Journal of Chromatography, 274 (1983) 187—199
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1618

DETERMINATION OF TRIMETHOPRIM, SULPHAMETHOXAZOLE AND ITS N^4-ACETYL METABOLITE IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

O. SPREUX-VAROQUAUX*, J.P. CHAPALAIN, P. CORDONNIER and C. ADVENIER

Département de Biologie Clinique et de Pharmacologie, Hôpital A. Mignot, 177 Rue de Versailles, 78150 Le Chesnay (France)

and

M. PAYS and L. LAMINE

Département de Chimie Analytique, Faculté des Sciences Pharmaceutiques et Biologiques, Caen Université, F-14032 Caen Cedex (France)

(First received August 31st, 1982; revised manuscript received December 27th, 1982)

SUMMARY

A normal-phase high-performance liquid chromatographic method was developed to determine therapeutic concentrations of trimethoprim, sulphamethoxazole, and its N_4-acetyl derivative in biological fluids. The compounds are extracted at pH 6.2 using ethyl acetate—chloroform in a single extraction. The detection limit is 15 ng/ml for trimethoprim, 20 ng/ml for sulphamethoxazole, and 10 ng/ml for its N^4-acetyl metabolite. The method is rapid, sensitive, precise, and well suited to clinical pharmacokinetic investigations.

INTRODUCTION

Cotrimoxazole, an association of trimethoprim (TMP) and sulphamethoxazole (SMZ) in a 5:1 ratio, is a powerful broad-spectrum antimicrobial agent used clinically for the treatment of a variety of infections in man [1, 2]. In order to study its pharmacokinetic parameters in the elderly, we decided to re-examine determination methods for the constituents of cotrimoxazole and the metabolite, N^4-acetyl sulphamethoxazole (N^4SMZ) in biological fluids.

SMZ determination has been described using gas—liquid chromatography (GLC) with flame-ionisation detection (FID) [3—5] and by high-performance

0378-4347/83/$03.00 © 1983 Elsevier Science Publishers B.V.

liquid chromatography (HPLC) with spectrophotometric detection [6–11]. TMP determination was carried out using GLC [12, 13] with thermoionic detection and, more easily and with more sensitivity, using HPLC [14]. Determinations using GLC–mass spectrometry [15], polarography [16, 17], and microbiological [18, 19] or isotopic [20, 21] assay have also been described.

TMP and SMZ determinations in the cotrimoxazole association can be done using spectrofluorimetry [22], but the method is long, requires two extractions, and is not free from interference. GLC determination with thermionic detection [23] also requires two extractions and preparation of a methyl derivative for separation of SMZ and N^4SMZ. The method is long and not free of interfering peaks after plasma extraction.

Different authors have reported HPLC methods requiring either two extractions and two chromatographic columns [24], or deproteinisation followed by a reversed-phase separation after an unsuitable dilution for TMP pharmacokinetic determinations [25], or separation using two elution solvents and two successive chromatographic operations [26]. A single extraction method proposed by Ascalone [27] with reversed-phase HPLC separation allows the simultaneous determination of TMP, SMZ, and N^4SMZ, with detection limits compatible with pharmacokinetic studies. In our experience, we were not able to obtain acceptable and reproducible plasma extracts using this method, or to reproduce the extraction yields described, which can be prejudicial for a technique developed without an internal standard.

More recently, Ascalone [28] proposed the simultaneous determination of TMP, sulfadiazine and its N^4-acetyl metabolite by normal-phase HPLC but without an internal standard. Gochin et al. [29] described the simultaneous determination of TMP, SMZ and its N^4-acetyl metabolite with reversed-phase HPLC and one internal standard for the sulfonamide derivatives. This method requires two different extractions for serum and urines samples and is time-consuming.

Taking all these facts into account, and also the adequate method described by Weinfeld and Macasieb [14] for TMP determination only, we described a normal-phase HPLC method for the simultaneous determination of TMP, SMZ and N^4SMZ.

The purpose of this study was to determine the solvent composition and optimum pH providing the best compromise for simultaneous extraction and efficient elution of the three products. This new method was applied to determinations in human plasma for therapeutic concentrations of cotrimoxazole.

EXPERIMENTAL

Chemicals

Trimethoprim, sulphamethoxazole, N^4-acetylsulfamethoxazole, and 2,4-diamino-5-(3,5-dimethoxy-4 methylbenzyl) pyrimidine, used as internal standard for TMP (IS-TMP), were supplied by Hoffmann-La Roche (Basle, Switzerland). Sulphamoxol (Justamil®) used as internal standard for the sulphonamides (IS-SMZ) was supplied by Amphar-Rolland (Paris, France). Chloroform, ethyl acetate, methanol suprapur, and 5 μm LiChrosorb Si-60 (Merck 9388) were purchased from E. Merck (Darmstadt, G.F.R.), or a ready-

to-use column Merck Hibar (ref. 50388); ammonia solution of 28–30% was from Prolabo (Paris, France). Phosphate buffer 0.2 *M*, pH 6.2, was prepared from potassium phosphate, monobasic (27.24 g/l) and sodium phosphate, dibasic dodecahydrate (71.6 g/l) (81.5:18.5, v/v), and purchased from Prolabo. Internal standards and calibration solutions were prepared and then diluted in methanol from the free bases.

Chromatography

The chromatography was performed on a Varian Model 5000 instrument with a fixed-wavelength detector (280 nm) and a 50-μl fixed-volume injector. The column was a stainless-steel tube (25 cm × 4 mm) filled with 5 μm LiChrosorb Si-60 (Merck 9388) using the balanced-density slurry packing

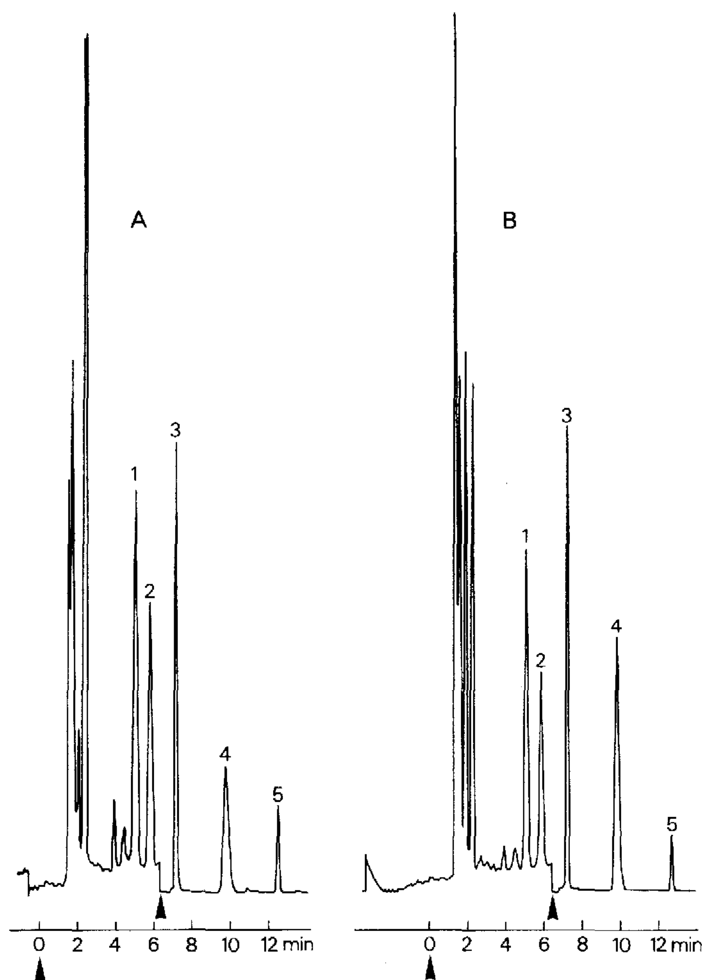

Fig. 1. Chromatograms corresponding to the extract of: (A) 1 ml of spiked plasma, with 1 = IS-TMP 2 μg, 2 = TMP 2.5 μg, 3 = IS-SMZ–N^4SMZ 40 μg, 4 = SMZ 25 μg, and 5 = N^4SMZ 12.5 μg; (B) 1 ml of patient plasma, with 1 = IS-TMP, 2 = TMP, 3 = IS-SMZ, 4 = SMZ, and 5 = N^4SMZ. Retention times are (min): peak 1 = 5, peak 2 = 5.8, peak 3 = 7, peak 4 = 9.6, peak 5 = 12.6.

technique. The slurry, 3.6 g of 5 μm LiChrosorb Si-60 dispersed in 15.6 ml of carbon tetrachloride, was forced into the column with methanol. Alternatively, a Merck Hibar column (ref. 50388) was used. The precolumn was a stainless-steel tube (4 cm × 4 mm) filled with 25—40 μm LiChroprep Si-60 (Merck 9390). The degassed mobile phase used at a flow-rate of 2 ml/min is a mixture of chloroform—methanol—distilled water—ammonia solution (94.5:5.0:0.25:

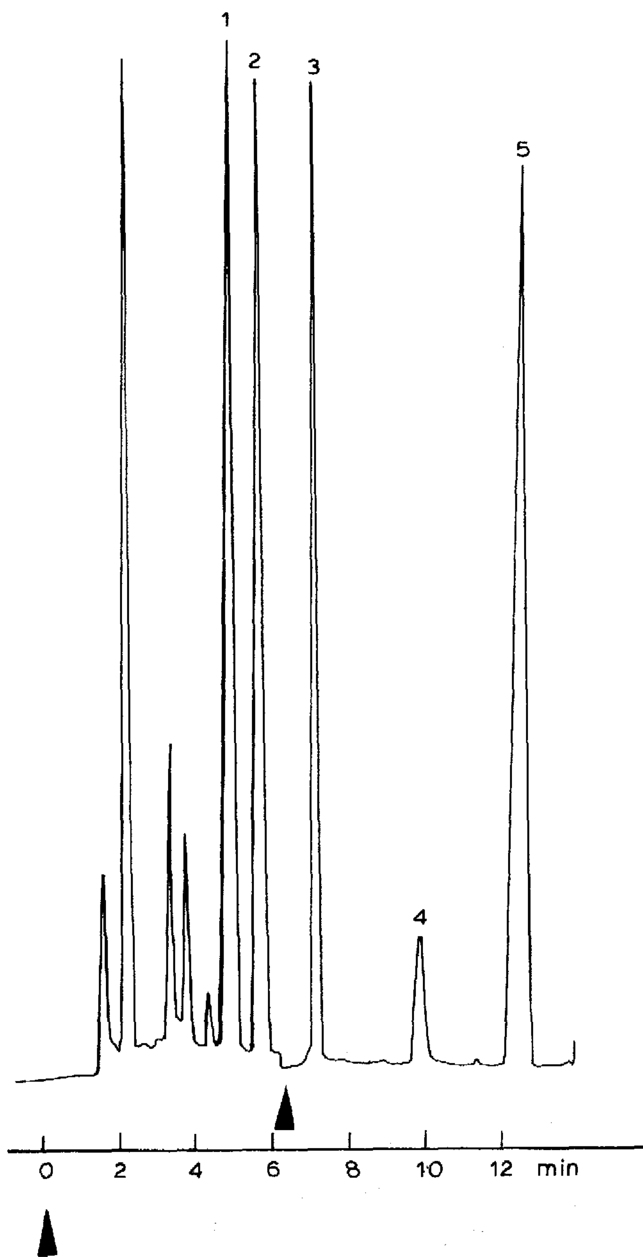

Fig. 2. Chromatogram of an extract of urine (subject 2) containing IS-TMP (1), TMP (2), IS-SMZ (3), SMZ (4), and N^4SMZ (5).

0.19, v/v, solvent A) during the first 3 min and (79:20:1:0.15, solvent B) during the following 8 min. Programming is: 0—3 min, solvent A; 3.5 min, solvent A + solvent B (50:50); 4—12 min, solvent B; 12.5 min, solvent A + solvent B (50:50); 13—16 min, solvent A.

TMP and its internal standard were eluted between 0 and 6 min; SMZ, N^4SMZ and their internal standard were eluted between 7 and 13 min.

The detector sensitivity is 0.02 a.u.f.s. between 0 and 6 min, and 0.5 a.u.f.s. beyond, which allows all five peaks that are being looked for to show on the same chromatogram, the differences in concentration to be detected between TMP and the sulphonamides being taken into account. Retention times are shown in Figs. 1 and 2. Maximum pressure was about 180 bars. After about 500 injections, the pressure had not increased, and the retention times were not modified. The mobile phase and the column were at room temperature.

Sample preparation

To 1 ml of plasma or urine (usually as a 1/2 to 1/5 aqueous dilution) in a 30-ml glass tube were added 20 μl of internal standard solutions (IS-TMP, 2 μg; IS-SMZ—N^4-SMZ, 40 μg), 5 ml of phosphate buffer, 0.2 *M* pH 6.2, and 12 ml of chloroform—ethyl acetate (75:25). The stoppered tube was shaken mechanically for 15 min (Kahn vibrator), and centrifuged at 3000 *g* for 10 min. Then 10 ml of the chloroform phase were transferred into another tube and evaporated to dryness under nitrogen at 50°C. The residue was dissolved in 300 μl of mobile phase A. After mixing for 20 sec on a Vortex mixer, 50 μl were injected.

Calibration curves

Plasma samples were prepared by adding 20 μl of each internal standard solution and 100 μl of methanolic solutions of increasing concentrations of TMP (0.5—10 μg/ml), SMZ (5—100 μg/ml) and N^4SMZ (2.5—50 μg/ml) to 1 ml of plasma from drug-free patients. Urine calibrations were established with 1 ml of dilute sample usually spiked with TMP, SMZ (2.5—50 μg/ml) and N^4SMZ (5—100 μg/ml). These samples were then worked up according to the procedure described above. The calibration curves were obtained by plotting the peak area ratio for each substance with its reference, against concentration.

RESULTS AND DISCUSSION

Choice of extraction solvent

This choice was guided by previous work. Weinfeld [14] extracted TMP, pH 10, in chloroform. Ascalone [27, 28] extracted TMP and the sulphonamides, pH 6.8, in ethyl acetate.

We have verified experimentally that TMP in a neutral or alkaline condition is more soluble in chloroform than in ethyl acetate, contrary to SMZ and its acetyl derivative. The influence of the proportions of the two constituents of the extraction solvent (chloroform and ethyl acetate) was studied for each product.

For each extraction solvent, 3—6 extractions were carried out on 1 ml of plasma loaded with 2 μg of TMP, 2 μg of IS-TMP, 40 μg of SMZ, 20 μg of

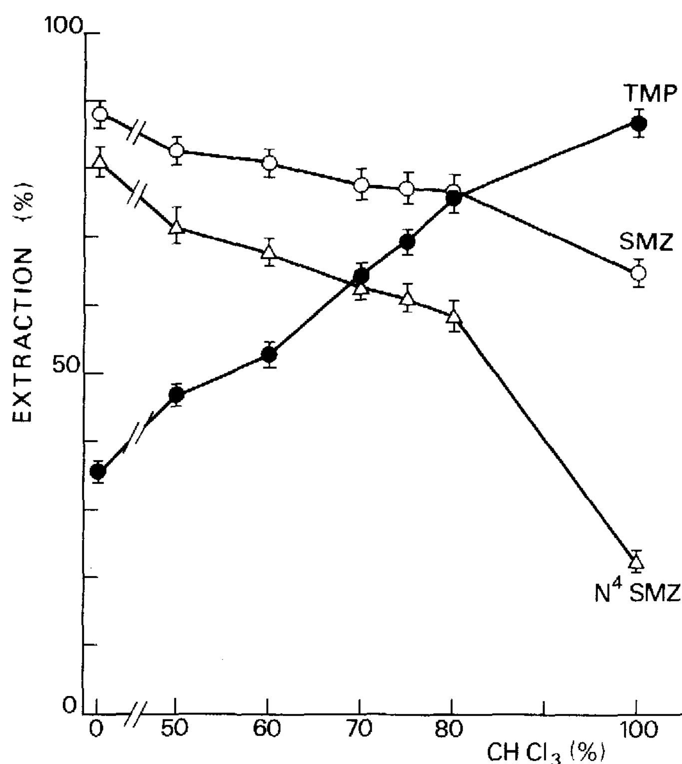

Fig. 3. Influence of the relative proportions of the two constituents of the extraction solvent (chloroform—ethyl acetate) on the percentage extraction of TMP (2 μg/ml), SMZ (40 μg/ml), and N^4SMZ (20 μg/ml) using a pH 6.2 buffer before extraction (mean ± S.E.M., n = 3—6).

N^4SMZ, and 40 μg of IS-SMZ. The extraction was worked up according to the procedure described above using a phosphate buffer, pH 6.2, and 12 ml of the extraction solvent. The mean extraction percentage results were obtained by comparing the amount added to the plasma with the same amount without extraction.

Fig. 3 shows that TMP extraction is considerably improved by an increased proportion of chloroform, while that of the sulphonamides is improved by an increased proportion of ethyl acetate.

In view of the relative proportions of the different products, TMP being present only in low concentrations must be determined more accurately. Only the range of the proportion of chloroform from 70 to 80% at pH 6.2 permits the acceptable simultaneous extraction of 60—80% of the three products and justifies the choice of the 75:25 proportions in the proposed method.

Choice of buffer pH before extraction

The effect of the pH of the buffer used before extraction has been studied for each product. Phosphate buffer solutions (0.2 *M*, at pH 5.4, 5.8, 6.2, 6.6, 7.0, 8.0, and 11) were prepared according to Sörensen's method [30].

For each pH, four extractions were carried out on 1 ml of plasma loaded

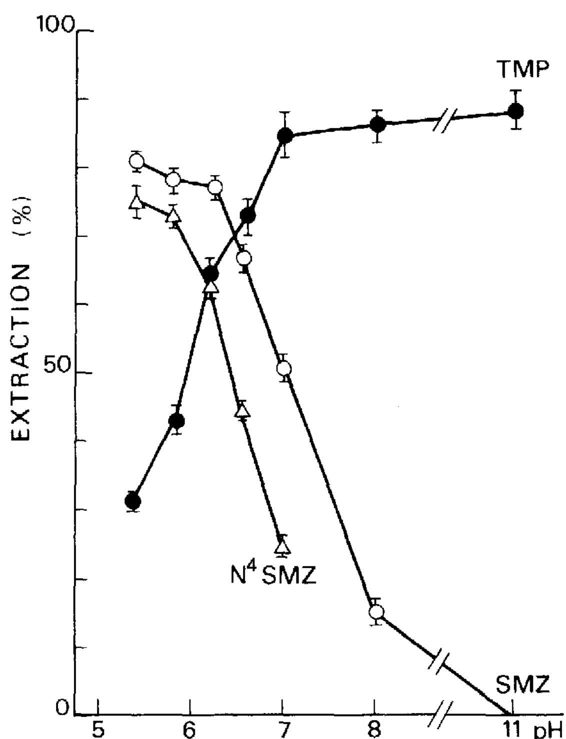

Fig. 4. Influence of buffer pH on the extraction of TMP (2 μg/ml), SMZ (40 μg/ml), and N^4SMZ (20 μg/ml) using 12 ml of chloroform—ethyl acetate (70:30) as the extraction solvent (mean ± S.E.M., n = 4).

with 2 μg of TMP, 2 μg of IS-TMP, 40 μg of SMZ, 20 μg of N^4SMZ, and 40 μg of IS-SMZ. The extraction was worked up according to the procedure described above using 12 ml of chloroform—ethyl acetate (70:30) as the extraction solvent. The mean extraction percentage results were obtained by comparing the amount added to the plasma with the same amount without extraction.

The choice of pH for the aqueous phase at the moment of extraction is also the result of a compromise that takes into account the differences in concentration being researched for TMP and SMZ. The extraction of SMZ is maximal at pH < 5.6, and that of TMP at pH > 8.0 (Fig. 4).

The choice of pH 6.2 ensures simultaneous extraction suitable for SMZ, its metabolite, and TMP. It should be noted that, in these experimental conditions, the choice of an extraction pH of 6.8, as recommended by Ascalone [27], would be prejudicial to the extraction of SMZ.

Optimal conditions for each product

The extraction yield of TMP was evaluated using the same procedure, but with a buffer solution of pH 11 and chloroform (100%) for the extraction of five series of three doses (1,2 and 4 μg/ml) of TMP, giving extraction efficiencies of 96.6, 98 and 95.4% with coefficients of variation of 1.4, 0.7 and 0.9%, respectively.

TABLE I

ACCURACY AND DAY-TO-DAY REPRODUCIBILITY FOR TMP, SMZ AND N^4SMZ (SPIKED HUMAN PLASMA SAMPLES, 1 ml)

	TMP			SMZ			N^4SMZ		
Amount added (μg/ml)	1	2	4	20	40	80	10	20	40
Amount found (μg/ml)	0.70	1.34	2.71	15.4	31.1	64.8	6.6	12.1	25.2
Replicates (*n*)	6	6	6	6	6	6	6	6	6
Coefficient of variation (%)	2.9	0.7	1.2	2.0	1.1	0.5	2.8	0.4	0.5
Mean recovery (%)	70.8	67.2	67.8	81	78.8	83.8	65.5	61	64.2

TABLE II

ACCURACY, PRECISION AND WITHIN-DAY REPRODUCIBILITY FOR TMP, SMZ AND N^4SMZ (SPIKED HUMAN PLASMA SAMPLES, 1 ml)

	TMP/IS-TMP				SMZ/IS-SMZ				N^4SMZ/IS-SMZ			
Amount added (μg/ml)	1	2	3	4	20	40	60	80	10	20	30	40
Mean ratio product/IS	0.24	0.45	0.66	1.0	0.87	1.35	1.99	2.53	0.34	0.63	0.93	1.15
n replicates	8	7	7	3	7	6	6	3	8	7	11	4
Coefficient of variation (%)	0.78	1.3	0.28	6.4	0.6	1.75	1.1	1.25	1.8	1.5	3.4	2.4

The extraction yields of SMZ and its metabolite were assessed with the same methodology, but using a buffer solution of pH 5.4 and ethyl acetate (100%) for the extraction of a series of 4–6 doses of SMZ (20, 40, and 80 μg/ml) and of N^4SMZ (10, 20, and 40 μg/ml). The extraction percentages were 97, 98.5 and 99.1% for SMZ and 99.3, 98.2 and 98.8% for N^4SMZ, respectively, with coefficients of variation of 0.8, 2.4 and 1.1% for SMZ and 1.4, 2.7 and 0.9% for N^4SMZ, respectively. In view of the analytical characteristics of these molecules, associated in cotrimoxazole, it would be illusory to envisage a quantitative yield for intermediate pH values. The choice of an intermediate pH is therefore a compromise that allows a simpler and more rapid investigation. It was validated by using two internal standards added before extraction insofar as these internal standards are homologous or structurally analogous to the two structures with similar analytical characteristics.

It should be noted that Weinfeld [14] for the determination of TMP alone, and Gochin et al. [29] for cotrimoxazole association, use this procedure, but the latter using only one internal standard for the sulfonamide derivatives.

Plasma interference

Theophylline and an unknown metabolite of caffeine were identified as interfering compounds with the same retention time (5.0 min) as the internal standard for TMP (IS-TMP), while caffeine has a shorter retention time (less than 2 min).

The ingestion of tea or coffee should therefore be prohibited at the time of biological measurements in humans.

Chromatographic conditions

Chromatographic separation is developed without an elution gradient, but with only one change in the polarity of the elution solvent. This solvent contains chloroform and two different proportions of polar solvent (methanol—water, 20:1) providing also a variable basicity (NH_4OH).

The increase in proportion of the polar solvent accelerates the elution of all the peaks on the chromatogram.

The solvent A used is the one proposed by Weinfeld [14] for TMP determination. Its basicity allows the separation of TMP and IS-SMZ. The more polar solvent B accelerates the elution of SMZ and N^4SMZ.

Linearity

The calibration curves were established as described above. A good linear relationship was obtained in the range of 0.1–20 μg/ml for TMP (r = 0.999), 2–150 μg/ml for SMZ (r = 0.999), and in the range of 1–100 μg/ml for N^4SMZ (r = 0.999).

For routine analysis, a calibration curve is established each day. The linear regression equations are $y = 0.049 + 0.28x$ for TMP, $y = 0.089 + 0.025x$ for SMZ, and $y = 0.043 + 0.012x$ for N^4SMZ.

Accuracy, precision and sensitivity

The day-to-day reproducibility for several series is shown in Table I. The variation coefficients are satisfactory. Extraction yields are weak but were expected in view of the opposing optimal conditions of the different products for the pH of the plasma buffer and for the extraction solvent.

The study of intraserial reproducibility is shown in Table II. The variation coefficients observed are satisfactory.

The sensitivity limit in 1 ml of plasma is 15 ng/ml for TMP, 20 ng/ml for SMZ, and 10 ng/ml for N^4SMZ, using the detector set at 0.01 a.u.f.s. for TMP and 0.05 a.u.f.s. for SMZ and N^4SMZ and a 100-μl sample of the evaporation residue instead of 300 μl. This corresponds to a signal-to-noise ratio of three for TMP and two for SMZ and N^4SMZ.

TABLE III

PHARMACOKINETIC PARAMETERS FOR TMP AND SMZ IN THREE NORMAL VOLUNTEERS

$K_{abs.}$ = Apparent absorption rate constant; K_e = elimination rate constant; $t_{1/2\beta}$ = elimination half-life; AUC = area under the curve; V_d = volume of distribution; and Cl_T = total plasma clearance.

	TMP	SMZ
$K_{abs.}$ (h^{-1})	2.36 ± 0.98	2.53 ± 1.38
K_e (h^{-1})	0.093 ± 0.023	0.068 ± 0.063
$t_{1/2\beta}$ (h)	8.74 ± 2.58	10.25 ± 0.50
AUC (mg l^{-1} h^{-1})	27.1 ± 6.2	826 ± 102
V_d (l kg^{-1})	72.2 ± 5.7	14.6 ± 1.4
Cl_T (l h^{-1})	6.44 ± 1.26	0.99 ± 0.11

Selectivity

N^4-Acetylation is a major hepatic pathway of the biotransformation of sulphonamides in man. The derivative N^4SMZ is usually found in the proportions of 30% in the blood and 60% in urine [31]. We were interested in being able to quantify it simultaneously with SMZ and TMP.

The biotransformation of TMP is less important, the proportion of its metabolites in the blood being of the order of 10% and of the order of 20—40% in urine [31].

Stability

The dry residue was dissolved in 300 μl of the mobile phase just before injection to ensure good reproducibility. The dry evaporation residue remains stable for 2 days when stored in the refrigerator; upward of two days was not tested.

Application

Pharmacokinetic constants of TMP, SMZ and N^4SMZ were studied in three elderly volunteers (two males and one female) aged 74 ± 3.6 years and weighing 58.6 ± 7.6 kg. They were all healthy subjects, gave their informed consent, and were not taking concurrent major medication.

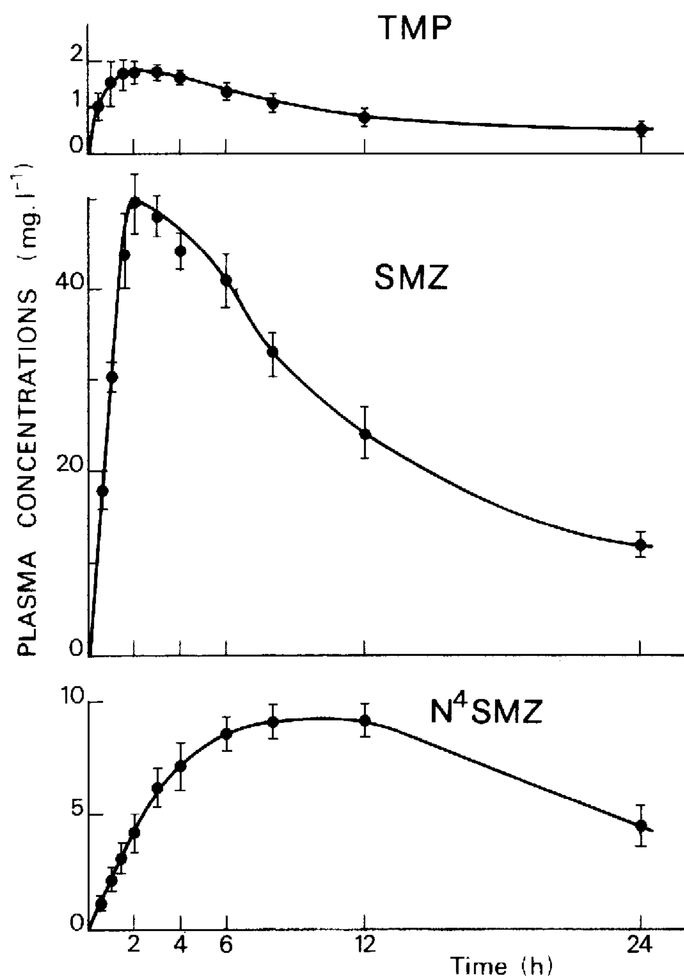

Fig. 5. Plasma TMP, SMZ and N^4SMZ concentrations (± S.E.M.) against time, after oral administration of TMP 160 mg, and SMZ 800 mg to three volunteers.

Each subject received orally, in a fasting state, TMP (160 mg) and SMZ (800 mg) (Bactrim forte®) and blood samples were collected 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24 h after administration. Plasma was immediately separated, frozen and kept at −15°C until analysed.

The plasma levels vs. time data following this administration are shown in Fig. 5, and the resultant pharmacokinetic parameters using a one-compartment model are represented in Table III. These pharmacokinetic data observed for TMP and SMZ are in good agreement with those reported in the literature [31].

Urine elimination of TMP, SMZ and N^4SMZ in the three patients represented in Table IV appears to be less than those reported in the literature for young adults (respectively, 58—71% and 70—84% for TMP and total SMZ) [32—35].

These preliminary results validate the analytical method. A further pharmacokinetic study in healthy and infected elderly patients will be published later.

TABLE IV

URINARY ELIMINATION (0—48 h) OF TRIMETHOPRIM (TMP), FREE SULFAMETHOXAZOLE (SMZ) AND N^4-ACETYLSULFAMETHOXAZOLE (N^4SMZ) IN THREE SUBJECTS

Subject	TMP		SMZ		N^4SMZ	
	Total mg excreted	Percentage dose excreted	Total mg excreted	Percentage dose excreted	Total mg excreted	Percentage dose excreted
1	22.5	14.0	3.8	0.4	112.6	14.3
2	41.7	26.1	42.8	5.4	181.9	22.9
3	53.0	33.1	37.1	4.6	395	49.1
Mean	39.0	24.4	27.9	3.4	229.8	28.7
S.E.M.	9.1	5.7	12.4	1.6	86.6	10.6

CONCLUSION

The present method with normal-phase HPLC permits pharmacokinetic studies and drug monitoring. The limit of detection, the use of internal standards for each of the studied structures and a rapid and identical protocol for the extraction of both urine and plasma samples [29] differentiate it from previously published methods [27—29].

REFERENCES

1 S.R.M. Bushby and G.H. Hitchings, Brit. J. Pharmacol., 33 (1968) 72.
2 A. Kucers and N. McK. Bennett, The Use of Antibiotics, William Heinemann Medical Books Ltd., London, 3rd ed., 1979, p. 687.
3 R.J. Daun, J. Ass. Offic. Anal. Chem., 54 (1971) 1277.
4 E. Roder and W. Stuhe, Z. Anal. Chem., 271 (1974) 281.
5 N. Nose, S. Kobayashi, A. Hirose and A. Watanabe, J. Chromatogr., 123 (1976) 167.
6 S.C. Su, A.V. Hartkopf and B.L. Karger, J. Chromatogr., 119 (1976) 523.
7 G.W. Peng, M.A.F. Gadulla and W.L. Chiou, Res. Commun. Chem. Pathol. Pharmacol., 18 (1977) 233.
8 K.L. Johnson, D.T. Jetter and R.C. Clairbone J. Pharm. Sci., 64 (1975) 1657.
9 J.P. Sharma, E.G. Perkins and R.F. Bevill, J. Pharm. Sci., 65 (1976) 1606.
10 M.H. Penner, J. Pharm. Sci., 64 (1975) 1017.
11 T.C. Kram, J. Pharm. Sci., 61 (1972) 254.
12 A.P. Lotter and A.P. Goossens, S. Afr. Pharm. J., 44 (1977) 282.
13 G. Land, K. Dean and A. Bye, J. Chromatogr., 146 (1978) 143.
14 R.E. Weinfeld and T.C. Macasieb, J. Chromatogr., 164 (1979) 73.
15 W.J. Irwin and J.A. Slack, J. Chromatogr., 139 (1977) 364.
16 M.A. Brooks, J.A.F. de Silva and L. D'Arconte, Anal. Chem., 45 (1973) 263.
17 M.A. Brooks, J.A.F. de Silva and L. d'Arconte, J. Pharm. Sci., 62 (1973) 1395.
18 S.R.M. Bushby, Postgrad. Med. J., 45 (Suppl.) (1969) 10.
19 J.C. Pechere, Pathol. Biol., 18 (1970) 343.
20 D.E. Schwartz, W. Wetter and G. Englert, Arzneim.-Forsch., 20 (1970) 1867.
21 P. Nielsen and R. Folke, Acta Pharmacol. Toxicol., 37 (1975) 309.
22 D.M. Lichtenwalner, B. Suh, B. Lorberg and A.M. Sugar, Antimicrob. Agents Chemother., 16 (1979) 579.

23 V. Ascalone, Boll. Chim.-Farm., 117 (1978) 176.
24 A. Bye and M.E. Brown, J. Chromatogr. Sci., 15 (1977) 365.
25 T.B. Vree, Y.A. Hekster, A.M. Baars, J.E. Damsma and E. van der Kleijn, J. Chromatogr., 146 (1978) 103.
26 R.W. Bury and M.L. Mashford, J. Chromatogr., 163 (1979) 114.
27 V. Ascalone, J. High Resolut. Chromatogr. Commun., 3 (1980) 261.
28 V. Ascalone, J. Chromatogr., 224 (1981) 59.
29 R. Gochin, I. Kanfer and J.M. Haigh, J. Chromatogr., 223 (1981) 139.
30 S.P.L. Sörensen, Ergeb. Physiol., 12 (1912) 393.
31 R.B. Patel and P.G. Welling, Clin. Pharmacokin., 5 (1980) 405.
32 D.E. Schwartz and J. Rieder, Chemotherapy, 15 (1970) 337.
33 S.A. Kaplan, R.E. Weinfeld, S. Cotler, C.W. Abruzzo and K. Alexander, J. Pharm. Sci., 59 (1970) 358.
34 P.G. Welling, A. Graig, G.L. Amidon and C.M. Kunin, J. Infect. Dis., 128 (Suppl.) (1973) 556.
35 H. Nolte and H. Büttner, Chemotherapy, 18 (1973) 274.