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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC QUANTITATION OF TRIMETHOPRIM, SULFAMETHOXAZOLE, AND N^4-ACETYLSULFAMETHOXAZOLE IN BODY FLUIDS

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SUMMARY

We describe a rapid, precise and simple procedure for the quantitative determination of trimethoprim, sulfamethoxazole, and N^4-acetylsulfamethoxazole in body fluids by reversed-phase high-performance liquid chromatography. This method utilizes antipyrine as an internal standard with the compounds detected by dual-wavelength monitoring at 225 nm and 254 nm after a single-step extraction. Precision, sensitivity, and accuracy of this assay are within the range of clinical utility; the coefficient of variation is $\leqslant 3\%$, sensitivity < 0.5 μg/ml for all compounds, and recovery $> 97\%$. The short time for performance and small sample size makes the assay ideal for clinical drug monitoring and pharmacokinetic studies.

INTRODUCTION

Trimethoprim [2,4-diamino-5(3,4,5-trimethoxybenzyl)pyrimidine] (TMP) in combination with sulfamethoxazole [N'-(5-methyl-3-isoazolyl)sulfanilamide]

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(SMX), often referred to as Co-Trimoxazole, is a widely used antibacterial agent which has excellent in vitro activity against a variety of aerobic gram-negative bacilli [1, 2].

A major metabolite of SMX is its corresponding N^4-acetylated sulfonamide: N^4-acetylsulfamethoxazole (acetyl-SMX). Acetylated sulfonamides have been associated with toxic reactions [3], particularly with deteriorating renal function [4, 5]. Crystalluria due to the low solubility of the acetylated metabolites has also been reported [6, 7].

It has been suggested that the monitoring of TMP and SMX is necessary to adjust dosing intervals to maintain therapeutic blood concentrations [8, 9] particularly in patients with changing renal function. A rapid, reliable, precise and accurate assay of TMP, SMX, and acetyl-SMX is desirable to limit toxic reactions; such an assay would also aid in devising individualized dosing regimens, and would facilitate clinical pharmacokinetic studies.

There have been several methods developed for the quantitation of TMP in body fluids: microbiological [10], gas–liquid chromatography [11], and ion-pair chromatography [8]. Sulfonamide concentrations are usually determined by a colorimetric assay based on the Bratton–Marshall reaction [12]. N^4-Acetylated metabolites have been quantitated with the same reaction, but with certain modifications [13].

High-performance liquid chromatography (HPLC), a reliable and rapid analytical tool in clinical laboratories has several advantages over conventional methods described above. HPLC techniques for the individual determination of TMP and SMX have been described [14, 15]. A normal-phase HPLC method for the simultaneous determination of TMP, SMX, and acetyl-SMX [16] and a reversed-phase HPLC method in which two determinations are necessary for quantitating TMP, SMX, and acetyl-SMX have also been reported [17]. Gochin et al. [18] describe a simultaneous determination of TMP, SMX, and acetyl-SMX by reversed-phase HPLC; however, this method requires 1.0 ml of serum and an extraction of serum and urine. These methods use only single-wavelength monitoring; absorbance ratios with dual-wavelength monitoring ensure greater degree of confidence in the specificity of the assay [19, 20]. We describe here a reversed-phase HPLC method using dual-wavelength monitoring for the simultaneous determination of TMP, SMX, and acetyl-SMX. The mobile phase is a simple aqueous buffer–solvent mixture, and the sample is prepared by a simple one-step precipitation procedure. The method is rapid and selective and its precision, reproducibility, accuracy, linearity, recovery and sensitivity are validated or documented. TMP was also measured with a normal-phase HPLC assay [21] and the values were compared with the reversed-phase technique.

EXPERIMENTAL

Materials

We used a high-pressure liquid chromatographic pump which could deliver a mobile phase at a constant flow-rate up to 31 MPa (Model Constrametric I, Laboratory Data Control, Riviera Beach, CA, U.S.A.). A sample injection loop with a loop filler port (Model 70-10 and Model 70-11, Rheodyne, Berkeley,

CA, U.S.A.) was fitted to the pump. We used a 10-μm, 30 cm × 3.9 mm I.D. Waters Assoc. (Milford, MA, U.S.A.) μBondapak C_{18} reversed-phase column (ASI 28N-S/N 44802, Analytical Sciences, Santa Clara, CA, U.S.A.). The mobile phase was monitored in series with a fixed-wavelength UV detector (UV III) at 254 nm and a variable-wavelength detector (Spectro Monitor III) at 225 nm. Both were purchased from Laboratory Data Control. A dual-pen recorder set at 0.5 cm/min (Omniscribe Recorder, Model B5217-5, Houston Instruments, Austin, TX, U.S.A.) received signals from both detectors. The flow-rate was 1.0 ml/min.

TMP, SMX, and acetyl-SMX (Lot 150, 754017, and RR-319-3, respectively) were kindly donated by Dr. R. Cleeland of Hoffmann-La Roche (Nutley, NJ, U.S.A.). Stock solutions of TMP and SMX were stored at −70°C at 100 μg/ml in distilled water and 1.9 mg/ml in 0.1 *M* sodium hydroxide, respectively. The acetyl-SMX stock solution was stored at −70°C as a 1.0 mg/ml solution in methanol—water (60:40, v/v).

Antipyrine (Aldrich, Milwaukee, WI, U.S.A.) was dissolved in acetonitrile (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) at 100 μg/ml, and was used as an internal standard and was stored at 5°C. It was stable under these conditions for three months.

The mobile phase consisted of methanol—0.067 *M* phosphate buffer (35:65, v/v). The phosphate buffer was made with 97% potassium phosphate, KH_2PO_4 (Mallinckrodt, Paris, KY, U.S.A.) and 3% sodium phosphate, Na_2HPO_4 (MCB Manufacturing Chemists, Cincinnati, OH, U.S.A.) and the pH was adjusted with phosphoric acid to 3.5. The buffer is stable for two weeks at 22°C. The mobile phase is prepared daily with Omnisolv glass-distilled methanol (MCB Manufacturing Chemists) and buffer by filtering through a 0.22-μm filter (Type GS, Millipore, Bedford, MA, U.S.A.) and degassed in vacuo.

Methods

Aliquots of 50 μl of standards, controls (stored at −70°C) and samples were added to 50 μl of acetonitrile containing the internal standard. The mixture was mixed vigorously for 15 sec on a Vortex mixer and centrifuged at approximately 10,000 *g* (Eppendorf Centrifuge Model No. 5412) for 2 min. A 20-μl aliquot of the supernatant was injected into the column. Absorbance unit full scale (a.u.f.s.) was changed between the TMP peak and SMX peak from 0.05 to 0.2. Then a.u.f.s. was changed to 0.1 after elution of the SMX peak. Peak heights were measured and the ratio of the drug (or metabolite) peak height to internal standard peak height was calculated. These ratios showed a direct linear correlation with the drug concentration of known standards. Drug concentrations of unknowns were calculated from standard curves obtained by least-squares regression.

Linearity

The stock solution of TMP was diluted to 20, 10, 5.0 and 2.5 μg/ml with distilled water. Similarly SMX was diluted to 190, 95, 47.5 and 23.75 μg/ml and acetyl-SMX was diluted to 100, 50, 25 and 12.5 μg/ml. Standards containing these concentrations were assayed and peak height ratio to the internal standard calculated. The standard curve was analyzed by linear regression

analysis to determine linearity. Acetyl-SMX standards were stable at 5°C for at least one month, and TMP and SMX were stable at −70°C for at least four months.

Precision

Stock solutions were diluted with normal pooled human sera, aliquoted and stored at −70°C. Two concentrations for both TMP and SMX were used and one concentration for acetyl-SMX. Seven aliquots at each concentration were assayed in one batch (within-assay precision); then an aliquot at each concentration was assayed daily over a four-month period (between-assay precision).

Accuracy

Known amounts of TMP, SMX, and acetyl-SMX were dissolved in normal human sera and assayed twice in an encoded fashion.

Recovery

Identical amounts of TMP, SMX and acetyl-SMX were dissolved in distilled water or human sera and assayed. Drug peak heights in serum were compared to drug peak heights in distilled water. Human sera, urine and cerebrospinal fluid (CSF) samples from patients not receiving TMP or SMX were also assayed.

Sensitivity

Working standards were serially diluted and assayed until a concentration was reached in which the peak height was twice the background noise.

Specificity

Peak heights at 225 nm were routinely used to determine concentrations. However, the ratio of peak height at 225 nm to peak height at 254 nm was constant: 4.25 for TMP, 0.85 for SMX, and 1.30 for acetyl-SMX. A value greater than 2 standard deviations (S.D.) from this ratio indicated an interfering substance.

Method comparison

A total of 312 serum samples were assayed for TMP by the reversed-phase HPLC method described here, and by the normal-phase HPLC described by Siber et al. [21].

RESULTS

Representative chromatograms of serum samples before and after Co-Trimoxazole administration are depicted in Fig. 1. Retention times of TMP, SMX, and acetyl-SMX are 4.6, 8.0, and 14.8 min, respectively. The retention time of the internal standard is 11.0 min.

A standard curve was calculated using aqueous solutions of five concentrations for TMP, SMX, and acetyl-SMX. Peak height ratios (described in Methods) were plotted versus concentrations. The squares of the correlation coefficients (r^2) were 0.99945, 1.0000, 0.99941 for TMP, SMX, and acetyl-

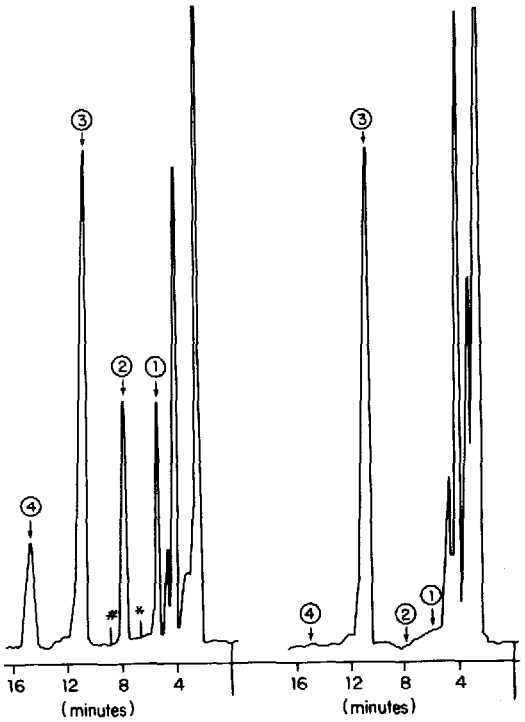

Fig. 1. Representative chromatogram of serum containing TMP, 1; SMX, 2; internal standard, 3; and acetyl-SMX, 4. Left, serum was obtained after the patient received Co-Trimoxazole with the absorbance measured at 225 nm; right, before drug administration. *Indicates a change of a.u.f.s. from 0.05 to 0.2; # indicates a change of a.u.f.s. from 0.2 to 0.1.

TABLE I

WITHIN-RUN REPRODUCIBILITY OF REVERSED-PHASE QUANTITATION OF TMP, SMX AND N-ACETYL SMX

Concentrations are given in μg/ml.

	TMP	SMX	Acetyl-SMX
Number (n)	7	7	10
Mean value	1.76	40.20	56.1
Standard deviation	0.053	0.40	0.95
Coefficient of variation (%)	3.0	1.0	1.7
Number (n)	7	7	
Mean value	9.56	185.8	
Standard deviation	0.22	1.19	
Coefficient of variation (%)	2.2	0.6	

SMX, respectively. Because of these excellent correlations, only three concentrations (including 0 μg/ml) were used to determine standard curves in routine analysis. Slopes of the peak height ratio to the standard curves (n = 30) were 0.0791 ± 0.0043, 0.0055 ± 0.0003, and 0.0055 ± 0.0003 for TMP, SMX, and

TABLE II

PRECISION OF THE QUANTITATION OF TMP, SMX AND ACETYL-SMX ON SUCCESSIVE DAILY DETERMINATIONS

Concentrations are given in μg/ml.

	TMP	SMX	Acetyl-SMX
Number (n)	41	41	40
Mean value	1.89	38.97	54.23
Standard deviation	0.11	0.70	1.79
Coefficient of variation (%)	5.8	1.8	3.3
Number (n)	41	41	
Mean value	9.80	188.40	
Standard deviation	0.24	2.87	
Coefficient of variation (%)	2.4	1.4	

acetyl-SMX, respectively. The y-intercepts did not differ significantly from zero in all cases ($p > 0.05$).

Precision analysis of seven identical aliquots (n = 10 for acetyl-SMX) of TMP, SMX, and acetyl-SMX are summarized in Table I. The coefficients of variation of quantitation of TMP < 3.0% and < 2.0% for SMX and acetyl-SMX indicate good reproducibility within therapeutic ranges. Precision analysis of day-to-day variation in assays is depicted in Table II. There was no detectable change in concentrations of aliquots stored at −70° C for four months.

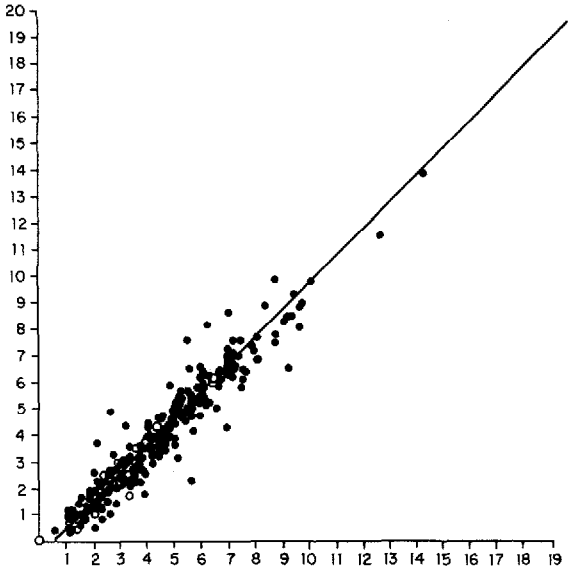

Fig. 2. Comparison of TMP quantitation by two techniques. The y-axis represents TMP quantitated by the reversed-phase method. The x-axis represents TMP quantitated by normal-phase HPLC [21]. TMP concentration is in μg/ml. The parameters of the regression line are: slope = 1.0293, y-intercept = −0.533, correlation coefficient = 0.9611 with 312 samples. Open circles represent more than one point having the same value.

TABLE III

ACCURACY OF REVERSED-PHASE QUANTITATION OF TMP, SMX AND ACETYL-SMX ON REPLICATE DETERMINATIONS (n = 14)

Concentrations are given in μg/ml.

TMP			SMX			Acetyl-SMX		
Actual concn.	Measured concn.	Percent difference	Actual concn.	Measured concn.	Percent difference	Actual concn.	Measured concn.	Percent difference
20	20	0	190	181.2	4.6	100	95.1	4.9
20	19.3	3.5	190	179.3	5.6	100	96.9	3.1
15	15.3	2.0	142.5	142.6	>0.1	75	75.7	0.9
15	15.1	0.7	142.5	137.7	4.7	75	72.7	3.1
10	9.8	2.0	95.0	92.1	3.0	50	46.7	6.6
10	10.0	0	95.0	90.6	4.6	50	47.9	4.2
7.5	7.4	1.3	66.5	65.5	1.5	35	34.9	0.3
7.5	7.4	1.3	66.5	65.1	2.1	35	34.6	1.1
5	5.1	2.0	47.5	47.0	1.1	25	24.6	1.6
5	4.9	2.0	47.5	47.0	1.1	25	25.0	0
2.5	2.4	4.0	114.0	111.2	3.3	60	59.4	1.0
2.5	2.3	8.0	114.0	112.1	2.5	60	59.4	1.0
1.0	1.0	0	74.0	74.0	0	30	20.1	3.0
1.0	1.0	0	74.0	73.8	0.3	30	29.4	2.0
Mean percent difference		1.9	Mean percent difference		2.45	Mean percent difference		2.34
Range of percent difference		0—8.0	Range of percent difference		0—5.6	Range of percent difference		0—6.6

Accuracy studies, depicted in Table III, demonstrate good accuracy over a wide range of concentrations for TMP, SMX, and acetyl-SMX. The mean percent difference between actual and observed values was < 3.0% for all three compounds.

Recovery studies comparing spiked sera to aqueous standards of TMP, SMX, and acetyl-SMX showed an average recovery of 97.3%, 99.4%, and 100.3%, respectively. Percent recovery was constant throughout the concentration range. Normal human sera, urine and CSF displayed no peaks on the chromatogram at the retention times of the indicated compounds.

Using a 20-μl injection, the sensitivity of detection (defined in Methods) of TMP, SMX, and acetyl-SMX is 0.05 μg/ml, 0.2 μg/ml and 0.5 μg/ml, respectively.

Out of 523 patient samples 63 samples were obtained from patients with renal failure. Of these, 2.5% had interference with SMX and internal standard peaks. Interference was present with acetyl-SMX in 6.1% of the samples. Interference with the TMP peak occasionally occurred in routine analysis; however, slight adjustment of pH or the percentage of methanol eliminated the interfering substances from the TMP peak, (when adjusting pH or percentage of methanol, the TMP standards must be reinjected to determine their retention time and for quantitation).

Comparison of TMP serum levels quantitated by reversed-phase HPLC and a normal-phase HPLC method [21] are depicted in Fig. 2. The correlation coefficient (r^2) was > 0.96 with a slope of 1.029.

TMP, SMX, and acetyl-SMX levels in serum, urine and CSF could be determined within 30 min after obtaining the samples.

DISCUSSION

A reliable, rapid and accurate assay is necessary for the routine clinical analysis of drugs and/or metabolite concentrations. The method described here meets these criteria. The monitoring by dual wavelength ensures specificity; when interfering substances are present, small changes in percentage of methanol or pH will separate interfering compounds from the TMP, SMX, acetyl-SMX peaks. The reversed-phase method negates the need of an extraction step, and the use of expensive organic solvents, which are used in normal-phase chromatography.

This method can be scaled down by using 10 μl of sample and 10 μl of internal standard. This allows quantitating the antibiotics in serum samples obtained by fingertip puncture or heel prick methods. To date, 500 samples have been assayed with the same column. A pre-column of large pore C_{18} μBondapak (37—50 μm) was used to increase the useful life span of the analytical column; however, after 500 injections, increases in back pressure and slight changes in peak shapes indicated column deterioration. Daily washing of the column was carried out with 30% methanol in water before and after. This method will allow the clinician to adjust the dose of Co-Trimoxazole to maintain therapeutic body fluid concentrations of TMP and SMX; it also allows quantitation of serum acetyl-SMX concentrations. Avoiding high acetyl-SMX concentrations may minimize toxicity.

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