# Specific TLC Determination of Trimethoprim and Sulfamethoxazole in Plasma

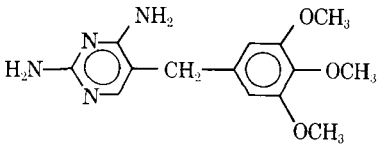
CARL W. SIGEL[x], MICHAEL E. GRACE, CHARLES A. NICHOL, and GEORGE H. HITCHINGS

**Abstract** □ A simple spectrodensitometric method for the direct determination of trimethoprim and sulfamethoxazole is based upon measurement of the absorbance of the two compounds on silica gel plates irradiated at 280 and 265 nm, respectively. Unlike the procedure for trimethoprim based on its fluorescence, which requires time for the development of the intensity of the spots, absorbance can be measured immediately. For both compounds, quantities as low as 0.005 μg can be detected and a linear relationship is observed between the integrated peak area and the amount on the plate between 0.02 and 0.5 μg. The percent recovery over the normal range of plasma concentrations for both compounds was 96–105%. This procedure for sulfamethoxazole is compared directly with a method based upon the Bratton–Marshall reaction.

**Keyphrases** □ Trimethoprim and sulfamethoxazole—analysis in plasma, TLC and spectrodensitometric method, compared to Bratton–Marshall reaction □ Sulfamethoxazole and trimethoprim—analysis in plasma, TLC and spectrodensitometric method, compared to Bratton–Marshall reaction □ TLC and spectrodensitometry—analysis, trimethoprim and sulfamethoxazole in plasma

Trimethoprim (I) and sulfamethoxazole (II) both inhibit the biosynthesis of folic acid in bacteria (1); the sulfonamide inhibits a step in the formation of dihydrofolic acid from *p*-aminobenzoic acid, and trimethoprim inhibits the conversion of dihydrofolic acid to tetrahydrofolic acid (1, 2). This relationship results in potentiation of the two activities and is the basis for a potent antibacterial combination[1] (3, 4). This report describes a new method that was applied to the specific determination of both drugs in plasma.

Previously reported analytical methods for the determination of trimethoprim in body fluids are based upon microbiological activity (5, 6), spectrophotometry (5), spectrofluorometry (7), scintillation spectrometry of $^{14}C$-labeled trimethoprim (8), differential pulse polarography (9), a combination of TLC and the microbiological assay (10), and fluorescence TLC (11). Of all of these methods, the only specific ones are those including a TLC separation. Isolation from the TLC adsorbent or the wait for the development of trimethoprim fluorescence, as used in previously reported methods, can be circumvented by the direct measurement of the absorbance of I on silica gel plates. Trimethoprim "quenching" can be visualized on fluorescing silica gel plates irradiated with UV light (254 nm); however, this determination is based upon measurement of the absorbance and can be done using either fluorescing or nonfluorescing TLC plates.



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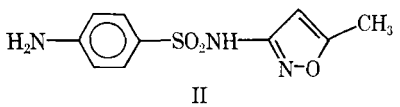

II

The most widely used method for the determination of sulfamethoxazole and other sulfonamides is a colorimetric procedure based upon the Bratton–Marshall (12) reaction. Falk and Kelley (13) and Kraml and Boudreau (14) reported adaptations of the method to automated systems. Various other improvements in the basic procedure have been introduced to improve specificity. Rieder (15) used the Bratton–Marshall reaction directly in an ethyl acetate extract of the nonhydrolyzed body fluid, thereby measuring what closely approximates the "bacteriostatically active fraction." His modification is generally applicable to the routine determination of sulfonamides in body fluids and potentially could be automated. Others have utilized various chromatographic methods including TLC (16–21), GLC (22), and partition chromatography (23, 24) to improve specificity for determinations of sulfonamides in both pharmaceutical preparations and biological samples. The present method is based upon the same principles as for the trimethoprim assay: separation on a silica gel thin-layer plate followed by direct determination by measuring the absorbance of sulfamethoxazole. A direct comparison between this TLC method and a modification of the Bratton–Marshall procedure was made and the results are discussed.

## EXPERIMENTAL

**Thin-Layer Plates**—Silica gel 60 F-254 plates (20 × 20 cm, 0.25 mm)[2] were used. Nonfluorescing silica gel 60 plates gave identical results.

**Solvents and Solutions**—All solvents were reagent grade[3] and all, except ammonia, were distilled before use. The buffer for the sulfamethoxazole determination was 0.3 *M* citric acid–0.6 *M* sodium phosphate, dibasic (pH 4.1).

**Standard Solutions**—The following were prepared: trimethoprim (2.0 mg) in 100 ml chloroform–methanol (9:1), and sulfamethoxazole (1.0 mg) in 100 ml chloroform.

**Instrumentation**—Plates were spotted with an automatic spotter[4] or by hand. Absorbance was determined by scanning TLC

[1] Trimethoprim–sulfamethoxazole (1:5 combination); Septra, Burroughs Wellcome Co.

[2] E. M. Laboratories.

[3] Mallinckrodt.

[4] Analytical Instrument Specialties Multi-spotter.

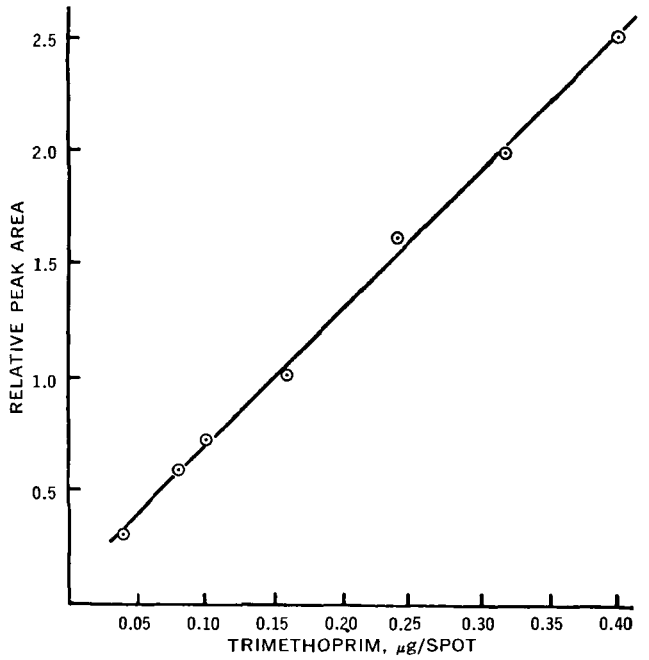


**Figure 1**—*Standard curve for trimethoprim.*

plates with a spectrodensitometer[5], using the reflectance mode and only the sample beam without a secondary barrier filter. Irradiation was at 280 nm for trimethoprim and at 265 nm for sulfamethoxazole. Absorbance was determined using a density computer[6] and the response was recorded[7]. Peak areas were electronically integrated[8] and standard curves were calculated using the method of least squares.

**Trimethoprim Determination**—Into a 50-ml glass-stoppered centrifuge tube, pipet 1.0 ml plasma and add 4 ml water and 0.1 ml 1 *N* NaOH. Add 20 ml isopropanol–dichloromethane (1:4), invert the tube gently 30 times, and centrifuge. Transfer 18 ml of the organic layer to a separate tube and decrease this volume to about 0.1 ml by heating the tube in a water bath at 40° under a stream of nitrogen. Transfer the residue to a 1-dram screw-capped vial using methanol–chloroform (1:9) and evaporate the solvent with a stream of nitrogen. Seal the vial with a septum-type lined screw-cap. With a syringe, add 100 μl of methanol–chloroform (1:9) to the vial, rotate it to dissolve the residue, and cool in an ice bath for a few minutes. Scan the TLC plate prior to spotting to check for uniformity of background. Apply 12 10-μl sample aliquots (up to about 50 μl can be spotted to each scored plate, 20 channels/plate) and a series of six concentrations of I (0.4–0.04 μg) by spotting appropriate volumes of the standard solution. Develop the plate in chloroform–*n*-propanol–28% aqueous ammonium hydroxide (80:20:1), allowing the solvent to migrate about 15 cm. Air dry the plate for 10 min and scan at 280 nm using only the sample beam. The trimethoprim peak ($R_f \simeq 0.42$) is detected by absorbance and appears on the recorder chart as a symmetrical peak well resolved from the trimethoprim metabolites, sulfamethoxazole and its metabolites, and common endogenous compounds.

**Sulfamethoxazole Determination**—Into a 15-ml glass-stoppered centrifuge tube, pipet 0.2 ml plasma and add 0.8 ml citrate–phosphate buffer and 4 ml chloroform–acetone (2:1). Invert the tube gently 30 times and centrifuge (1500×*g* for 3 min). Apply 80 μl of the organic phase (12 samples/plate) and a series of six sulfamethoxazole standards (0.50–0.05 μg) to each scored plate using an automatic spotter. Develop the plate in isopropanol–benzene (1:4), allowing the solvent to migrate about 15 cm. Air dry the plate for 10 min and scan at 265 nm in the same manner as for trimethoprim. The sulfamethoxazole spot appears at $R_f \simeq$ 0.5. The Bratton–Marshall procedure followed was based on the adaptation of Falk and Kelley (13).

[5] Schoeffel model SD 3000 using a reflectance mode assembly, SDA325.
[6] Schoeffel SDC30.
[7] Honeywell Electronik 194.
[8] Hewlett-Packard calculator, model 9100B.

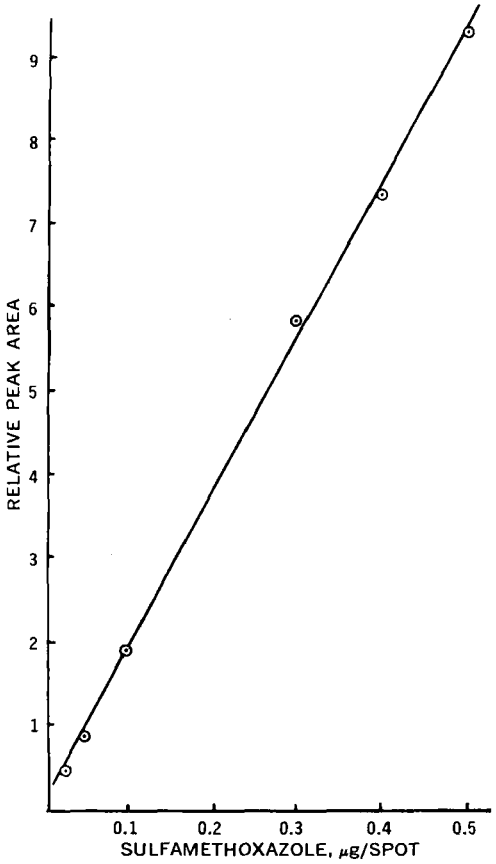


**Figure 2**—*Standard curve for sulfamethoxazole.*

## RESULTS

Calibration curves for both compounds using the described procedures are shown in Figs. 1 and 2. A linear relationship between the integrated area under the peak and the amount on the plate

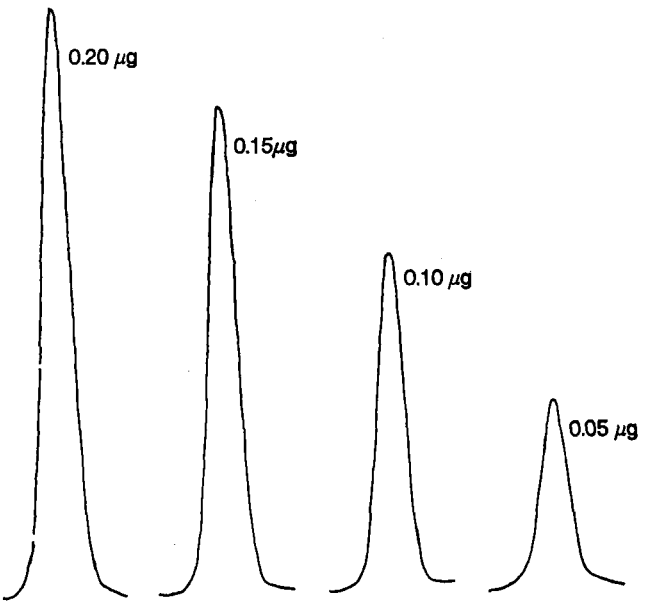


**Figure 3**—*Typical peaks observed when scanning the absorbance of trimethoprim standards on silica gel 60 F-254 plates.*

**Table I**—Reproducibility of Recovery of Trimethoprim from Spiked Human Plasma (1 ml)

| Quantity Added, μg | Number of Samples | Mean Recovery | Percent | Standard Deviation |
|---|---|---|---|---|
| 2.00 | 5 | 2.08 | 104 | 0.07 |
| 1.20 | 5 | 1.23 | 103 | 0.04 |
| 0.80 | 5 | 0.77 | 96 | 0.035 |
| 0.40 | 5 | 0.40 | 100 | 0.008 |

**Table II**—Reproducibility of Recovery of Sulfamethoxazole from Spiked Human Plasma (0.2 ml)

| Quantity Added, μg/ml | Number of Samples | Mean Recovery | Percent | Standard Deviation |
|---|---|---|---|---|
| 40.0 | 5 | 40.42 | 105 | 0.44 |
| 20.0 | 5 | 19.28 | 96 | 0.12 |
| 8.0 | 5 | 8.15 | 102 | 0.16 |

was observed between 0.02 and 0.5 μg. Typical peaks observed on the recorder chart after scanning four trimethoprim standards are shown in Fig. 3. Quantities as low as 0.005 μg can be detected for both I and II.

Both I and II appeared as well-resolved symmetrical peaks. Occasionally, an endogenous compound with $R_f$ 0.5 was observed and overlapped slightly with trimethoprim. Development of the plate a second time in the same solvent separated the two peaks to recorder baseline.

Reproducibility and recovery data for a normal range of plasma concentrations for I and II are shown in Tables I and II, respectively. The percent recovery from plasma varied from 96 to 105%. An application of the method is summarized in Table III, which shows trimethoprim and sulfamethoxazole plasma concentrations for a human subject during the 25-hr period following an oral dose of 160 mg trimethoprim and 800 mg sulfamethoxazole[9]. In this case, 1 ml plasma was used for trimethoprim; however, volumes as low as 0.1 ml can be employed by spotting larger aliquots of the redissolved sample. Sample application to the plate can be done manually but is accomplished more readily with an automatic multispotter. For sulfamethoxazole, 0.2 ml plasma was used routinely.

## DISCUSSION

For trimethoprim, the outlined method has several advantages over previously reported assays. It is specific, accurate, and rapid; 20 samples can be determined in 1 day by one person. The microbiological techniques are reproducible and sensitive but not specific. During therapy, when there is a change from another antibacterial to trimethoprim-sulfamethoxazole, the microbiological assay for I can be complicated by the presence of other agents. Spectrofluorometry is also sensitive but includes measurement of the metabolites or endogenous materials to some extent. The fluorescence TLC method reported earlier from these laboratories is specific and direct and can be used for the determination of I in plasma, urine, and tissues or the metabolites in urine (25). The fluorescence procedure, however, requires time for development of sufficient intensity of the spots for detection whereas absorbance can be measured immediately. Sensitivities of the two modes of operation are comparable. Application of the absorption procedure to the determination of urinary metabolites was not attempted because of the presence of endogenous compounds that also absorb.

Perhaps the only feature of the described method that might be considered as a limitation is the requirement for a good scanning spectrodensitometer. Considering the potential applications of this technique to assays in the clinical laboratory, however, such instrumentation will undoubtedly become standard in the near future. This technique can be applied to drugs of diverse structure (as in this instance, I and II). Also, in recent experience the alternative of using the fluorescence or absorbance mode has made possible the analysis of different types of drugs in body fluids.

For the routine assay of sulfamethoxazole levels in the clinical laboratory, the automated procedures based upon the Bratton-Marshall reaction are fast and provide a reliable measure of the bacteriostatically active fraction. A comparison between sulfonamide levels for both spiked and actual blood samples as determined by the TLC method and the Bratton-Marshall reaction as described by Falk and Kelley (13) was conducted and close agreement between the results of the two methods was observed (Table IV). The length of time required for both studies was similar; however, for a large number of samples, use of an automated Bratton-Marshall procedure would save considerable time. The agreement between two such different methods provides evidence that both procedures give reasonably accurate measures of the "active," nonconjugated sulfonamide fraction.

The major advantage of the TLC method is that one can be more confident that only the sulfonamide is being determined and not another drug or endogenous material that furnishes a chromophore with the Bratton-Marshall reagent. This problem of specificity, particularly for tissue level studies and for drug assay of preparations composed of sulfonamide combinations, has caused

[9] Two tablets of Septra.

**Table III**—Plasma Concentrations of Trimethoprim and Sulfamethoxazole following Oral Dose of 160 mg Trimethoprim and 800 mg Sulfamethoxazole

| Hours | Trimethoprim Concentration, μg/ml | Sulfamethoxazole Concentration, μg/ml |
|---|---|---|
| 0 | — | — |
| 0.5 | 1.95 | 26.33 |
| 1 | 1.77 | 47.53 |
| 2 | 1.67 | 60.33 |
| 3 | 1.43 | 46.33 |
| 5 | 1.19 | 47.66 |
| 13 | 0.73 | 23.00 |
| 22 | 0.41 | 15.50 |
| 25 | 0.29 | 14.4 |

**Table IV**—Comparison of the Determination of Plasma Concentrations of Sulfamethoxazole using Quantitative TLC and the Bratton-Marshall Reaction[a]

**Spiked Plasma Samples**

| Sample | Quantity Added, μg/ml | TLC Assay | Colorimetric Assay[b] |
|---|---|---|---|
| Blank | — | — | 0.3 |
| 1 | 10.0 | 10.8 | 10.4 |
| 2 | 10.0 | 11.3 | 10.8 |
| 3 | 10.0 | 10.8 | 11.1 |
| 4 | 10.0 | 10.0 | 10.7 |
| 5 | 40.0 | 42.9 | 39.2 |
| 6 | 40.0 | 39.9 | 39.1 |
| 7 | 40.0 | 39.6 | 39.7 |
| 8 | 40.0 | 39.2 | 40.3 |

**Plasma Samples from Patients Receiving Trimethoprim/Sulfamethoxazole**

| | TLC Assay, μg/ml | Colorimetric, μg/ml |
|---|---|---|
| Pretreatment sample | — | 0.6 |
| 1 | 33.2 | 28.9 |
| 2 | 52.5 | 51.4 |
| 3 | 33.8 | 31.5 |
| 4 | 28.8 | 28.9 |
| 5 | 31.0 | 31.4 |
| 6 | 26.0 | 26.9 |

[a] All samples were 0.2 ml. [b] Bratton-Marshall assay results corrected by subtraction of value for plasma blank or pretreatment sample.

others to devise various chromatographic procedures for sample preparation prior to spectrophotometric determination. The current method is an advancement over those procedures since the sulfonamide is determined directly on the separation medium. Presumably, the TLC method could be adapted for measurement of specific sulfonamide drug levels when sulfonamide mixtures are used. TLC conditions for separation of up to 15 different sulfonamides on one plate have been reported (19).

Finally, the lower limit of sensitivity of the TLC procedure is at least 20 times lower than is required for normal sulfonamide plasma concentration levels. This factor enabled the development of the very simple extraction procedure. In most laboratories, TLC has been utilized mainly as a qualitative tool. These new assay procedures for trimethoprim and sulfamethoxazole in plasma demonstrate that TLC can also provide a rapid, sensitive, accurate, and specific means for quantitative determinations.

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# Interaction of 8-Hydroxyquinoline Sulfate with Components of Tuberculin Purified Protein Derivative Solutions III: Binding of 8-Hydroxyquinoline by Tuberculin Purified Protein Derivative


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**Abstract** □ The interaction between the preservative 8-hydroxyquinoline sulfate and macromolecules present in tuberculin purified protein derivative solutions such as tuberculoprotein, nucleic acid, and polysaccharide was studied. In buffered solution (pH 7.38), 8-hydroxyquinoline sulfate is dissociated to 8-hydroxyquinoline and sulfuric acid, and it is the base 8-hydroxyquinoline that forms a reversible association with these macromolecules. The degree of binding of 8-hydroxyquinoline to tuberculoprotein, nucleic acid, and polysaccharide was shown to be a function of the concentration of these macromolecules. In commercial solutions of tuberculin purified protein derivative, the low concentrations of purified protein derivative used have little effect on the concentration of 8-hydroxyquinoline sulfate. Thus, for all practical purposes the antimicrobial activity of 8-hydroxyquinoline sulfate was not affected.



**Keyphrases** □ 8-Hydroxyquinoline sulfate—interaction with components of tuberculin purified protein derivative □ Tuberculin purified protein derivative—interaction of 8-hydroxyquinoline sulfate with solution components, binding of 8-hydroxyquinoline □ Binding—8-hydroxyquinoline to tuberculoprotein, nucleic acid, and polysaccharide (components of tuberculin purified protein derivative) □ Antimicrobial agents—interaction of 8-hydroxyquinoline sulfate with components of tuberculin purified protein derivative


8-Hydroxyquinoline sulfate[1] is added to tuberculin purified protein derivative solutions as an antimicrobial agent (2). In previous reports, the authors described how 8-hydroxyquinoline sulfate disappeared from these solutions when dispensed in glass vials stoppered with rubber closures. They showed that, in

[1] Chinosol (1), Eastman Organic Chemicals, catalog number 1776.