Use of the water-filled buret technique eliminated the possibility of errors due to air leakage about the site of cannulation. If air entered, it would pass by the needle, go up through the liquid in the inverted container, and become visible as bubbles.

RESULTS AND DISCUSSION

The results from the experiments are shown in Tables I and II. Statistical evaluation of these data yields evidence that temperature fluctuation and agitation treatments have no detrimental effect on vacuum seal.

The greatest difference detected between average vacuums for test and control groups was found in the temperature fluctuation experiments. Table I shows that the 5–40°, 500-ml. Type A group had average vacuums of 85.75 ml. water for the test bottles and of 88.99 ml. water for the control bottles. A standard "pooled" or two-sample t test on this test-control pair of samples indicated that the differences were insignificant since the calculated t value is 2.0 or less than 2.413 at the 1% level.

The natural variation in vacuum from bottle to bottle among the Type A and Type B control samples is given by their standard deviations: 4.54 ml. water for Type A and 4.37 ml. water for Type B. When using the F test, it was found that there was no significant difference in the vacuum variance between the two types of control samples.

It is apparent from analysis that the conditions imposed upon the test containers did not affect the vacuum in these systems. The test conditions were strenuous to the point where they exceeded the normally expected environments to which the containers would be exposed. It may be concluded that normally expected amounts of agitation experienced in transport or fluctuations in temperature as in transport or storage are not primarily responsible for a loss of vacuum in these types of containers. When loss of vacuum is suspected, other factors such as decomposition of ingredients and faulty glassware or stoppers should be considered.

REFERENCES

(1) N. M. Davis, S. Turco, and E. Sivelly, *Bull. Parenteral Drug Ass.*, **24**, 257(1970).

(2) N. M. Davis, S. Turco, and E. Sivelly, *Amer. J. Hosp. Pharm.*, **27**, 822(1970).

(3) N. M. Davis and S. Turco, *ibid.*, **28**, 620(1971).

(4) A. Das, *Mfg. Chem. Aerosol News*, **June 1972**, 21.

(5) T. J. Macek, *Bull. Parenteral Drug Ass.*, **26**, 18(1972).

(6) J. T. Mayernik, *ibid.*, **26**, 205(1972).

(7) T. R. Arnold and C. D. Hepler, *Amer. J. Hosp. Pharm.*, **28**, 614(1971).

(8) E. N. Deeb and G. A. Natsios, *ibid.*, **28**, 764(1971).

(9) M. B. Pinckney, Jr., L. A. Luzzi, and T. E. Needham, Jr., *J. Pharm. Sci.*, **62**, 80(1973).

(10) R. J. Duma, J. F. Warner, and H. P. Dalton, *N. Engl. J. Med.*, **284**, 257(1971).

(11) K. W. Ashcraft and L. L. Leape, *J. Amer. Med. Ass.*, **212**, 454(1970).

(12) B. McGovern, *Mil. Med.*, **135**, 1137(1970).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 16, 1973, from the *School of Pharmacy, University of Georgia, Athens, GA 30602*

Accepted for publication June 27, 1973.

* Present address: Manor Pharmacy, Warner-Robbins, Ga.

▲ To whom inquiries should be directed.

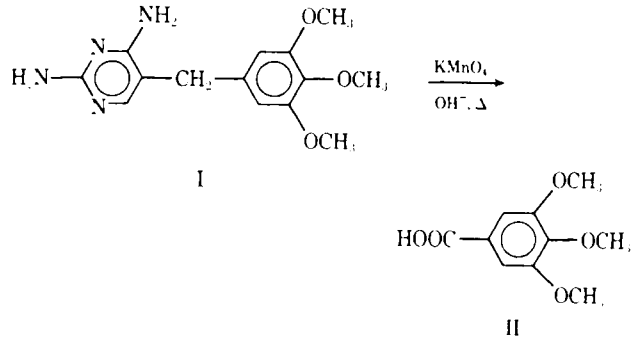
Semiautomated Spectrophotofluorometric Determination of Trimethoprim in Biological Fluids

S. A. KAPLAN▲, R. E. WEINFELD, and T. L. LEE

Abstract □ A semiautomated spectrophotofluorometric method for the determination of trimethoprim in blood, urine, and tissue is described. The initial extraction procedures are performed manually. The subsequent alkaline permanganate oxidation and the chloroform extraction of the fluorescent trimethoxybenzoic acid are performed by the automated system at a rate of 30 specimens/hr. The fluorescence is measured by a microflow cell in a spectrophotofluorometer. The method exhibits the same specificity and precision as the manual procedure, with a sensitivity limit in blood of 0.2 mcg./ml.

Keyphrases □ Trimethoprim in blood, urine, and tissue—semiautomated spectrophotofluorometric analysis □ Spectrophotofluorometry—analysis, semiautomated, trimethoprim in biological fluids

Trimethoprim[1] [2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine, I] is an inhibitor of dihydrofolate reductase which potentiates the activity of sulfonamides against a wide variety of bacterial species (1, 2). The previously reported manual method for determining I in blood and urine (3) met the requirements for specificity, sensitivity, accuracy, and precision. However, the generation of a large number of specimens required



Scheme I

[1] Trimethoprim is an active ingredient in Bactrim, F. Hoffmann-La Roche and Co., Basle, Switzerland.

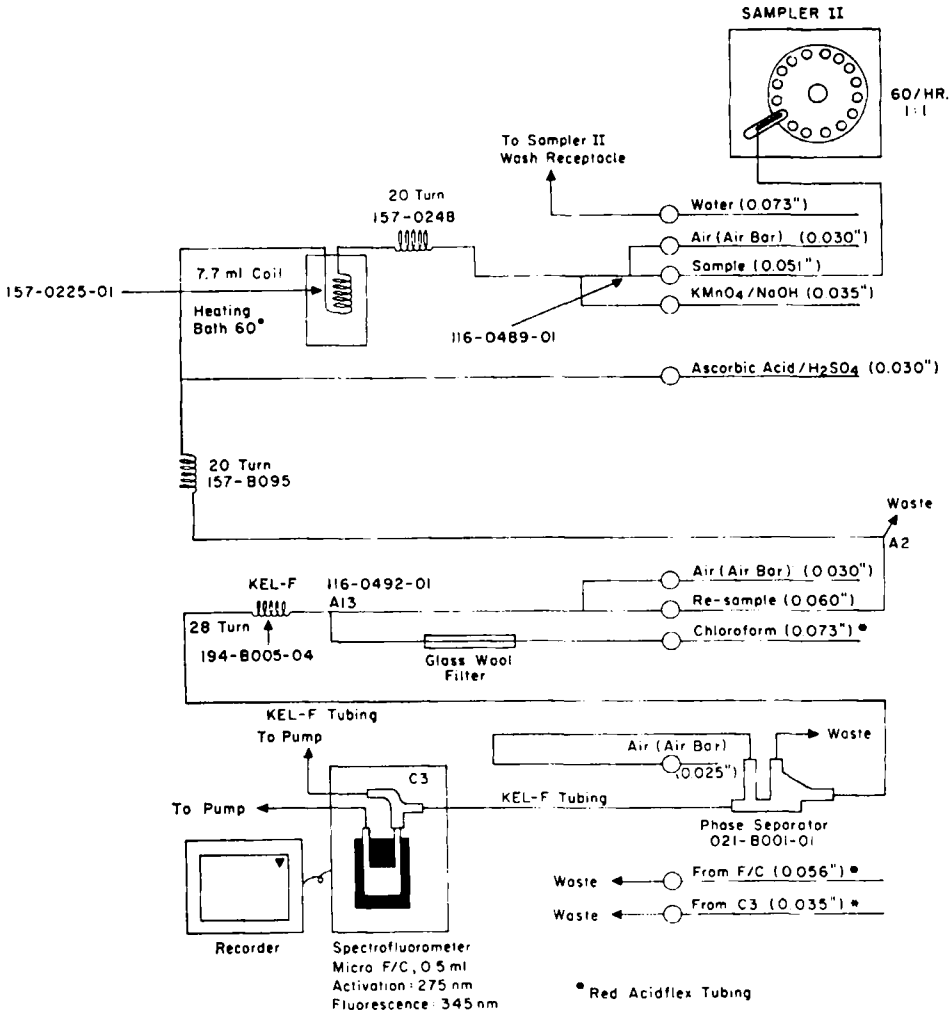


Figure 1—*Flow diagram for the automated determination of trimethoprim. Pump tubing is given with the internal diameter; Tygon tubing was used, except where noted. Technicon part numbers are also indicated.*

speed of analysis. Studies were undertaken to modify the manual spectrophotofluorometric method to make it compatible with continuous-flow automated techniques.

The manual procedure involves the extraction of I from biological specimens at pH 11.0 with chloroform, back-extraction into dilute sulfuric acid, and oxidation with alkaline potassium permanganate at 60° for 20 min. to the fluorescent trimethoxybenzoic acid, II (Scheme I). Following removal of excess permanganate with formaldehyde and subsequent acidification with 1 *N* sulfuric acid, II is extracted into chloroform and the fluorescence is measured at an activation wavelength of 275 nm. and a fluorescence wavelength of 345 nm.

Several modifications of the manual procedure were required to design an automated system using continuous-flow techniques. The manual, 20-min. oxidation step at 60° had to be shortened to approximately 4 min. at 60° for the automated system, and a 12% ascorbic acid solution in 1 *N* sulfuric acid was substituted for the 35% formaldehyde solution to destroy the excess potassium permanganate. The resulting clear acidified aqueous solution was extracted with chloroform in the automated procedure and, following phase separation, the organic phase was passed through the microflow cell of a fluorescence spectrophotometer[2] at the appropriate wavelengths. The finalized automated manifold utilizes automated analysis components[3] and allows for an analysis rate of 30 specimens/hr.

EXPERIMENTAL[4]

Reagents All chemicals are either reagent grade or USP: sodium carbonate, 0.2 *N* aqueous solution; sulfuric acid, 0.01 and 1 *N* aqueous solutions; potassium permanganate, 0.1 *M* in 0.1 *N* NaOH; ascorbic acid USP; 12% (w/v) ascorbic acid solution in 1 *N* H_2SO_4 (prepared fresh daily); and chloroform.

Standards—All standard solutions should be stored in a refrigerator and prepared fresh monthly. The following were used: trimethoprim, 100 mcg./ml. (dissolve 10.0 mg. of I in 0.01 *N* H_2SO_4 in a 100-ml. volumetric flask), and diluted standards of I ranging from 0.05 to 4.0 mcg./ml. in 0.01 *N* H_2SO_4. The standards are required for establishing external standard curves and for preparing internal standards.

[2] Perkin-Elmer.

[3] Technicon AutoAnalyzer II.

[4] The equipment and instruments used were: Technicon Sampler II, Technicon Instruments Corp., Tarrytown, NY 10591; Technicon proportioning pump model III; Techne thermostatic bath model TWB-5 with circulation pump, fitted with special cover for use with Technicon glassware, Techne, Inc., Princeton, NJ 08540; Perkin-Elmer model 204 fluorescence spectrophotometer with 150-w. xenon lamp light source, xenon lamp power supply, and R212 photomultiplier, Perkin-Elmer Corp., Norwalk, CT 06850; Technicon recorder with chart speed of 45.7 cm. (18 in.)/hr.; recorder interface accessory, Perkin-Elmer No. 010-0130; flow cell, rectangular, quartz, 0.5-ml. chamber capacity, 10-mm. light path, Perkin-Elmer No. 220-1461; and Technicon AutoAnalyzer II glassware.

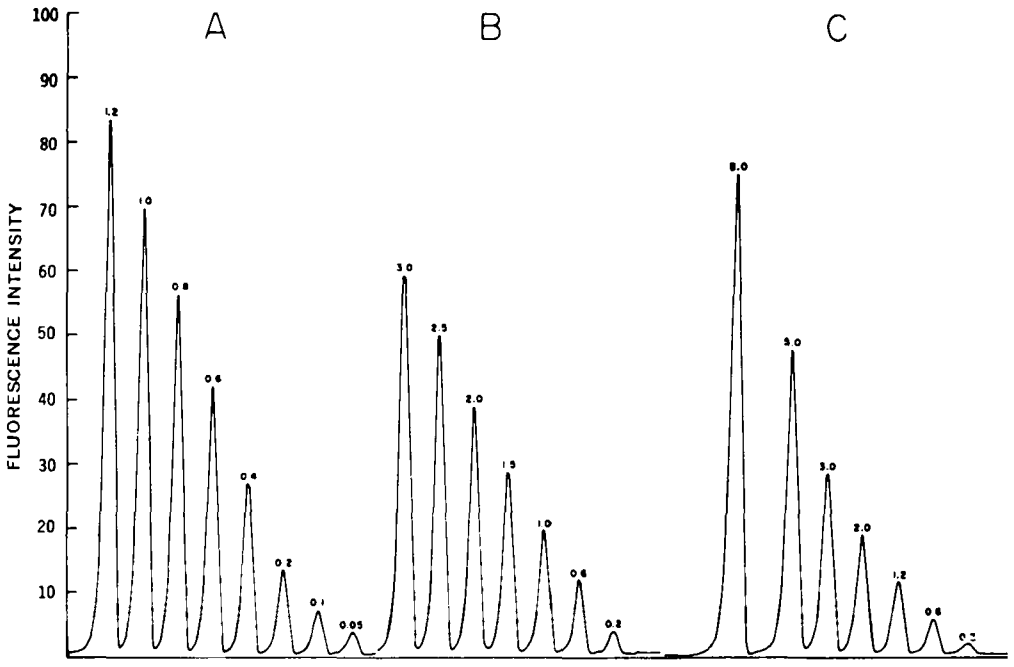


Figure 2—*Chart recordings relating trimethoprim concentration and fluorescence intensity. Instrument sensitivities were: A, 8 × 1; B, 6 × 1; and C, 5 × 1. Flow cell had 0.5-ml. capacity; recorder chart speed was 45.7 cm. (18 in.)/hr.*

Sample Preparation (Manual Extraction)—Pipet 1.0 ml. of blood, urine[5], or 10 ml. tissue homogenate[6] into a 40-ml. glass-stoppered, round-bottom centrifuge tube. Add 5 ml. of 0.2 *N* sodium carbonate solution for blood and urine specimens, and mix[7]. Extract the mixture with 10 ml. chloroform by shaking *slowly* for 15 min. on a mechanical reciprocating shaker. Centrifuge samples for 10 min. at 2000 r.p.m. with stoppers removed, and aspirate aqueous and most of the protein interface. Transfer a fixed aliquot of chloroform (7 or 8 ml.) to a 40-ml. glass-stoppered centrifuge tube, and extract with 5.0 ml. of 0.01 *N* sulfuric acid for blood[5] or tissue and with 10.0 ml. of 0.01 *N* sulfuric acid for urine due to higher concentrations of I[8] in urine. Shake for 10 min. at moderate speed, and centrifuge for 10 min. with stoppers removed. Transfer approximately 95% of the acid phase to 15-ml. glass-stoppered tubes and retain for automated analysis. Control (drug-free) blood, to which 5 or 10 mcg. of I[8] has been added, is analyzed along with the unknown specimens and serves as the internal drug standard. In the case of urine specimens, 30 mcg. of I is added.

Automated Analysis Procedure—The flow diagram for the automated manifold is presented in Fig. 1. Approximately 1 hr. prior to pumping any reagents, the spectrophotofluorometer, heating bath[9], and recorder (not the chart drive) should be turned on. The reagents are then pumped through the system for about 10 min., prior to establishing a reagent blank baseline on the recorder. The sampling cam used is 60/hr. [sample–wash ratio (1:1)] with a water wash cup between extracted samples. Sample cups[10] are used for extracts, standards, and washes.

Prior to introducing unknown extracts, two external standards of I are analyzed in the desired range (0.05–1.6 mcg./ml. for blood or 0.2–4.0 mcg./ml. for urine). The spectrophotofluorometer is adjusted to about 40% of full scale for a midconcentration standard using the instrument sensitivity control. The entire series of external standards of I can then be analyzed[11], placing a water wash cup between each sample.

The acid extracts are transferred to sample cups and placed on the sampler tray, with a water wash between extracts. When analyses of several hours are anticipated, the internal drug standard extract

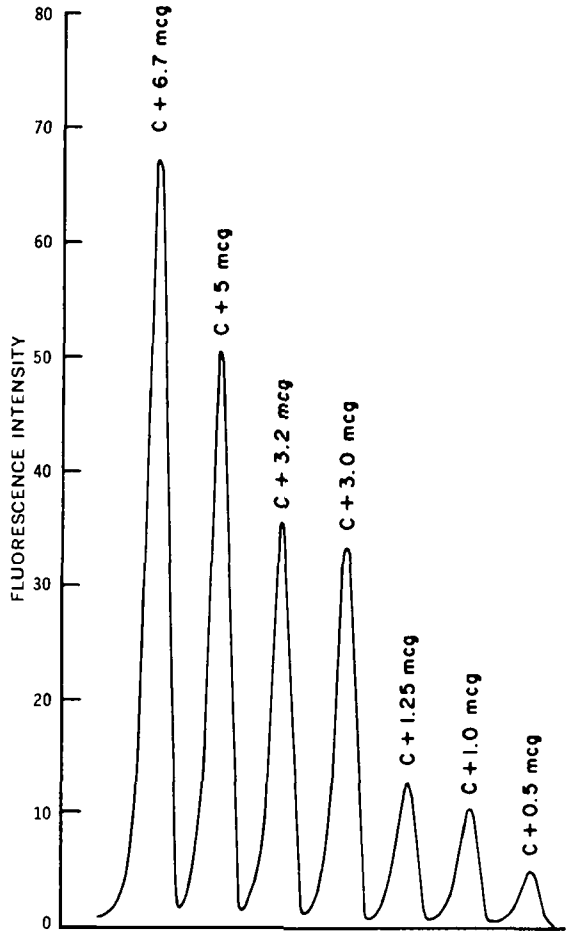


Figure 3—*Chart recording of indicated amounts of trimethoprim added to control (C) blood and analyzed by the described procedure. Acid extract volume = 5 ml.; instrument sensitivity was at 8 × 1.*

[5] Plasma, spinal fluid, or other biological fluids can also be analyzed by the same procedure.

[6] Homogenize a weighed tissue specimen directly with 0.2 *N* sodium carbonate solution. Use approximately 10 ml. sodium carbonate solution/g. tissue.

[7] In the analysis of urine, it is suggested that the pH of this mixture be checked so that the resultant pH is 11.0. Add additional sodium carbonate solution, if required.

[8] If necessary, the acid extracts can be further diluted with 0.01 *N* sulfuric acid to bring them into the range of the standard curve.

[9] Heating bath filled with ethylene glycol.

[10] Technicon.

[11] For routine analyses, it is unnecessary to run the entire standard curve. Duplicate determinations of the midconcentration standard are satisfactory for calculating percent recovery of the internal standard samples.

Table I—Concentration of Trimethoprim in Blood Determined by Manual and Automated Methods

Sample	Methods: Automated (A), mcg./ml.	Manual (B) Using Ascorbic Acid, mcg./ml.	Manual (C) Using Formaldehyde, mcg./ml.	Ratios: A/B	A/C	B/C
1	5.35	5.19	5.06	1.03	1.06	1.03
2	5.37	5.68	5.44	0.95	0.99	1.04
3	4.64	4.76	4.80	0.97	0.97	0.99
4	4.54	4.72	4.31	0.96	1.05	1.10
5	3.95	3.67	3.90	1.08	1.01	0.94
6	3.49	3.32	3.35	1.05	1.04	0.99
7	2.59	2.61	2.77	0.99	0.94	0.94
8	1.39	1.52	1.35	0.91	1.03	1.13
9	2.97	2.75	3.26	1.08	0.91	0.84
10	5.07	5.68	5.00	0.89	1.01	1.14
11	4.48	4.51	4.49	0.99	1.00	1.00
12	4.15	4.38	4.31	0.95	0.96	1.02
13	3.85	3.82	3.81	1.00	1.01	1.00
14	3.38	3.18	3.49	1.06	0.97	0.91
15	2.37	2.26	2.55	1.05	0.93	0.87
16	1.12	1.30	1.26	0.86	0.89	1.03
17	1.71	1.60	1.87	1.06	0.91	0.86
18	2.17	2.46	2.44	0.88	0.89	1.00
19	2.84	2.92	2.87	0.97	0.99	1.02
20	2.54	2.88	2.90	0.88	0.88	0.99
21	2.46	2.68	2.64	0.92	0.93	1.02
22	2.32	2.31	2.50	1.00	0.93	0.92
23	1.97	2.27	2.09	0.87	0.94	1.09
24	1.16	1.27	1.33	0.91	0.87	0.95
Mean ± *SE*				0.971 ± 0.014	0.963 ± 0.015	0.993 ± 0.016

should be reevaluated several times to check for any significant change in instrumental response.

The manifold (Fig. 1) performs the following steps. Acid extracts are aspirated from the sampler into the system, segmented with air, and mixed with alkaline potassium permanganate solution in a 20-turn coil. Oxidation is accomplished by passing the sample stream into a 7.7-ml. capacity coil (2.0 mm. i.d.) placed in a heating bath at 60°. The sample stream is then mixed with the ascorbic acid–sulfuric acid solution in a 20-turn coil to remove the excess potassium permanganate and acidify the stream, which is then debubbled, resampled through the pump, and resegmented with air. Chloroform is pumped directly with red pump tubing[12] through a glass-wool filter and mixed with the sample stream in a 28-turn coil[13] [0.10 cm. (0.040 in.) i.d.] to accomplish extraction of II into the chloroform. The mixture is passed through a four-point phase separator[3] with Teflon insert. The lower chloroform phase is resegmented with air and transferred through tubing to the flow cell in the spectrophotofluorometer. The sample stream is debubbled prior to entrance to the flow cell where the fluorescence of the chloroform stream is measured. The total time of analysis from sample aspiration to appearance on the recorder is about 12 min.

At the conclusion of the day's analyses, all tubing carrying aqueous reagents are washed through with water, while tubing carrying solvents are washed through with methanol and pumped with air until dry.

RESULTS

Standard Curves—Recorder tracings of external standards of I obtained by the automated procedure are shown in Fig. 2. Spectrophotofluorometer sensitivity settings are indicated in each case. Extracts of control blood specimens to which known amounts of I were added and a series of unknown human blood specimens following administration of the drug analyzed by the automated procedure are shown in Figs. 3 and 4. Extracts of control urine specimens to which known amounts of I were added and a series of unknown human urine specimens analyzed by the automated procedure are shown in Fig. 5. A plot of concentration of I (external and internal drug standards) *versus* fluorescence intensity is shown in Fig. 6 for the 0.05–1.2-mcg./ml. range and in Fig. 7 for the 0.2–4-mcg./ml. range. The fluorescence is linear up to 8 mcg./ml. of I, which is equivalent to approximately 4.10 mcg./ml. of II[14] under the described conditions.

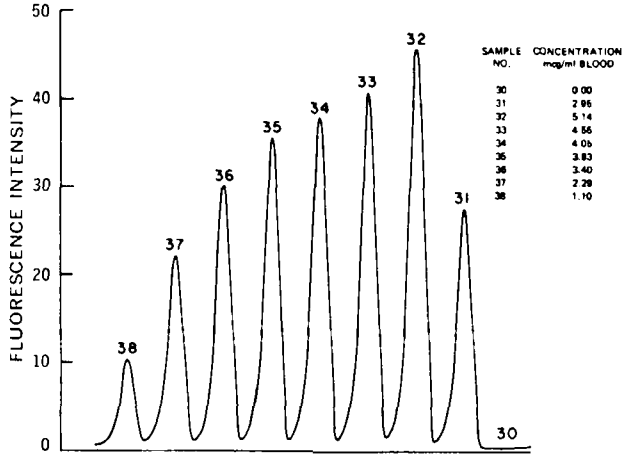


Figure 4—*Chart recording and corresponding concentrations of trimethoprim blood specimens following a single oral dose of the combination trimethoprim and sulfamethoxazole (400 and 2000 mg., respectively) formulation in man. Specimens correspond to 0, 1, 2, 3, 4, 6, 8, 12, and 24 hr. Acid extract volume = 5.0 ml.; instrument sensitivity was at 8 × 1.*

Extracts with a fluorescence intensity higher than the 8 mcg./ml. of I standard (or higher than the most concentrated standard being analyzed) must be diluted with 0.01 *N* sulfuric acid and rerun to bring the fluorescence into the linear portion of the standard curve.

Calculations—The fluorescence intensity at the apex of each peak is read directly from the chart recording. Reagent or control blood fluorescence is subtracted to give the net fluorescence due to I present. The concentration of I in the unknown specimens is di-

[12] Acidflex, Technicon.
[13] Kel-F, Technicon.
[14] Trimethoprim is oxidized to trimethoxybenzoic acid to the extent of about 70%; thus, the theoretical yield of 5.85 mcg./ml. trimethoxybenzoic acid is not attained.

Table II—Concentration of Trimethoprim in Urine Determined by Manual and Automated Methods

Sample	Methods: Automated (A), mcg./ml.	Manual (B) Using Ascorbic Acid, mcg./ml.	Manual (C) Using Formaldehyde, mcg./ml.	Ratios: A/B	A/C	B/C
1	4.4	4.1	4.0	1.02	1.11	1.08
2	7.1	6.9	6.4	1.07	1.09	1.02
3	173.3	155.6	216.1	1.11	0.80	0.72
4	34.6	33.3	32.4	1.04	1.07	1.03
5	175.7	156.4	171.5	1.12	1.02	0.91
6	28.7	33.3	28.3	0.86	1.01	1.18
7	72.8	68.7	74.8	1.06	0.97	0.92
8	29.2	30.2	29.3	0.97	1.00	1.03
9	9.8	8.5	9.7	1.15	1.01	0.88
10	75.4	75.3	75.1	1.00	1.00	1.00
11	45.7	41.3	47.2	1.11	0.97	0.88
12	162.2	160.9	179.4	1.01	0.90	0.90
13	36.5	38.0	35.0	0.96	1.04	1.09
Mean ± *SE*				1.037 ± 0.022	0.999 ± 0.022	0.972 ± 0.033

rectly proportional to the internal drug standard carried through the entire procedure. If further dilutions are necessary to bring the unknown extracts into the concentration range of the standards, they must be considered in calculating the final results. For urine specimens, dilution will be encountered more frequently since a much wider range of I values are encountered as compared to blood specimens.

The reproducibility of the automated steps of the procedure was determined by performing 34 replicate samplings of a 0.6 mcg./ml. I standard solution, at the spectrophotofluorometric sensitivity setting of 8 × 1 required for blood level studies, and was found to be 0.60 mcg./ml. ● 0.030 (*SD*).

The precision of the overall method including the manual sample preparation, manual extraction steps, automated steps, and fluorescence measurements was determined by the preparation and analysis of an internal drug standard, 10 mcg. I/ml. blood. This evaluation was based on the determination of the internal standard, in duplicate, by the automated section of the procedure on 11 separate days. The results of these analyses show that the precision of a single determination is approximately ±9.1%. This value was developed from analysis of variance which estimated both the variation within a day and between days. Comparable data evaluation based on single-day multiple determinations of drug internal standards using the manual method was reported to be ±6.2% (3).

Specimens of blood and urine containing I were analyzed by the manual and automated procedures to compare results. The published manual procedure (3) using formaldehyde to remove the excess potassium permanganate and the manual version of the automated procedure substituting 12% ascorbic acid were compared. This was necessary to demonstrate that the use of ascorbic acid in the automated procedure did not alter the chemical basis of the assay.

The results for 24 separate blood specimens analyzed by the three methods are shown in Table I, and results for 13 separate urine specimens containing a wide range of I concentrations are shown in Table II. Correlation of the results of these assays was tested by fitting a line to the joint determinations (A *versus* B, A *versus* C, and B *versus* C) and developing a 95% confidence region for the intercept and slope. The confidence limits for the intercept when the slope is fixed at unity are shown in Table III.

Significant differences would be reflected in confidence limits of the same sign. However, in those instances where this occurs, the calculated significance appears to be marginal (Table III).

Control bloods and urines exhibited no increase in fluorescence

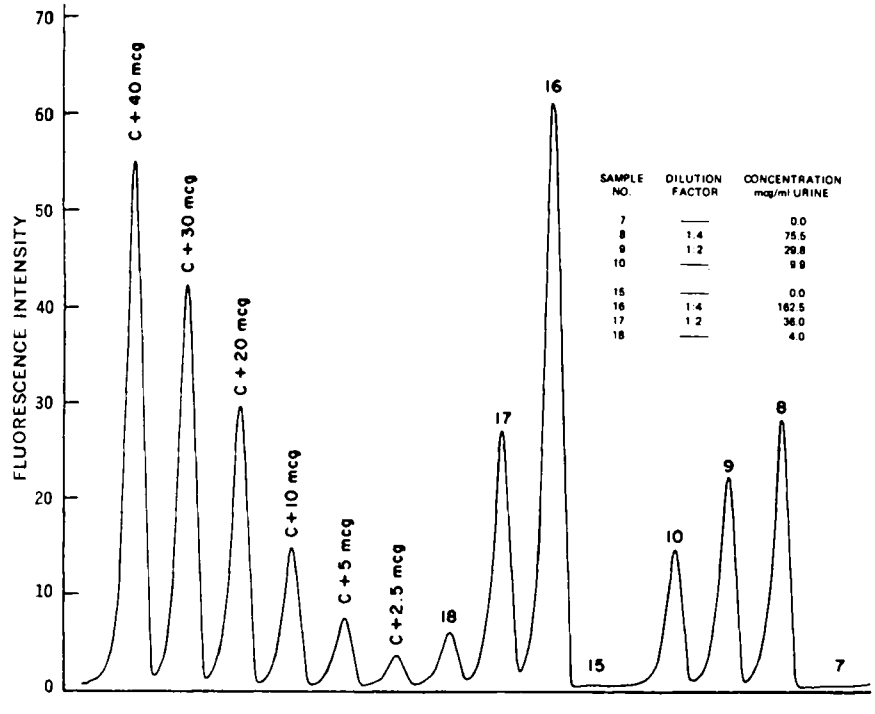


Figure 5—*Chart recording and corresponding concentrations of trimethoprim urine specimens following a single oral dose of the combination trimethoprim and sulfamethoxazole (400 and 2000 mg., respectively) formulation in two subjects and urine internal standards of the indicated amounts of trimethoprim added. Specimens correspond to control, 0–24, 24–48, and 48–72 hr. in each subject. Acid extract volume = 10.0 ml. (with appropriate dilutions); instrument sensitivity was at 6 × 1.*

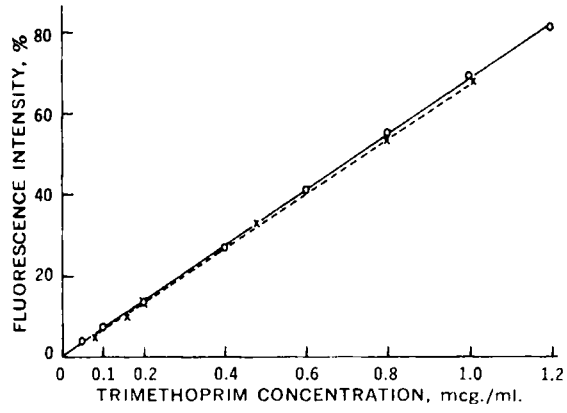


Figure 6—*Plot of trimethoprim standards (direct and recovered from blood) versus fluorescence intensity by automated procedure. Trimethoprim concentration, mcg./ml. acid expected. Instrument sensitivity was at 8 × 1. Key: O—O, direct standards; and ×- - -×, added to blood.*

compared to a reagent blank when analyzed by the automated procedure. No shifting of baseline was noted for control bloods, urines, and reagent blank.

The specificity of the automated procedure is the same as that previously reported for the manual procedure (3). In both the automated and manual procedures, it was demonstrated that physiological components of human plasma or blood do not interfere with the determination of I. Sulfonamides normally administered along with trimethoprim do not interfere with the analytical procedure (3).

DISCUSSION

The direct automation of the manual procedure for the determination of trimethoprim in blood and urine presented several major problems. Use of formaldehyde in the automated system resulted in the formation of the particulate brown manganese dioxide which clogged the mixing coil after several specimens passed through the system. Attempts to break up the precipitate proved difficult due to the large amounts formed. The same procedure was attempted using a solution of sodium bisulfite in place of formaldehyde. Although this process eliminated much of the precipitate problem, the resulting dissolved sulfur dioxide interfered with the fluorescence of II in the chloroform. To overcome this problem, a solution of sodium tetrachloromercurate was unsuccessfully used to complex the large amounts of sulfur dioxide produced (4). Ascorbic acid proved to be an excellent reagent for removing excess potassium permanganate from the reaction mixture. A 12% aqueous solution of ascorbic acid produced negligible particulate manganese dioxide, which was completely dissolved after several turns through the mixing coil. Preparation of the ascorbic acid in 1 *N* sulfuric acid proved to be more efficient than sequentially adding each reagent to the reaction mixture.

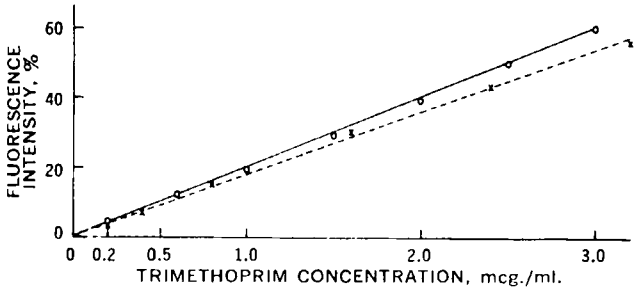


Figure 7—*Plot of trimethoprim standards (direct and recovered from blood) versus fluorescence intensity by automated procedure. Trimethoprim concentration, mcg./ml. acid expected. Instrument sensitivity was at 6 × 1. Key: O—O, direct standards; and ×- - -×, added to blood.*

Table III -Confidence Intervals

Fluid Analyzed	Method Y	Method X	95% Confidence Interval for Intercept when Slope = 1	Correlation Coefficient (*r* Value)
Blood	A	B	(−0.26, 0.01)	0.987
Blood	A	C	(−0.09, −0.02)	0.994
Blood	B	C	(−0.05, 0.16)	0.985
Urine	A	B	(0.7, 7.0)	0.997
Urine	A	C	(−15.0, 3.8)	0.990
Urine	B	C	(−18.4, −0.5)	0.987

Working with Kel-F tubing yielded better results than Teflon tubing, particularly for the extraction coil and less importantly for the transfer lines, due to their surface tension properties.

In developing the automated manifold for I, it was found that the use of AutoAnalyzer II glassware (mixing coils, injection fittings, phase separators, and tubing) resulted in superior overall reproducibility as compared with AutoAnalyzer I components. The former system was the most efficient regarding reagent consumption, reproducibility of bubble pattern, and phase separation and is considered the system of choice.

Development of a fully automated manifold for the determination of trimethoprim is being investigated. This will involve direct extraction of the buffered biological fluid with chloroform on the manifold and back-extraction of the chloroform with dilute sulfuric acid prior to the automated procedure described.

SUMMARY

A semiautomated spectrophotofluorometric method for the determination of trimethoprim in blood and other biological specimens is described. The automated procedure has the same specificity and precision as the manual procedure and allows for the analysis of 30 specimens/hr.

REFERENCES

(1) E. Grunberg and W. F. DeLorenzo, *Antimicrob. Ag. Chemother.*, **1966**, 430.

(2) S. R. M. Bushby and G. H. Hitchings, *Brit. J. Pharmacol. Chemother.*, **33**, 72(1968).

(3) D. E. Schwartz, B. A. Koechlin, and R. E. Weinfeld, *Chemotherapy (Suppl.)*, **14**, 22(1969).

(4) W. West and G. C. Gaeke, *Anal. Chem.*, **28**, 1816(1956).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 22, 1973, from the *Department of Biochemistry and Drug Metabolism, Hoffmann-La Roche Inc., Nutley, NJ 07110*

Accepted for publication June 14, 1973.

▲ To whom inquiries should be directed.