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DETERMINATION OF TRIMETHOPRIM IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid, sensitive, and specific high-performance liquid chromatographic assay was developed for the determination of trimethoprim in blood, plasma, and urine using normal-phase (adsorption) chromatography on a microparticulate silica column and UV monitoring at 280 nm. Trimethoprim is selectively extracted from the biological sample matrix at alkaline pH with chloroform, providing nearly quantitative extraction (>95%) and a sensitivity limit of 0.01 to 0.02 μg/ml blood or plasma, without interference from sulfonamides.

INTRODUCTION

Trimethoprim (TMP, Fig. 1) is a synthetic antibacterial, marketed in combination with sulfamethoxazole (SMZ)* and used extensively in the treatment of a variety of infections in man [1—3].

Analytical methods for the quantitation of trimethoprim in biological fluids have included microbiological assay [2], spectrofluorometry [4, 5], differential pulse polarography [6], gas—liquid chromatography [7], thin-layer chromatography [8], and high-performance liquid chromatography (HPLC) [9—11]. Many of these procedures involve tedious sample preparation steps and have sensitivity limits of about 0.1 μg TMP per ml of plasma or blood.

Following a single therapeutic dose of TMP in 24 normal subjects (400 mg TMP in combination with 2.0 g SMZ), the reported mean blood levels ranged from 3.21 μg TMP per ml at 2 h to 1.12 μg TMP per ml at 24 h [12]. These concentrations are easily determined by the existing methodology [2, 4—11]. However, in various clinical situations, such as limited sample size (<1.0 ml plasma) from pediatric patients, low single oral doses of TMP (100 mg or

*Bactrim ™ (Roche Laboratories Division, Hoffmann-La Roche), and Septra™ (Burroughs Welcome); trimethoprim—sulfamethoxazole (1:5).

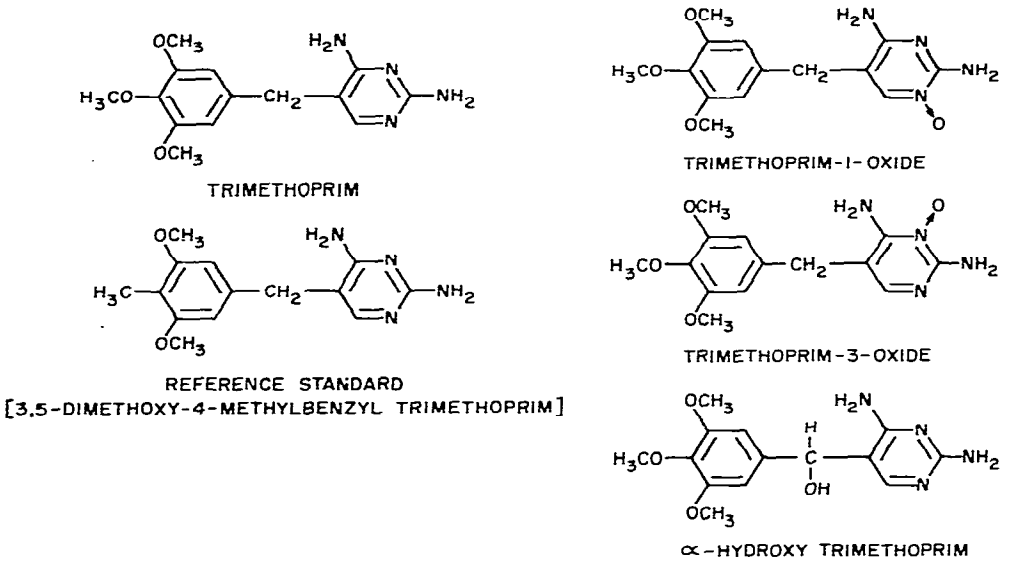

Fig. 1. Structure of trimethoprim, trimethoprim metabolites and the reference standard.

less), and in bioequivalency and pharmacokinetic studies, many of these methods are not sufficiently sensitive (0.01 μg/ml) for the reliable determination of a plasma level—time curve from 0 to 48 h. An HPLC method that is specific, precise, rapid, simple to perform, and with a sensitivity limit of 0.01 to 0.02 μg/ml (using a 2-ml blood or plasma specimen) has been developed. The assay utilizes adsorption (normal-phase) chromatography with isocratic elution at ambient temperature and measurement of the UV absorbance of the eluant at 280 nm. Quantitation is performed using the peak height ratio of TMP to a reference standard, the 2,4-diamino-5-(3,5-dimethoxy-4-methylbenzyl)-pyrimidine analog of TMP. The assay can also be applied to the measurement of the urinary excretion of TMP.

MATERIALS AND METHODS

Reagents

Reagent grade chloroform (a) for the extraction solvent was obtained from Fisher Scientific (Pittsburgh, Pa., U.S.A.). Methanol and chloroform (b), which was used for the chromatography solvent, preserved in 1% ethanol, were obtained from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.). 0.2 *N* aqueous sodium carbonate and 0.01 *N* aqueous sulfuric acid were prepared. Concentrated ammonium hydroxide (Mallinckrodt, St. Louis, Mo., U.S.A.) was 28—30%, sp. gr. 0.90.

The mobile phase was composed of chloroform (b) and a mixture of methanol—water—ammonium hydroxide (150:9:1, v/v) in a 500:25 (v/v) ratio. Prepare fresh for each chromatographic run, and vacuum degas for approximately 5 min with ultrasonic vibrating.

Apparatus

A Waters Assoc. high-performance liquid chromatographic system consisting of the following components: Model 6000A solvent delivery system, Model U6K sample injector, and a Model 440 UV detector operated at 0.01 or 0.02 a.u.f.s. with a 280-nm wavelength kit (Waters Assoc., Milford, Mass., U.S.A.), in conjunction with a 10-mV recorder (Model 56 Perkin-Elmer) and a 10-μm silica gel column (μPorasil, Waters Assoc.), 30 cm × 3.9 mm I.D., was used for chromatographic analysis. The mobile phase flow-rate was 1.5 ml/min at a pressure of 500 p.s.i.

Under these conditions, the reference standard and TMP have retention times (t_R) of approximately 4.8 and 5.3 min, respectively, and 100 ng of the reference standard and TMP give about 50% full-scale response at 0.02 a.u.f.s.

Standard solutions (aqueous) for preparation of blood, plasma, and urine standards

TMP. A stock solution of 100 μg/ml was prepared by accurately weighing 10.0 mg of pure material (pharmaceutical grade, >99% purity) and dissolving in 100 ml of 0.01 *N* sulfuric acid in a volumetric flask. A 1:10 dilution of the stock solution in distilled water yielded a working standard solution of 10 μg TMP per ml.

Reference standard

A TMP analog, 2,4-diamino-5-(3,5-dimethoxy-4-methylbenzyl)-pyrimidine (Fig. 1), was used as the reference standard. A stock solution of 100 μg/ml in 0.01 *N* sulfuric acid was prepared, followed by a 1:10 dilution of the stock solution in distilled water to yield a 10 μg/ml working standard solution.

HPLC standards (external standards)

A stock solution of TMP, 100 μg/ml in chloroform (b), and a stock solution of the reference standard, 100 μg/ml in chloroform (b) were prepared. A series of standard solutions are then prepared by diluting aliquots of the stock solutions to 10 ml in volumetric flasks with chloroform (b) as shown below for use in establishing a calibration curve from 0.020 to 0.200 μg of TMP containing 0.100 μg of reference standard per 10 μl injected:

Solution	TMP (stock) (ml)	Conc. TMP (μg/ml)	Conc. TMP (μg/10 μl)	Reference standard (stock) (ml)
1	0.2	2	0.020	1.0
2	0.5	5	0.050	1.0
3	1.0	10	0.100	1.0
4	2.0	20	0.200	1.0

Plasma or blood sample preparation

Into 50-ml glass-stoppered round bottom centrifuge tubes, 1.0 μg of the reference standard (100-μl aliquot of working standard solution in 0.01 *N* sulfuric acid) was added. A 2.0-ml aliquot (or less) of plasma or blood was taken for each unknown and added to the reference standard followed by 5 ml of 0.2 *N* sodium carbonate solution. The samples were mixed well on a

Vortex mixer, and then 12 ml of chloroform was added to each tube using a Repipet (Lab Industries, Berkeley, Calif., U.S.A.). Internal standards of 0.2, 0.5, 1.0, and 2.0 μg of TMP were included, by the addition of 20-, 50-, 100-, and 200-μl aliquots, respectively, of the 10 μg/ml working standard solution of TMP in 0.01 *N* sulfuric acid along with 1.0 μg of reference standard (100 μl of the working standard solution in 0.01 *N* sulfuric acid) to separate 2-ml specimens of control plasma or blood. A control plasma, blood or urine sample was also included without addition of the internal standard to determine if interfering endogenous substances were present in the extracts.

All samples were stoppered with PTFE No. 16 stoppers and shaken for 10 min on a reciprocating shaker at a slow to moderate speed. The samples were then centrifuged for 10 min at 1000 *g* at 5 to 10° in a refrigerated centrifuge

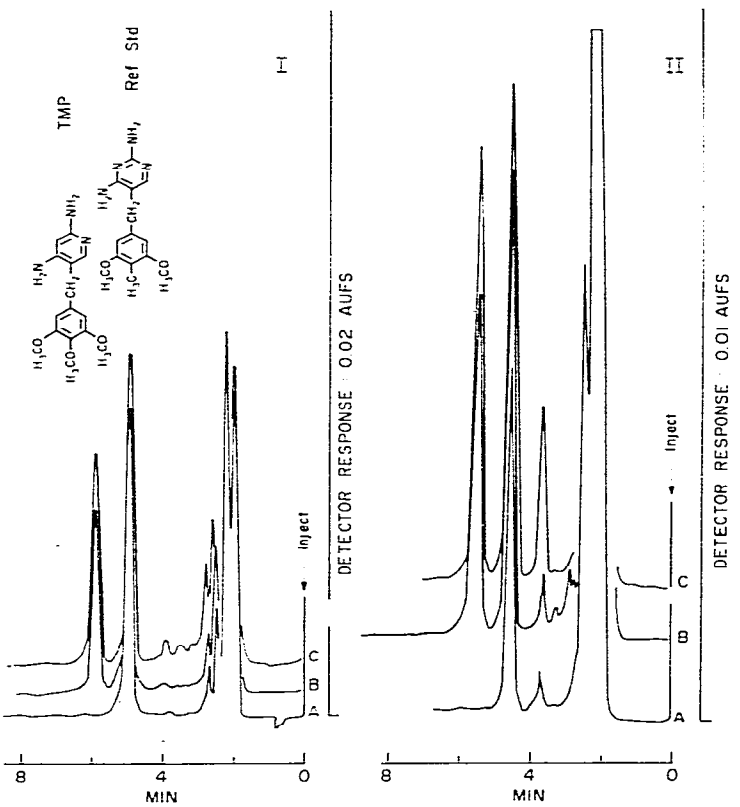

Fig. 2. Chromatogram I: Extracts of: (A) control human blood extract with reference standard (1.0 μg added), 10 μl/100 μl injected; (B) control human blood extract with 1.0 μg TMP and 1.0 μg reference standard added, 10 μl/100 μl injected; and (C) 8-h blood sample extract following administration of 160 mg TMP orally, with 1.0 μg reference standard added, 10 μl/100 μl injected. Chromatogram II: Extracts of: (A) control human plasma extract with reference standard (1.0 μg added), 10 μl/100 μl injected; (B) control human plasma extract with 1.0 μg TMP and 1.0 μg reference standard added, 10 μl/100 μl injected; and (C) 8-h plasma sample extract following administration of 100 mg TMP orally, with 1.0 μg reference standard added, 10 μl/100 μl injected.

(Model PR-J with a No. 253 horizontal head; Damon/IEC Division, Needham, Mass., U.S.A.). The aqueous layer was aspirated and discarded and a 9—10 ml aliquot of chloroform was carefully removed from each sample and transferred to a clean 15-ml conical centrifuge tube. The chloroform was evaporated to dryness under a stream of nitrogen in a N-EVAP assembly (Organomation Assoc., Worcester, Mass., U.S.A.) at 50°. The residues were dissolved in 100 μl (or less) of mobile phase and 10 μl routinely injected for HPLC analysis. Typical chromatograms of plasma and blood extracts are shown in Fig. 2.

Urine sample preparation

For urine specimens, the 24-h and 48-h fractions were diluted 1:10 with water prior to analysis (usually there is sufficient intact drug excreted up to 48 h to permit this dilution and still be able to quantitate the levels). Urine fractions collected beyond 48 h were not diluted. A 1.0-ml aliquot of diluted or undiluted urine specimen was taken for analysis and treated exactly in the manner described for plasma and blood with the exception of the addition of 2 μg of reference standard (200 μl of the working standard solution in 0.01 *N* sulfuric acid) to the unknowns. Internal standards of 1, 2, 5, and 10 μg of TMP per ml (addition of 0.1, 0.2, 0.5, and 1.0 ml working standard solution in 0.01 *N* sulfuric acid, respectively, to diluted or undiluted control urine) and 2 μg of reference standard (200 μl of the working standard solution in 0.01 *N* sulfuric acid) added to each tube were included with each set of unknowns. Typical chromatograms of urine extracts are shown in Fig. 3.

Calculations

The concentrations of TMP in the unknown plasma, blood or urine samples were determined by a computer program using a least-squares best fit straight line established from the experimentally determined peak height ratio of TMP to the reference standard in the internal standards and the unknowns. The equation of a typical calibration curve from 0.1 to 5.0 μg/ml was $y = 0.343\ x + 0.0803$ and the coefficient of correlation was 0.9993.

Recovery and sensitivity limits

The recovery of TMP was found to be 95 ± 5% S.D. over the range 0.1 to 10 μg/ml plasma, blood, and urine (using 2 ml of plasma, blood, and 1.0 ml of undiluted or diluted urine and the injection of 10 μl from 100 μl of the reconstituted residues). The sensitivity limit can be increased to 0.01 μg of TMP per ml of plasma or blood by the injection of a 10-μl aliquot from 25 μl of a reconstituted extract, operating the detector at an attenuation of 0.005 a.u.f.s.

Specificity of the method

The method was found to be specific for TMP, without interference from endogenous plasma or blood extracted materials or three known metabolites of TMP (Fig. 1). Authentic standards of three identified metabolites of TMP in plasma, the N-1-oxide, N-3-oxide, and the α-hydroxy metabolite were chromatographed to determine their retention times (t_R) under the conditions used for the analysis of TMP. The retention times of these metabolites and two sulfonamides are presented in Table I. It appeared that the N-1-oxide of

TMP, if present in the extracts would interfere with the quantitation by affecting the peak height of the reference standard. However, when a series of blood extracts from a subject who had received 400 mg TMP were prepared without addition of the reference standard, no indication of the presence of the N-1-

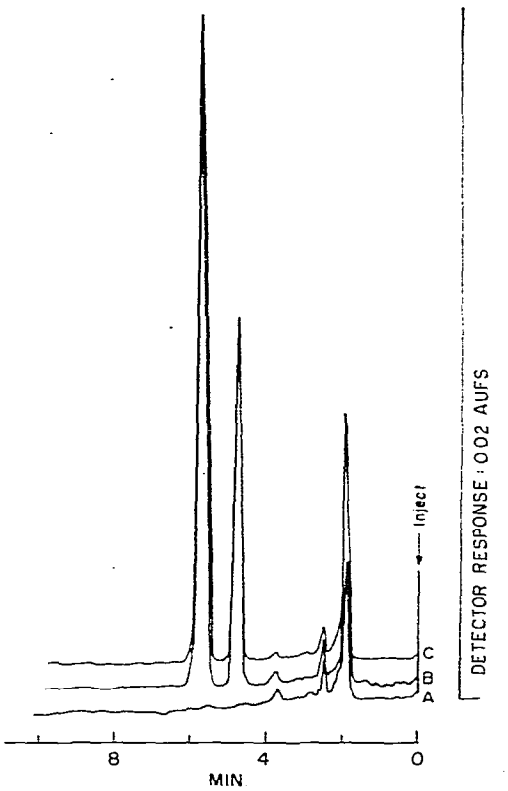

Fig. 3. Chromatograms of (A) control human urine extract (24-h collection) diluted 1:10 prior to extraction; (B) control urine extract with 5.0 μg TMP and 2.0 μg reference standard, 10 μl/200 μl injected; and (C) human urine sample extract from 0—24-h collection following a 160-mg single oral dose of TMP diluted 1:10 prior to extraction, 10 μl/200 μl injected.

TABLE I

RETENTION TIMES OF TMP AND RELATED COMPOUNDS BY HPLC

Compound	t_R (min)
TMP	5.6
Reference standard	4.73
N-1-oxide of TMP	4.80
N-3-oxide of TMP	29
α-Hydroxy-TMP	18
SMZ	7.1
N_4-Acetyl-SMZ	27

oxide was observed in the chromatograms of specimens taken from 1 h to 24 h following TMP administration.

Interference in the quantitation of the urine extracts as a result of the presence of the N-1-oxide metabolite was not encountered, although levels of that metabolite have been reported following oral administration of TMP in man [9, 13]. In these reports only about 2.1% of the administered dose was excreted as the N-1-oxide in 24 h, and in addition, authentic standards of the N-1-oxide are poorly recovered under the extraction conditions of this assay (<50%). Therefore, the method presented here is specific for TMP using the reference standard as described.

SMZ and the N4-acetyl SMZ metabolite do not interfere with the analysis of TMP since they are not extractable from blood, plasma or urine at pH 11 into chloroform, and in addition, they are chromatographically separated in this system (Table I). Possible interference from other classes of compounds was not evaluated in this system.

Application of the method to biological specimens

One normal adult volunteer was administered two different formulations of Bactrim™ (TMP, 160 mg and SMZ, 800 mg) on separate occasions. Oxalated whole blood specimens were collected prior to dosing, and at 1, 2, 3, 4, 6, 8, 12, and 24 h following dosing. In addition, urine specimens were collected prior to dosing and at 0 to 24, 24 to 48, and 48 to 72 h following dosing. The period between administration of the two formulations was two weeks to ensure a sufficient drug washout period. The blood and urine specimens were assayed by the HPLC method described and also assayed by a previously reported spectrofluorometric method [5]. The data obtained for these analyses are given in Tables II and III. The coefficients of correlation for the two sets of blood level data were 0.957 and 0.994, respectively. The coefficients of correlation for the two sets of urine data in Table III were 0.999 and 0.988, respectively.

TABLE II

COMPARISON OF TMP BLOOD LEVELS (μg/ml) IN ONE SUBJECT FOLLOWING ORAL ADMINISTRATION OF TWO BACTRIM FORMULATIONS* BY SPECTROFLUOROMETRIC AND HPLC METHODS

Time (h)	Formulation A		Formulation B	
	Fluorometric	HPLC (UV)	Fluorometric	HPLC (UV)
0	0	0	0	0
1	0.75	0.69	0.71	0.71
2	1.12	1.11	1.72	1.64
3	1.45	1.26	1.69	1.74
4	1.29	1.34	1.60	1.65
6	1.18	1.10	1.44	1.47
8	1.00	1.12	1.26	1.27
12	0.91	0.89	0.89	1.00
24	0.33	0.47	0.39	0.41

*160 mg TMP and 800 mg SMZ administered.

TABLE III

COMPARISON OF TMP URINARY EXCRETION LEVELS (mg) IN ONE SUBJECT FOLLOWING ORAL ADMINISTRATION OF BACTRIM FORMULATIONS* BY SPECTROFLUOROMETRIC AND HPLC METHODS

Time period (h)	Formulation A		Formulation B	
	Fluorometric	HPLC (UV)	Fluorometric	HPLC (UV)
Control	0	0	0	0
0—24	56.4	51.3	63.0	55.0
24—48	20.7	19.8	28.2	31.7
48—72	2.4	1.7	3.4	3.0
cumulative 0—72	79.5	72.8	94.6	89.7
% of administered dose	49.7%	45.5%	59.1%	56.1%

*160 mg TMP and 800 mg SMZ administered.

Another study involving administration of 100-mg TMP formulations to a volunteer and collection of plasma specimens prior to administration, and at 0.5, 1, 2, 3, 4, 8, 12, 24, and 48 h following dosing, was analyzed using the HPLC method. The data for that subject are shown in Table IV. The HPLC method was successfully used to quantitate TMP plasma levels as low as 0.01 μg/ml and demonstrates the utility of the method on a routine basis.

TABLE IV

TMP PLASMA LEVELS (μg/ml) IN ONE SUBJECT FOLLOWING ORAL ADMINISTRATION OF TWO TMP FORMULATIONS (100 mg TMP) BY HPLC ASSAY

Time (h)	Formulation	
	A	B
0	0	0
0.5	0.91	0.57
1	0.84	0.86
2	0.79	0.92
3	0.77	0.80
4	0.69	0.64
8	0.51	0.55
12	0.32	0.36
24	0.10	0.10
48	0.02	0.01

RESULTS AND DISCUSSION

A rapid, sensitive, and specific HPLC assay was developed for the determination of TMP in blood, plasma, and urine using normal-phase (adsorption)

chromatography on a microparticulate silica column. TMP is selectively extracted from the biological sample matrix at alkaline pH into chloroform, providing nearly quantitative extraction (>95%) and a sensitivity limit of 0.01 to 0.02 μg/ml of blood or plasma.

The UV absorption spectrum for TMP in the HPLC mobile phase used is shown in Fig. 4. The absorbance maximum is at 290 nm, and is sufficiently sensitive to allow for detection of nanogram quantities of TMP in that mobile phase at 280 nm using a fixed-wavelength detector. TMP, being a weak difunctional base with a pK_a of 7.2, required a basic modifier in an organic solvent to reduce its elution time from a silica column. The mobile phase used in the development of this method is a modification of a system reported by Bye and Brown [10]. The use of small percentages (<1%) of ammonium hydroxide and distilled water in a chloroform—methanol mobile phase on a silica column was found to be effective in reducing the elution time of TMP, without resorting to high percentages of polar organic solvents (e.g., methanol) or organic amines (e.g., diethanolamine), which could result in column deterioration or reduction in column life. This HPLC mobile phase has been used daily for six months, without any signs of deterioration of column performance. Typical chromatograms of blood and plasma extracts are shown in Fig. 2, and under these conditions of analysis, the efficiency of the column was calculated to be approximately 20,000 plates per meter. After each use of the column with this mobile phase, chloroform is pumped through the column to remove

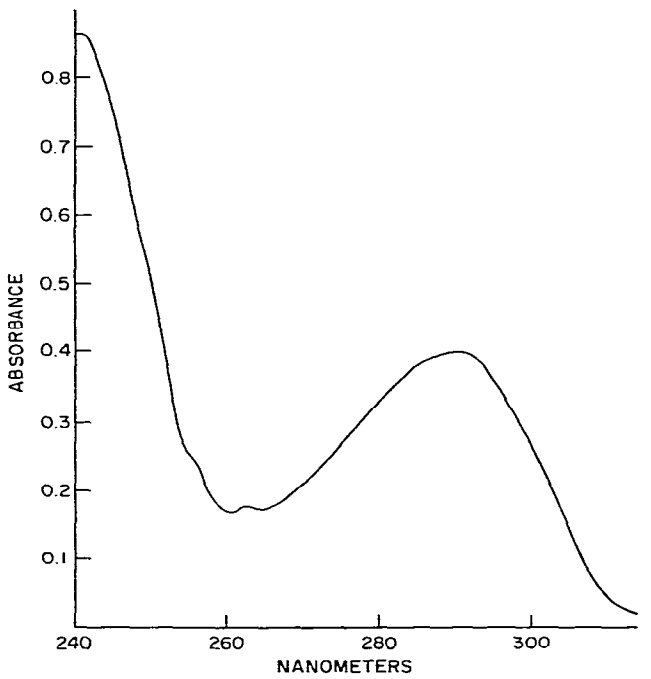

Fig. 4. UV absorption spectrum of TMP (10 μg/ml) in the HPLC mobile phase [500 ml chloroform + 25 ml of a mixture of methanol—water—ammonium hydroxide (150:9:1)]. The reference cell contained the mobile phase.

the trace ammonium hydroxide and water and avoid column degradation by long-term exposure at basic pH.

A TMP analog, 2,4-diamino-5-(3,5-dimethoxy-4-methylbenzyl)-pyrimidine was chosen as the reference standard based on its similar extractability and chromatographic behavior enabling a short total time of analysis (<10 min) between injections. The endogenous co-extracted biological material is eluted primarily in the void volume of the column, and no other interfering substances were observed in the measurement of TMP and the reference standard.

Further interest in enhancing the sensitivity of TMP detection in biological fluids, led to the investigation of UV detection of the compound below 254 nm, using a variable-wavelength detector*. It was observed that the UV absorbance of TMP increases markedly below 250 nm (see Fig. 4); however, the HPLC mobile phase described could not be effectively used below 254 nm, since the UV cut-off of chloroform is 245 nm. Although modification of the mobile phase to substitute chloroform with a more suitable UV-transparent solvent (e.g., hexane) is possible, such an approach was not undertaken.

However, reversed-phase chromatography was investigated as an alternative to the normal-phase separation, since such separations for TMP have been reported in the literature [9, 11, 14]. A rapid separation of TMP on a μBondapak C_{18} column (Waters Assoc.) was accomplished (Fig. 5) by modification of an acetonitrile—phosphate buffer (pH 6) system [14]. The conditions for the reversed-phase chromatography were: mobile phase, 20% acetonitrile (Burdick & Jackson Labs.) in pH 6 phosphate buffer (0.05 *M* prepared as a mixture of KH_2PO_4 and K_2HPO_4), flow-rate, 2.0 ml/min at a pressure of 1500 p.s.i. Although the absolute sensitivity of TMP at 225 nm was 3.7 times that at 254 nm, and increased to 4.25 times at 210 nm, the additional sensitivity could not be fully exploited, due to the necessity of dissolving biological extracts in a minimum of 750 μl of a 50% acetonitrile—pH 6 buffer solution. Thus, as seen in Fig. 5, a typical extract required 750 μl of solvent to completely dissolve the residue of the plasma extract and the injection of a 50-μl aliquot to attain a sensitivity comparable to normal-phase separation, thus offering no advantage over that system.

The intrinsic fluorescence reported for TMP in solution [15] was confirmed in the HPLC mobile phase used in the method described. A scan of the corrected fluorescence spectrum using a Farrand MK-1 spectrofluorometer (Farrand Optical Co., Valhalla, N.Y., U.S.A.) showed an emission maximum at 335 nm with excitation at 285 nm. The effluent from the UV detector was connected in series to a dual monochromator spectrofluorometer** equipped with a HPLC flow cell (20 μl; Perkin-Elmer Part No. 010-0137) for a comparison of the sensitivity limits using both modes of detection. A typical chromatogram of the fluorescence detection mode for a pure standard and a blood sample

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*Model 785; Micromeritics, Norcross, Ga., U.S.A.

**Model 204 fluorescence spectrophotometer (Perkin-Elmer, Norwalk, Conn., U.S.A.). The instrumental parameters were as follows: a 150-W xenon lamp light source, xenon lamp power supply and a R212 photomultiplier were used. The sensitivity controls were set at 11 and the selector set at 10. Excitation monochromator set at 290 nm, with emission monochromator set at 340 nm.

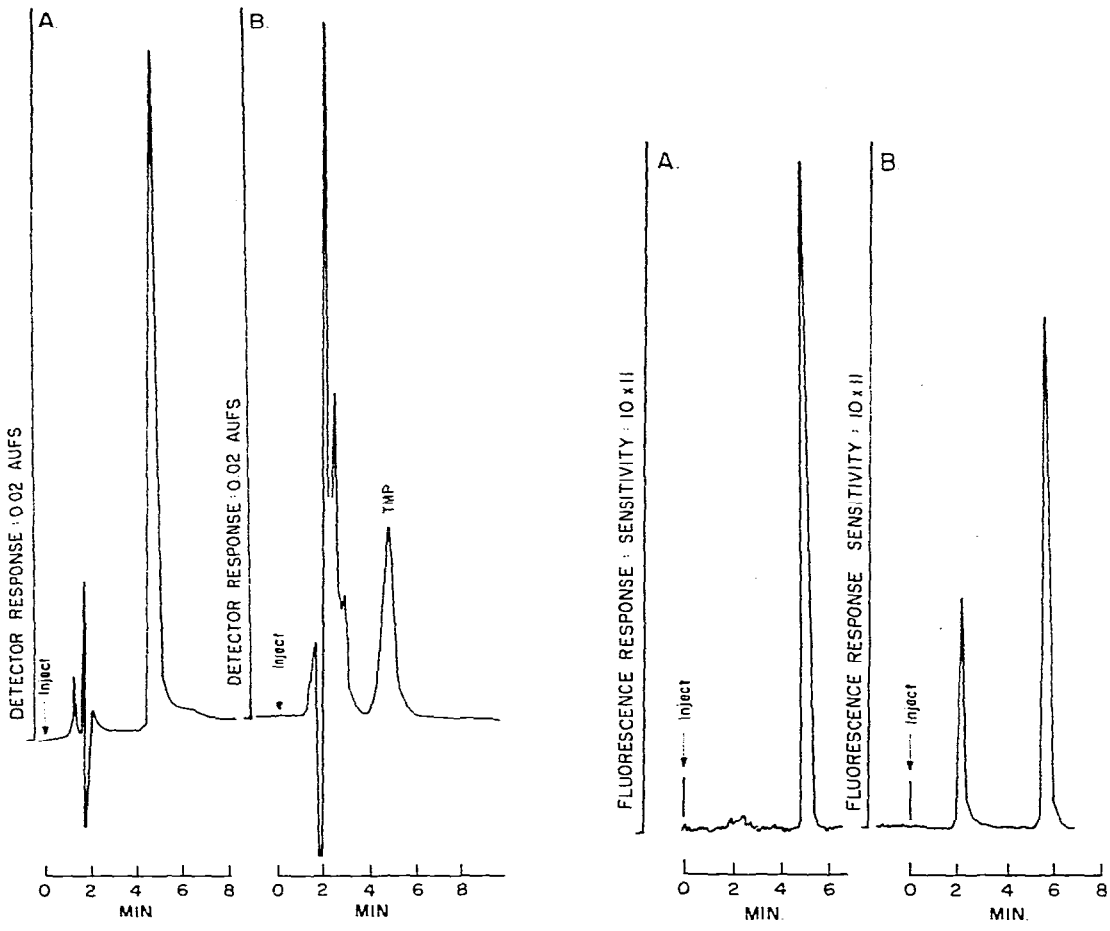

Fig. 5. Chromatogram of a reversed-phase separation on μBondapak C_{18} column using a variable-wavelength UV detector at 225 nm of (A) 100 ng TMP standard and (B) control plasma extract containing 1.0 μg TMP, 50 μl/750 μl injected.

Fig. 6. Chromatograms using fluorescence detection at 290 nm (activation) and 340 nm (emission) of (A) 100 ng TMP standard and (B) control blood extract containing 1.0 μg TMP, 10 μl/100 μl injected.

are shown in Fig. 6A and B. A 100-ng TMP standard gave nearly full scale response with the fluorescence detector operating at a signal-to-noise ratio of 2:1, which is no more sensitive than the UV detector at 280 nm, using an attenuation of 0.005 a.u.f.s. However, the potential advantage of the fluorescence mode is in those instances where additional specificity is required, (e.g., coadministration of other drugs).

In conclusion, the HPLC analysis of TMP in biological fluids is a viable technique that represents a significant improvement over many of the previously used procedures. The method is simple, rapid, sensitive, reproducible, readily automatable, and has been used on a routine basis for many months without

any column degradation. It was used to quantitate TMP levels following low single oral doses and in cases of limited sample size.

A comparison of adsorption vs. reversed-phase chromatography and the use of variable-wavelength UV vs. fluorescence detection yielded no advantage in sensitivity. Furthermore, the reversed-phase mode introduced problems due to poor solubility of the lipophilic sample residue of the chloroform extract which are not encountered using adsorption mode for chromatographic analysis.

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