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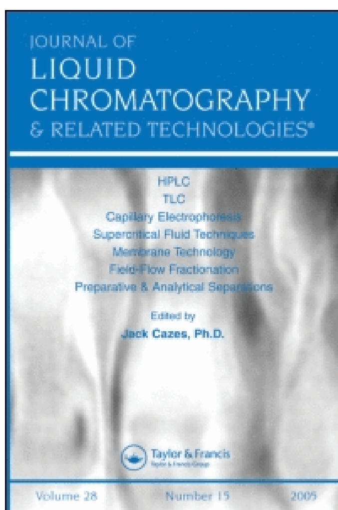

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Determination of Trimethoprim and Sulphamethoxazole in Serum by Reversed-Phase and Ion Pair HPLC

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DETERMINATION OF TRIMETHOPRIM AND SULPHAMETHOXAZOLE IN SERUM BY REVERSED-PHASE AND ION PAIR HPLC

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ABSTRACT

HPLC serum assay methods for trimethoprim and sulphamethoxazole are described. Octadecylsilane coated silica is used as the stationary phase in both analyses. The determination of trimethoprim involves extraction and chromatography employing reversed phase ion-pair HPLC with u.v. detection at 229nm. Using 1ml of serum, trimethoprim at levels of 10ng/ml can be measured. Sample preparation of sulphamethoxazole consists of protein precipitation only. Chromatography is by ion-supression and monitoring at 254nm. The sulphamethoxazole can be detected in serum at concentrations in the region of 0.1μg/ml. Results are presented to show the variation of trimethoprim and sulphamethoxazole in serum of eight healthy subjects over a 72 hour period following ingestion of 20ml co-trimoxazole suspensions from two manufacturers.

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INTRODUCTION

Co-trimoxazole, a combination of sulphamethoxazole (SMZ) and trimethoprim (TMP) in a 5:1 ratio is a highly effective broad spectrum antibacterial formulation. The determination of TMP and SMZ in serum and other biological samples by HPLC has been the subject of several reports (1-5). Due to strong retention of polar substances on normal phase columns, which results in long column recovery time, reversed phase support is preferred in routine analysis of these two drugs in serum samples.
In the reported assays, chromatographic retention and separation of SMZ and TMP have been achieved using aqueous and organic modifier eluents with or without the addition of hydrophobic pairing ions.

However, quantitation of these two drugs in biological samples by HPLC is not without problems. Retention of the weakly acidic sulphemethoxazole (pK_a = 5.6) is usually achieved by ionic supression using a low pH mobile phase. In such cases trimethoprim, which is a weak base with a pK_a of 7.2 (6), is fully protonated and will elute rapidly from the column. In some investigations the pH of the eluent is adjusted so as to achieve retention of the two compounds. These procedures usually result in chromatograms with poor resolution. Furthermore, many of these methods involve time consuming sample preparation steps and/or lack adequate sensitivity or specificity for pharmacokinetic studies or routine drug monitoring.

In the present study two relatively rapid and reliable isocratic methods are described. A reversed phase ion-pair chromatographic method is presented for the determination of TMP and a reversed phase system is described for SMZ. These procedures have been applied to a study comparing the bioavailability of two co-trimoxazole suspensions of the same strength but from different manufacturers.

EXPERIMENTAL

Apparatus

A waters Associates (Milford, MA, USA) liquid chromatography system consisting of a M6000A pump, a M441 selectable wavelength

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uv detector and an U6K injector was employed. Chromatographic columns for TMP and SMZ were 90mm x 4.6mm i.d. and 150mm x 4.6mm i.d. respectively. Both of these columns were slurry packed with 5μm Hypersil ODS (Shandon Southern Products, London, UK) and typically gave a plate efficiency in excess of 5000 per 100mm column length. Retention time data were obtained directly from chromatograms as recorded on a potentiometric recorder (Toshim electron, Tokyo, Japan). Peak height was used for quantitation.

Materials

TMP, SMZ and sodium lauryl sulphate (SLS) were obtained from Sigma Chemicals (St. Louis, Missouri, USA). Dichloromethane, ether, perchloric acid, disodium hydrogen phosphate dodecahydrate, orthophosphoric acid (88%) and 4-dimethyl-amino-benzaldehyde (4DAB) were purchased from BDH (Poole, UK). Acetonitrile (HPLC grade) and absolute methanol were obtained from J.T. Baker (Phillipsburg, N.J., USA). The glassware silanizing agent, Aquasil(R), was from Pierce Chemical (Rockford, USA). Timolol maleate (TIM) was kindly donated by Merck Sharp & Dohme (West Point, Pennsylvania, USA). Bactipront(R) suspension (Lot No:343-13009) was supplied by Pfizer Laboratories (Bangladesh). Septrin(R) suspension (batch 9357) was manufactured by Wellcome New Zealand Ltd. Both suspensions contain 40mg TMP and 200mg SMZ per 5ml. Water was glass-distilled and MilliQ(R) filtered. All reagents were of Analar or equivalent grade.

Study Design

Eight healthy adult male volunteers aged between 20 and 31, participated in a two way cross-over bioavailability study. The subjects were divided into two groups. Group one received 20ml of Septrin(R) suspension first, followed by 20ml of Bactipront(R) suspension, and group two received 20ml of Bactipront(R) suspension first, followed by 20ml of Septrin(R) suspension. At least one week separated the administration of the two dosage forms. Ten millilitre blood samples were collected from 0 to 72 hours after

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the dose at scheduled intervals via intravenous cannulae or by venepuncture. All blood samples were allowed to clot. Serum was collected and frozen at -15°C until assayed.

Sample Preparation

Trimethoprim.

All glassware was silanized before use. To 1ml of serum in a centrifuge tube, 100μl of internal standard (TIM) at a concentration of 40 or 250μg/ml in methanol, and 500μl of 0.5M sodium hydroxide were added and vortexed for 10 seconds. After adding 5ml of dichloromethane: ether mixture (1:1.5), the contents were shaken gently for 5 minutes and centrifuged to separate the phases. The organic layer was then transferred to another centrifuge tube and evaporated to dryness at 37°C under a stream of nitrogen. The residue was dissolved in 100μl of 0.1M hydrochloric acid. The sample was further purified by vortexing briefly with 500μl of ether then centrifuging for 5 minutes at 3000rpm. Ten to 25μl of the aqueous solution were injected into the chromatograph.

Sulphamethoxazole.

Fifty microlitres of internal standard (4-DAB) at a concentration of 50 or 500μg/ml in absolute methanol were added to 1ml of serum in a centrifuge tube and vortexed for 10 seconds. The serum was deprotienated by adding 250μl of perchloric acid (60%w/v) and vortexed for 10 seconds. The tube was certifuged at 3000rpm for 10 minutes. Ten to 25μl of the clear supernatent were then injected onto the column.

Chromatography

The mobile phase for TMP was a mixture of acetonitrile aqueous buffer (35:65 v/v) containing 30mM SLS, 50mM disodium hydrogen phosphate and adjusted to pH2 with orthophosphoric acid. A flow rate of 2ml/min and u.v. detection at 229nm with a cadmium light source was employed.

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The eluent for SMZ consisted of acetonitrile aqueous buffer (25:75v/v) which contained 50mM disodium hydrogen phosphate and adjusted to pH 2 with orthophosphoric acid. A flow rate of 2ml/min and uv detection at 254nm with a mercury light source was used.

RESULTS AND DISCUSSION

Extraction Procedure

Several extraction methods for TMP from serum were investigated. Single step extraction by protein precipitation using various precipitants was found to be ineffective. This method provided neither adequate sensitivity nor selectivity for TMP. The conventional liquid-liquid extraction, evaporation and reconstitution procedures were then adopted. When ether alone was used as the extraction solvent, a sufficiently "clean" extract that required no further purification was obtained. However, recoveries of TMP from serum were poor (40-50%). Substitution of dichloromethane for ether gave excellent recoveries (>90%), nevertheless, high background interference in blank was observed. Of the solvent systems studied a dichloromethane:ether mixture (1:1.5) provided the optimal extraction efficiency (88-95%) and selectivity. A chromatogram of a subject's blank serum is shown in Figure 1A.

As SMZ possesses good uv absorption and was present in relatively high concentrations it did not, therefore, require any preconcentration of the samples for more sensitive analysis. Direct injection of the sample after protein precipitation provided satisfactory results. For SMZ, protein precipitants, such as tungstic acid (20% w/v sodium tungstate in 2M sulphuric acid), trichloroacetic acid (60% w/v), perchloric acid (60% w/v) and acetonitrile were studied for their sample clean-up and extraction efficiency. Trichloroacetic and perchloric acid were found to be superior to the other chemicals. For convenience perchloric acid was used. A specimen chromatogram of a volunteer's blank serum sample is shown in Figure (1B).

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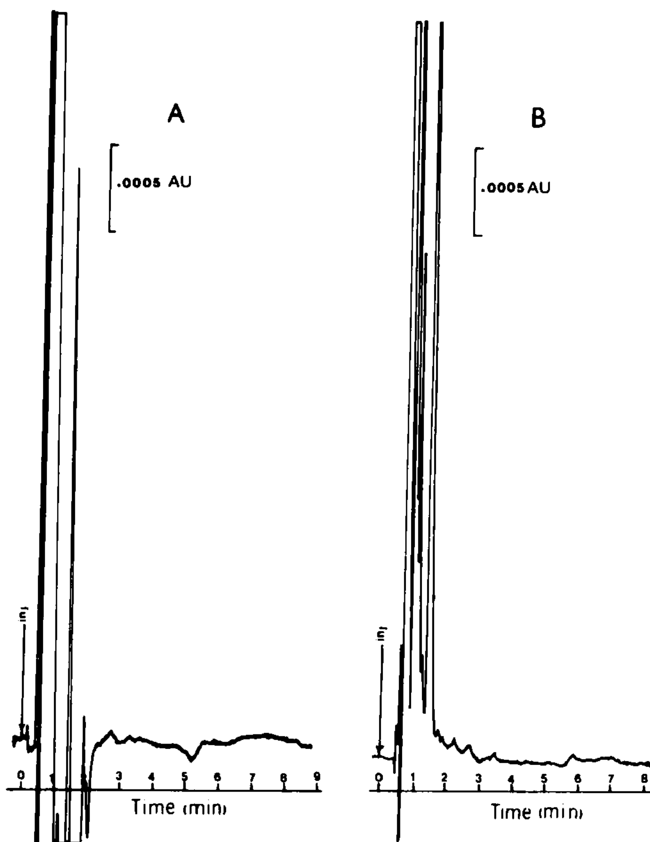

Figure 1 Representative chromatograms of serum blank abstracts from volunteers (A) TMP analysis, (B) SMZ analysis. Chromatographic conditions: see text.

Chromatography

Trimethoprim.

Ionic supression techniques, which are often applied to the analysis of acidic compounds, are less attractive when used in the assay of basic compounds like TMP. This is because the high pH value used for ionic suppression will have a devastating effect on the silica support. Reversed phase ion-pair HPLC is obviously the alternative for the quantitative determination of compounds such as TMP.

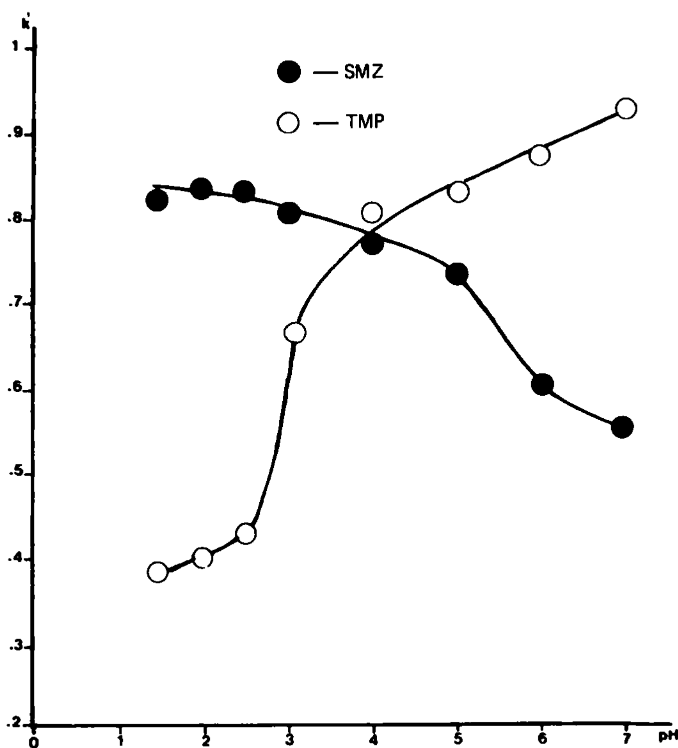

Figure 2 Effect of pH on k' values of TMP and SMZ. Chromatographic conditions: 150 x 4.6mm Hypersil - ODS column; mobile phase of 50% v/v acetonitrile - buffer of 20mM Na_2HPO_4.

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There are several variables peculiar to the technique of ion-pair chromatography, and they can be adjusted to modify the capacity factor (k') of both ionic and neutral solutes. How they are varied will depend upon the purpose of the chromatography. The intention of this study was to develop an ion-pair system which could display high sensitivity and selectivity for TMP in the presence of larger quantities of SMZ in the plasma.

The variables considered in selecting a chromatographic system are interrelated. For example the organic modifier concentration will alter pairing ion adsorption. These variables are tested as follows.

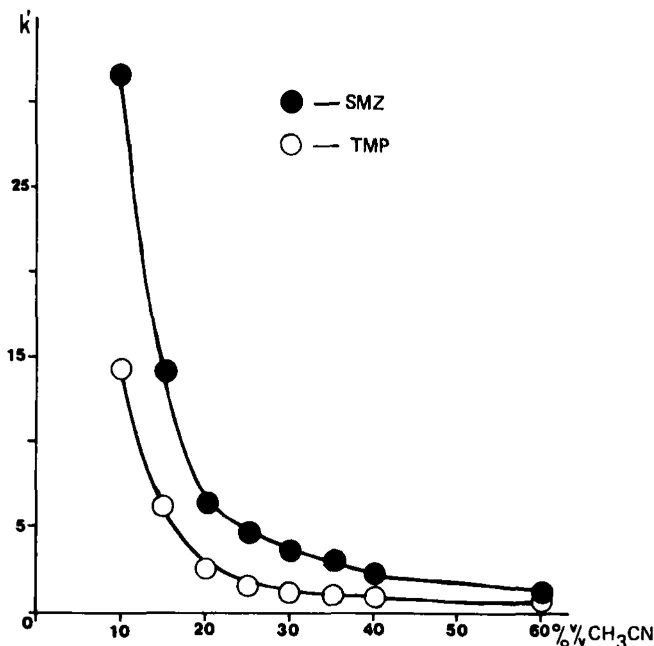

Figure 3 Influence of the acetonitrile content of the eluent on k' of TMP and SMZ. Chromatographic conditions: 150 x 4.6mm Hypersil - ODS column; solvent : acetonitrile - buffer of 20mM Na_2HPO_4 at pH 2.

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Effect of pH: This can markedly affect retention by changing the dissociation state of a solute. In ion-pair chromatography, it is general practice to assume that the solute is fully ionized in order to utilize retention based on electrostatic interaction (7). Figure 2 demonstrates the effects of altering pH of the eluent on the k' of TMP and SMZ. It is observed that the k' value of TMP increases as pH increases. When pH is above 5 the peak shape becomes distorted due to severe peak tailing. The pH of the eluent was thus fixed at pH 2, since at this pH the peak shape is sharp and the k' of TMP is insensitive to the change of hydrogen ion concentration.

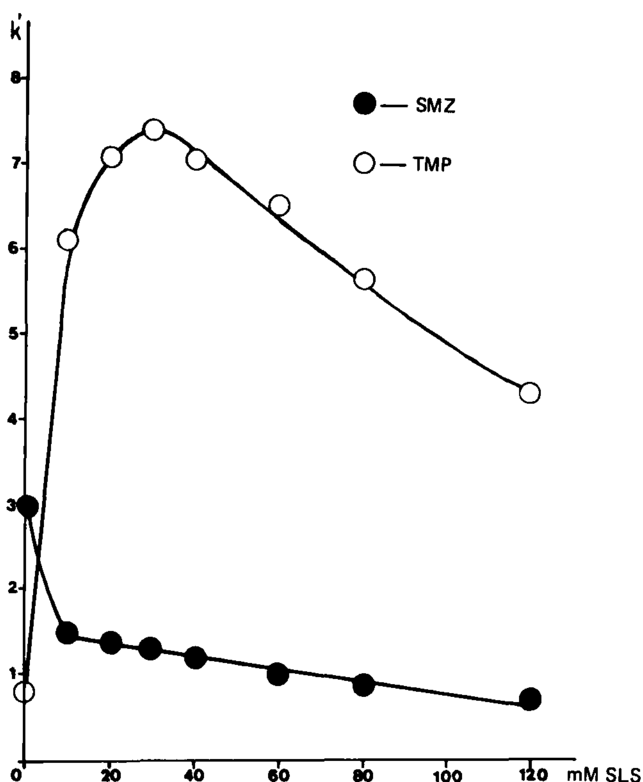

Figure 4 Variation of k' with mobile phase SLS concentration for TMP and SMZ. Column 90 x 4.6mm Hypersil ODS; solvent 35% v/v acetonitrile - buffer of 50mM Na_2HPO_4 at pH 2.

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Effect of the Organic Modifier Concentration: In general, increasing organic modifier concentration will produce shorter retention times with consequent loss of separation. Figure 3 demonstrates the effect of acetonitrile on the protonated TMP and the neutral SMZ at a mobile phase pH value of 2. Below 25% v/v acetonitrile concentration, the k' of TMP and SMZ are seen to depend very strongly on the organic modifier content. Therefore, the use of acetonitrile to control retention will result in a unstable system and would inevitably give low reproducibility of

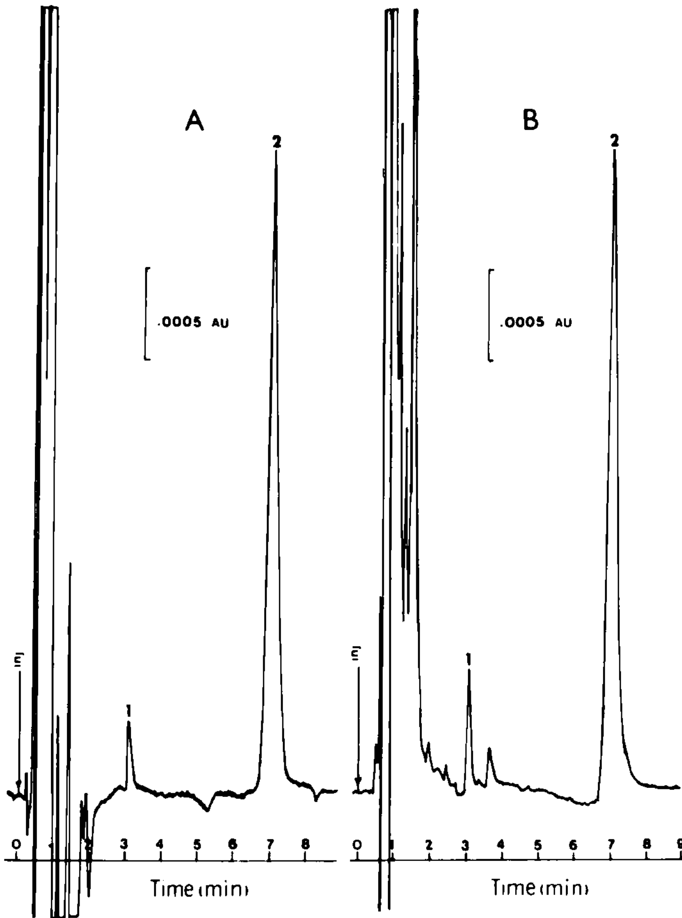

Figure 5 Representative chromatograms of a serum sample at 72 hours after ingestion of 160mg TMP and 800mg SMZ in a 20ml dose of co-trimoxazole suspension: (A) analysis of TMP (1) with the internal standard TIM (2); (B) analysis for SMZ (1) with the internal standard 4DBA (2). Chromatographic conditions: see text.

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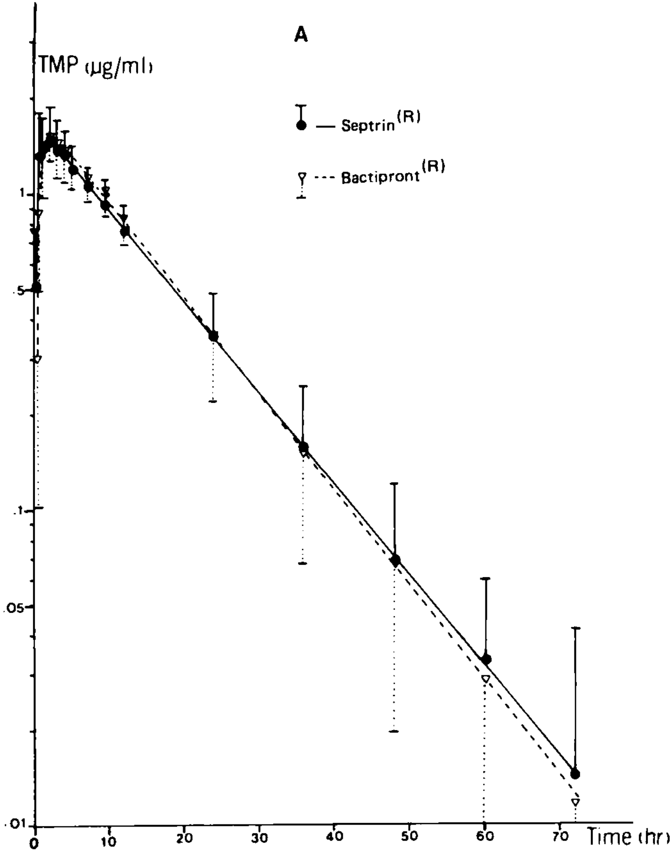

Figure 6 Mean serum concentration of TMP and SMZ resulting from the oral administration of 20ml of Septrin(R) and Bactipront(R) suspension to eight subjects: (A) mean serum TMP concentration (B) mean serum SMZ concentration. The bars indicate one standard deviation from the mean.

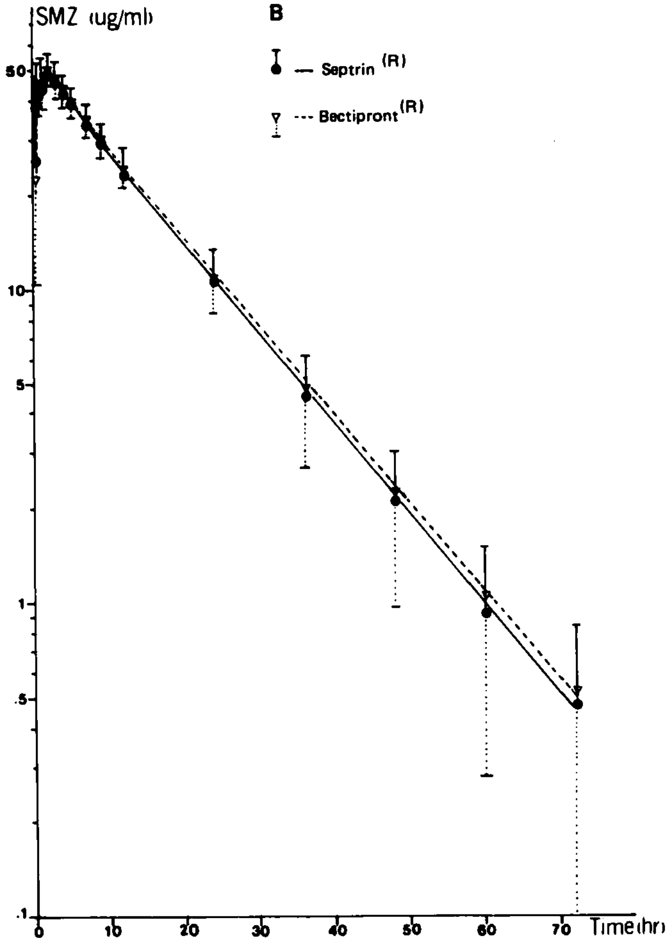

Figure 6B

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the assay. Acetonitrile of 35% v/v was thus chosen as the modifier content.

Effect of Buffer Salt Concentration: In ion pairing systems, the effect of inorganic buffer salts will be twofold;

a) they may act as a counter ion and reduce solute retention as their concentration increases (8),

b) they also act to fix the state of ionisation of the solute (9).

In order to optimise the separation of TMP and SMZ and other plasma constituents, the above variables were examined independently. It was observed that addition of buffer salt causes a decrease in the retention of TMP with a consequent increase in peak sharpness. Above 50mM disodium hydrogen phosphate further increases in the salt concentration causes little reduction of the retention of the solute. The buffer salt concentration in the eluent was therefore fixed at 50mM.

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Effect of Mobile Phase Pairing Ion Concentration: SLS was chosen as the pairing ion because of its ready availability and is relatively inexpensive. It has also been demonstrated that SLS at 35% v/v actonitrile will still be appreciably absorbed onto the C-18 surface and will thus function effectively as a pairing ion in increasing k' values (10). The variation of k' of TMP and SMZ as a function of mobile phase SLS concentration is demonstrated in Figure 4. The k' of the protonated TMP goes through the predicted maximum, while the retention of SMZ is reduced by increasing SLS concentration. Such chromatographic phenomena can be adequately explained by the ion-exchange-desolvation mechanism previously described (10,11). A mobile phase SLS concentration of 30mM was chosen as it provides acceptable retention of TMP, with a k' of 7.5, and maximum separation between TMP and SMZ. Figure 5A shows the chromatogram of an extracted serum sample of a subject at 72 hours after taking a dose of co-trimoxazole.

Sulphamethoxazole.

It was decided on the basis of the above experimental findings that the most satisfactory eluent for the analysis of SMZ in the

TABLE 1

Regression Data for the Calibration Curves of TMP and SMZ. Wavelength of Detection for TMP was at 229nm and SMZ at 254nm.

Drug	Range (μg/ml)	Internal Standard	Internal Standard Concentration (μg/ml)	Slope	Intercept	R
TMP	0.01 to 1	TIM	40	10.733	-0.036	0.9998
	1 to 10		250	1.747	-0.079	0.9997
SMZ	1 to 10	4-DAB	50	0.455	-0.025	0.9974
	10 to 100		500	0.090	-0.012	0.9971

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presence of TMP in serum was 25% v/v acetonitrile-buffer containing 50mM disodium hydrogen phosphate at pH of 2. The chromatogram of SMZ is the deproteinated serum sample of a volunteer at 72 hours after taking 20ml of co-trimoxazole suspension is shown in Figure 5B.

Calibration

All standard curves were prepared by adding known amounts of TMP and SMZ to human plasma. Two calibration curves for each drug were prepared and concentrations of TMP and SMZ in serum samples were calculated from the plot of peak height ratio (drug to internal standard) versus the concentration of the drug under investigation. The regression data and correlation coefficients are listed in Table 1. In all cases, good linearity was observed.

TABLE 2

Accuracy and Precision of the Assays for TMP and SMZ in Human Plasma.

Drug	Spiked Concentration (μg/ml)	Within Day			Between Day		
		n	Mean	%CV	n	Mean	%CV
TMP	0.02	5	0.019	7.0	6	0.021	6.5
	0.5	5	0.53	6.2	6	0.051	7.0
	1	4	0.95	4.0	5	1.02	2.9
	5	3	4.80	5.5	6	4.75	6.4
SMZ	0.5	4	0.46	4.5	6	0.47	3.9
	5	4	4.86	1.8	6	4.95	2.4
	10	4	10.01	2.5	6	10.50	3.0
	60	4	59.90	1.9	4	60.23	4.2

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Accuracy and Precision

Blank human plasma samples with 0.02, 0.5, 1, and 5μg/ml of TMP and 0.5, 5, 10 and 60μg/ml of SMZ were analysed by the above methods. The results expressed as mean values of concentration found are given in Table 2. The relative standard deviation of the samples for both within day and between days demonstrates the precision of the methods for routine purposes.

Bioavailability Studies

Figures 6A and B show the mean plasma concentrations and SMZ in eight subjects after oral administration of 160mg of TMP and 800mg SMZ in a single 20ml dose of co-trimoxazole suspension from the two manufacturers. Comparison of the maximum serum concentration, time of maximum concentration and area under the serum concentration-time curve for TMP and SMZ using a paired t-test indicates no difference at the 5% level. Therefore,

it can be concluded that there is no difference in the bioavailability of TMP nor SMZ from these two dosage forms.

The flexibility of the chromatographic systems, together with the simplicity of sample preparation and the satisfactory precision, make the proposed method suitable for pharmacokinetic studies and for routine drug monitoring in medical laboratories.

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