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ASSAY OF TRIMETHOPRIM, SULFADIAZINE AND ITS N^4-ACETYL METABOLITE IN BIOLOGICAL FLUIDS BY NORMAL-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A normal-phase high-performance liquid chromatographic method was developed to determine the concentration of timethoprim, sulfadiazine and its N^4-acetyl derivative in human serum and urine. The unchanged compounds and the metabolite are extracted in organic solvent by a single extraction. The method is accurate and sensitive and suited for pharmacokinetic studies in man.

INTRODUCTION

A combination of trimethoprim (TMP) and sulfadiazine (SDZ) is used as an antibacterial preparation for therapy in humans, following that of trimethoprim and sulfamethoxazole (SMZ), co-trimoxazole. SDZ distributes well into lung tissues, bronchial secretion and saliva [1] thus having a useful chemotherapeutic effect in respiratory tract infections [1]. The proportion of the sulfonamide (TMP/SDZ, ratio 1:3) has been reduced in comparison with co-trimoxazole where TMP/SMZ is in the ratio 1:5.

A number of analytical methods have been employed for TMP determination in biological fluids: microbiological [2], radiochemical [3] or spectrofluorimetric assay (most often used) [4], and thin-layer chromatography (TLC) with densitometry [5].

Recently gas—liquid chromatography (GLC) has been used for the determination of TMP [6] and for TMP, SMZ and the SMZ N^4-acetyl metabolite [7]. Both methods use a nitrogen—phosphorus detector. The most widely used assay for the determination of sulfonamides in biological fluids is a colorimetric assay based on the Bratton—Marshall reaction [8]. Rieder [9] has improved such a method in order to distinguish total sulfonamide (free

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+ N^4-acetyl metabolite) and the "bacteriostatically active fraction" (the free fraction). Other authors describe GLC determination with a flame ionisation detector [10].

Various authors have reported high-performance liquid chromatographic (HPLC) methods for sulfonamide determination in biological liquids [11, 12], for TMP [13], and for determination of both TMP and SMZ [14] by two different chromatographic assays. Recent papers [15, 16] have dealt with direct reversed-phase HPLC for the determination of TMP and SMZ in serum and plasma after protein precipitation, as well as for a mixture of sulfonamides [15], among them SDZ.

A recent paper describes the simultaneous HPLC determination of TMP, SMZ and its N^4-acetyl metabolite [17] after a single extraction from biological fluids. Working in reversed-phase under chromatographic conditions used for TMP and SMZ [17] we obtained a poor separation between SDZ and its metabolite which was not separated from TMP. Using a more polar eluent mixture (15% ethanol and 85% phosphate buffer, pH 4.5) the separation between substances was slightly improved, but TMP, the last to be eluted, showed a broadening tailing peak with a poor response in the UV detector (variation of the pH of the buffer in the range 4—7 did not improve the chromatographic behaviour of the drugs).

Instead, working in normal phase, under the conditions described in Experimental, a good separation between substances was achieved; TMP eluted first, and gave a high response in the UV detector with a symmetrically shaped peak. In this paper we describe a new method for normal-phase HPLC determination of the drugs and the sulfonamide metabolite in human serum and urine. The organic substances are extracted into ethyl acetate by a single and rapid liquid—liquid extraction at pH 6.8. The assay is performed in the presence of an internal standard, sulfadimethoxine (SDM).

EXPERIMENTAL

Chromatographic system

The determinations were carried out using the following chromatographic system: Altex Model 110 A solvent metering pump, back pressure up to 350 bar (Altex Scientific, Berkeley, CA, U.S.A.); UV—visible (200—850 nm) Kontron Uvicon 725 spectrophotometric detector with a cell volume of 8 μl and path length of 6 mm (Kontron, Zürich, Switzerland) operating at a wavelength of 289 nm with a sensitivity of 0.1 a.u.f.s.; Rheodyne Model 7120 sample injector with a 20-μl loop capacity (Rheodyne, Berkeley, CA, U.S.A.); Hibar® chromatographic column filled with LiChrosorb Si 60 (10 μm), stainless steel, 250 mm × 4 mm I.D. (E. Merck, Darmstadt, G.F.R.); precolumn filled with LiChroprep Si 60 (25—40 μm) (Merck). The detector was coupled through an interface to a chromatographic computer (Sigma 10 data system, Perkin-Elmer, Norwalk, CN, U.S.A.). All calculations were performed according to the "internal standard method".

The eluent was a mixture of dichloromethane—methanol—25% aqueous ammonia (80:19:1). The procedure was carried out at a constant flow-rate of 1.5 ml/min (about 50 bar).

Chromatograms were recorded on a Tarkan 600, W + W recorder, with an input of 10 mV (Kontron).

Under the above conditions the retention times of test substances are as follows: TMP, 2.6 min; SDM (internal standard), 3.7 min; SDZ, 6.7 min; SDZ N^4-acetyl metabolite, approx. 10.4 min (see Fig. 6).

Reagents and solvents

Dichloromethane and methanol (Lichrosolv), ethyl acetate (nanograde), and ammonia solution (25% Suprapur) were all from Merck. Phosphate buffer (pH 6.8, 1 *M*) of USP type prepared with KH_2PO_4 RPE-ACS was from Carlo Erba, Milan, Italy; water was double-distilled on glass. TMP, SDM, SDZ, and the N^4-acetyl SDZ derivative (all approx. 100% purity) were supplied by F. Hoffmann-La Roche, Basle, Switzerland.

Extraction from human serum and urine

A 1-ml volume of human serum is place in a screwcap test tube (for urine 1 ml is diluted with 5 or 10 ml of distilled water, and 1 ml of this solution is used at the start); 0.2 ml of phosphate buffer (1 *M*, pH 6.8) and 6 ml of ethyl acetate are added; the mixture is agitated for 3 min with a Vortex apparatus (Whirlimixer, Fisons, Loughborough, Great Britain) then centrifuged for 5 min at 2400 *g*, and 5 ml of supernatant are carefully collected. The organic extract is evaporated in a thermostated water-bath at 50 ± 1°C under a light stream of pure nitrogen. The residue is redissolved with 200 µl of

TABLE I

RECOVERY OF TMP, SDZ, AND SDZ N^4-ACETYL METABOLITE ADDED TO HUMAN URINE

Results are expressed as the mean of 5 determinations ± standard deviation, from 5 urine samples with the same nominal concentration. The urine was pooled from healthy volunteers who had taken no drugs for at least two weeks.

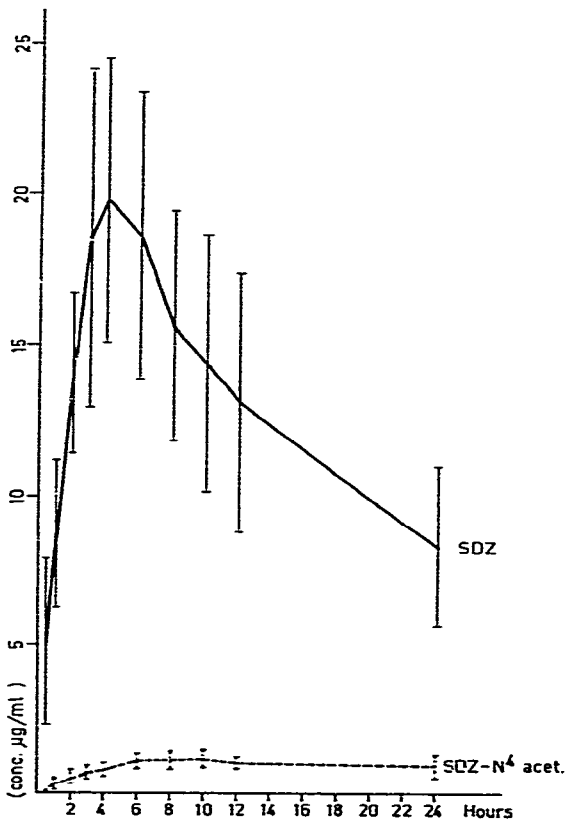
Compound	Quantity added (µg/ml)	Recovery (µg/ml)	Recovery (%)
TMP	45	44.8 ±0.9	99.5 ± 2
	40	39.73± 0.63	99.3 ± 1.6
	35	32.66±0.5	93.3 ± 1.5
	20	18.3 ±0.158	91.5 ± 0.86
SDZ	165	164 ±0.4	99.4 ± 2
	160	155 ±1.0	96.7 ± 0.64
	130	129.3 ±1.5	99.4 ± 1.16
	100	98.94±1.74	98.9 ± 1.75
N^4-acetyl SDZ	60	52.6 ±1.87	87.7 ± 3.5
	55	48.3 ±1.26	87.8 ± 2.6
	45	38.2 ±1.2	85 ± 3.1
	40	35.34±0.54	88.3 ± 1.5

eluent mixture containing internal standard (30 μg/ml). Parallel tests are run with a working standard solution obtained by adding the drugs to blank serum or urine organic extracts and with blank fluid sample.

RESULTS AND DISCUSSION

We have found a linear relationship between concentration of the test substances and the ratio of their peak areas to the internal standard peak area within the following concentration ranges: TMP, 0.03—10 μg/ml; SDZ, 0.1—200 μg/ml; N^4-acetyl SDZ derivative, 0.05—60 μg/ml. (This refers to a 1-ml serum specimen in all cases.) The minimum amounts detectable were about 0.03 μg/ml, 0.1 μg/ml and 0.05 μg/ml for TMP, SDZ and N^4-acetyl SDZ, respectively, working at 0.01 a.u.f.s. under the above conditions.

In chromatography of human serum extract we found a few times a small peak close to that of TMP, on its tail and partly overlapping; in such a case



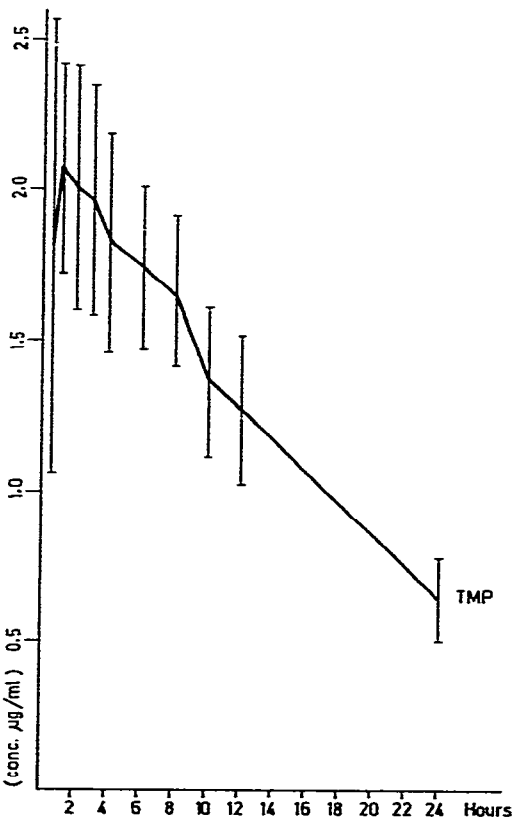


Fig. 1. Serum profile (average of 8 subjects) of free sulfadiazine (SDZ) and its N^4-acetyl metabolite (SDZ N^4-acet.) after oral administration of two tablets (900 mg SDZ + 300 mg TMP).

Fig. 2. Serum profile (average of 8 subjects) of trimethoprim (TMP) after oral administration of two tablets (900 mg SDZ + 300 mg TMP).

it is necessary in chromatographic calculations to use peak height rather than area.

The column shows no evidence of deterioration after about 1000 injections. Before a period of non-use (for example, a weekend) the column is treated first with 30 ml of dichloromethane—methanol—water (80:19:1), then with 30 ml of dichloromethane—methanol (90:10), 30 ml of dichloromethane and finally with 50 ml of *n*-hexane.

We have performed statistical recovery of drugs from biological fluid blanks to which concentrations simulating therapeutic and subtherapeutic amounts were added. The results are listed in Tables I and II.

TABLE II

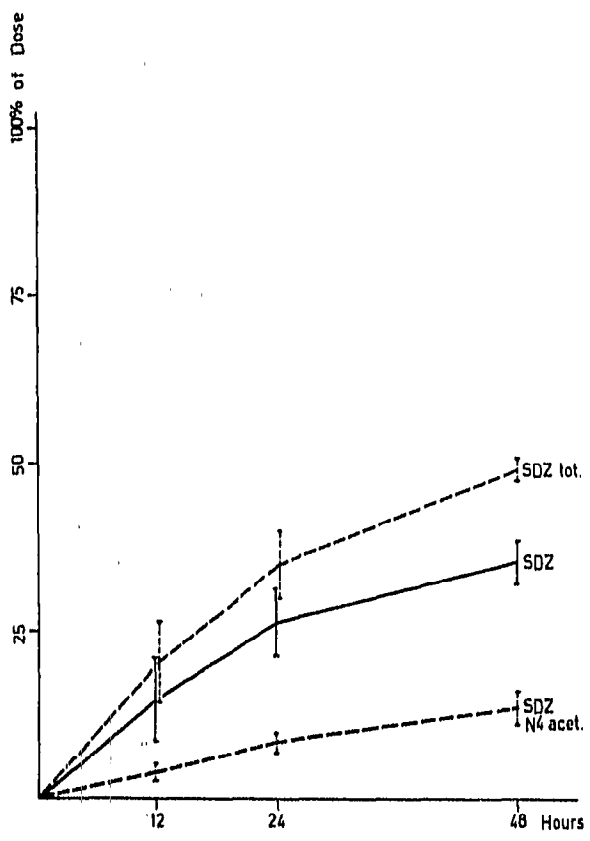
RECOVERY OF TMP, SDZ, AND SDZ N^4-ACETYL METABOLITE ADDED TO HUMAN SERUM

Results are expressed as the mean of 5 determinations ± standard deviation from 5 serum samples with the same nominal concentration. Other values represent the mean of two determinations. Serum was pooled from healthy volunteers who had taken no drugs for at least two weeks.

Compound	Quantity added (μg/ml)	Recovery (μg/ml)	Recovery (%)
TMP	0.2	0.17 ± 0.006	85 ± 3.5
	0.3	0.259 ± 0.008	86.7 ± 3.2
	1.0	0.975 ± 0.012	97.5 ± 1.2
	1.8	1.724	95.8
	2.0	1.850	92.5
	3.0	2.750	91.7
SDZ	3	2.9 ± 0.04	96.3 ± 1.4
	4	4.75 ± 0.07	95 ± 1.5
	10	9.88 ± 0.11	98.8 ± 1.1
	20	20	100
	30	29.5	98.3
N^4-acetyl SDZ	0.2	0.155 ± 0.007	77.5 ± 4.5
	0.5	0.395 ± 0.014	79.0 ± 3.7
	1.0	0.83 ± 0.02	83.0 ± 2.3
	2.0	1.64	82.0
	3.0	2.50	83.3

Clinical application of the method

The behaviour of SDZ and TMP in man can be described by a one-compartment model as also mentioned elsewhere [18]. Pharmacokinetic parameters obtained in a clinical study performed on healthy volunteers agree with those reported by other authors [18, 19]. Figs. 1—4 represent serum and urine excretion profiles of SDZ and TMP in man after oral administration of a solid (tablet) dosage combination.



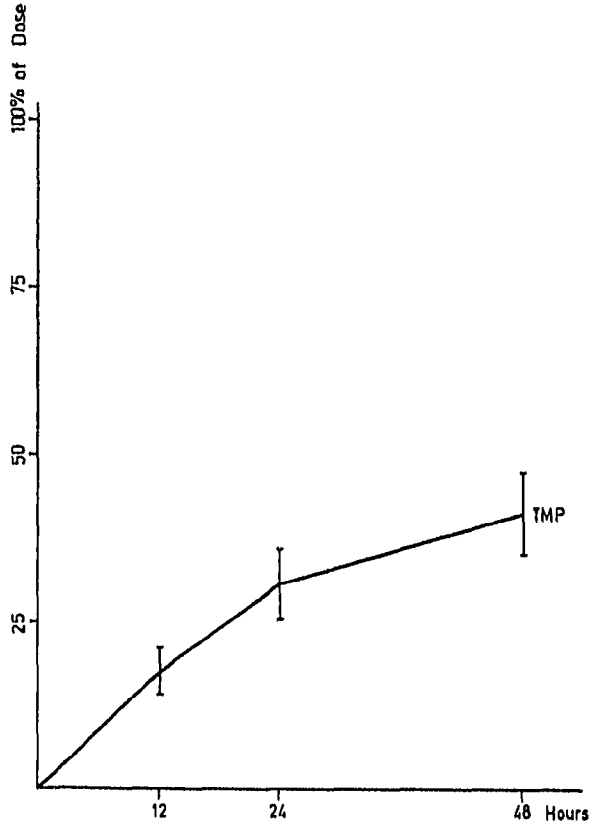


Fig. 3. Cumulative renal excretion profile (in percentage dose) of free sulfadiazine (SDZ) and total sulfadiazine (SDZ + N^4-acetyl metabolite) (average of 8 subjects) after oral administration of two tablets (900 mg SDZ + 300 mg TMP).

Fig. 4. Cumulative renal excretion profile (in percentage dose) of trimethoprim (TMP) (average of 8 subjects) after oral administration of two tablets (900 mg SDZ + 300 mg TMP).

The method described here has been successfully applied in the determination of plasma levels of sulfamethoxazole, its N^4-acetyl metabolite and trimethoprim, after oral administration of co-trimoxazole (Fig. 5). It is well suited too for the determination of drugs and metabolite plasma (and urine) levels in man, after administration of a drug combination of tetroxoprim (TXP) and sulfadiazine (Figs. 6 and 7).

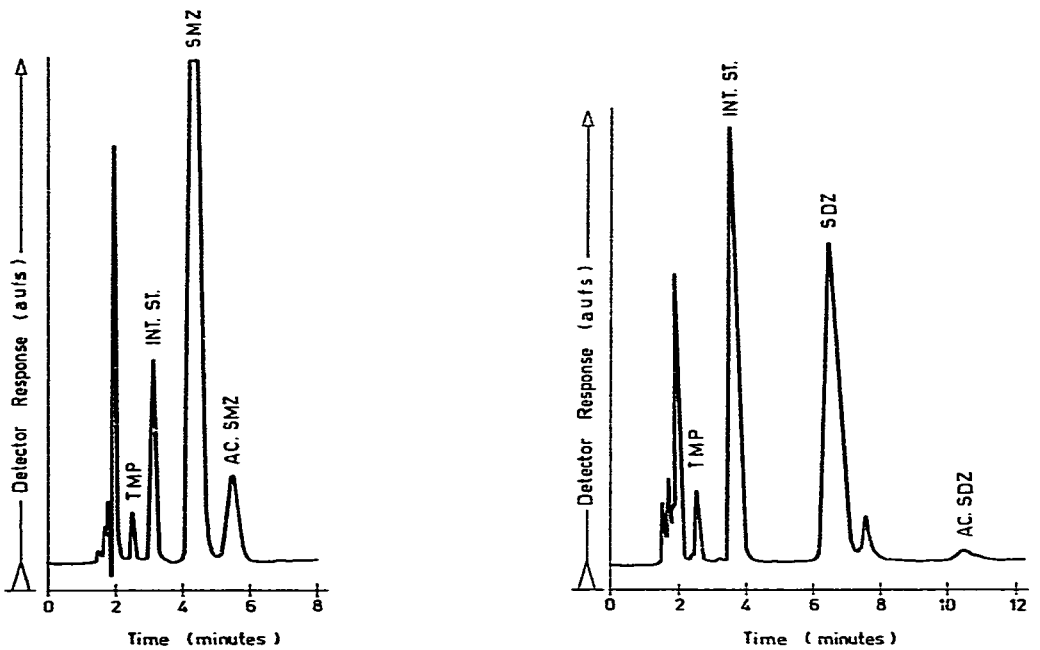


Fig. 5. Chromatogram of human plasma extract from a patient receiving two tablets of co-trimoxazole (Bactrim®; 160 mg TMP + 800 mg SMZ). First administration of a chronic treatment; plasma sample taken at 6 hours.

Fig. 6. Chromatogram of human serum extract from a volunteer receiving two tablets of TMP—SDZ combination (300 mg TMP + 900 mg SDZ; single oral administration, serum sample at 10 hours) (S = 0.1 a.u.f.s.).

Tetroxoprim [2,4-diamino-5-3′,5′-dimethoxy-4′-(β-methoxyethoxy)benzyl-pyrimidine] is a newly developed, highly active inhibitor of procaryontic dihydrofolate reductase, possessing marked antibacterial activity [20]. Another method has been reported [21] for the determination of TXP and SDZ in biological fluids by reversed-phase HPLC. However, the method requires two different types of liquid—liquid extraction for the drugs and different chromatographic conditions for each drug determination.

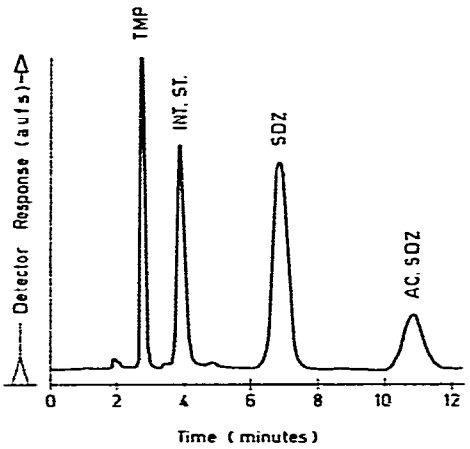


Fig. 7. Chromatogram of human urine extract (1 ml diluted to 10 ml) of a volunteer (same conditions as in Fig. 6). Urine fraction (400 ml) collected from 12 to 24 hours) (S = 0.1 a.u.f.s.).

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