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SIMULTANEOUS DETERMINATION OF TRIMETHOPRIM, SULPHAMETHOXAZOLE AND N^4-ACETYLSULPHAMETHOXAZOLE IN SERUM AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The simultaneous determination of trimethoprim, sulphamethoxazole and N^4-acetylsulphamethoxazole in serum and urine by high-performance liquid chromatography using sulphafurazole as internal standard is described. The separation was achieved on a reversed-phase column employing acetic acid—methanol as the mobile phase with spectrophotometric detection at 230 nm. Precise simultaneous quantitative analysis of the relative components has been achieved at levels of 0.1 μg/ml for trimethoprim and 1.0 μg/ml for both sulphamethoxazole and its N^4-acetyl metabolite using 1 ml of serum or urine.

INTRODUCTION

Co-trimoxazole, a combination of trimethoprim (TMP) and sulphamethoxazole (SMZ) in a 1:5 ratio is a highly effective broad spectrum chemotherapeutic formulation [1].

Several high-performance liquid chromatographic (HPLC) assay methods for TMP, SMZ and other related sulphonamides in dosage forms or as pure drugs have been reported [2—8]. HPLC has also been used for the determination of sulphamethoxazole [9—11] and trimethoprim independently [10—15], whereas several different analytical procedures for the simultaneous determination of both drugs in pharmaceutical preparations [16, 17] and in biological fluids [18, 19] have also been described. Recently, however, HPLC methods have been reported describing the simultaneous determination of SMZ and N^4-acetyl-SMZ [20] and TMP, SMZ and N^4-acetyl-SMZ [21, 22] in biological fluids.

The HPLC method of Vree et al. [21] gives no account of the precision of the assay and lists the lower limit of sensitivity for TMP in serum and urine as

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0.75 μg/ml, whereas the method of Ferry et al. [22] does not completely resolve all the relevant components. In addition, the former method involves the use of 225 nm as the detection wavelength which results in the sulphonamide peaks being too large in relation to the TMP peak, making simultaneous determinations in serum difficult. The latter method utilizes a wavelength of 270 nm resulting in a reduction in the limits of sensitivity.

The HPLC method described here has a detection limit of 0.1 μg/ml TMP and, in addition, involves the use of an internal standard, the advantages of which have been well established [23, 24]. Using this procedure, the concentrations in serum and urine of TMP, SMZ and N^4-acetyl-SMZ, covering the entire concentration range normally encountered during therapy, can be simultaneously determined. Furthermore, this method can be used to quantitatively determine the relevant drugs and major sulphonamide metabolite in serum and urine after a single dose of co-trimoxazole as would be the case during a bioavailability study.

MATERIALS AND METHODS

Apparatus

A Perkin-Elmer Model 601 liquid chromatograph was used, equipped with a Perkin-Elmer LC-55 variable-wavelength detector and a strip chart recorder. A reversed-phase column (μBondapak C_{18}, particle size 10 μm, 30 cm × 3.9 mm, from Waters Assoc., Milford, MA, U.S.A.) housed in a temperature controlled oven was used and samples were injected using a Rheodyne Model 70-10 sample injection valve equipped with a 20-μl loop.

Reagents

TMP and SMZ were supplied by Wellcome (Kempton Park, South Africa), and the sulphafurazole (SFZ), which was used as the internal standard, by Maybaker (Port Elizabeth, South Africa). The N^4-acetyl-SMZ was prepared by acetylation of SMZ as described by Sharma et al. [9] (theoretical: %C, 48.81; %H, 4.44; %N, 14.23; found: %C, 48.75; %H, 4.50; %N, 14.25). All solvents and other reagents were purchased from E. Merck (Darmstadt, G.F.R.) and were of analytical grade. The water used was de-ionized and then glass distilled.

Mobile phase

The mobile phase was methanol—acetic acid, and was prepared by mixing methanol (200 ml) with 1% v/v acetic acid (800 ml), the latter being prepared from glacial acetic acid and water. The solvent mixture (pH 2.9) was simultaneously filtered and degassed through a 0.45-μm HA filter (Millipore, Bedford, MA, U.S.A.). This mobile phase was used for the analysis of both serum and urine samples.

Chromatography

For both serum and urine samples the flow-rate of the mobile phase was set at 1.5 ml/min at a pressure of 100 bar and the wavelength of detection was 230 nm. The oven temperature was set at 30°C for the analysis of serum extracts whereas urine samples were analysed at ambient temperature (20—24°C). Peak

height ratios were used for quantitation based upon calibration curves established on the same day. The calibration curves were prepared from the results of assays on serum and urine spiked with known quantities of TMP, SMZ, N^4-acetyl-SMZ and the internal standard, SFZ.

Extraction

Serum samples. Acetonitrile (3 ml) containing SFZ (6 μg/ml) as internal standard was added to serum (1 ml). This was mixed in a tube (Vacutainer, Becton-Dickinson, Parsippany, NJ, U.S.A.) using a vortex mixer for 0.5 min. The mixture was allowed to stand for 15 min to ensure complete protein precipitation. Ethyl acetate (3 ml) was then added and mixed on a vortex mixer set at a low speed for 1 min. The sample was allowed to stand for 30 min and then mixed again. Sodium chloride (0.4 g) was added to the mixture in order to saturate the aqueous layer. The tube was vortexed briefly and allowed to stand for a further 30 min. Finally the tube was mixed again and then centrifuged at 1700 *g* for 15 min. Five millilitres of the clear upper layer were then transferred to a finely tapered centrifuge tube and evaporated to dryness at 50°C under a gentle stream of nitrogen. The sides of the tube were carefully washed down with methanol during drying. The dry extract was reconstituted in 50 μl of mobile phase, mixed, placed in an ultrasonic bath for 1 min and allowed to stand for 15 min. After centrifugation at 1700 *g* for 15 min, 1—10 μl of the clear supernatant were injected onto the column. Relevant chromatograms are depicted in Fig. 1.

Urine samples. The sample (1 ml) was prepared by adding Sorensen's phosphate buffer (1 ml, pH 7.2) [25]. The mixture was vortexed briefly and the pH

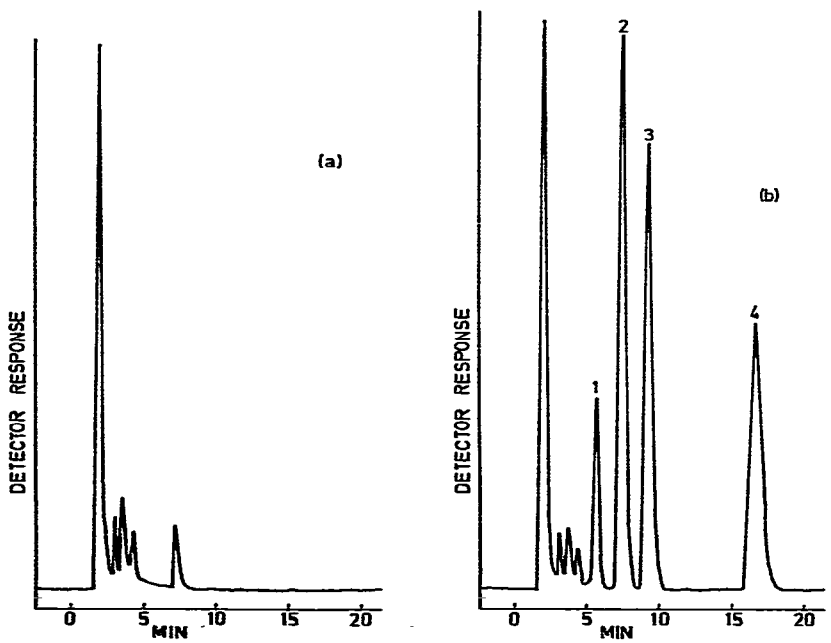

Fig. 1. (a) Chromatogram of blank serum extract. (b) Chromatogram of an extract of serum containing TMP (1), SMZ (2), SFZ (3) and N^4-acetyl-SMZ (4).

readjusted to 7.2 by the addition of a suitable quantity of 0.1 *M* sodium hydroxide. Acetonitrile (2 ml) containing SFZ (75 μg/ml) was added as internal standard. This was mixed for 1 min on a vortex mixer and allowed to stand for 30 min. Sodium chloride (1 g) was added and the mixture was agitated again. It was left to stand for 30 min and then remixed and centrifuged at 1700 *g* for 15 min. The upper acetonitrile layer (1 ml) was removed, placed in a tapered centrifuge tube and evaporated to dryness at 50°C under a gentle stream of nitrogen. The sides of the tube were carefully washed down with methanol during drying. The dry extract was reconstituted in 200 μl of mobile phase, mixed thoroughly and then allowed to stand for 30 min. Finally the solution was centrifuged at 1700 *g* for 15 min and 1—10 μl injected onto the column. Relevant chromatograms are depicted in Fig. 2.

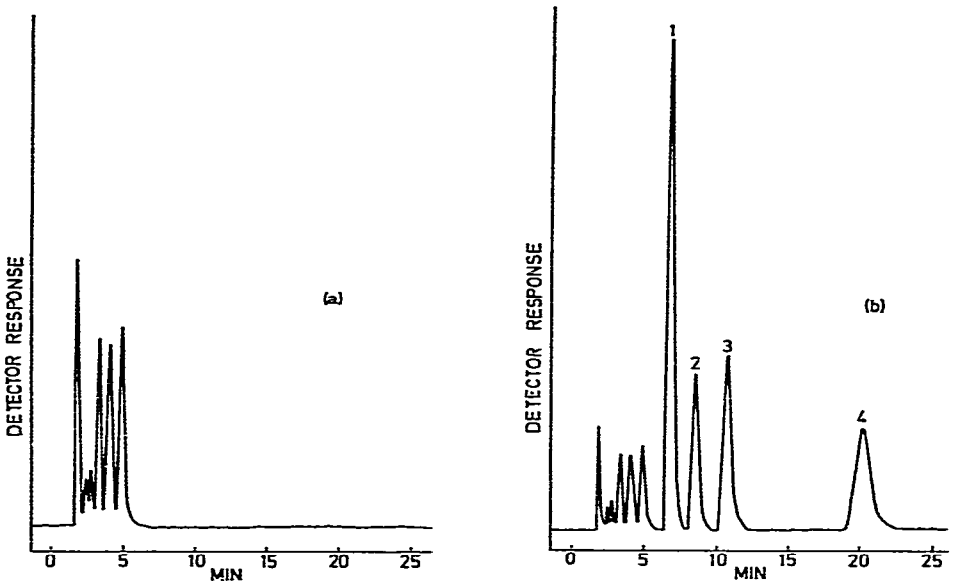

Fig. 2. (a) Chromatogram of blank urine extract. (b) Chromatogram of an extract of urine containing TMP (1), SMZ (2), SFZ (3) and N^4-acetyl-SMZ (4).

RESULTS

Extraction efficiency

Known amounts of each component were added to drug-free serum and urine. All samples were extracted as previously described except that the internal standard was added as a final step to each extract. Three different concentrations of each component in both serum and urine were assayed in triplicate. The results were compared with those obtained from the injection of equivalent concentrations of pure drugs in mobile phase solution. Mean values obtained were 101.0%, 76.3% and 72.3% recovery for TMP, SMZ and N^4-acetyl-SMZ, respectively. The concentration ranges were 1—3 μg/ml for TMP, 6—60 μg/ml for SMZ and 3—30 μg/ml for N^4-acetyl-SMZ.

Precision

The within-run precision was established by spiking serum and urine with TMP, SMZ and N^4-acetyl-SMZ at two different concentrations corresponding, approximately, to the lower and upper limits likely to be encountered after the administration of a single oral dose of 160 mg TMP and 800 mg SMZ. The coefficients of variation for the analysis were determined to be 3.78% at a concentration of 0.6 μg/ml and 2.83% at a concentration of 1.6 μg/ml for TMP, 3.09% at a concentration of 12 μg/ml and 3.99% at a concentration of 35 μg/ml for SMZ and 2.60% at a concentration of 6 μg/ml and 3.52% at a concentration of 16 μg/ml for N^4-acetyl-SMZ. Six samples were assayed at each concentration of the three compounds.

Linearity

Calibration curves obtained by plotting the ratio of the peak height of each compound to that of the internal standard, SFZ, versus their respective concentrations using five different concentrations of serum and urine were linear over all the concentration ranges studied.

Interferences

Chromatograms of blank serum and urine extracts are shown in Figs. 1 and 2. Whereas no interfering peaks were evident in the urine control, a small peak corresponding to the elution time for caffeine was evident in the serum blank. This peak is not present if the volunteer refrains from consuming caffeine-containing beverages. Solutions of acetylsalicylic acid and salicylic acid were also tested for possible interference. Peaks for both compounds gave an identical retention time which did not interfere with any of the compounds of interest.

Sensitivity and detection limit

Under the conditions of this assay, the detection limits for TMP and the sulphonamides (SMZ and N^4-acetyl-SMZ) were established as 0.1 and 1.0 μg/ml respectively.

Patient serum and urine profiles

Application of the assay method was carried out by analyzing serum and urine from a volunteer. A single oral dose of 160 mg TMP and 800 mg SMZ (2 tablets Septran, Wellcome) was administered to the volunteer after an overnight fast. Blood samples were collected from a forearm vein at scheduled intervals and the serum separated by centrifugation and then frozen. Urine samples were collected until 48 h after administration of the dose and representative aliquots were frozen until analysis. The serum and urine concentration—time profiles of TMP, SMZ and N^4-acetyl-SMZ are listed in Table I. The cumulative amounts excreted in the urine over 48 h were TMP 98.46 mg, SMZ 98.20 mg and N^4-acetyl-SMZ 386.92 mg.

DISCUSSION

The method is well suited for routine application in the clinical laboratory because of the relatively simple extraction procedure which allows the simulta-

TABLE I

SERUM AND URINE CONCENTRATION–TIME PROFILES OF TMP, SMZ AND N^4-ACETYL-SMZ

All values are expressed in μg/ml.

Time (h)	TMP		SMZ		N^4-Acetyl-SMZ	
	Serum	Urine	Serum	Urine	Serum	Urine
1	1.57		54.68		2.11	
2	1.34	51.13	49.29	75.90	4.55	65.83
3	1.30		44.57		5.87	
4	1.22	42.54	47.82	50.22	7.43	118.23
6		81.47		53.68		167.11
8	0.78	89.56	32.60	73.81	7.36	246.56
12	0.55	62.37	25.14	97.57	6.43	310.56
19		65.82		19.75		230.71
22		41.72		20.55		251.29
24	0.18	10.42	11.66	13.75	3.45	64.09
25		3.02		6.19		20.12
29		24.67		27.62		169.58
31		11.18		16.81		62.52
32		2.86		4.61		16.01
33		5.39		9.35		32.52
36		12.94		8.37		78.05
47		9.19		5.57		92.23
48		1.93		3.95		36.01

neous determination of the three compounds. The assay provides adequate sensitivity and precision for monitoring therapeutic steady state concentrations as well as the subtherapeutic concentrations which are encountered following the administration of a single solid oral dose of co-trimoxazole for bioavailability assessment.

In view of the fact that the mean serum concentration ratio of SMZ to TMP is approximately 20:1 [26], it was necessary to have a detection system that is considerably more sensitive to TMP in order to obtain responses of a similar magnitude for all the relevant compounds. A detection wavelength of 230 nm proved to be optimal since at this wavelength the absorbance of TMP is about five times that of SMZ on a weight basis. This enabled the use of a single internal standard to quantitate both drugs and the major metabolite and also resulted in easy measurement of peak heights and a simultaneous analysis without the need to selectively attenuate certain peaks. Although the SMZ to TMP ratio in urine is approximately 1:1 [27], the same wavelength gave excellent results for the simultaneous analysis of all three components.

Reconstitution of the final dry extracts with mobile phase as opposed to methanol or acetonitrile markedly improved resolution and peak shapes. Band broadening occurred when the samples were reconstituted in either methanol or acetonitrile.

In summary, the HPLC method presented here is specific, highly sensitive, reliable, reproducible and extremely suitable for the simultaneous analysis of TMP, SMZ and N^4-acetyl-SMZ in both human serum and urine.

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