell Laboratories; and Rorasul, Rorer.

BACKGROUND

Das and coworkers (1–3) established the relationship among the sulfasalazine metabolites (II, III, VI, and VII) in serum, acetylation phenotype, and sulfasalazine therapeutic efficacy and side effects. Total serum concentrations of II, III, VI, and VII between 20 and 50 mg/liter were associated with disease remission (1). Side effects were more likely to occur in slow acetylators when total sulfapyridine metabolite concentrations were greater than 50 mg/liter (2). Among the several metabolites, only II and III are present in sufficient concentrations in serum (3) and saliva (5) to be important in acetylator phenotyping and to be clinically useful as a guide to dosage adjustment.

Compound I and its metabolites were identified previously by TLC and by colorimetric methods. Schroder and Campbell (6) used TLC to help establish the metabolic pathway of sulfasalazine in humans. Subsequent work by Das and coworkers (1, 2, 4) made use of a modified Bratton–Marshall colorimetric assay (7) that measures only free II. Therefore, all metabolites of II must be hydrolyzed with acid or with enzymes to release free II for quantitation. These hydrolyses require temperature control. It is then necessary to diazotize the free II to produce the color for colorimetric analyses. Two of the several reagent solutions must be made fresh weekly because of their instability. Although the Bratton–Marshall method is reliable for investigative study, the procedural complexity limits its utility for routine clinical monitoring of patient samples on a daily basis and, of course, it is relatively nonspecific.

Current work to identify and quantitate sulfonamides utilizes high-pressure liquid chromatography (HPLC). HPLC was used in measuring I in tablets and powders (8). Sharma *et al.* (9) measured various sulfas and acetyl metabolites in animal urine by HPLC. Cobb and Hill (10) determined the relative retention times of various sulfonamides eluted from an HPLC column. The measurement of II and III from the serum

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and saliva of inflammatory bowel disease patients by HPLC has not been reported. This paper reports the development of an HPLC assay for II and III, including comparison to the Bratton–Marshall procedure.

EXPERIMENTAL

Materials—Reagent grade acetic acid, acetic anhydride, dioxane[2], perchloric acid, and sodium hydroxide[3] were used. Pure samples of II[4,5], III, I[4], and sulfamethoxazole[6] were used as standards. Very pure water was used. Reagents were prepared with distilled and deionized water; the HPLC mobile phase was prepared with degassed, distilled, deionized, and filtered water.

Acetylsulfapyridine was prepared in this laboratory using a procedure similar to that described by Sharma *et al.* (9). Pure acetic anhydride, 100 μl, was added dropwise to 100 mg of sulfapyridine in a 10-ml beaker and mixed to wet all of the sulfapyridine. After 50 min, the reaction mixture was rinsed with 40% dioxane–water into a 50-ml volumetric flask. A 2-ml aliquot of 0.1 *N* NaOH was added to the solution to dissolve any remaining sulfapyridine, and enough 40% dioxane–water was added to attain 50 ml. The yield was 97% acetylsulfapyridine and 3% sulfapyridine. The properties of the synthesized acetylsulfapyridine were the same as authentic acetylsulfapyridine[7].

Human plasma was obtained from a hospital blood bank and cleaned of chromatographically interfering substances using activated charcoal. The charcoal was shaken with the plasma for 10 min and then separated from the purified plasma by centrifugation at 20,000 rpm overnight at 10°.

Standards—Standards in distilled, deionized water or in purified plasma were prepared at concentrations of 2.5, 5, 10, 15, 20, and 25 mg/liter by dilutions of a 100- or a 10-mg/liter II and III stock solution (in water or in plasma) in 10-ml volumetric flasks. The standards were frozen in small aliquots (0.65 ml) in 12 × 75-mm glass test tubes covered with plasticized paper[8].

Internal Standard—A 20% perchloric acid–250-mg/liter sulfamethoxazole solution was prepared daily by adding 400 μl of a 60% perchloric acid solution to 800 μl of a 375-mg/liter sulfamethoxazole solution.

Apparatus—A high-performance liquid chromatograph[9] with a 254-nm UV detector and a reversed-phase, high efficiency, CN column[10] (4 mm i.d. × 30 cm) was used. The mobile phase consisted of an aqueous solution of 0.4% sodium acetate and 4% acetic acid. Flow rates varied between 2.0 and 2.3 ml/min under a pressure of approximately 140.6 kg/cm^2. The HPLC attenuation was set between 0.01 and 0.08 with a 1-mv recorder.

Analytical Procedure—Of the 13 sulfonamides tested on the CN column, the retention time of sulfamethoxazole (X), about twice that of II, was the shortest without chromatographically interfering with II or III. Therefore, X was chosen as the internal standard.

To reduce potential serum protein accumulation in the column and to minimize chromatographic interference, protein was precipitated from the samples with a 20% perchloric acid solution containing 250 mg of the internal standard/liter. Plasma standards (three) were used for patient serum samples. Into a 10 × 75-mm glass test tube, 0.02 ml of the perchloric acid–X solution was added to 0.2 ml of the sample or standard. After vortexing and then centrifuging at 2000 rpm for 12 min, the clear supernate was transferred to a clean 10 × 75-mm glass test tube. A 10-μl aliquot of this supernate was injected onto the column.

For patient saliva samples, water standards (three) were used. A 20-μl aliquot of a 250-mg of X/ml aqueous solution was added to 0.2 ml of the sample or standard. Ten microliters of this solution was injected onto the column.

Bratton–Marshall Assay—Compounds II and III also were measured by the Bratton–Marshall assay, modified to yield a three- to fourfold increase in analytical sensitivity over a previous method (7) and to allow convenient second extraction and color development steps for II at room temperature rather than at refrigeration temperatures. Compounds II and III were coextracted from 0.2–1.0 ml of biological specimen (mixed with 1.0 ml of 1 *M* pH 4.8 acetate buffer and 0.5 g of sodium chloride) into 5.0 ml of methyl isobutyl ketone and then reextracted from 3.0 or 4.0 ml of the ketone into 3.0 ml of 2 *N* HCl (acidic phase).

To assay for II, 100-μl quantities of aqueous solutions of sodium nitrite (0.12%), ammonium sulfamate (0.8%), and *N*-(1-naphthyl)ethylenediamine dihydrochloride (0.8%) were added sequentially at 3-min intervals to a 1.0-ml aliquot of the acidic phase. The mixture was stored in the dark for 30 min for maximum color development before measuring absorbance at 540 nm[11]. Total II and III were analyzed similarly after heating a second 1.0-ml aliquot of the acidic phase at 100° for 30 min to effect deacetylation of III.

Recovery—To test for drug recovery after precipitation, concentrations of II, III, and X were analyzed from water, saliva, and 4% human serum albumin in pH 7.4 phosphate-buffered saline and from plasma standards.

Stability—Concentrations of 10 and 20 mg of II and III/liter in plasma were stored at 4° and determined each day for 10 days and at 37° for 48 hr to assess the effect of temperature on short-term stability.

Reproducibility—The reproducibility of determining the concentrations of II and III was obtained by running 20-mg/liter plasma standards, stored in aliquots at 0° in glass tubes, 10 times in 1 day. Furthermore, plasma standards at 10 and 20 mg/liter were run once daily for 10 days over 3.5 weeks. Thus, both the "within" day (intraday) and the "day to day" (interday) variations of the assay could be determined. Measurements were made using peak heights (height of the chromatographic peak) and peak height ratios (drug peak height to internal standard peak height) for comparison as to the most reliable measurement method.

Comparative Assays—Five spiked plasma samples of known concentrations were determined by both the Bratton–Marshall procedure and the HPLC method. To assess clinical reliability, the concentrations of II and III in 35 serum samples from 19 ambulatory patients receiving sulfasalazine were determined independently by both methods. Concentrations of II and III were determined, and the percent acetylation was calculated.

Interferences—The concurrent medications received by the 19 patients were recorded to assess any chromatographic interference that would affect method specificity. Thirteen sulfonamides, sulfasalazine, and 5-aminosalicylic acid also were checked for interference.

RESULTS AND DISCUSSION

Figure 1 shows the typical chromatograms of a 15-mg/liter standard concentration of II and III and a serum sample from a patient receiving 0.5 g of sulfasalazine twice daily. The HPLC method has good specificity, as indicated by the separate peaks for each metabolite and by a lack of overlap with most other drugs taken by the patients as well as with most of the sulfonamides tested on the column. The retention times for II, III, and X varied with the mobile phase flow rate; at a flow rate of 2.20 ml/min, the retention times were 2.8, 4.0, and 4.7 min, respectively.

The concentrations of II and III were derived from a linear regression standard curve using peak height ratio (peak height of II or III to peak height of the internal standard, X). Because of the wide range of II and III concentrations from patient samples, the recorder absorptivity scale was changed by fourfold. Initial studies indicated that the detector response of 2.078, 3.898, and 7.910 correlated fairly well with the changes in the HPLC attenuator of two-, four-, and eightfold, respectively.

Recovery—Since essentially no protein precipitates out of saliva upon addition of 20% perchloric acid, saliva standards were injected directly onto the HPLC column. Saliva standards were compared to water standards, each being injected directly onto the column. The peak heights were nearly equal (saliva II was 103% of water II, and saliva III was 93.6% of water III), and the peak height ratios were identical.

During sample deproteinization, approximately 98, 62, and 71% of II, III, and X, respectively, were recovered from human plasma standards compared to aqueous standards as determined from the slopes of the peak heights (with concentrations of 20 and 50 mg/liter) and from the peak heights themselves. Because of the similar recoveries of III and X during deproteinization, use of the peak height ratio for III is nearly independent of protein concentration. Because II is less bound to serum proteins than X, their peak height ratios may be dependent on protein concentration.

Recoveries of II in 4% human serum albumin appeared to be greater than in water. This result was due to the concentrating effect of precipitating the protein volume out of solution, leaving the free II in a smaller

[2] Fisher Scientific Co., Springfield, N.J.
[3] J. T. Baker Chemical Co., Phillipsburg, N.J.
[4] Pharmacia, Piscataway, N.J.
[5] Eli Lilly and Co., Indianapolis, Ind.
[6] Hoffmann-La Roche, Nutley, N.J.
[7] Provided by Dr. K. M. Das, Albert Einstein College of Medicine, New York, N.Y.
[8] Parafilm.
[9] Model 848, DuPont Instruments, Wilmington, Del.
[10] μBondapak CN, Waters Associates, Milford, Mass.
[11] Beckman DB-6, Beckman Instruments, Fullerton, Calif.

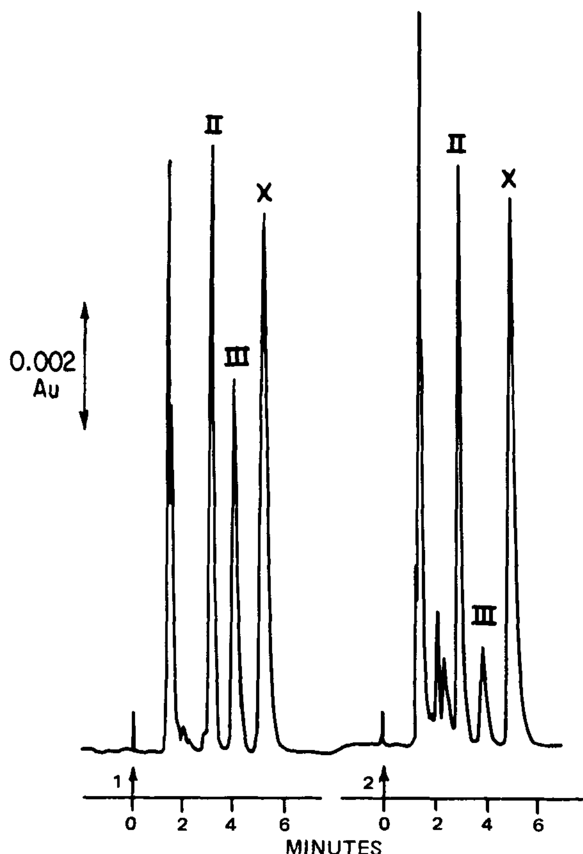

Figure 1—*Chromatogram of sulfapyridine (II),* N-*acetylsulfapyridine (III), and sulfamethoxazole as the internal standard (X). The arrows denote times of sample injection. Chromatogram 1 was obtained from a standard plasma concentration of 15 mg of II and III/liter. Chromatogram 2 was obtained from a patient sample containing 14.7 mg of II/liter and 3.94 mg of III/liter. The initial peaks are plasma effects.*

supernatant volume. Recoveries of III in 4% human serum albumin were reduced from those in water, although not as much as in plasma (77% in 4% albumin *versus* 62% in plasma). Therefore, to simulate patient samples, standards were made in purified plasma.

Peak height ratios were linearly related to the concentrations of II and III over a range of 2.5–100 mg/liter. Below 6 mg/liter, there was some loss of sensitivity, which could be increased tenfold by decreasing the HPLC attenuation from 0.02 to 0.01 aufs and by increasing injection volume from 10 to 50 μl. This step would allow detection of 0.25 mg of II and III/liter.

Stability—There was less than 3.3% variation in concentrations of II and III stored at 4° and measured once a day on 10 different days. The means and standard deviations determined for the two standard concentrations (10 and 20 mg/liter) tested were 10.4 ± 0.3 and 20.3 ± 0.3 mg/liter for II and 10.1 ± 0.3 and 19.8 ± 0.3 mg/liter for III. Heating a 20-mg/liter sample to 37° did not significantly change the concentration over 48 hr, with the resultant mean concentrations and standard deviations being 21.7 ± 1.5 mg/liter for II and 20.4 ± 0.5 mg/liter for III. Standard plasma samples were stored in glass tubes at 0° until use.

Reproducibility of HPLC Analysis—Table I shows the excellent reproducibility (less than 3% variation) for concentration determinations of both II and III in intraday and interday (over 10 days) determinations using peak height ratios. A 20-mg/liter standard was used for the intraday run, and 10- and 20-mg/liter standards were used for the interday run. Using peak heights alone without the internal standard resulted in variations up to 7% for II and 8% for III for the interday series. As the concentrations of II decreased to less than 5 mg/liter, with a corresponding peak height of 40 mm, the peak height ratio variations increased (5.6% variation at 2.5 mg/liter or at a 20-mm peak height). This ratio variation was also seen with III concentrations less than 10 mg/liter or with less than a 55-mm peak height (6.8% variation at 5 mg/liter or at a 25-mm peak height). The use of peak height ratios and attenuations of the chromatograph and recorder to produce peak heights greater than 25 mm is recommended for optimum results.

Table I—Reproducibility of the HPLC Analysis of II and III by Peak Ratios

Statistical Data	II			III		
	Intraday Series	Interday Series		Intraday Series	Interday Series	
Number	10	10	10	10	10	10
Mean concentration, mg/liter	20.8	10.12	19.92	20.7	10.0	19.99
SD	0.3	0.28	0.31	0.3	0.27	0.35
CV, %	1.5	2.7	1.5	1.5	2.7	1.8

Assay Comparison—Figure 2 compares the results of the HPLC and Bratton–Marshall spectrophotometric analyses of 35 serum samples obtained from patients receiving sulfasalazine maintenance therapy for ulcerative colitis and Crohn's disease. The graphs demonstrate an excellent correlation between concentrations of II and III as determined by the two methods, with respective correlation coefficients of 0.981 and 0.991. In 30 of the 35 assay comparisons, the concentrations of the sum of II and III ("total sulfapyridine") determined by the two procedures were within 20% of each other.

On the average, the HPLC method yielded concentration values lower than those derived from the spectrophotometric method. By regression analysis of the 35 patient serum specimens analyzed by both the HPLC assay and the Bratton–Marshall spectrophotometric assay (Fig. 2), the resulting slopes indicate that the HPLC method concentrations of II and III were 9.2 and 7.7% lower, respectively, than those of the spectrophotometric method. Similarly, when a set of plasma standards was run by both HPLC and the spectrophotometric methods (Table II), the five standards gave slopes indicating that the HPLC method yielded concentrations of II and III 4.5 and 2.7% lower than those of the spectrophotometric method. Compared to the *actual* spiked plasma concentrations listed in Table II, HPLC determinations gave consistently lower values and the spectrophotometric determinations gave consistently higher values. The maximum differences in concentrations between the methods of 5% for the spiked plasma and of 9% for the patient samples are of little clinical significance.

Figure 3 compares the percent acetylation, III × 100%/(II + III), as calculated from the HPLC and Bratton–Marshall assays of 32 patient samples. The average of two samples from each of the 16 patients was used in Fig. 3. There was an excellent correlation between the methods, with a correlation coefficient of 0.994. The HPLC method produced values 5.1% higher than the Bratton–Marshall method. The higher percent acetylation values may reflect the lower concentrations of II obtained using the HPLC method relative to III concentrations. The population was also differentiated by both methods into two distinct groups: rapid acetylators and slow acetylators. There was complete agreement between the assays on which patients were fast and slow acetylators.

Interference—Of all of the patient samples analyzed, only one contained an interfering material, which may have been a metabolite of propoxyphene. The patients sampled were on thiazide diuretics, oral and rectal steroids, vitamins, iron, folic acid, diazepam, meprobamate, oral contraceptives, diphenoxylate hydrochloride[12], diphenhydramine hydrochloride[13], aspirin, and cholestyramine[14]. Of the 13 sulfonamides

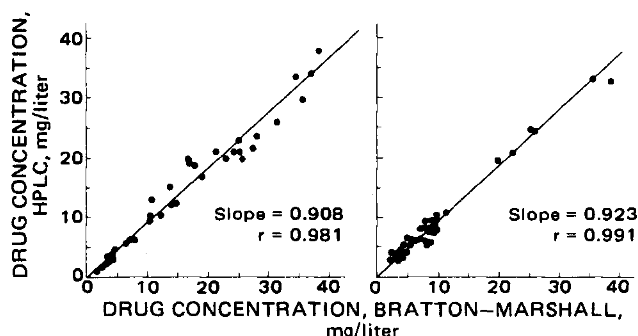

Figure 2—*Correlation between the concentrations of sulfapyridine (left) and acetylsulfapyridine (right) in 35 patient serum specimens as measured by the HPLC assay and the Bratton–Marshall spectrophotometric assay.*

[12] Lomotil, Searle & Co.
[13] Benadryl, Parke, Davis & Co.
[14] Questran, Mead Johnson Laboratories.

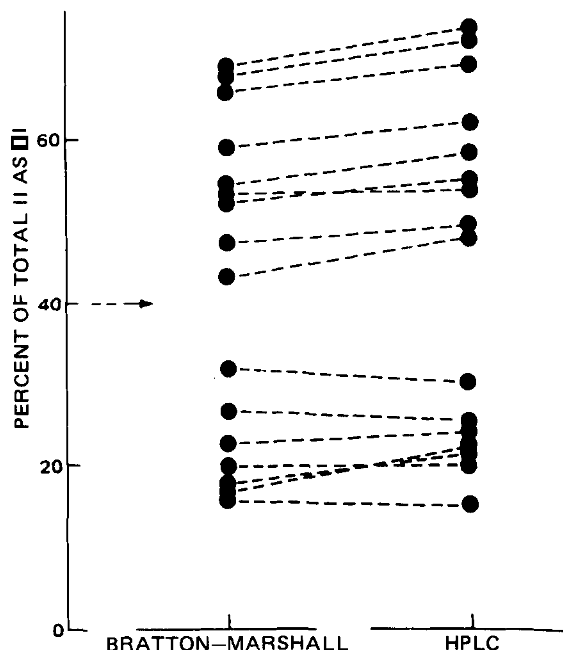

Figure 3—*Acetylation phenotyping in 16 patients by HPLC and the Bratton–Marshall spectrophotometric assay. The points represent the average of two acetylation percent determinations for each patient.*

tested on the column, sulfisomidine, sulfadiazine, sulfamerazine, sulfameter, sulfamethizole, and sulfamethoxypyridazine may interfere. Neither sulfasalazine nor 5-aminosalicylic acid interfered with the assay. Thus, there was minimal interference by other drugs, especially in the actual patient samples.

DISCUSSION

The described method for the HPLC assay of sulfapyridine is useful in the monitoring of patient samples. Although the pharmacokinetic characteristics of sulfasalazine and its clinical toxicity do not necessitate rapid serum level determinations, as may be important with drugs such as theophylline and procainamide, the routine monitoring of a small number of samples is facilitated by a simple method that can be readily added to existing laboratory procedures. Sulfapyridine samples may be injected into the same column and HPLC system as used for procainamide and quinidine with virtually no adjustments (11). Results can be obtained in less than 1 hr.

The method is specific in that it measures III directly whereas the Bratton–Marshall procedure requires hydrolytic cleavage to II and calculation of the amount of II contributed by the acetyl metabolite. Of the concurrent medications given the patients sampled with the HPLC serum level determination and acetylation phenotyping, only one interfered.

An additional feature is the relatively small volume of sample required for the HPLC determination. Less than the 200 μl of plasma may be analyzed if necessary. This advantage is particularly applicable to the pediatric population when only small amounts of serum can be obtained.

Table II—Analysis of Spiked Plasma Standards Containing Mixtures of II and III by the Bratton–Marshall Method and the HPLC Method

II, mg/liter			III, mg/liter		
Bratton–Marshall	Standard	HPLC	Bratton–Marshall	Standard	HPLC
0.53	0.26	0.16	7.81	7.5	7.3
1.05	0.51	0.37	14.90	15.0	15.2
4.24	4.00	3.80	8.13	8.0	7.8
11.60	11.00	10.30	4.44	4.0	3.5
21.70	21.00	20.90	0.94	0	0

The close correlations between the Bratton–Marshall and HPLC assay results clearly demonstrate the utility of the HPLC assay for II, III, and acetylator phenotyping with sulfasalazine. Its superiority over existing methods is evidenced by the small sample volume required, rapid chromatography times, good reproducibility, high sensitivity and specificity, and a minimum of procedural manipulation and technical functions required for analysis. There is the added benefit of minimal environmental pollution in the HPLC method with its lack of organic solvents.

The clinical monitoring of sulfapyridine concentrations and acetylation phenotyping in inflammatory bowel disease patients receiving sulfasalazine is a relatively new advance and has only been extensively reported to date in one series of patients. HPLC analysis facilitates such measurement in patient samples and provides a basis for further clinical research as well as service assay capabilities.

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