DEVELOPMENT OF A HIGH PRESSURE LIQUID CHROMATOGRAPHIC METHOD FOR THE SIMULTANEOUS ANALYSIS OF SULPHA= METHOXAZOLE AND TRIMETHOPRIM AND ITS APPLICATION TO BIOLOGICAL FLUIDS AND DISSOLUTION RATE STUDIES ON SOLID ORAL DOSAGE FORMS

by

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PREFACE

Co-trimoxazole, a combination of a 5-to-1 ratio of Sulphamethoxazole (SMZ) and Trimethoprim (TMP), is a highly effective, broad-spectrum antibacterial agent. Since its introduction in 1968, it has been extensively used in infections of the respiratory and urinary tracts[1].

Co-trimoxazole was developed by the systematic investigation of a series of compounds whose mechanism of action was already known[2]. As early as 1950 synergy between sulphon= amides and 2,4-diaminopyrimidines was reported. This was to be expected as both groups of drugs exert their anti= bacterial activity by interfering with the same biochemical pathway in bacteria. TMP was chosen from among many 2,4-diaminopyrimidines tested[3] because of its good anti= bacterial activity and low toxicity. SMZ was chosen from the sulphonamides available for combination with TMP because of similarity of their biological half-lives.

The widespread use of the combination coupled with the fact that monitoring of the levels of all drugs in the body is becoming increasingly important has stimulated research into rapid and efficient methods for the analysis of TMP and SMZ in biological fluids. Another consequence of the immense popularity of the combination is the appearance on the market of several generic preparations of Co-trimoxazole. It is now generally recognized that drug products from different manufacturers which are chemically equivalent may not be therapeutically equivalent[4]. This is due to the fact that

the absorption rate and/or bioavailability (extent of absorption) of a poorly soluble drug may be markedly affected by its release rate from the product and by its subsequent dissolution rate in gastrointestinal fluids. Hence bioequivalence of these various products should be established.

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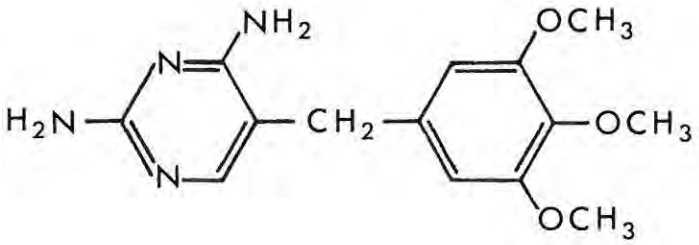
CHAPTER 1

INTRODUCTION

1.1 PHYSICO-CHEMICAL PROPERTIES OF TMP AND SMZ:

A. Trimethoprim:

Chemically trimethoprim is 2,4-diamino-5-(3,4,5-trimethoxy=benzyl)pyrimidine. Its structural formula is indicated in Fig. 1.1.



$C_{14}H_{18}N_4O_3$

Figure 1.1 Trimethoprim

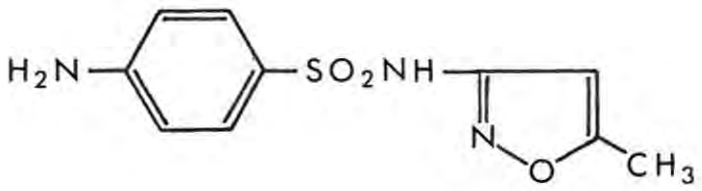
It appears as a white, odourless, bitter powder. The molecular weight of TMP is 290,3 and it has a melting point of 199-200° C[5]. TMP is a weak difunctional base with both basic groups titrating almost simultaneously with a pKa of 7,2[6], and is lipid soluble. It forms stable salts with a variety of acids.

TMP is very slightly soluble in water, soluble in 550 parts of 0,01 N HCl, in 300 parts of alcohol (95%), in 55 parts of chloroform, in 80 parts of methanol and in 640 parts of methylene chloride. It is practically insoluble in solvent ether[7a,8].

The ultraviolet absorption spectrum of TMP in methanol shows a maximum at 289 nm (E 1%, 1 cm 223)[8].

B. Sulphamethoxazole:

Chemically sulphamethoxazole is 3-(4-aminobenzenesulphonamido)-5-methylisoxazole (Fig. 1.2).



$C_{10}H_{11}N_3O_3S$

Figure 1.2 Sulphamethoxazole

It is a white or yellowish-white, crystalline powder, odourless and slightly bitter. The molecular weight is 253,28 and the melting point 169 - 170°C[5]. SMZ is a weak acid with a pKa value of 5,6[9].

SMZ is very slightly soluble in water, soluble in 50 parts of alcohol (95%) and in 3 parts of acetone and is soluble in solutions of the alkali hydroxides[7b]. It is almost insoluble in ether and in chloroform[10].

The ultraviolet absorption spectrum of SMZ in sodium hydroxide solution (1 in 250) exhibits a maximum at about 257 nm[10].

It has been shown that a physico-chemical interaction occurs

between TMP and SMZ[5]. This report indicates that TMP and SMZ will, in aqueous solution or suspension, interact to form a 1:1 molecular compound. Linkage may be due to the amino group in position 2 or 4 of the TMP pyrimidine ring and to the - SO_2 - grouping of the sulphonamide.

The effect of such interactions on solubility, dissolution, methods of analysis, membrane transport and pharmacological response is not known. Thin-layer chromatography (TLC) was found to readily separate the TMP-SMZ compound into its components so that its formation is unlikely to interfere with chromatographic methods of analysis. These methods could possibly be useful in studying the unknown effects of this interaction.

1.2 MODE OF ACTION:

TMP and SMZ sequentially interfere with the biosynthesis of tetrahydrofolic acid in bacteria. SMZ, like all sulphon= amides, produces its antibacterial effect by competing with the natural precursor para-aminobenzoic acid in the synthesis of dihydrofolic acid. In man the folate pathway requires exogenous folate as its initial substrate so the step blocked by the sulphonamides is absent, thus forming the basis for their selective toxicity for bacteria. The next step in the folate pathway is the conversion of dihydrofolic acid to tetrahydrofolic acid by the enzyme dihydrofolate reductase. In bacteria TMP binds strongly to this enzyme and blocks this reduction. The TMP is bound at least 10 000

times more strongly by the bacterial than by the mammalian enzyme[11]. The blocking at two sequential stages in the folate pathway leads to a halt in the synthesis of DNA, RNA and protein and, therefore the death of the bacterium[9,12-14].

It is this sequential blockade in the synthesis of tetra= hydrofolic acid that generally has been held responsible for the high degree of synergy observed when these two drugs are administered together. The combination TMP - SMZ is more rapidly bactericidal and is less susceptible to the develop= ment of resistance than either of the component drugs[2]. That the antibacterial potentiation of TMP by SMZ can be explained by sequential blockade has been doubted by Poe[15]. He has found SMZ to be a moderately potent inhibitor of E. coli dihydrofolate reductase and suggests that potentiation may occur by inhibition of this single enzyme.

As may be expected from its mode of action, trimethoprim potentiates and is potentiated by all of the sulphonamides[3]. SMZ was chosen for combination with trimethoprim because of the similarity of their biological half-lives. Other sulphonamides, however, have been proposed and utilized as partners for TMP because of improved concentrations under certain conditions and similar pharmacokinetic behaviour, e.g. sulphadiazine[16], sulphamoxole[17,18], sulphamethoxypyrazine[19]. The sulphadiazine-trimethoprim combination (Co-trimazine) has only recently been marketed. Sulphadiazine may have certain advantages over SMZ, viz., lower protein-binding

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and a lower degree of metabolism[20] and may potentially be more suitable for administration to patients with reduced renal function[21]. The minimum inhibitory concentrations of the two sulphonamides are identical[16].

1.3 ANTIMICROBIAL ACTIVITY:

The ultimate effect of TMP and SMZ on bacteria is to deprive them of folate coenzymes. The spectra of *in vitro* activity of both drugs are therefore similar although TMP is usually 20-100 times more active than SMZ[22]. Multiple studies have confirmed that the combination, TMP-SMZ, has a wide spectrum of activity against both Gram-positive and Gram-negative bacteria[14,22-24]. The only outstanding exceptions are *Pseudomonas aeruginosa*, *Mycobacterium* species, *Treponema pallidum* and *Mycoplasma* species[14].

The mutual potentiation of TMP and SMZ when used in combination, as measured by a reduction in the minimum inhibitory concentration (MIC) of each individual drug, has been demonstrated both *in vivo* and *in vitro*[3,22,25,26]. Examples of the increased *in vitro* activity are shown in in Table 1.1. An important point is that the degree of potentiation which occurs depends on the ratio of the concentrations of SMZ and TMP used and tends to be maximal when this ratio is similar to that of their respective MIC's when acting alone[22]. Thus for a range of organisms sensitive to both drugs the ratio would be about 20 parts of SMZ (MIC's 5-50 μg/ml) to one part of TMP (MIC's

TABLE 1.1

EFFECT ON MIC OF COMBINING ONE PART TRIMETHOPRIM WITH TWENTY PARTS SULPHAMETHOXAZOLE.[a]

Organism	MIC (µg/ml)			
	Sulphamethoxazole		Trimethoprim	
	Alone	Mixture	Alone	Mixture
Streptococcus pyogenes	>100	1	1	0,05
Streptococcus pneumoniae	30	2	2	0,1
Staphylococcus aureus	3	0,3	1	0,015
Haemophilus influenzae	10	0,3	1	0,015
Bordetella pertussis	50	4	3	0,2
Klebsiella pneumoniae	>100	4	1	0,2
Klebsiella aerogenes	>100	4	1	0,2
Escherichia coli	3	1	0,3	0,05
Salmonella typhimurium	10	1	0,3	0,05
Shigella sonnei	10	1	0,3	0,05
Proteus vulgaris	30	3	3	0,15

[a] from Bushby[22].

0,25 - 2 µg/ml), although it may vary widely with individual species. Significant potentiation does, however, occur over a fairly wide range of ratios on either side of the optimum[14]. Neisseria gonorrhoeae is an exception in which the ratios of the MIC's of SMZ and TMP are completely reversed, the organism being more sensitive to sulphonamides than to TMP[22].

Administration of TMP and SMZ in combination allows for a high degree of synergy and enables the conversion of the bacteriostatic activity of both drugs when administered individually to a bactericidal action in the combination[9]. It also makes possible the administration of a lower dose of both drugs, thus reducing the incidence of side-effects.

Resistance to one of the drugs does not abolish synergy; synergy is maximal however, when the organism is susceptible to both drugs[22]. Administration of the drugs in combination plays a role in preventing the acquisition of resistance to TMP[27].

Great care must be taken in the selection of media and conditions for sensitivity testing and for microbiological assay methods for TMP and SMZ. The presence of even small amounts of thymidine can reverse the antimicrobial action of the combination in vitro[11]. Often discrepancies in susceptibility data in the literature are due to a lack of standardization of these parameters[14,17].

1.4 CLINICAL PHARMACOLOGY OF CO-TRIMOXAZOLE:

Due to the sequential blockade achieved by SMZ and TMP a quantitatively fixed combination has become available. A 5:1 ratio of SMZ to TMP has been found most suitable in patients with normal organ functions and this has, besides a bacteriological explanation, also pharmacokinetic reasons. In many cases, however, the fixed ratio may not provide ideal therapy. Hence, monitoring of drug levels in patients with serious and difficult infections could play a valuable role in optimizing therapy.

1.4.1 Absorption:

Most pharmacokinetic studies have indicated that both TMP and SMZ are rapidly and completely absorbed following oral administration[6,9,28]. Invasion phase kinetics of both compounds are of the same order[29]. More important, uptake of both drugs in patients under treatment[30] and in non-fasting volunteers[31] is also satisfactory.

After absorption TMP is metabolized only to a small extent and most of these metabolites are antibacterial, whereas SMZ is metabolized to inactive products[14]. Thus SMZ levels are usually expressed as "active" SMZ which refers to the unchanged drug, both free and protein-bound, and as total SMZ which is the sum of the active drug and its inactive biotransformation products. Of these inactive metabolites, the N^4-acetylated derivative of SMZ predominates.

Peak blood levels occur for TMP, active SMZ and for total SMZ between 1 and 4 hours following administration of all dose levels studied[29,32,33].

There have been some reports however that suggest incomplete or delayed intestinal absorption of SMZ. Baethke et al.,[34] have postulated that approximately 85% of ingested SMZ is absorbed. Kaplan et al.,[32] found that the absorption of SMZ was somewhat slower when the drug was administered in combination tablets than when it was administered in a separate tablet. It was also found[32] that the mean peak concentrations of TMP from combination tablets were slightly lower than those after administration of separate tablets. These absorption problems may indicate non-ideal formulations.

1.4.2 Protein Binding:

Trimethoprim has been shown to be 42-46% bound to plasma proteins[21,33,35]. About 66% of unchanged SMZ is bound to proteins[21,33]. Of the total sulphonamide present in the plasma, about 70% is in the active form[36]. About 84% of the metabolized SMZ is bound to plasma proteins[27].

The addition of 10 mg % SMZ to plasma, the concentration expected after a single therapeutic dose, decreased the binding of TMP by only 3-4%. TMP does not influence the protein binding of SMZ at the range of concentrations usually encountered in therapy[35].

Hence 34% SMZ and 55% TMP (i.e. the non-protein-bound portions) will remain in a diffusible state and equilibrate with the interstitial fluids[33]. In addition, only these unbound portions will be available for chemotherapeutic activity. Thus, protein binding should be accounted for when quoting plasma drug levels.

In the evaluation of drug concentrations in biological fluids it is important to consider whether the assay method utilized will measure the protein bound or unbound portions of the drug. Microbiological methods of analysis will measure the non-protein-bound part[37]. If ultrafiltration is utilized for the removal of proteins during sample preparation[38a] this will also give a measure of unbound, rather than total, drug. In contrast most chemical methods involving extractions and protein precipitation always measure the sum of the bound and unbound drug. Good coincidence has, however, been found between chemical and microbiological results[34,39,40] especially in the case of SMZ[41]. This coincidence may be explained by the high dose of this drug used in these studies which would be clearly above the protein-binding capacity saturation level of this drug[41].

Protein binding figures may, however, be altered under certain conditions. Certain disease states affect the protein binding of sulphonamides and will influence the amount of protein present in plasma[42,43]. Binding also

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depends on renal function (see Section 1.4.6). Displacement of protein-bound sulphonamides can occur by compounds such as phenylbutazone, sulfinpyrazone, salicylate, etc.[42,44] and these may also influence binding of TMP. If the non-protein bound fraction is required with great accuracy, it must be evaluated in each patient. Monitoring of drug levels could also play a role here in ensuring optimum levels. Ultrafiltration of the sample will yield the non-protein bound fraction which is bacteriostatically active.

1.4.3 Distribution:

The distribution of TMP and SMZ in the body can, to a large extent, be explained on the basis of their physicochemical properties. TMP is a base with a pKa of 7,2 and hence in plasma constitutes nearly an equilibrium mixture of uncharged molecules of free TMP base and cations. The uncharged base is lipid soluble and seems to diffuse throughout the tissues of the body. TMP will concentrate in areas where the pH is lower than that in the plasma, e.g. prostatic fluid[45,46], urine[33], vaginal fluid[47]. There also appears to be an active localization of the drug in certain tissues, e.g. kidney and liver[9]. In general, TMP levels in serum are lower than those found simultaneously in other biological fluids and tissues. The relative concentrations of TMP in most body tissues has been described by Hansen[48] and Wormser[14].

SMZ, on the other hand, has a pKa of 5,6 and hence is

essentially ionized at plasma pH. Less of this drug also is unbound to plasma proteins and hence free to diffuse. The volume of distribution of SMZ is thus much smaller than that of TMP[9]. Concentrations of active SMZ in all tissues and fluids except urine are always lower than those in the plasma[33]. Detailed reviews have appeared concerning SMZ distribution throughout the body[14,48].

Bergan and Brodwall[29] found the mean distribution coefficient to be 1,75 ℓ/kg for TMP but only 0,2 for total and 0,28 for active SMZ. Data for concentrations of TMP and SMZ in some body fluids and tissues are shown in Table 1.2.

This difference in relative distribution volumes between SMZ and TMP will influence the ratios between the two drugs in the blood and various tissues of the body. After administration of the standard preparation which provides a fixed 5:1 ratio of SMZ to TMP there will be a gradual rise of the SMZ : TMP ratio during the absorption phase in plasma[33]. After such a dose, the mean ratio in the plasma between non-protein bound non-metabolized SMZ to active non-protein bound TMP is approximately 20:1; a range of values between 10:1 and 30-40:1 can be encountered[33,36]. Thus therapeutic doses of a combination containing 5 parts SMZ and 1 part TMP will yield, in the plasma, a concentration ratio that is practically optimal for their reciprocal potentiation for a large spectrum of bacterial species (see Section 1.3).

TABLE 1.2.

TRIMETHOPRIM AND SULPHAMETHOXAZOLE CONCENTRATIONS IN BODY FLUIDS AND TISSUES RELATIVE TO SERUM LEVEL.[a]

	TMP Concentration	Active SMZ Concentration
Cerebrospinal fluid	0,3-0,5:1	0,2:1
Aqueous humour	0,1-1,0:1	0,25:1
Breast milk	1,25:1	0,1:1
Prostatic fluid	2,0-2,3:1	0,3-0,5:1
Vaginal fluid	1,0-3,0:1	very low
Bile fluid	2,0:1	0,4-0,7:1
Saliva	1,13:1	0,02:1
Urine	100:1 (approx.)	1:1 (approx.)
Amniotic fluid	0,75:1	0,45:1
Foetal tissues	0,5:1	0,04:1
Erythrocytes	1,0:1	0,25-0,5:1
Lung tissue	2-6:1	0,3:1
Liver tissue	6-7:1	0,2-0,5:1
Spleen	2:1	0,2-0,5:1
Skin	2:1	0,2-0,5:1
Kidney	7:1	0,2-0,5:1

[a]Data from Hansen[48], Stamey and Condy[47] and Eatman et al.[49].

This optimal ratio, however, will certainly not be present in the tissues and since organisms are more usually in other tissues or body fluids may present a serious problem. Thus in the treatment of individual tissue infections it may be necessary to supplement the sulphonamide dosage if a satisfactory ratio between the two drugs is to be maintained. However some sources doubt whether this argument has any relevance to the chemotherapeutic efficacy of the drug, since the concentrations in interstitial fluid should equal those in plasma water[34].

TMP was found to be equally distributed between plasma and erythrocytes but the concentration of SMZ was 2-4 times higher in plasma[30]. Thus TMP concentrations will be similar in serum and whole blood but whole blood concentrations of SMZ will therefore be lower than equivalent serum concentrations[50]. Thus care must be taken when quoting "blood" or "serum" levels of the drugs which are often used interchangeably in the literature. In addition care must be taken in the assay methodology utilized. Some methods, e.g. a colorimetric method for sulphonamides[51] and a differential pulse polarographic method for TMP analysis[52] use whole blood, whereas most other analyses determine levels in plasma or serum.

1.4.4 Serum levels:

Peak serum levels after a single oral dose of 160 mg TMP and 800 mg SMZ, the usual adult dose, are 1-2 $\mu g\ ml^{-1}$ for

TMP, 40-60 μg ml^{-1} for total SMZ and 30-50 μg ml^{-1} for active SMZ[29,36]. Nolte and Büttner[28] encountered wide variations in the peak concentrations of TMP and in the time in which they are reached. These are thought to be due to different dissolution and absorption conditions among the individuals. Plasma levels 1 hour after dosage are especially variable[33]. Mean levels at 8 hours after a single oral dose of 160 mg TMP and 800 mg SMZ are still higher than the MIC's for the majority of bacteria[30,48]. Of the total SMZ, an average of 70% is in the active form[36].

The mean peak serum concentration is proportional to the dose administered[50]. Each drug has no appreciable effect on the concentrations achieved in the blood by the other[35].

Steady state blood levels for TMP, active SMZ and total SMZ are reached within 2-3 days after chronic administration of 160 mg TMP and 800 mg SMZ every 12 hours[6,32,36]. These levels are approximately 1,5-2 fold greater than the levels observed following the first dose. These steady state levels are pharmacokinetically predictable based on the blood level data obtained following a single dose[6,32]. Steady state levels further substantiate the constancy of absorption, distribution, metabolism and excretion with each administration of the TMP-SMZ combination[9]. The steady state profile precludes unpredicted drug cumulation and suggests no enzyme induction by either drug. Since one can successfully predict plateau levels in blood for

each drug after multiple dosing, based on pharmacokinetic data following a single dose, a patient may be titrated for an individual dosing regimen. The mean ratio of active SMZ:TMP in serum is approximately 20:1 at steady state[36]. The slow build-up to steady state levels could be obviated by using an initial double dose[1].

The pharmacokinetic profile of both drugs remains the same whether the drugs are administered separately or in combination in single or in multiple dosing regimens[28,32]. Both TMP and SMZ exhibit physiologic-disposition characteristics which follow first order kinetics[28,32].

TMP and SMZ blood levels appear earlier and are higher after intra-muscular administration than after oral administration. After intra-muscular administration to humans peak serum concentrations are reached, in the case of TMP, about 40 minutes after injection and in that of SMZ, about 2 hours after injection[41]. After such administration blood levels of both drugs are regular and have a constancy that is not observed after oral administration. The optimum 20:1 ratio is for the most part maintained, although it has been suggested that reducing the SMZ:TMP ratio to 4:1 in intra-muscular preparations will improve the serum ratio[41].

1.4.5 Metabolism and Excretion:

The metabolic pathways of TMP and SMZ are shown in Figures 1.3 and 1.4 respectively.

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Fig. 1.3. Metabolism of trimethoprim. (Reproduced from Rieder[37]). In pooled human urine, collected during the first 24 hr after an oral dose of 2 mg ^{14}C-labelled TMP per kg of body weight, Schwartz et al.,[53] found 77,5% of the total radioactivity in the form of the unaltered drug, 5,8% as free extractable metabolites, 5,5% as conjugates, and 11,2% as other water soluble metabolites.

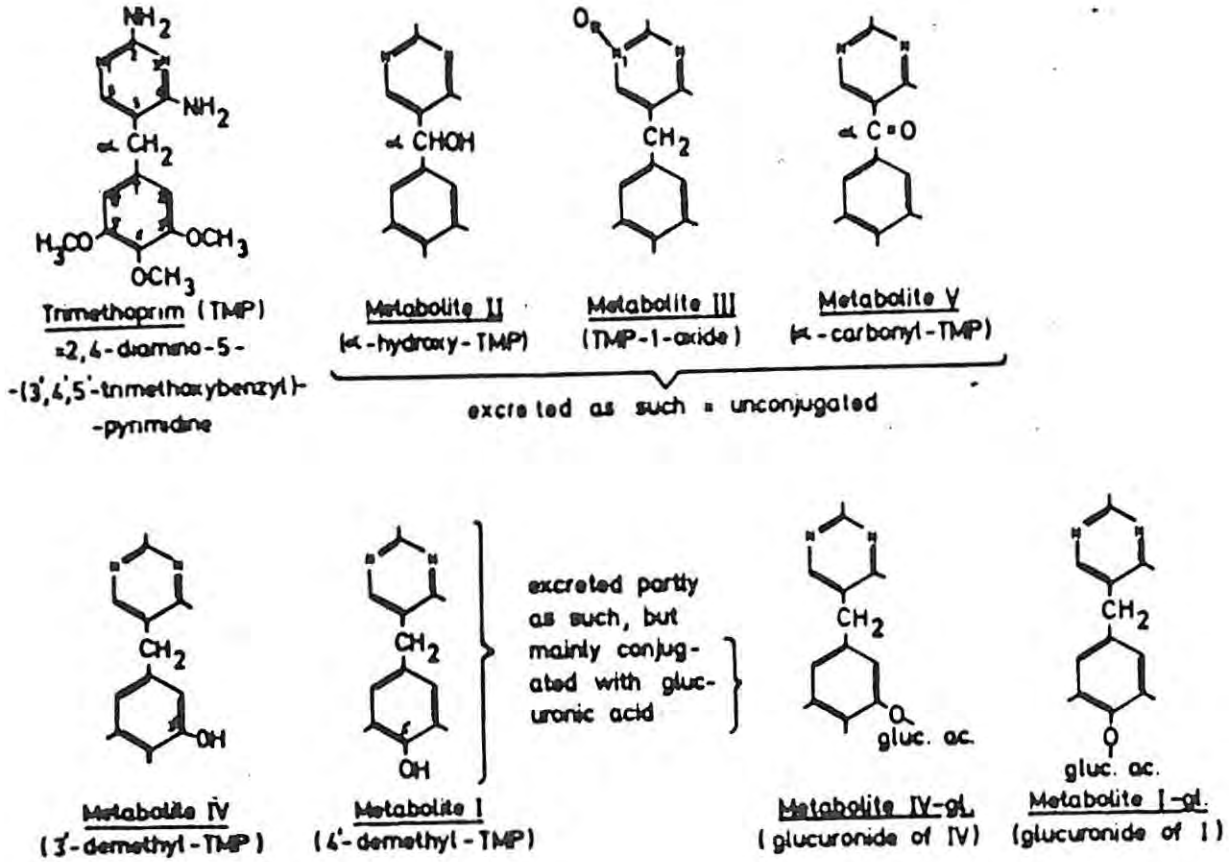
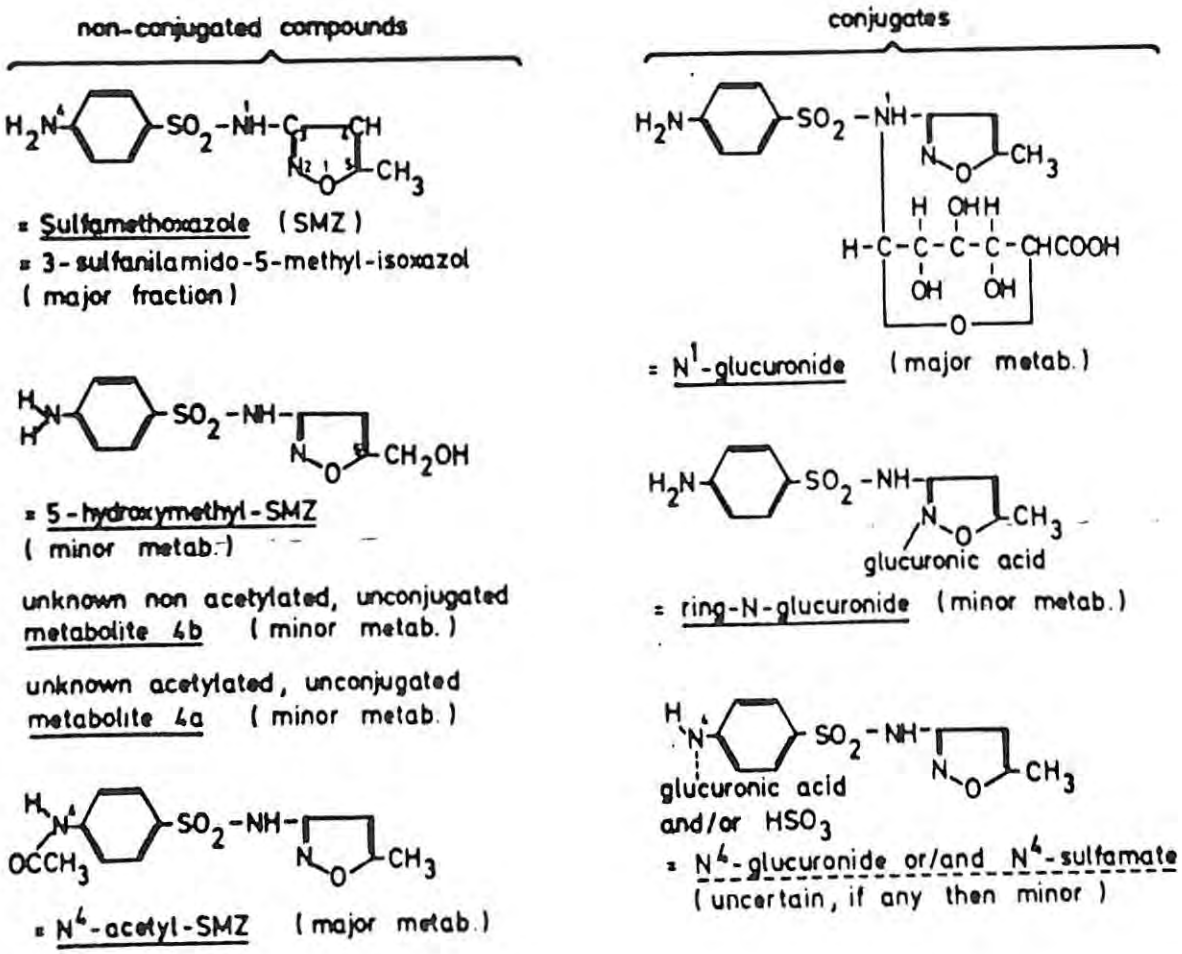


Fig. 1.4. Metabolism of sulphamethoxazole. (Reproduced from Rieder[37]). About 20% of all renal excretion of SMZ occurs in the unaltered form. The N^4-acetyl derivative and the different N-glucuronides account for about 60-65% and 15% respectively of the total urinary excretion. Other metabolites are present only in minor amounts[37].



TMP is more slowly metabolized than SMZ[33]. In the serum, metabolites account for approximately 10-15% of the total TMP concentration[48] and most have antibacterial activity similar to TMP[14]. In contrast SMZ undergoes rapid bio= transformation to inactive products. 25-35% of total plasma SMZ consists of various transformation products, among which the inactive N^4-acetylsulphamethoxazole (N^4-acetyl-SMZ) predominates[37].

SMZ and TMP are completely eliminated from the body of humans with unimpaired liver and kidney functions[33]. Both are excreted for the most part in the urine with mean recoveries after 96 hours of 80-100% of the administered dose excreted as total SMZ and 65-75% as intact TMP[32,33,36]. 20-30% of the total sulphonamide is excreted as intact SMZ[14,32,36] with the remainder of the dose excreted for the most part as N^4-acetylated SMZ. The approximate proportions of other metabolites of TMP and SMZ found in the urine are indicated in Figures 1.3 and 1.4 respectively.

Thus it can be seen that the N^4-acetyl derivative constitutes the major metabolite for SMZ (and for most other sulphon= amides). The difference between the total and active SMZ values is considered to represent the quantity of N^4-acetyl-SMZ present in assay methods based on the Bratton-Marshall[51] reaction, which has been utilized for the majority of pharmacokinetic studies on Co-trimoxazole (see Section 2.2). This is of course an oversimplification as it disregards the

fact that acetylation is not the only metabolic pathway and that it is unlikely to be an irreversible reaction[34].

Because of this relatively extensive metabolism of active SMZ to the inactive N^4-acetylated metabolite, differences in the rate at which this acetylation occurs is bound to affect blood levels of active SMZ. Some drugs are polymorphically acetylated in man, some persons being genetically constituted as slow acetylators (Mendelian recessives) and others as rapid acetylators (dominants). Examples of drugs acetylated by this enzymatic system are isoniazid, sulphadimidine, hydrallazine, dapsone, sulphapyridine[55], sulphamethazine, sulphamethoxypyridazine[56,57] and procainamide[58]. The determination of the acetylator phenotype may be of importance in establishing a suitable dosage regimen for a particular drug. It is not known whether this will apply to SMZ but it should be borne in mind that this could influence blood levels of active SMZ and N^4-acetyl-SMZ. Certainly a wide inter-subject variation of active and total SMZ levels has been reported[29,30] which may possibly be due to differences in acetylator status. Vree et al.,[59,60] however, suggest that no such acetylation differences occur in the case of SMZ and that the extent of acetylation depends on the amount of SMZ available for acetylation. Inter-subject variability may be due to differences in urinary pH and flow which will affect SMZ excretion (see below).

The mean elimination half-lives of SMZ and TMP have been

calculated following single dose administrations. In adults with normal renal functions, these values have been reported to be 6-17 hours for TMP, 7-11 hours for active SMZ and 12-14 hours for total SMZ[14,28,29,36]. In all these studies the mean values of the half-lives for each drug are similar. This was a prime reason for choosing SMZ as a companion sulphonamide for TMP in the marketed preparations. The half-life corresponds closely with the dosing interval which is 12 hours.

The half-life of both drugs is usually shorter in children, but will vary greatly according to the age of the child[48]. Monitoring of drug levels in children would be an invaluable aid in optimizing therapy.

Concentrations of intact SMZ and TMP in the urine of subjects receiving the combination are high. The concentration of TMP in the urine parallels that in the blood but is 10-100 times greater[30]. Concentrations of active SMZ in plasma and urine are approximately equal[48]. The ratio of the concentrations of active SMZ to TMP in urine is variable but in general it approximates 1:1[14,30] after both single and multiple dosing. This ratio is far from ideal. However, due to the high concentrations present in the urine, the overall antibacterial activity is usually more than adequate to deal with urinary pathogens.

The renal handling of SMZ and TMP differs. TMP is subjected

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to passive non-ionic tubular diffusion in the kidneys[61]. Being a weak base, TMP dissociates increasingly by a reduction in pH and thus this tubular transport will be dependent on urinary pH. With urinary pH values below 6,2 there is generally net tubular secretion, whereas there is net reabsorption above this point[21,61]. Salvesen and Frønum[62] have suggested that the recovery of unchanged TMP in urine is dependent not only on urine pH but also on urine flow. Thus when doing bioavailability studies with TMP formulations, they have stressed the need for acidic urine and high urinary output.

In a similar manner, acidic urine causes a reduction in the excretion of SMZ which is an acidic drug whereas alkalinization of the urine increases the renal clearance and cumulative renal excretion of this compound[59]. There is also a linear relationship between urine flow and renal clearance of SMZ[59,61]. The renal clearance of the metabolite N^4-acetyl-SMZ is not influenced by urine pH or urine flow[59,60]. However, if renal excretion of SMZ is suppressed, e.g. acidic urine, the concentration of the metabolite in the plasma is higher and the half-life is slightly longer[59].

Thus the ratio of urinary concentration of SMZ to TMP is dependent on urine pH and flow[63], i.e. alkalinization results in higher active SMZ:TMP ratios and acidification results in lower ratios. Care should be taken when

acidic urines are encountered due to increased risk of precipitation of N^4-acetyl-SMZ in the tubules under these conditions.

Urinary pH also has some influence on the absorption rate of SMZ. Under alkaline urine conditions the compound was absorbed faster than under acidic conditions[59]. Differences in the plasma half-life of SMZ under acid and alkaline urinary conditions were small.

Uncontrolled urinary conditions have led to certain discrepancies in the literature in pharmacokinetic para= meters, e.g. Schwartz and Ziegler[35] found 80-90% unchanged TMP excreted within 48 hours, but Kaplan et al.[6] could only find 30-60% excreted unchanged in the same interval.

This variation in the urinary levels will influence serum levels and may result in non-ideal values in some instances. Closer monitoring of body levels may provide more information about the extent of this variation.

1.4.6 Impaired Renal Function:

SMZ-TMP has found extensive application in the treatment of urinary tract infections, especially for the long-term therapy of recurrent bacteriuria. Inevitably patients with widely different degrees of kidney function will receive this combination. The effect of impaired renal function on pharmacokinetic characteristics has been well

documented[29,34,61]. All patients with creatinine clearance values above 60 ml min^{-1} are considered, from a pharmaco= kinetic point of view, to have normal renal functions[29,61] The clearance of TMP decreases markedly in renal insufficiency (see Figure 1.5a). A marked rise in the elimination half-life ($t_{\frac{1}{2}}$)of TMP is seen at a creatinine clearance value of about 20 ml min^{-1}[29]. For creatinine clearances of less than 10 ml per minute, the $t_{\frac{1}{2}}$ of TMP rises to 1,5 to 3 times normal[14]. However the urine concentration of TMP remains at therapeutic levels irrespective of the renal function[61]. Even in chronic haemodialysis patients, unmetabolized TMP will be effectively eliminated and urine levels will be in the therapeutic range[14]. The fate of TMP metabolites in renal failure has not yet been determined.

The mean $t_{\frac{1}{2}}$ for active SMZ is practically uninfluenced by alterations in kidney function (see Fig. 1.5.a - the slope of the unchanged sulphonamide fraction is not significantly different from zero). Renal insufficiency, however, entails a larger variation in the $t_{\frac{1}{2}}$ of active SMZ[34]. Thus therapeutic concentrations of unchanged SMZ may be achieved in the urine even in severe renal insufficiency[34]. The active SMZ:TMP ratio in urine remains constant for all practical purposes, even in dialysis patients[34].

For total SMZ however, there is a precipitous increase in $t_{\frac{1}{2}}$ as creatinine clearances become successively reduced below

Fig. 1.5. The effect of impaired renal function on the pharmacokinetic characteristics of trimethoprim and sulphamethoxazole. (Reproduced from Baethke et al.[34])

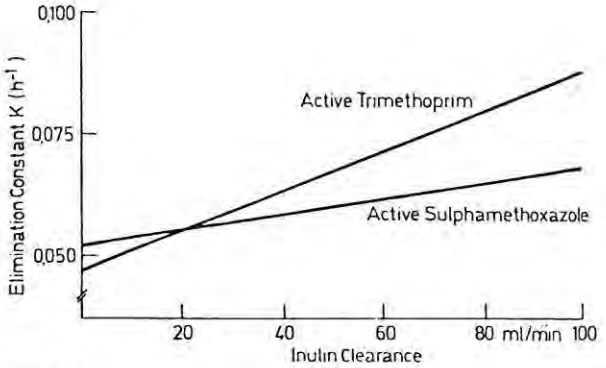

Fig. a. Linear regression analysis of renal function vs. rate constants for over-all elimination:
$K_{SMZa} = 0.0520 + 0.0002\ Cl_{In}$ ($r = +0.316$; $p < 0.1$)
$K_{TMP} = 0.0473 + 0.0004\ Cl_{In}$ ($r = +0.524$; $p < 0.05$)

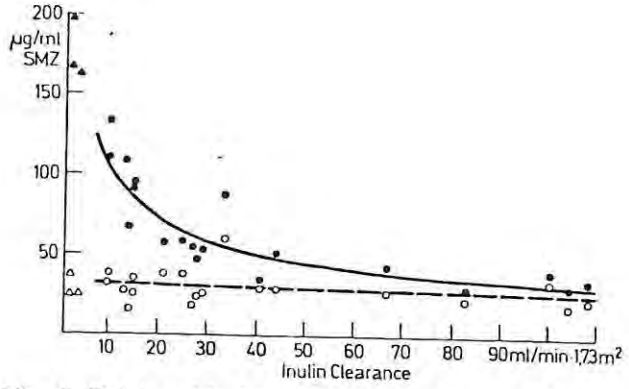

Fig. b Relationship between renal function and mean plasma concentration at equilibrium state. Solid symbols represent total sulphamethoxazole, open symbols the unchanged sulphonamide fraction. Results obtained from dialysis patients are indicated by triangles

approximately 30 ml min^{-1} [29] (see Fig. 1.5.b). Thus, in renal insufficiency, accumulation of SMZ metabolites especially N^4-acetyl-SMZ occurs. Accumulation of these metabolites increases hyperbolically and their elimination half-life approaches infinity in severe renal failure. The N^4-acetyl derivative of SMZ has been implicated in renal damage associated with Co-trimoxazole therapy[64] and thus levels of this metabolite should be closely monitored in patients with reduced renal function.

Renal insufficiency is reflected by delayed excretion but does not affect the cumulative amount of total sulphonamides eliminated by the kidneys[34].

The plasma concentration of TMP rises with a deterioration in renal function[34]. Active SMZ concentrations also increase slightly due to displacement from protein binding sites caused by rising metabolite levels[34,61]. These metabolites have a higher affinity for serum proteins. It is possible that they may also interfere with binding of TMP although this has not been determined. The ratio of the chemotherapeutically active components in plasma remains almost unchanged unless renal insufficiency becomes very severe[34]; at low creatinine clearance values (e.g. dialysis patients) the ratio between active SMZ and TMP is reduced[29,34,61]. The volume of distribution of TMP is unaffected by renal impairment but that of active SMZ increases with reduction in renal function[34].

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It is apparent that the limitations in the use of TMP-SMZ in renal insufficiency are due primarily to the kinetic behaviour and potentially toxic effects of their metabolites. Since the retention of SMZ metabolites becomes marked at creatinine clearance values of less than 30 ml min^{-1}, it is this range of renal failure at which routine prescription should be abandoned. In cases of drug overdosage, conditions also arise which favour metabolism to inactive and possibly toxic compounds.

The recommended dosage must be altered for patients with renal insufficiency to guard against toxic accumulation[65]. The most probable half-life for each drug component can be estimated from creatinine clearance values to allow selection of appropriate dosage intervals although prediction of the half-life value is made more difficult because greater variations are found in patients with reduced renal function[29]. Lower maintenance doses are required for subjects with marked renal impairment; dosage schedules have been postulated[48,66]. It would be advisable here to monitor levels of drugs, especially the SMZ metabolites, and possibly one should refrain from using the fixed combination altogether. The half-lives become so unpredictable and dissimilar that the two components cannot be given with equal time intervals. The use of the fixed combination, Co-trimoxazole, is not recommended in patients where the creatinine clearance is below 15 ml/minute[65]. However, with careful monitoring of blood levels, it could be used

in such cases because active drugs remain within the therapeutic range.

The use of the full recommended dose in the elderly, who often have diminished renal function, could lead to excessively high blood concentrations, which might in turn account in part for the higher incidence of adverse haematological reactions in this group of patients[1].

From the above it can be seen that SMZ metabolites are excreted almost exclusively through the urine. Since the half-life for active SMZ is largely uninfluenced by the renal function, the elimination of this compound must therefore depend largely on non-renal mechanisms[29]. Thus one would expect that malfuncton of other vital organs, e.g. liver, would change the half-life for active SMZ. At impaired hepatic function the peak levels of active SMZ and TMP in plasma were lower by an average factor of 1,5 - 2,0[48]. The effect of other organ malfunctions on the pharmacokinetic profile of TMP-SMZ is unknown.

1.5 THERAPEUTIC USES OF CO-TRIMOXAZOLE:

Lower respiratory and urinary tract infections are the commonest indications for Co-trimoxazole. Other recognized uses are the treatment of Gonorrhoea, brucellosis, enteric fevers, some types of endocarditis and septicaemia, coliform meningitis, pneumocystis pneumonitis and febrile episodes in patients with leucopenia[1,65].

There are some aspects of Co-trimoxazole dosage and duration of therapy that need careful re-assessment[65]. Some authorities have questioned whether TMP alone would not be equally satisfactory treatment in urinary tract infections. The latter has been used successfully in Finland for some time[1]. Different ratios of TMP-SMZ could be useful for treating infections caused by some bacteria (for example gonococci) or infections in certain sites, but this is impractical. A useful method for determining serum and urine levels of these drugs would be an invaluable aid in the re-examination of dosages.

1.6 DOSAGE:

TMP-SMZ is supplied as a standard tablet containing 80 mg TMP and 400 mg SMZ; as a double-strength tablet containing 160 mg TMP and 800 mg SMZ; as an oral suspension containing the equivalent of 40 mg TMP and 200 mg SMZ per teaspoonful and as an ampoule for intravenous administration containing 80 mg TMP and 400 mg SMZ per 5 ml. The usual adult dosage is two standard tablets twice a day.

1.7 ADVERSE EFFECTS:

In the recommended dosage, Co-trimoxazole is well tolerated. Nausea, vomiting and drug rash may occur; the latter is due to hypersensitivity to the sulphonamide component[1]. Haematological changes (mainly leucopenia and thrombocytopenia) have been reported in isolated cases but are generally re= versible. TMP may induce folate deficiency especially in

patients with pre-existing deficiency and this may be implicated in blood dyscrasias[27]. Sulphonamides are nephrotoxic[64], and crystalluria has been considered a potential hazard related to N^4-acetyl-SMZ insolubility[67]. Current evidence suggests that creatinine clearance is diminished by Co-trimoxazole, probably by the TMP component[65].

1.8 THE ROLE OF DRUG MONITORING IN CO-TRIMOXAZOLE THERAPY:

Fixed combinations of chemotherapeutic agents should usually be avoided. However it can be seen that TMP-SMZ is a drug combination with reproducible and predictable drug levels under normal circumstances that can be maintained at fixed dose-levels and intervals. Thus the fixed combination has become available for general therapeutic use.

There are, however, certain instances, as has been shown above, where close monitoring of levels in the body and supplementation of either drug if necessary could play a great role in optimizing and providing for more effective therapy. This could be invaluable where a serious infection is encountered; in these cases optimization of SMZ and TMP ratios and concentrations according to the particular organism and the site of infection could provide maximum therapeutic activity. Observations on concentrations in numerous tissues and secretions strongly suggest that physico-chemical factors alone, e.g. pKa, pH, protein-binding, generally govern the disposition of TMP and SMZ[46,47,49,68]. Thus it may be possible to predict, from serum or urine data, or from

available distribution data, the amounts of each drug in certain tissues and hence one could theoretically calculate the dosage regimen necessary to create an optimum ratio and concentration in that tissue with respect to the particular invading organism.

There are many factors, e.g. volume of distribution, acetylator status, urine pH, etc., that will affect blood and tissue levels of both drugs and will result in inter- and intra-individual differences. In many cases, patients to whom the combination is administered may have pharmacokinetic parameters deviating from normal as a result of certain organ malfunctions. Monitoring of drug levels will improve therapy in these cases and may also play a part in delaying the development of resistance. Non-ideal ratios and concentrations for a certain organism may promote the development of resistance.

It is also theoretically possible that use of the drug could be extended to patients with severe renal failure (creatinine clearance less than 15 ml/minute) provided that close control is kept over drug levels, especially of N^4-acetyl-SMZ. Monitoring of the levels of this metabolite especially is important as it constitutes by far the major amount of any of the metabolites and it has been implicated in kidney damage and crystalluria especially in cases where renal function is impaired.

Thus monitoring of drug levels can potentially play a greater

role. Hence a quick and accurate method for the assay of these drugs in biological fluids is desirable. Care must be taken in the chemical or biological method utilized in the assessment of drug concentrations. Many reports have been based on analytical methods which did not permit unequivocal determination of the chemotherapeutically active substances. Problems of specificity and speed of assays are becoming increasingly predominant with the advent of bioavailability determinations and the large numbers of samples requiring processing.

In addition, since Co-trimoxazole is marketed under several trade names, it is essential to ensure bioequivalence of all these products and ensure adequate absorption of both components to yield the optimum ratios required. In the assessment of bioavailability in human volunteers, many parameters would have to be strictly controlled, e.g. urine pH, urine flow, etc. A more specific determination of SMZ and N^4-acetyl-SMZ would be useful to discriminate poor biological availability from fast metabolic degradation in cases of low blood levels.

CHAPTER 2

ANALYTICAL METHODS FOR THE DETERMINATION OF TMP AND SMZ IN BIOLOGICAL FLUIDS

Several methods are available for the quantitative determination of TMP and SMZ in biological fluids[69-96]. The majority of these methods involve a separate analysis of these two compounds. This is effected, in microbiological assays, by the use of certain antagonists or organisms to nullify the effect of one component or, as in the more commonly used chemical methods, by separation of the two drugs through an extraction procedure. In most cases, determination of the major metabolite of SMZ, N^4-acetyl-SMZ, and of other meta= bolites of these drugs, requires additional analytical steps.

Many of the earlier, long-established methods available suffer from one or more limitations. They are either non-specific, involve lengthy sample preparation and/or analysis time or are not amenable to simultaneous determination. Attempts to improve specificity and speed have in recent years led to a growth in chromatographic methods of analysis. High pressure liquid chromatography (HPLC) has become recognized as an invaluable tool for the routine analysis of drugs in physiological samples[97,98]. Over the past two or three years and during the course of this present investigation, several methods have appeared where the application of HPLC to the determination of TMP and SMZ, both separately and in combination, in pharmaceuticals and in biological fluids, has

been investigated[90-96,99]. The HPLC determination of N^4-acetyl-SMZ has also been reported[59,94-96].

The present investigation was undertaken in the attempt to develop a method for the simultaneous extraction and determination of TMP, SMZ and also N^4-acetyl-SMZ in biological fluids by HPLC.

2.1 EXTRACTION OF TMP AND SMZ FROM BIOLOGICAL FLUIDS:

The vast majority of literature methods available for the determination of TMP and SMZ depend upon a separate analysis of these two compounds. Hence emphasis has been placed on extraction methodology that will enable their separation. Because of their diverse characteristics, TMP and SMZ can, in fact, be extracted selectively from aqueous samples using water-immiscible solvents following the classical approach of Brodie et al.,[100] i.e. basic drugs are made alkaline before extraction into water immiscible organic solvents and acidic drugs are made acidic, the principle being that the unionized form is more lipophilic than the ionized form.

Thus, TMP, a basic drug with a pKa of 7,2, is extracted at alkaline pH into a variety of water-immiscible solvents. Most published methods involve the addition of sodium carbonate or occasionally sodium hydroxide to the biological fluid or tissue in order to adjust the pH to 11, followed by extraction into chloroform[3,70,80]. Isopropanol - dichloro=

methane (1:4)[81], chloroform - isopropanol (95:5)[92] and dichloromethane[87] have also been utilized as solvents for the extraction of TMP. It has been found that a pH of 11 is satisfactory with regard to the selectivity and efficiency of extraction into chloroform[91]. Under these conditions fairly good recoveries of TMP are obtained even if a very small amount of chloroform is used to extract a larger volume of aqueous phase[85] . One serious disadvantage, however, associated with the use of chloroform is emulsification when extracting from biological fluids, especially urine at alkaline pH. Thus care must be taken when samples are shaken. Unconjugated metabolites will be extracted together with unchanged TMP when these methods are utilized[101].

The extraction methodology described above that is commonly utilized for TMP will not extract SMZ which is an acidic drug (pKa 5,6) and hence will be ionized under these conditions. Often SMZ is analysed directly in the remaining aqueous layer[51]. Extraction of SMZ from plasma or urine is usually effected by adjusting each fluid to an acidic pH, making the SMZ essentially non-ionized and hence lipophilic. SMZ can be extracted into several water-immiscible solvents, e.g. chloroform[94], ethyl acetate[78], ether[84b]. Addition of acetone to the aqueous phase prior to extraction with chloroform[81] or ether[84b] greatly increases the yield. The choice of pH of extraction is important. An extreme pH offers little selectivity in extraction and also may result in ionization of secondary groups thus producing water-

soluble species. The appropriate pH which would allow an almost quantitative extraction was found to be pH 3 for SMZ[91]. However, pH values of 5,5[78] and 4,1[81] have also been used. The N^4-acetyl derivative of SMZ and other non-conjugated metabolites will also be extracted under these conditions[78].

SMZ, like most sulphonamides is highly protein-bound[35]. The above extraction methodology is likely to be effective in releasing all the bound drug for analysis[79]. The procedure utilized for the extraction of TMP will also remove protein-bound and unbound drug[102].

These methods of extraction are unsuitable for the simultaneous determination of SMZ and TMP. A sample preparation system was required which would quantitatively extract both drugs in a single operation. No methods for the simultaneous extraction of TMP and SMZ were available at the time of this investigation. Vree et al.,[96] have since, however, employed a perchloric acid protein precipitation procedure for biological samples in order to determine both SMZ and TMP, and also N^4-acetyl-SMZ. In addition, Bury and Mashford[93] have recently utilized tri=chloroacetic acid to prepare plasma samples for both SMZ and TMP analyses.

Thus various solvent systems and protein-precipitation methods were evaluated for their extraction efficiencies and ability to separate the relevant compounds from impurities.

A simple and rapid method of extraction is preferable since simplicity of the method reduces manipulative errors and hence enhances reproducibility and precision. A rapid method is highly desirable especially if the method is to be applied to a bioavailability or pharmacokinetic study of Co-trimoxazole where large numbers of samples require analysis. It is very often the extraction that takes the longest time in an analysis. Simultaneous extraction of TMP and SMZ would save time as both drugs would be extracted in one procedure.

The extraction of SMZ and TMP simultaneously poses certain problems because of their widely differing characters. The pH of extraction would have to be closely controlled as changes in pH will affect recoveries of both drugs. In this particular case a neutral or near-neutral pH is the most logical choice. Several reviews dealing with sample preparation techniques have appeared[38,91,102].

It must also be borne in mind that TMP presents a problem in that assays must be extremely sensitive to determine the very low levels (0,1-2 µg/ml) found in serum during treatment with the drug. Urine levels are higher (10-100 times) and hence less of a problem in this respect. TMP has a low absorptivity[8] and hence for HPLC analysis where UV detection is most commonly employed the low TMP levels will not be detected unless large volumes of biological fluid or adequate concentration of extracts is done. Thus the ability to readily concentrate the sample extract is an

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important factor that must be considered. For SMZ, plasma and urine levels are high, so little concentration of the original plasma or urine level is needed.

2.2 METHODS AVAILABLE FOR THE DETERMINATION OF TMP, SMZ AND THEIR METABOLITES IN BODY FLUIDS:

Methods for the analysis of TMP were first outlined by Bushby and Hitchings in 1968[3]. They utilized a microbiological procedure with Bacillus pumilus as the test organism. A second microbiological assay method for TMP appeared in 1970[69]; in this method para-aminobenzoic acid was added to the medium to neutralize any sulphonamide present. For the microbiological assay of SMZ a TMP-resistant strain is used[46]. The microbiological techniques are reproducible and sensitive but not specific. Active metabolites and other antibacterial agents, if present, will interfere. These methods will measure only the immediately active, i.e. non-protein-bound portion of the drug (see Section 1.4.2). Microbiological procedures, however, have been shown to correlate well with chemical determinations[24,40] and have even been used recently for many pharmacokinetic studies[46].

Bushby and Hitchings[3] also outlined a spectrophotometric method for the determination of TMP in biological fluids. This method is not sufficiently sensitive to determine the very low concentrations encountered in the serum. In addition it is not very specific although metabolites interfering here are only formed in very small amounts.

In 1969 a spectrofluorimetric method[70] was developed which was sufficiently sensitive for biological samples (sensitivity 0,1 µg/ml TMP). The method was later modified to enable semi-automation[71]. Despite being rather intricate and time-consuming, this method has become the most commonly used analytical technique for the routine determination of intact TMP in biological fluids. The majority of pharmacokinetic studies, even recently[49], carried out on TMP or TMP-SMZ have utilized this technique. The method is not absolutely specific for the unaltered drug. Small amounts of metabolites are also extracted and contribute slightly to the fluorescence measured, but they do not affect the determination to any considerable extent[37]. (More interference from metabolites may, however, be encountered in animal species who metabolize TMP to a far greater extent than man[35,101].

Urinary concentrations of unchanged TMP can be measured by a simple and specific method based on the native fluorescence of the drug[72]. This method is not, however, sufficiently sensitive to allow for the determination of serum levels.

TMP and its metabolites have also been determined by scintillation spectrometry of ^{14}C labelled TMP[53] and by differential pulse polarography[52,73]. The latter method is considerably less sensitive but more strictly specific for unaltered TMP than the spectrofluorimetric procedure[70].

The most widely used method for the determination of SMZ and

other sulphonamides is a colorimetric procedure based upon the Bratton-Marshall[51] reaction which was originally described in 1939. This method consists of diazotizing the aromatic amino group of the sulphonamide followed by coupling to form a coloured azo compound. It gives a measure of the formerly incorrectly called "free sulphonamide", i.e. the sulphonamide fraction reacting directly with Bratton-Marshall reagents, but includes, together with unaltered SMZ, several metabolites which possess a free amino group[37]. The quantitatively important metabolite of SMZ, N^4-acetyl-SMZ, can be assayed by repeating the analysis after hydrolysis so that "total" sulphonamide is measured. The difference between "total" and "free" SMZ gives a measure of the N^4-acetyl-SMZ present.

Despite its lack of specificity, the Bratton-Marshall method has been widely used for pharmacokinetic studies and is still being used[41]. It has been adapted to automated systems to provide speed[44,74-77]. Rieder[78,79] modified the procedure to include an extraction step and hence improved specificity. His modification is generally applicable to the routine determination of sulphonamides and their metabolites in body fluids and potentially could be automated.

Recently the use of various chromatographic techniques for the determination of drugs in biological fluids has increased. The major advantage of these methods is that specificity is increased, particularly for tissue level studies where other drugs, metabolites or endogenous materials may interfere.

Various thin-layer chromatographic (TLC) methods were developed for the determination of TMP in biological fluids. A fluorescence TLC method developed in 1973[80] enabled quantitative determination of TMP in plasma and urine and its metabolites in urine. Other TLC methods for the determination of TMP in plasma[81] and in both plasma and urine[82] followed.

Chromatographic techniques have also been used to improve specificity for the determination of SMZ and N^4-acetyl-SMZ in both pharmaceutical preparations and biological samples. TLC has been used for the determination of SMZ in plasma[81], both plasma and urine[82], and for the simultaneous determination of SMZ and N^4-acetyl-SMZ in urine[83]. These TLC methods provide a rapid, sensitive, accurate and specific means for quantitative determinations.

TMP has been determined by gas chromatography (GC). A GC method for the analysis of TMP was first outlined by Clark[84a]. Recently a number of GC methods for the determination of TMP in biological fluids have appeared[62,85-87]. These methods are sensitive, selective and rapid.

Various attempts have been made to develop GC methods for the determination of sulphonamides[88,89]. They are not, however, readily determined by GC and extensive derivatization is required which is too time consuming for routine analyses.

The advent of HPLC has led to increased speed, specificity and accuracy in the quantitative determination of drugs in biological fluids. HPLC has been used for the simultaneous determination of vancomycin, anisomycin and TMP lactate in antibiotic mixtures[90]. Bye and Brown[91] carried out preliminary studies in an attempt to devise an HPLC assay for TMP in biological fluids but experienced difficulty in quantitating results. A recent sensitive method describing the determination of TMP in biological fluids using HPLC and fluorescence detection has appeared[92]. Bury and Mashford[93] have also described an HPLC analysis of TMP in plasma employing ultraviolet (254 nm) detection. These two methods do not utilize an internal standard for the quantitation nor do they allow for the simultaneous determination of SMZ.

Sulphonamides are well suited to assay by HPLC. HPLC has been used for the determination of pure sulphonamides[103-106] and for the determination of sulphonamides in biological fluids[107-109]. The determination of SMZ alone[93] as well as the simultaneous determination of SMZ and its N^4-acetylated metabolite[59,94,95] in biological fluids by HPLC have been reported.

It must be of great value to be able to determine simultaneously, in a single operation, the concentration of each component from a small volume of sample. Various methods have been reported where a simultaneous determination of TMP and SMZ is described. TMP and SMZ have been determined from a single volume of

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biological fluid using a microbiological procedure following separation of the two drugs by electrophoresis[40]. NMR has also been used for the simultaneous determination of TMP and SMZ in pharmaceuticals[110,111] but this technique is not sensitive enough to determine the low concentrations found in biological fluids.

Helboe and Thomsen[99] used HPLC for the simultaneous determination of TMP and SMZ in pharmaceuticals, but did not adapt the system to biological fluids. Vree et al.,[96] have recently reported the simultaneous determination of TMP, SMZ and N^4-acetyl-SMZ in biological fluids by HPLC. However, this method is only sensitive down to 0,75 µg/ml of TMP and hence is unsatisfactory for clinical pharmacokinetic studies. In addition, it does not incorporate the use of an internal standard for the quantitation.

The value of simultaneously determining TMP, SMZ and N^4-acetyl-SMZ is clearly apparent. HPLC seems to be the ideal technique to use for this purpose. Hence this study was undertaken to develop an HPLC method for the simultaneous determination of all three compounds. The method described by Vree et al.,[96] appeared during the course of the present investigation and will be compared and discussed in relation to the present studies.

2.3 CHOICE OF HPLC AS A TECHNIQUE FOR THE DETERMINATION:

Of the techniques discussed above, the only specific ones

are those including a chromatographic separation. In addition, since a mixture of compounds is to be determined, a chromatographic procedure would facilitate the simultaneous analysis of the relevant compounds. As a technique, HPLC offers superior resolving power to TLC combined with the quantitative precision similar to that obtained with GC. GC, however, requires prior derivatization of the sulphonamides with its attendant disadvantages. A great advantage of HPLC is that low concentrations of drugs may be analysed without any necessity to derivatize the compounds.

For this particular analysis HPLC seems the most logical choice. The technique itself provides specificity, speed, good accuracy and precision. It is versatile and can be applied to drugs of diverse characteristics as in this case. In addition it offers the possibility of a simplified method of sample preparation[38a] as compared with other analytical techniques, since extraction is followed by another separation. Often removal of proteins from biological fluids before injection will suffice so that speed and accuracy are increased.

Ultra-violet (UV) detection is the most widely used technique in HPLC and was utilized for the present studies. However, the use of this detector has an associated disadvantage since it is totally dependent upon the absorptivity of the relevant compounds being analysed. Thus, it has limited sensitivity

in the case of TMP which has a low absorptivity. It was necessary, therefore, to develop methods of extraction that will enable concentration of the sample to be effected.

2.4 OBJECTIVES IN THE DEVELOPMENT OF AN HPLC ASSAY FOR THE SIMULTANEOUS DETERMINATION OF TMP AND SMZ IN BIOLOGICAL FLUIDS:

The objectives were as follows:

(i) The selection of suitable conditions, i.e. column, mobile phase, etc., for the separation of TMP and SMZ.

(ii) The choice of a suitable detection system for both drugs and a method of ensuring relatively similar responses from both drugs present in widely differing proportions.

(iii) The development of a suitable extraction procedure from biological fluids where these drugs, of differing chemical nature, may be simultaneously extracted and where reproducibility and extraction efficiency are satisfactory.

(iv) Suitable concentration of the sample extract to enable detection of low concentrations of drugs, especially TMP.

(v) The choice of a suitable internal standard if possible.

(vi) The simultaneous quantitation of the major metabolite of SMZ, N^4-acetyl-SMZ.

(vii) The application of the method to the analysis of samples of dissolution medium taken during dissolution rate studies of various Co-trimoxazole dosage forms.

CHAPTER 3

PREPARATION OF N^4-ACETYLSULPHAMETHOXAZOLE

The N^4-acetyl derivative of sulphamethoxazole was prepared by acetylation with acetic anhydride in acetic acid. The method employed is outlined by Sharma, et al.[107]

3.1 REAGENTS:

Sulphamethoxazole (Lot No. 3262) was obtained from Wellcome (Pty) Ltd., S.A., and was used without further purification. It complied with BP standards. Other reagents used were all of analytical grade and included acetic anhydride (E. Merck), extra pure glacial acetic acid (E. Merck), dioxane (E. Merck) and ethyl acetate (Hopkin and Williams). The water used was deionized and then glass distilled.

3.2 APPARATUS:

Infrared spectra were obtained using a Beckman AccuLab 4 Infrared spectrophotometer. Pre-coated silica gel 60F-254 plates (20 x 20 cm, 0,25 mm; E. Merck) were used for the thin-layer chromatography. Melting points were obtained using the apparatus specified in the British Pharmacopoeia[7c].

3.3 METHOD:

To 2 g SMZ was added 20 ml of acetic anhydride. The SMZ dissolves almost immediately and a fine precipitate of the product, N^4-acetyl-SMZ, appears after a few minutes. 8 ml

of glacial acetic acid was then added; this increases the yield of the product. The reaction mixture was then allowed to stand overnight at room temperature.

The product was filtered off by suction, washed thoroughly with cold distilled water and then recrystallized from dioxane-water (1:1 vol/vol). Finally it was dried in an oven at 50° C.

3.4 TESTS FOR PURITY:

The following tests were performed to confirm the identity and purity of the N^4-acetyl-SMZ formed:

(i) Carbon, Hydrogen, Nitrogen Analysis[a]:

The results of this analysis are shown in table 3.1

TABLE 3.1

C,H,N ANALYSIS OF N^4-ACETYL-SMZ ($C_{12}H_{13}N_3O_4S$)

	RESULTS FOUND	EXPECTED VALUES
% C	48,75	48,81
% H	4,50	4,44
% N	14,25	14,23

[a]This analysis was performed by the Microanalytical Section, Department of Organic Chemistry, University of Cape Town by the courtesy of Dr. G. Cragg.

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(ii) Infrared Absorption Spectra:

The infrared (i.r.) spectra of both SMZ and its N^4-acetyl derivative were obtained (see Figures 3.1 and 3.2). Both compounds were examined as nujol mulls.

(iii) Thin-layer Chromatography:

Solutions of SMZ and N^4-acetyl-SMZ in methanol were applied to a pre-coated fluorescent silica gel plate (Merck). The plate was developed in a solvent system consisting of ethyl acetate-dioxane-glacial acetic acid (8:2:0,1)[83] to a height of 13 cm. Once dry, the plate was viewed under an ultraviolet lamp (254 nm) and the R_f values of each compound determined.

(iv) HPLC:

An aliquot of a solution of the product in methanol was injected into a high-pressure liquid chromatograph under the conditions described in Section 4.3.2, and the retention time was noted.

(v) Melting Point Determination:

The melting point of a finely powdered sample of the product was determined using the method specified in the British Pharmacopoeia[7c].

3.5 RESULTS AND DISCUSSION:

The previously described method was found to give a high yield (> 90%) of N^4-acetyl-sulphamethoxazole. The product

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Fig. 3.1. Infrared spectrum of sulphamethoxazole.

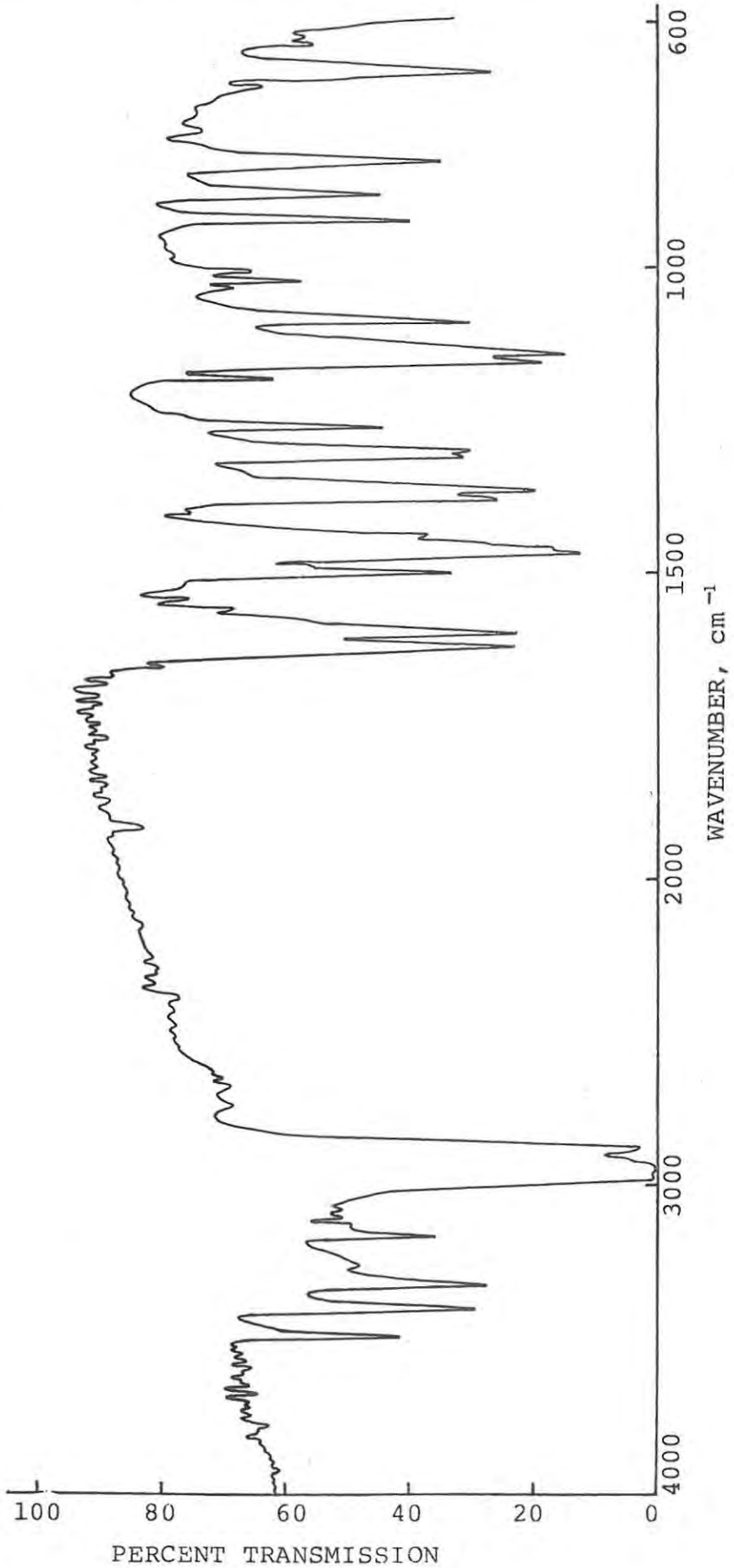

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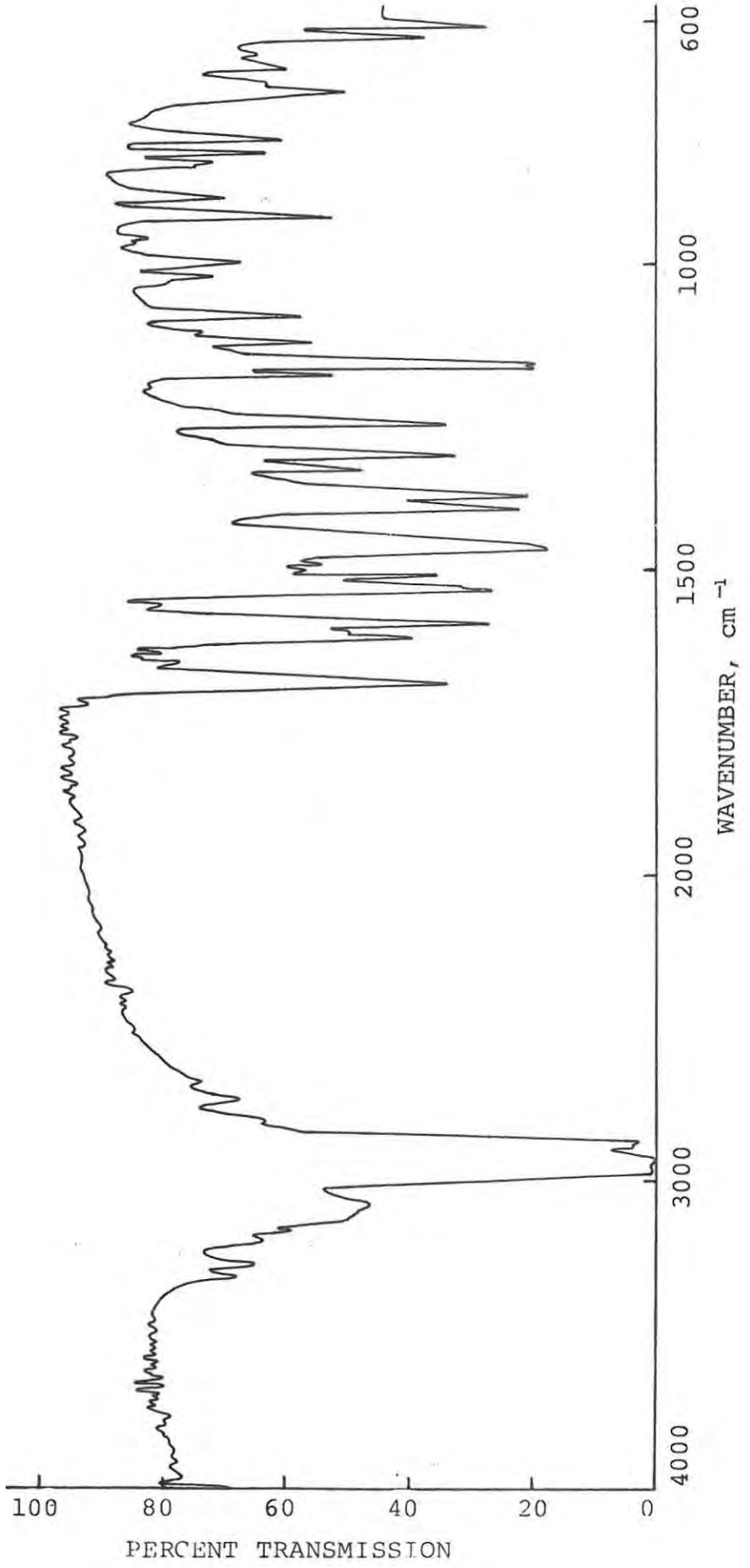
Fig. 3.2. Infrared spectrum of N^4-acetylsulphamethoxazole.



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appeared as white crystals which melted at 224 - 225°C. The reaction goes to completion without any application of heat; heating, in fact, causes decomposition of the product.

The structure of N^4-acetyl-SMZ is shown in Figure 3.3.

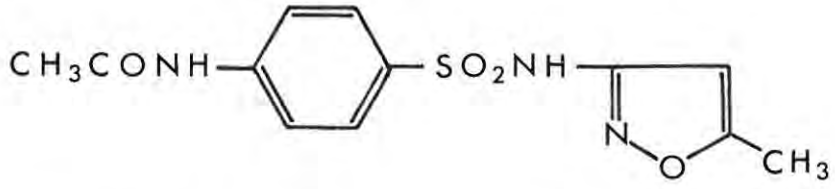

Figure 3.3. N^4-acetylsulphamethoxazole

The C, H, N analysis on the product agreed very closely with values expected on the basis of the molecular formula of N^4-acetyl-SMZ.

Two important features are apparent when comparing the i.r. spectra of SMZ and N^4-acetyl-SMZ. The first is the significance reduction in the intensity of absorptions in the region 3600 - 3200 cm^{-1} seen in the spectrum of N^4-acetyl-SMZ. These absorptions correspond to stretching frequencies of N-H bonds. The reduction in their intensity is due to the monosubstitution of the primary $-NH_2$ group in SMZ that has occurred. The second feature of interest is the strong carbonyl group absorption at ca. 1700 cm^{-1} that is seen only in the spectrum of N^4-acetyl-SMZ and is due to the acetyl group at the N^4 position. These features further confirm the identity of the product formed.

Chromatographic methods were used to check the purity of the product. Both SMZ and N^4-acetyl-SMZ appeared as single spots on the thin-layer chromatogram. There was no trace of the parent compound, SMZ, nor of any other impurity in the sample of N^4-acetyl-SMZ.

The R_f-value of SMZ was found to be 0,815 and that of N^4-acetyl-SMZ was 0,662. No positive identification can be made on the basis of this TLC separation as an authentic specimen was not available; the product was, however, shown to be apparently pure.

Under the HPLC conditions described in Section 4.3.2, the N^4-acetyl-SMZ produced was found to have a retention time of 16,7 minutes. A single well-defined peak was observed indicating a pure sample. No SMZ was observed in the chromatogram. HPLC analysis of serum and urine extracts from patients after ingestion of Co-trimoxazole show a peak at the same retention time which can only correspond with the N^4-acetylated metabolite of SMZ.

These tests confirm the identity and purity of the synthesized N^4-acetyl-SMZ. This product was thus used as a standard to allow for quantitative analysis of N^4-acetyl-SMZ in serum and urine.

The identity and purity of further batches of N^4-acetyl-SMZ synthesized were confirmed by i.r., melting point, mixed melting point and retention time when injected under the specified HPLC conditions.

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CHAPTER 4

CHROMATOGRAPHIC ANALYSIS

4.1 MATERIALS AND APPARATUS:

4.1.1 Reagents:

Trimethoprim (Lot No. 3156) and Sulphamethoxazole (Lot No. 3262) were supplied by Wellcome (Pty) Ltd., S.A. Sulphafurazole (Lot No. RFA 2258) was obtained from Maybaker (Pty) Ltd., S.A. and Sulphasomidine (Batch No. 81/10) from Noristan Laboratories (Pty) Ltd., S.A. All of these drugs complied with B.P. standards and were used without further purification. The N^4-acetylsulphamethoxazole was prepared by acetylation of SMZ (see Chapter 3).

All other solvents and materials used were of analytical grade and were purchased from E. Merck, Darmstadt, with the exception of acetonitrile which was "synthesis" grade (E. Merck), whereas chloroform (A.R.) and ethyl acetate (A.R.) were obtained from Hopkin and Williams (Searle Co., S.A.). The water used was deionized and then glass-distilled.

4.1.2 Source of Biological Samples:

Drug-free serum was obtained from polycythaemia patients. Whole blood without anticoagulants was centrifuged at 3000 rpm for 10 minutes and the serum removed and stored frozen at -20° C. The serum was thawed at room temperature before use. Human urine was obtained daily from a volunteer.

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4.1.3 Apparatus:

4.1.3.1 High-Pressure Liquid Chromatographic Systems:

System A.

A Perkin-Elmer Model 601 liquid chromatograph was used, equipped with a Perkin-Elmer Model LC-55 variable wavelength ultraviolet absorption detector and a Model 123 strip-chart recorder. A temperature controlled column oven was also included. Samples were injected using a Rheodyne Model 70-10 Sample Injection Valve equipped with a 20 µl loop.

System B.

A Spectra-Physics Model 3500B liquid chromatograph was used. Two detectors were included for monitoring the column effluent, a Model 770 spectrophotometric detector (Schoeffel Detector) and a Spectra-Physics dual channel U.V. absorbance detector Model 230. Recording and integration were performed using a Hewlett-Packard Automation System 3385A. A Pantos Unicorder U-125 M strip-chart recorder was also included. Samples were injected using a Valco CV-6-UHPa sample injection valve equipped with a 10 µl loop.

System C.

A Varian Aerograph 8500 liquid chromatograph was used, equipped with a stop-flow septumless injector, a Vari-chrom variable wavelength U.V. detector and a CDS III electronic integrator. A Perkin-Elmer Model 56 recorder was also used.

4.1.3.2 Columns:

(i) Amino-bonded Column.

An Aerograph(R) Micropak(R) NH_2 - 10 bonded phase column (25 cm x 2 mm x $\frac{1}{4}$ inch O.D.; Varian Associates) was used.

(ii) C_{18} Reverse-phase Column.

A 30 cm x 3,9 mm (i.d.) μBondapak C_{18} reserve-phase column (Waters Associates) was employed.

4.1.3.3 Other Instruments Used:

(i) Bransonic 220 ultrasonic bath.

(ii) Whirlimixer(R), Fisons.

(iii) Roto-Uni II, BHG, centrifuge.

(iv) Techne Dri-Block DB-3.

(v) Sartorius Precision 5-figure Analytical Balance, Type 2474.

(vi) Edwards "Modulyo" Freeze Drier.

(vii) Orion Research Model 801A digital ionalyzer.

(viii) A Hewlett-Packard Model 9810A desk-top calculator was used for the linear least squares regression analysis.

4.1.3.4 Additional Equipment:

Finely tapered 15 ml conical Pyrex centrifuge tubes were used as well as Vacutainer(R) tubes (Becton-Dickinson, U.S.A.). Tubes were sealed with Parafilm(R) M (American can company). Grade A volumetric glassware was used.

All glassware was cleaned in chromic acid, thoroughly rinsed with distilled water and dried, before use.

Millipore type HA(0,45 µm) and type FH(0,5 µm) membrane filters (Millipore Corp., Bedford, Mass., U.S.A.) were also employed.

4.2 PRELIMINARY TESTS USING AMINO-BONDED COLUMN:

During the development of a high-pressure liquid chromatographic method for the determination of TMP and SMZ two types of columns were used. The first column employed was an amino-bonded column (see Section 4.1.3.2). This column has a high polarity, strongly basic surface functionality created by chemically bonding a monomolecular layer of 3-aminopropyl silane to 10 µm silica particles. This phase is bonded through the stable siloxane bond (Si-O-Si) and is claimed to be thermally and hydrolytically stable[112]. During the present studies, however, a lack of stability was observed (see later). This type of column can be used for normal phase, reverse phase or ion-exchange chromatography.

The use of an amino-bonded reverse phase column has been reported for the HPLC determination of sulphamethazine, sulphamerazine, sulphathiazole and their N^4-acetylated metabolites in cattle urine[107].

A preliminary method for the determination of TMP and SMZ in urine was developed using this column and recovery

studies were carried out to evaluate a few sample preparation techniques that were of possible use in simultaneously extracting these drugs.

A. Experimental:

(i) Standard solutions:

Trimethoprim Stock Solution: 125 mg TMP per 100 ml water, dissolved with the aid of 20 ml 0,1 N HCl.

Sulphamethoxazole Stock Solution: 125 mg SMZ per 100 ml water, dissolved with the aid of 20 ml 0,1 N NaOH.

Internal Standard Solution: This solution consisted of 0,5 mg ml^{-1} sulphasomidine in methanol.

All standard solutions were freshly prepared.

(ii) Preparation of "spiked" urine samples:

The method utilized for fortifying urine with known quantities of TMP and SMZ consisted of adding suitable small aliquots of the aqueous stock solutions (see above) to larger volumes of urine in order to obtain the required urine concentration. For each urine sample equal volumes of the acidic TMP and alkaline SMZ solutions were added so that, in effect, the resulting solution had a neutral pH.

(iii) Chromatographic conditions and quantitation:

The following chromatographic conditions were utilized:

Column: Amino-bonded (Micropak$^{(R)}$ NH_2 - 10)

HPLC system: C (see section 4.1.3.1)

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Detection wavelength: 290 nm

Sensitivity: Vari-chrom 0,1 A

CDS III 4

Recorder input 5 mV

Chart speed: 5 mm min^{-1}

Flow rate: 40 ml hr^{-1}

Pressure: 1000 p.s.i.

Temperature: Ambient (20 - 24° C)

Mobile phase: Acetonitrile - methanol 80:20 (vol/vol)

Preparation of mobile phase:

The mobile phase was prepared by mixing 80 parts by volume of acetonitrile with 20 parts by volume of methanol. No correction was made for volume changes as a result of mixing. The mobile phase was recycled twice and then discarded.

Quantitation:

The areas of all peaks were recorded on the electronic integrator. The ratios of the peak areas of TMP and SMZ to that of the internal standard were determined. These ratios were quantitated by comparison with the ratios obtained from the analysis of standards of known concentration.

(iv) Extraction procedure:

To a 5 ml urine sample in a separating funnel was added approximately 7 ml of chloroform-ethyl acetate 85:15 (vol/vol). The funnel was carefully shaken by continuously inverting gently for 15 minutes. This usually avoided emulsification

and allowed easy separation of the phases without centrifugation. Occasional emulsions which formed were broken by centrifuging at 2000 rpm for 10 minutes. The extraction was repeated twice and the organic layers combined. The aqueous phase was discarded.

To the combined organic phases was added 1 ml sulphasomidine internal standard (see above) and this was then made up to a volume of 25 ml. Aliquots of 1 to 5 µl were injected into the liquid chromatograph.

(v) Calibration curves:

Control urine was fortified with TMP and SMZ to yield six different concentration levels varying between 25,0 and 250,0 $\mu g\ ml^{-1}$ of both drugs. Each urine pool was assayed as described above. Calibration curves were then constructed by plotting the ratios of the peak areas of each drug to that of the internal standard, sulphasomidine, versus their respective concentrations. Each sample was assayed in triplicate and the mean value obtained.

(vi) Extraction efficiency:

Recoveries of TMP and SMZ from urine were assessed at five different concentration levels. Drug-free urine samples, spiked with known quantities of TMP and SMZ, were taken through the extraction procedure, the internal standard was added and peak area ratios were computed. Results were expressed as percentages of the peak area ratios

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obtained by the injection of equivalent concentrations of pure drugs in chloroform-ethyl acetate (85:15) solution. Since the internal standard, which was added to both standards and urine extracts, was not carried through the extraction procedure it was used to correct for volumetric error in sample injection.

B. Results and Discussion:

Chromatograms of a blank urine extract and an extract of a urine sample containing TMP, SMZ and the internal standard, sulphasomidine, are shown in Figures 4.1(a) and (b) respectively. Under these conditions the retention times were as follows:

TMP	2,3 minutes
SMZ	4,0 minutes
Sulphasomidine	8,0 minutes

No interfering peaks were evident in the urine control. In addition, a urine sample obtained from a volunteer, who had ingested 160 mg TMP and 800 mg SMZ (two tablets Septran$^{(R)}$) four hours before sampling, showed no interfering peaks when subjected to the above analysis.

(i) Linearity:

Calibration curves obtained were linear over the entire range of concentrations tested. Table 4.1 lists the linear regression data for the calibration curves.

Fig. 4.1. Chromatograms of (a) a blank urine extract and (b) an extract of urine containing 98,2 µg ml^{-1} TMP and 99,3 µg ml^{-1} SMZ. Chromatographic conditions as in Section 4.2. The arrows denote times of sample injection (2,5 µl injected).

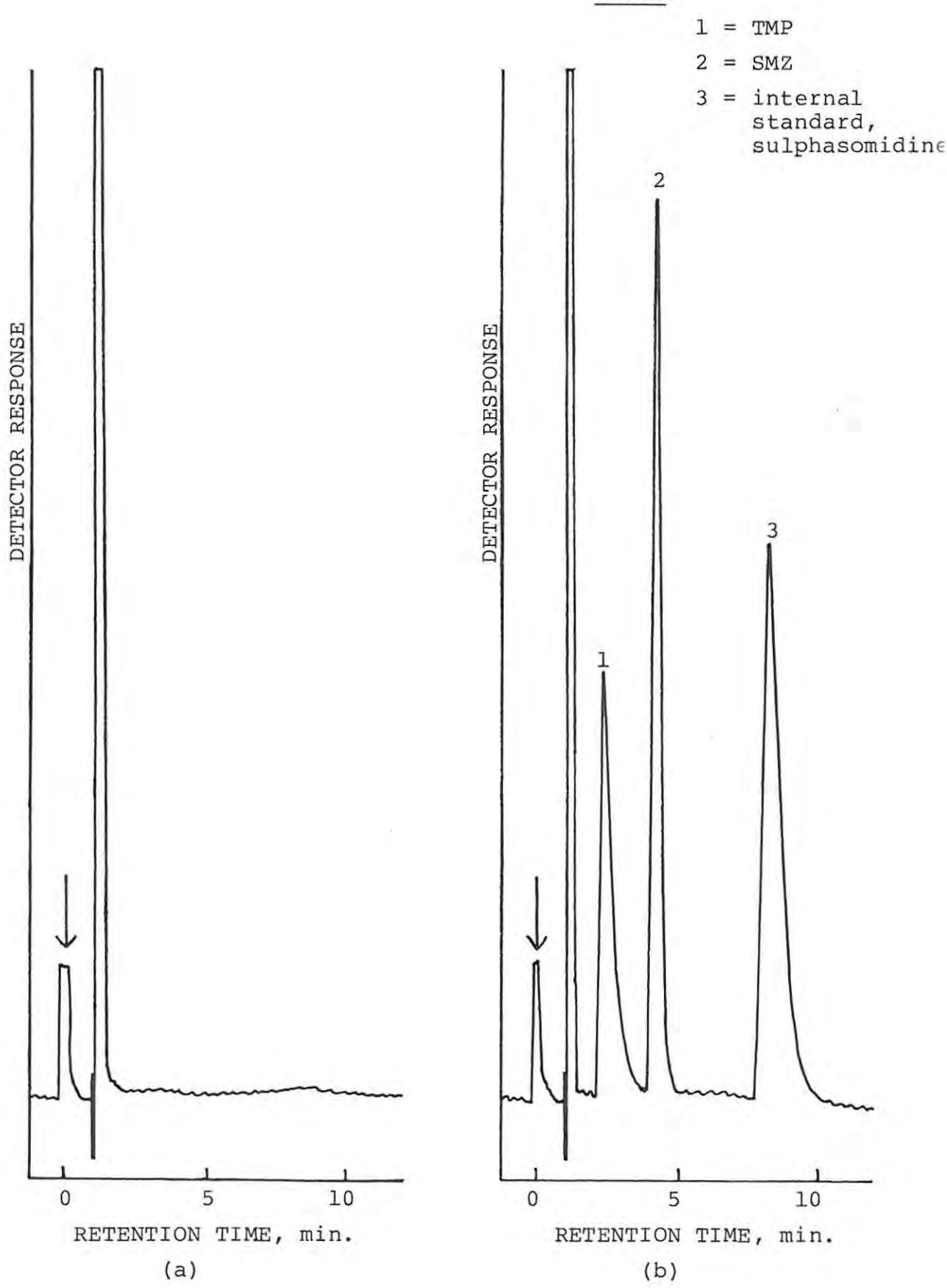

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TABLE 4.1:

LINEAR REGRESSION DATA FOR CALIBRATION CURVES[a]

DRUG	CONCENTRATION RANGE, µg ml^{-1}	SLOPE	Y-INTERCEPT[b]	CORRELATION COEFFICIENT
TMP	25,0 - 250,0	0,0035	-0,0342	0,9952
SMZ	25,0 - 250,0	0,0066	-0,0339	0,9998

[a]Analysis using NH_2-bonded column.

[b]Peak-area ratios are plotted on the y-axis and urinary drug concentrations in µg ml^{-1} on the x-axis.

Drug concentrations normally encountered in the urine during therapy will fall within the linear range of the calibration curves.

(ii) Extraction Efficiency:

The results of the analytical recovery of TMP and SMZ from urine are listed in Table 4.2.

TABLE 4.2:

ANALYTICAL RECOVERY OF TMP AND SMZ FROM URINE[a]

DRUG	CONCENTRATION, µg ml^{-1}		RECOVERY %
	ADDED	FOUND	
SMZ	24,8	24,4	98,4
	49,6	49,2	99,1
	99,3	98,2	98,9
	148,9	148,3	99,6
	248,2	249,2	100,4
TMP	24,5	20,0	81,8
	49,1	40,6	82,6
	98,2	79,8	81,3
	147,2	120,6	81,9
	245,4	202,0	82,3

[a]Analysis using NH_2-bonded column.

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Recoveries of both drugs are fairly consistent over a 10-fold range in concentration and the values obtained are sufficiently high for analytical work[38c, 102].

(iii) Sensitivity and detection limit:

Under the conditions of this assay, the detection limits for TMP and SMZ were established as 10,0 and 5,0 µg ml^{-1} respectively. The method is adequately sensitive for all clinical applications. The detection limits may, however, be improved by increasing the injected sample volume and also by employing a lower wavelength for the detection (see Figure 4.2). However, it must be borne in mind that background absorbance also increases at lower wavelengths.

(iv) Extraction procedure:

Various solvent extraction techniques were investigated in the search for a suitable system which would quantitatively extract both TMP and SMZ from urine. The development of an appropriate technique is largely empirical although general guidelines on the methodology and requirements of extraction procedures have appeared[38c,102]. Jones[38c] has listed the properties of the ideal solvent. The preferred solvent is usually the least polar one which will efficiently extract the required substance[102] as this will also extract minimal amounts of impurities.

Several liquid-liquid extraction methods were tested and the suitability of a range of solvents of varying polarity

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was assessed. Preliminary studies were carried out by extracting aqueous solutions (neutral pH) containing known amounts of SMZ and TMP with various organic solvents in a similar manner to that described above for urine samples. In order to ascertain the efficiency of extraction, aliquots of both the organic and remaining aqueous phase were injected into the liquid chromatograph.

It was found that non-polar solvents, i.e. iso-octane, cyclohexane and benzene, extract very little of either drug. Ether extracts approximately 56% of the SMZ from an aqueous solution but very little TMP. SMZ is completely and efficently extracted by both ethyl acetate and methyl= isobutyl ketone but these solvents remove very little TMP. Use of chloroform results in total extraction of TMP and about 72% extraction of the SMZ from an aqueous solution. Addition of acetone to chloroform in the proportion 1:4[81] yielded a solvent mixture which improved SMZ recovery but not to a significant extent. Various chloroform-ethyl acetate combinations were then tested for suitability. A mixture of 85 parts by volume of chloroform and 15 parts by volume of ethyl acetate was found to be optimal with respect to efficiency and completeness of extraction of both SMZ and TMP from an aqueous solution. A reduction of the ethyl acetate proportion in this mixture results in lower SMZ recoveries whilst increased proportions yield lower TMP recoveries.

When applied to urine samples, extraction with the chloroform-

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ethyl acetate 85:15 solvent system was found to give adequate recoveries of both drugs. This was the final sample preparation method chosen for use. The extract was found to be analytically 'clean', permitting high detector sensitivity, and it could be injected directly since solvent and drug peaks were well resolved. Concentration of the extract is not required for the determination of urine levels of TMP and SMZ. However, should this be necessary, e.g. for the evaluation of lower concentrations found in serum, it is possible to evaporate the extract to dryness and reconstitute the residue in a small quantity of a suitable solvent or mobile phase.

Incorporation of ethyl acetate reduces the liklihood of emulsion formation compared to chloroform when used alone. Possibly the presence of a high concentration of an inorganic salt would be beneficial for the extraction[38c]. This further lowers the incidence of emulsification by reducing the solubility of the organic solvent in the aqueous phase. It may also aid the transfer of TMP and SMZ from aqueous to solvent phase and may thus obviate the need for repeated extraction of a single sample. A single extraction step is optimal for a solvent extraction process[102].

Simplified techniques for sample preparation, e.g. removal of proteins by precipitation, will often suffice for HPLC analysis[38a]. For the determination of sulphonamides in urine using an amino-bonded packing, Sharma et al.,[107]

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pretreated urine samples with methanol before injection. Precipitation of urine components with organic solvents, e.g. acetonitrile and methanol[38a,91], was investigated as a possible approach to the TMP-SMZ analysis. Four parts of methanol or acetonitrile were added to 1 part urine. The gelatinous precipitate formed was removed by centrifugation and the supernate was injected. However, in this case organic solvent precipitation of urine samples was found to be inadequate as endogenous urinary constituents interfered with the TMP and SMZ peaks and could not be separated without very large increases in analysis times. These components may also be detrimental to column life.

Liquid-solid extraction methods were also considered for this assay since these are often particularly suitable for compounds which are not easily extracted from aqueous solutions[38c]. Biological samples can be freeze-dried before extraction as used, for example, by Dell[113] for the determin= ation of cephalosporins. Potentially, freeze-drying of samples has several advantages for this type of assay where drugs of differing character are required to be efficiently and selectively extracted. In addition, emulsification would be avoided, no control of pH would be necessary, storage of samples would be facilitated and samples may be easier to handle.

In this case, urine samples fortified with TMP and SMZ were freeze-dried and the solid residue was then extracted with

chloroform-ethyl acetate 85:15. The solvent was filtered, sulphasomidine was added as internal standard and an aliquot of the resulting solution was chromatographed. However, extraction was found to be non-quantitative by this method since the recoveries of both drugs decreased with increasing concentrations.

Several factors may have contributed towards this non-quantitative recovery. The drugs may have been volatile or unstable and thus the conditions utilized for freeze-drying may require more careful control. In addition, the suitability of a range of solvent extractants should be assessed since chloroform-ethyl acetate may not be ideal in this case. Possibly the use of polar solvents miscible with water, e.g. methanol, as used by Dell[113] who did not encounter these variations, may prove advantageous. Soxhlet extraction may be necessary to dissolve the drugs fully as proposed by Bye and Brown[91]. Filtration of samples after extraction could have been a source of drug loss; centrifugation may be a better method for the removal of unwanted solid material. Jones[38c] has suggested that when urine is to be freeze-dried it is convenient to add a quantity of a cellulose filtering aid so that a fine dried powder results and this may also improve extraction efficiency. Incomplete recovery may be due to adherence of the drugs to urinary constituents. The use of an internal standard is probably essential if a freeze-drying technique were to be employed.

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(v) Chromatographic conditions:

Mobile Phase

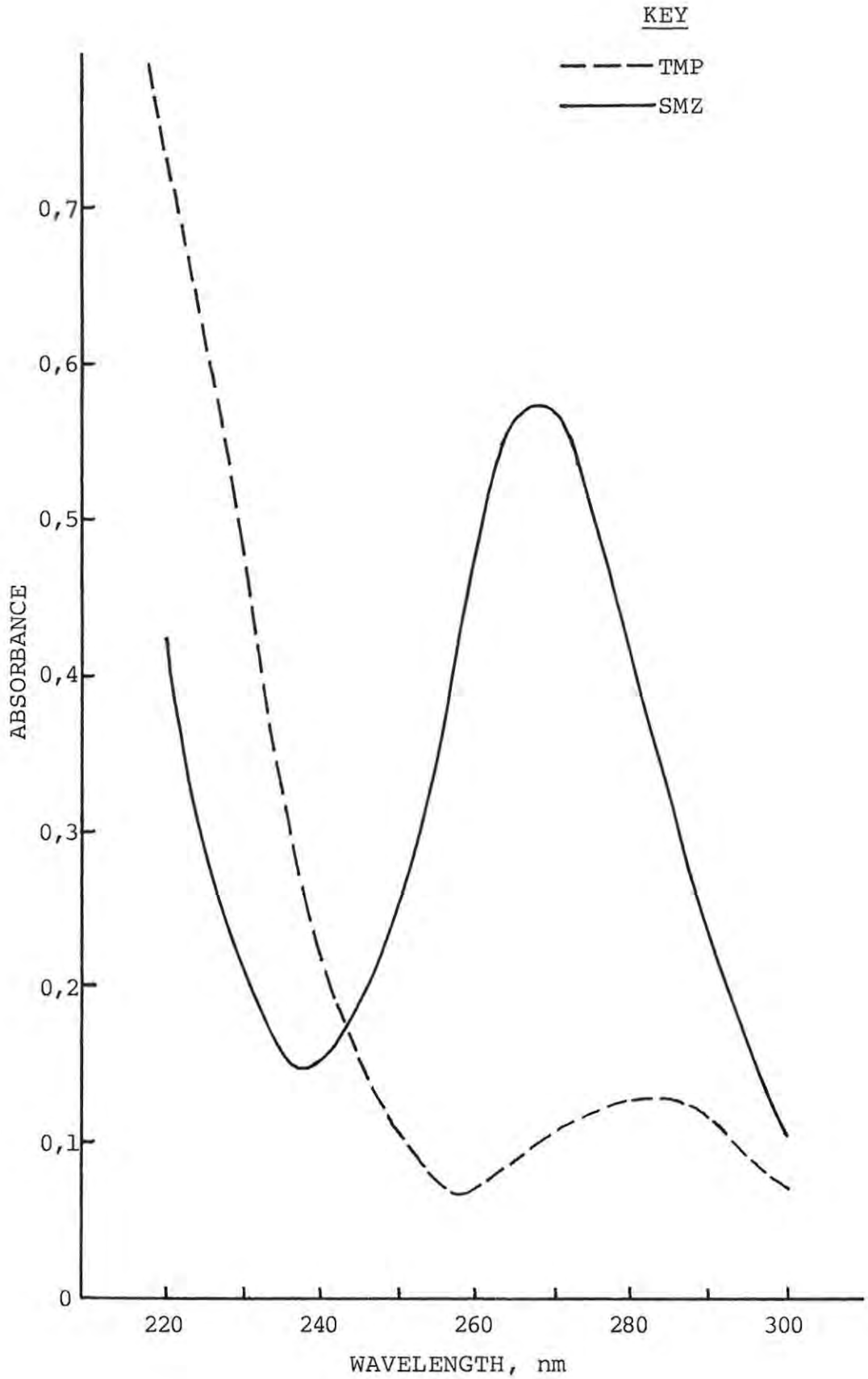
No restrictions are imposed on solvents which may be used with this column provided the pH does not go below two or above nine[112]. Several solvent combinations, containing varying proportions of each component, were investigated in the search for a mobile phase suitable for the analysis. Water/methanol, dilute acetic acid/methanol, chloroform/methanol and chloroform/acetonitrile mixtures were tested as mobile phases but were found to be unsuitable as TMP eluted within the void volume. Improved separation was obtained using acetonitrile/methanol mixtures. A mobile phase consisting of acetonitrile-methanol 80:20 with a flow rate of 40 ml hr^{-1} was found to be optimal and was finally selected for use. When applied to urine assays, there was no interference from urinary constituents or metabolites.

The mobile phase was recycled twice before being discarded. Trace amounts of TMP and SMZ present in the recycled solvent did not affect the quantitative aspects of the analysis.

Detection

The ultraviolet absorption spectra of TMP and SMZ measured under the conditions of this assay (acetonitrile-methanol 80:20 solvent mixture) are shown in Figure 4.2. Since the mean ratio of TMP to SMZ in urine is approximately 1:1, it would be advantageous to select a wavelength where

Fig. 4.2. Ultraviolet absorption spectra of 5 µg ml^{-1} TMP and SMZ in acetonitrile-methanol 80:20 (vol/vol).



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similar absorption occurs for both drugs at the same concentration.

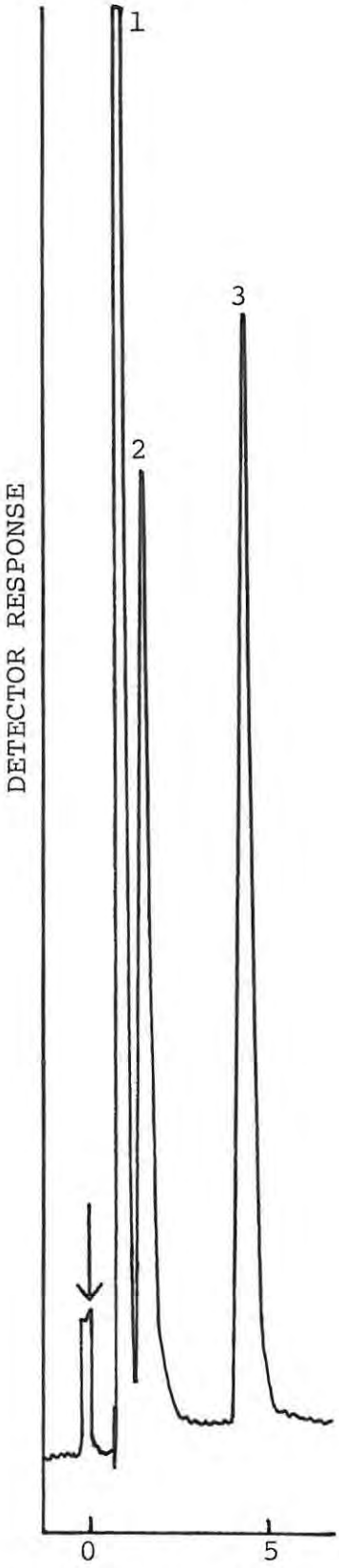
A wavelength of 290 nm was utilized for these preliminary studies. This wavelength is less than optimal since responses for SMZ exceeded those of TMP. However, despite this discrepancy in relative peak heights, both drugs were easily quantitated at this wavelength, especially since peak areas were being employed. In addition, use of this wavelength resulted in low background noise and enabled adequate sensitivity for both drugs at the levels encountered in urine.

The use of lower wavelengths may be more ideal in terms of relative responses but problems of increased background noise and greater absorbance of injected solvents were encountered at these wavelengths.

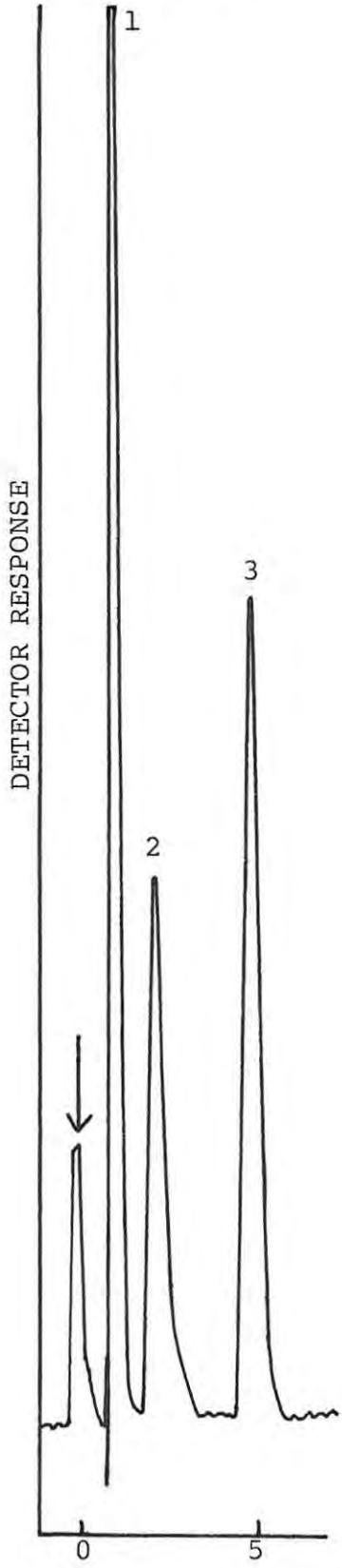
(vi) Column deterioration:

Deterioration and alteration of column characteristics were apparent within a short space of time. The separations and retention times were found to be non-reproducible under the experimental conditions previously described. Progressive migration of the TMP and SMZ peaks occurred until these finally reversed in position. The gradual changes that took place are depicted by the chromatograms in Figures 4.3 (a) - (i). These were all run under identical chromatographic conditions. The numbers of samples

Fig. 4.3(a)-(i): Stages in the deterioration of the amino-bonded column. Chromatographic conditions as in Section 4.2. Peaks: 1 = solvent (chloroform-ethyl acetate 85:15); 2 = TMP; 3 = SMZ. The arrows denote times of sample injection.



(a)



(b)

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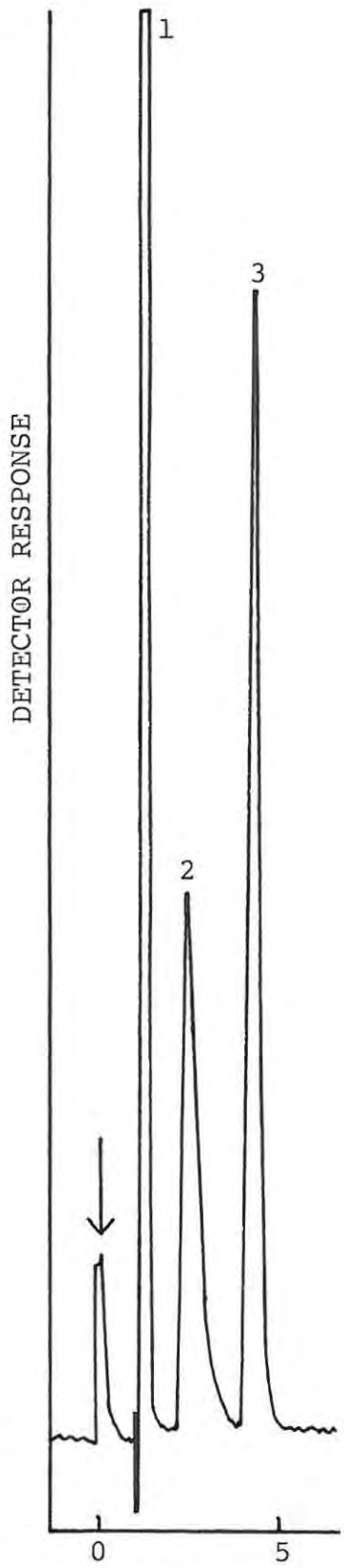
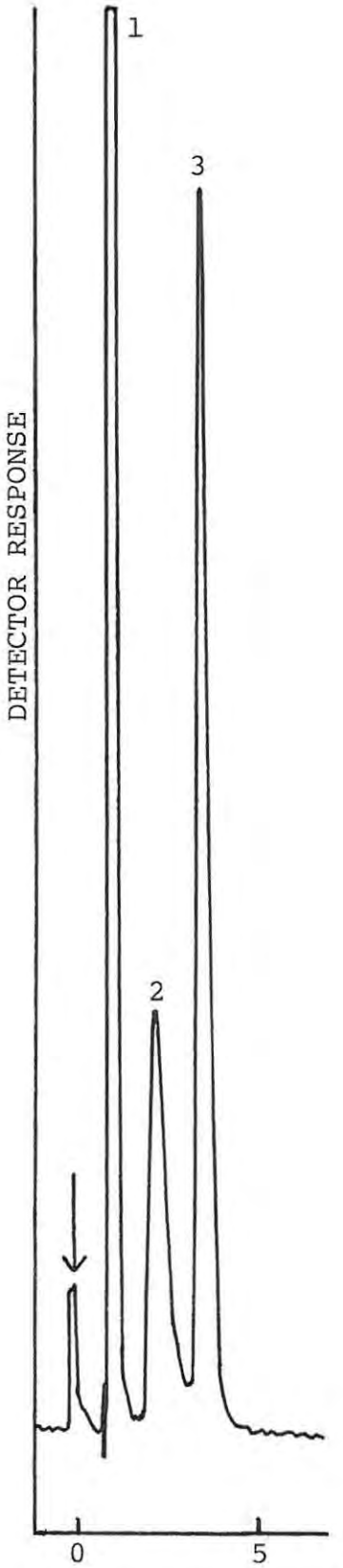
Fig. 4.3 (continued):

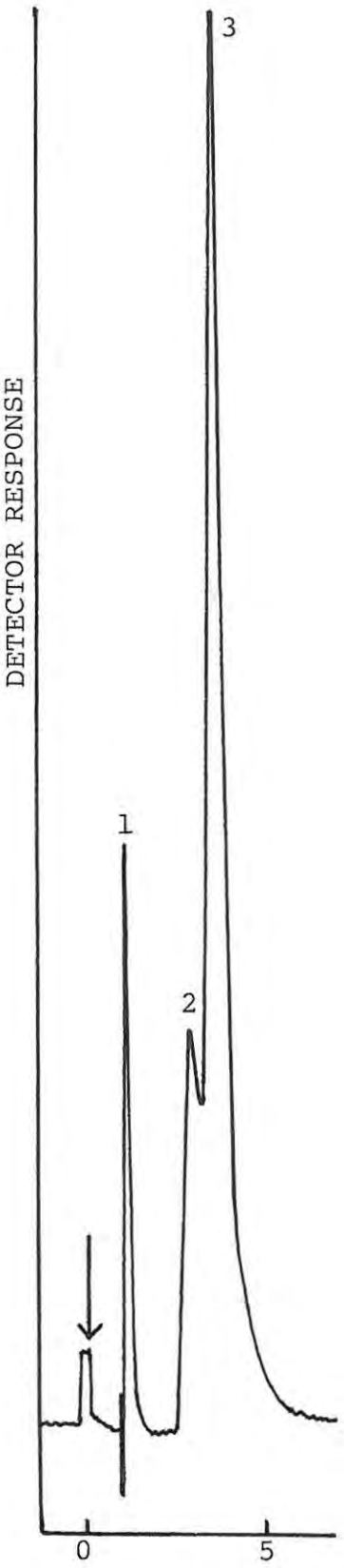


(c)

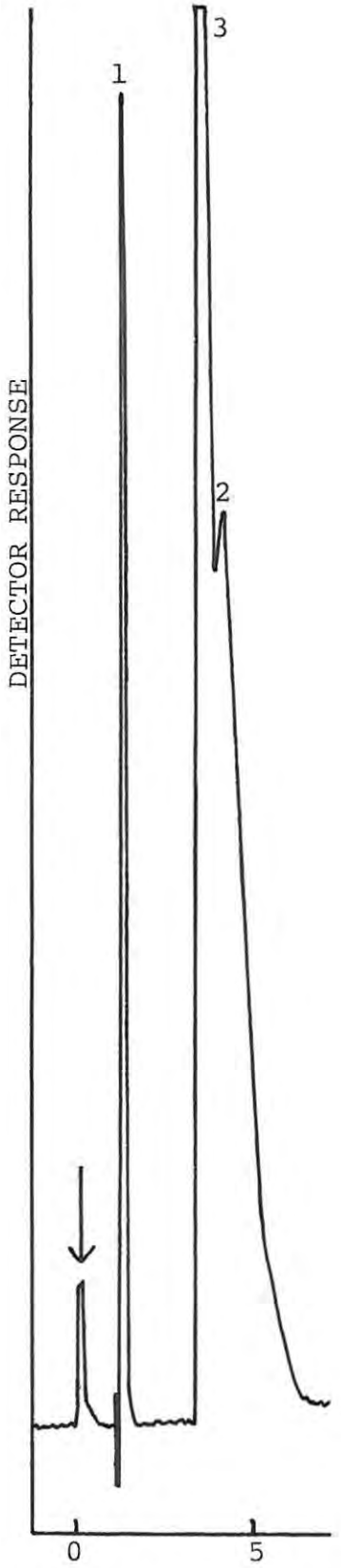


(d)

Fig. 4.3 (continued):

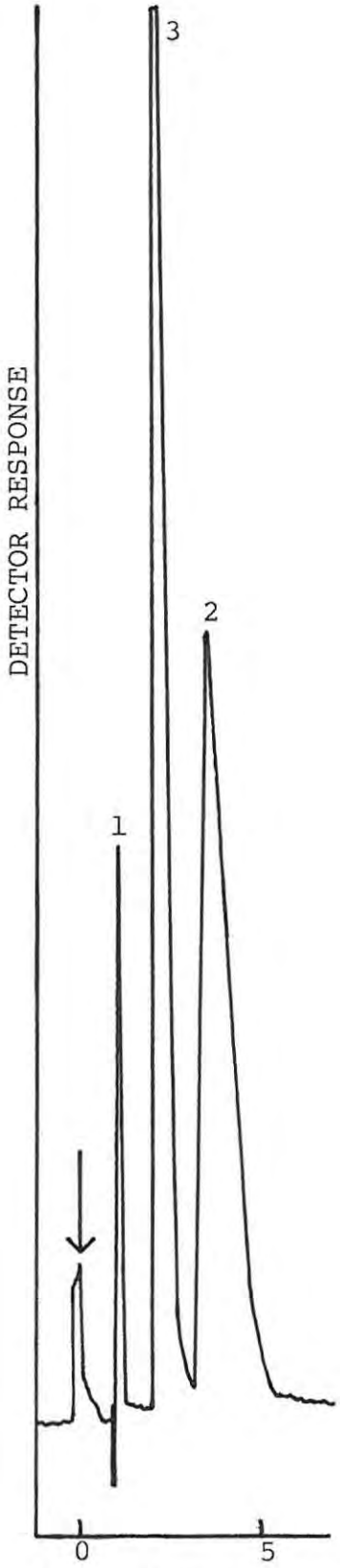


(e)

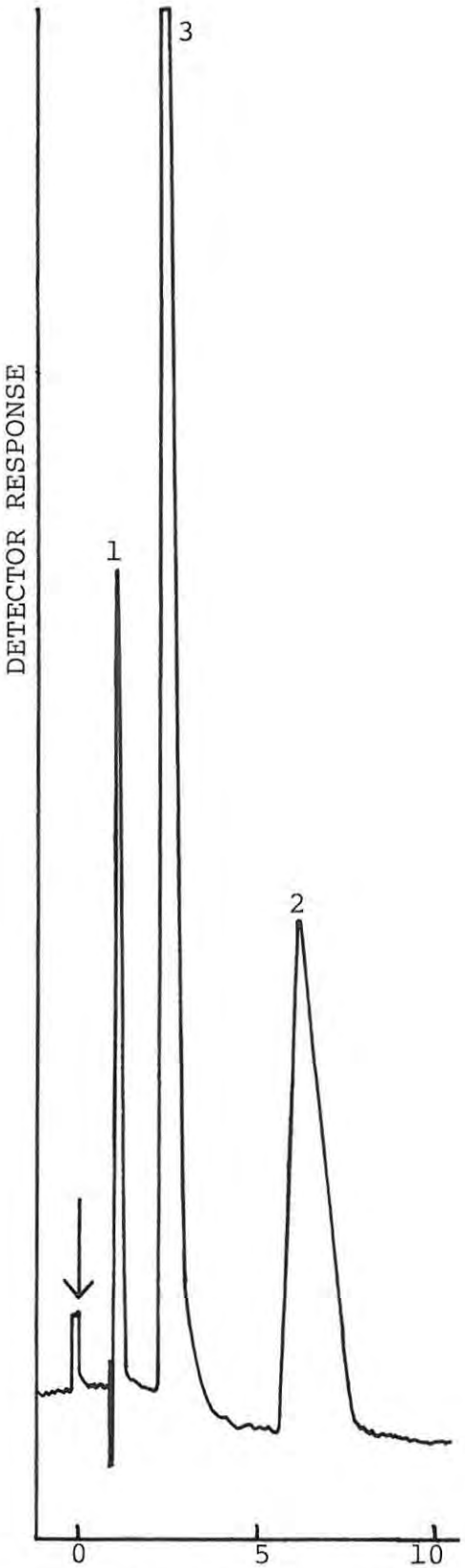


(f)

Fig. 4.3 (continued):



(g)



(h)

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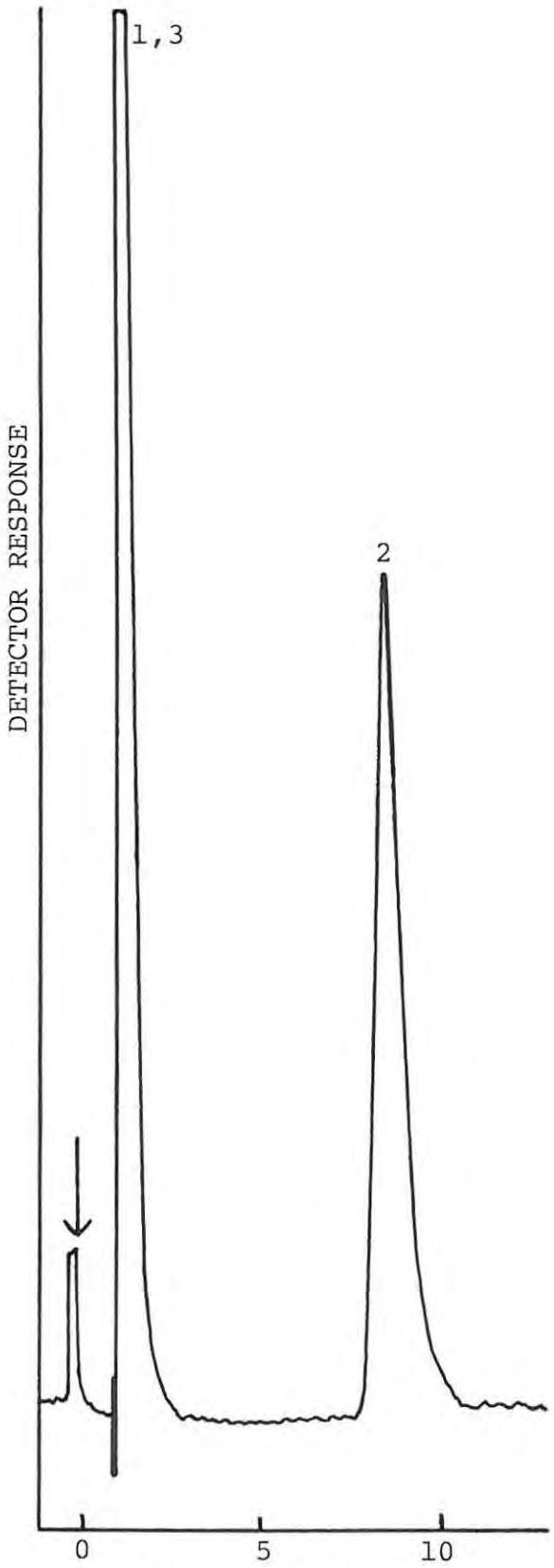
Fig. 4.3 (continued):



(i)

injected during the course of this deterioration were approximately 50 during the initial stages (a) and (b), approximately 300 during stages (c) and (d) and approximately 40 during stages (e) to (i). Thus changes were apparent when the column was initially used, although it seemed to have stabilized somewhat during stages (c) and (d). During the latter stages, all the previously described quantitation, recovery, etc., determinations were undertaken. In the final stages ((e) to (i)) the alterations in the chromatogram were apparent in successive injections. The retention time of TMP continued to increase until it reached a value of 12,1 minutes, whereas that of SMZ gradually decreased until this compound eluted within the void volume.

The efficiency of the column at each stage ((a) to (i)) was assessed by calculation of the height equivalent to a theoretical plate (HETP) for the SMZ peak in several chromatograms. The HETP was fairly constant at all stages and was found to have a value of 0,04078 ± 0,00530 cm (Mean ± S.D., n = 12). Thus, there was no apparent decrease in column efficiency but possibly a change in the actual nature and polarity of the bonded phase itself.

Initially it was thought that a weak acid may have become permanently bound to the NH_2 groups in the column. This would result in increased retention times for a basic compound, e.g. TMP, and decreased retention times for

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acidic compounds, e.g. sulphonamides. A number of sulphonamides had been injected under the same chromatographic conditions (see Chapter 6). Whilst changes in column characteristics had resulted in a reversal in the relative positions of the TMP and SMZ peaks, there was no alteration in the elution sequence of the various sulphonamides tested although their retention times had shortened considerably. Thus the fact that an acid may have become bound to the NH_2 groups seemed to be possible.

Hence, in an attempt to regenerate the column, it was rinsed with 100 ml triethylamine (0,1% vol/vol) in chloroform and then with pure chloroform. This appeared to reverse the change to a limited extent in that the TMP retention time was shortened to 7,8 minutes and SMZ was retained slightly longer although it remained incompletely resolved from the solvent (methanol) peak. A repeat of this treatment failed to improve these conditions. Possibly the change was irreversible at this stage.

A complete explanation for the sudden column deterioration is not however known. It appeared that a chemical change occurred resulting in an alteration of the chemical nature of the bonded NH_2 groups. These groups are highly reactive and hence this type of column tends to be rather unstable and must be treated with extreme caution. With this in mind, recycling of the mobile phase may have been a contributory factor towards the instability found when using this column.

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It may be possible also that trace amounts of water were present in the solvent system and caused a reduction in separating efficiency. Water has been found to produce changes in the characteristics of amino-bonded columns[107]. The NH_2 group, because of its high reactivity, will react with compounds containing aldehyde or ketone groups to form Schiff bases[114]. In addition, the group is readily oxidized and therefore the use of peroxides should also be avoided with this packing material[115]. Thus, great caution should be exercised, in the selection of solvents and these should possibly have been further purified and redistilled before use.

Failure of the sample preparation method utilized to sufficiently purify the biological samples and remove endogenous constituents may have contributed towards column degradation. Nonabsorbing inorganic ions in urine may have been inadvertantly extracted and the gradual accumulation of these ions on the column could influence column characteristics and lead to a change in retention times[107,113]. An NH_2-bonded phase column was employed by Sharma et al., for sulphonamide analyses[107]. They found retention times to be reproducible with the column but a 3 hour column regeneration step was required after only 15 to 30 urine samples were injected (samples pretreated with methanol to precipitate unwanted urine components). Thus the NH_2 column is clearly very sensitive to impurities in biological samples. Possibly, a pre-column should always

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be included to safeguard this type of column when biological samples are injected so that the above effects are minimized.

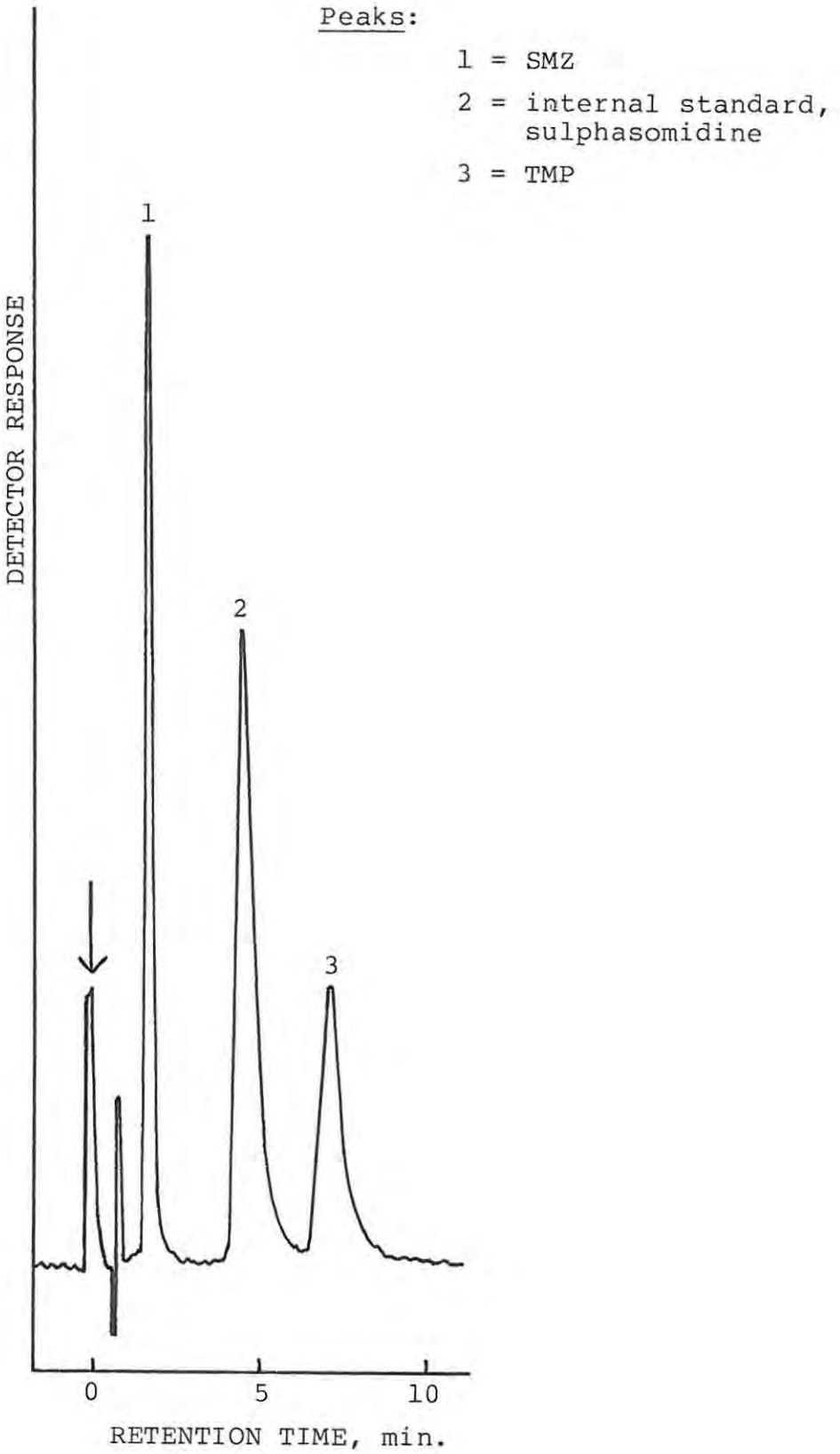
Regeneration of the column was attempted by utilizing the method employed by Sharma et al.,[107] and also by rinsing with a variety of solvents covering the range from low to high polarity. These were, however, unsuccessful.

Attempts were made to restore some column performance and enable separation of TMP and SMZ by alteration of the mobile phase. A three-component mobile phase comprising chloroform-acetonitrile-methanol 45:50:5 (parts by volume) at a flow rate of 60 ml hr^{-1} was found to adequately separate TMP and SMZ. Sulphasomidine could be used as internal standard (see Figure 4.4). The addition of a less polar third component to the mobile phase is a useful means of adjusting retention and selectivity[116].

However, no further use was made of this column. In all liklihood further changes would have occurred. In addition, it is doubtful whether the analysis could ever be reproduced if another, similar column were utilized.

A second, unused amino-bonded column (obtained from the same manufacturer) was tested under similar conditions as described for the first column. Separation of TMP and SMZ was achieved using an acetonitrile-methanol 40:60 (vol/vol) mobile phase at a flow rate of 80 ml hr^{-1}. Sulphamoxole

Fig. 4.4. Separation of TMP, SMZ and the internal standard, sulphasomidine, on the amino-bonded column using chloroform-acetonitrile-methanol 45:50:5 as the mobile phase at a flow rate of 60 ml hr^{-1}. The arrow denotes the time of sample injection.



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could be employed as internal standard. However, once again, the separation changed drastically during the course of approximately 50 injections. Large increases in the retention times of all components were observed. The mobile phase was not recycled in this case so this seems an unlikely reason for rapid column deterioration. Rinsing with triethylamine (0,1%) in chloroform worsened the situation further.

C. General Conclusions:

A method was devised for the simultaneous extraction and determination of TMP and SMZ in human urine which, at first, seemed potentially useful insofar as recovery, linearity of calibration curves and sensitivity were concerned. However, the amino-bonded column employed for the analysis was found to be non-reproducible and unstable under the conditions used and eventually was abandoned, before any attempt was made to assess precision or to adapt the assay to serum or to the determination of the sulphonamide metabolite, N^4-acetyl-SMZ. Thus, extreme caution must be exercised when utilizing this type of column for the purposes of analysing biological samples and possibly its use for these samples should be avoided altogether. Solvents must be carefully selected and purified, strict attention should be paid to sample preparation methods and back-extractions may be required. Pre-columns should also be considered for inclusion to safeguard the main column.

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4.3 SIMULTANEOUS DETERMINATION OF TRIMETHOPRIM, SULPHAMETHOXAZOLE AND N^4-ACETYLSULPHAMETHOXAZOLE IN SERUM AND URINE USING AN OCTADECYLSILANE REVERSE-PHASE COLUMN:

The possibility of employing reverse phase chromatography for the Co-trimoxazole analysis was investigated. In its most commonly practiced form, this mode of chromatography utilizes a hydrophobic bonded phase packing, usually possessing an octadecyl or octyl functional group, and a polar mobile phase. In general, polar substances have a higher affinity for the mobile phase and elute first. As the hydrophobic character of the solute increases retention becomes longer[117]. For these determinations, the column packing consisted of a monomolecular layer of octadecylsilane chemically bonded to fully porous 10 µm silica particles. A pre-packed column was obtained commercially (see Section 4.1.3.2).

The reverse phase technique is presently the most widely used mode in HPLC[117]. The majority of the currently available HPLC methods for the analysis of TMP, SMZ and N^4-acetyl-SMZ both separately and in combination, in pharmaceuticals and in biological fluids, have utilized either C_8 reverse-phase columns[92,95,96,99] or C_{18} reverse-phase columns[90,93]. Several advantages are associated with this technique which account for its high degree of popularity[117]. Efficient separation of a wide variety

of compounds can be accomplished, solvents commonly utilized are relatively cheap and, in addition, these columns are known to be stable provided certain precautions are taken.

When applied to biological fluids, reverse-phase micro= particulate columns offer the possibility of minimum sample preparation prior to actual injection into the chromatograph[38a]. This mode of HPLC is preferred because endogenous compounds in biological samples are predominantly hydrophilic and hence it would be advantageous to have these components eluting first. This would avoid excessively long retention of polar constituents associated with normal phase silica columns (polar stationary phase-nonpolar mobile phase). Thus, for routine clinical applications, the use of reverse-phase HPLC is preferable.

The C_{18} bonded reverse-phase column was successfully employed for the determination of TMP, SMZ and N^4-acetyl-SMZ in human serum and urine. This was the final method chosen for the analysis. A number of additional methods of sample preparation were evaluated using this column since a wide range of procedures can potentially be utilized.

4.3.1 Development of Methods for the Simultaneous Extraction of TMP and SMZ from Serum Samples:

A. Experimental:

(i) Standard solutions:

TMP stock solution. This solution contained 10,00 mg TMP per 100 ml methanol.

SMZ stock solution. This contained 20,00 mg SMZ per 100 ml methanol.

Internal Standard solution. This contained 20,0 $\mu g\ ml^{-1}$ sulphafurazole (SFZ) in methanol.

All standard solutions were freshly prepared.

(ii) Preparation of 'spiked' serum samples:

A bulk quantity of serum (40 ml) was fortified with 5,0 $\mu g\ ml^{-1}$ TMP and 3,0 $\mu g\ ml^{-1}$ SMZ. This was prepared by transfering 2,0 ml of the TMP stock solution and 600 µl of the SMZ stock solution to a ground-glass stoppered flask and evaporating to dryness at 50° C under a gentle stream of nitrogen. Once dry, 40,0 ml serum was added and the flask was then gently shaken for 4-5 hours to ensure complete dissolution of the drugs in the serum. This bulk spiked serum was utilized for all extraction procedures.

(iii) Chromatographic conditions:

The following chromatographic conditions were utilized:

Column: C_{18} reverse-phase (µBondapak C_{18}).

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HPLC system: B (see Section 4.1.3.1)

Detection wavelength: 270 nm (Schoeffel detector) and 280 nm (Spectra Physics Model 230 detector).

Sensitivity: 0,040 Absorbance Units Full Scale (A.U.F.S.)

Chart speed: 5 mm min^{-1}

Flow rate: 2,2 ml min^{-1}

Pressure: 1950 p.s.i.

Temperature: Ambient (~20° C)

Mobile Phase: 1% Acetic acid-methanol 80:20 (vol/vol).

Preparation of mobile phase:

The mobile phase was prepared by mixing 20 parts by volume of methanol with 80 parts by volume of a 1% vol/vol acetic acid solution, the latter solution being prepared from glacial acetic acid and water. The solvent mixture (pH 2,9) was simultaneously filtered and degassed before use by vacuum filtration through a Millipore type HA (0,45 μm) membrane filter.

(iv) Extraction Procedures:

Generally for each method, 1,0 ml of the bulk spiked serum was extracted. Unless otherwise indicated, Vacutainer(R) tubes were employed for the extractions. These were sealed with Parafilm(R) after each addition of reagent and, in addition, solutions were mixed for approximately 30 second intervals on a vortex mixer. Centrifugation was performed at 3000 r.p.m. for 15 minutes.

In all extraction procedures investigated, a specified aliquot of the final extract was placed in a tapered centrifuge tube. Subsequent treatment was identical for each and was carried out as follows:

The extract was evaporated to dryness at 50° C under a gentle stream of nitrogen. The sides of all tubes were carefully washed down with methanol during drying. Each dry extract was then reconstituted in 100 µl of the internal standard solution, and the tube was sealed. The reconstituted extracts were mixed thoroughly on a vortex mixer and were then centrifuged at 3000 r.p.m. for 15 minutes. An 8 µl sample of each was injected into the liquid chromatograph.

The following extraction procedures were assessed.

I. Acetonitrile/NaCl. To the serum sample was added 3 ml acetonitrile. The solution was thoroughly mixed and left to stand for 15 minutes to ensure complete protein precipitation. Sufficient sodium chloride (0,36 g) was added to saturate the aqueous phase, and the sample was re-mixed thoroughly. It was then centrifuged and 2 ml of the clear upper layer was transferred to a tapered centrifuge tube.

II. Acetonitrile/$(NH_4)_2SO_4$. The serum sample was treated as in extraction I except that, in place of sodium chloride, ammonium sulphate (0,6 g) was utilized to saturate the

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aqueous phase.

III. Acetonitrile/Na_2CO_3. The serum sample was treated as in extraction I except that, in place of sodium chloride, sodium carbonate (0,36 g) was utilized to saturate the aqueous phase.

IV. Acetonitrile/Na_2SO_4. The serum sample was treated as in extraction I except that, in place of sodium chloride, anhydrous sodium sulphate (0,36 g) was utilized to saturate the aqueous phase.

V. Dilute/acetonitrile/NaCl. The serum sample was initially diluted by the addition of an equal volume of pH 7 buffer. It was then treated as in extraction I with the exception that 0,72 g sodium chloride was required to saturate the aqueous phase.

VI. NaCl/acetonitrile. In this case, solid sodium chloride (0,36 g) was added to the serum sample initially and dissolved with the aid of thorough mixing. 3 ml acetonitrile was subsequently added, the mixture was vortexed briefly and it was then allowed to stand for 15 minutes. It was finally re-mixed and centrifuged. 2 ml of the clear upper layer was transferred to a tapered centrifuge tube.

VII. Pentane sulphonate sodium (0,005 M)/acetonitrile/NaCl. To the serum was added 1 ml aqueous pentane sulphonate

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sodium solution (0,005 M). The sample was subsequently treated as in extraction I with the exception that it was necessary to add 0,72 g sodium chloride to saturate the aqueous phase.

VIII. Pentane sulphonate sodium (1 g)/acetonitrile/NaCl. Preliminary treatment of the serum sample involved the addition of 1 g solid pentane sulphonate sodium. Subsequently the sample was treated exactly as described in extraction I.

IX. Isopropanol/NaCl. The sample was treated as in extraction I, except that 3 ml isopropanol was utilized in place of acetonitrile as the protein precipitating agent.

X. Ethanol/NaCl. In this case, 3 ml ethanol was used to replace the acetonitrile employed in extraction I. Subsequent treatment of the sample was performed as described in extraction I.

XI. Acetone/NaCl. The method utilized was similar to extraction I with the exception that 3 ml acetone was employed in place of acetonitrile.

XII. Acetone-Ethanol (1:1)/NaCl. The sample was treated as in extraction I, except that 3 ml of an acetone-ethanol 1:1 (vol/vol) mixture was utilized in place of acetonitrile to precipitate proteins.

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XIII. Trichloroacetic acid (14%)/acetonitrile/NaCl.
To the serum sample was added 0,5 ml of a 14% (wt./vol.) aqueous solution of trichloroacetic acid. The tube was mixed and left to stand for 15 minutes. 3 ml acetonitrile was added, the sample re-mixed thoroughly and finally 0,54 g sodium chloride was transferred to the tube. The whole was then vortexed briefly, centrifuged and a 2 ml portion of the clear supernatant was transferred to a tapered centrifuge tube.

XIV. Perchloric acid (2%)/acetonitrile/NaCl. The sample was treated as in extraction XIII but with the following modifications: (a) in place of 0,5 ml 14% trichloroacetic acid solution, 1 ml of a 2% (wt./vol.) aqueous solution of perchloric acid was added, and (b) 0,72 g sodium chloride was required to saturate the aqueous phase.

XV. Perchloric acid (5%)/acetonitrile/NaCl. The serum sample was treated as in extraction XIII but with the following modifications: (a) 1 ml of a 5% (wt./vol.) aqueous perchloric acid solution was utilized in place of the trichloroacetic acid solution, and (b) 0,72 g sodium chloride was required to saturate the aqueous phase.

XVI. Na_2SO_4/sodium tungstate (10%)/sulphuric acid (0,1 N)/acetonitrile. To the serum sample was added sodium sulphate (0,9 g) and 1 ml of 10% (wt./vol.) aqueous sodium tungstate. The mixture was thoroughly shaken, 1 ml 0,1 N sulphuric acid

was added, and, after further shaking the mixture was allowed to stand for 15 minutes. The aqueous layer was then extracted by shaking with 3 ml acetonitrile. After centrifugation, 2 ml of the organic layer was transferred to a finely tapered centrifuge tube.

XVII. NaCl/Chloroform-ethyl acetate (85:15). To the serum sample in a glass-stoppered, round-bottom centrifuge tube was added 1 ml pH 7 buffer, 0,72 g sodium chloride and 6 ml of an 85:15 (vol/vol) mixture of chloroform and ethyl acetate. The mixture was extracted by shaking slowly for 30 minutes on a mechanical reciprocating shaker. The samples were centrifuged and the upper aqueous layer and most of the protein interface were aspirated. 4 ml of the organic layer was then removed and transferred to a tapered centrifuge tube.

XVIII. NaCl/Chloroform-ethyl acetate-methanol (6:2:2). The sample was treated as in extraction XVII except that 6 ml of an organic solvent mixture composed of chloroform-ethyl acetate-methanol 6:2:2 (parts by volume) was utilized as the extractant in place of chloroform-ethyl acetate (85:15).

XIX. Acetonitrile/ethyl acetate (1:1)/NaCl. To the sample was added 3 ml of acetonitrile, the solution was thoroughly mixed and it was then allowed to stand for 15 minutes. 3 ml ethyl acetate was transferred to the tube and it was

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again shaken thoroughly. To the mixture was added 0,36 g sodium chloride. The sample was shaken and then centrifuged. 4 ml of the clear supernatant was transferred to a tapered centrifuge tube.

XX. Acetonitrile/ethyl acetate (1:1)/Na_2CO_3. The serum sample was treated as in extraction XIX except that, in place of sodium chloride, sodium carbonate (0,36 g) was utilized to saturate the aqueous phase.

XXI. Acetonitrile/ethyl acetate (1:1)/Na_2SO_4. The serum sample was treated as in extraction XIX except that 0,36 g sodium sulphate was employed in place of sodium chloride to saturate the aqueous phase.

XXII. Isopropanol/ethyl acetate (1:1)/NaCl. The sample was treated as in extraction XIX except that 3 ml isopropanol was utilized in place of acetonitrile.

XXIII. Ethanol/ethyl acetate (1:1)/NaCl. The method utilized was similar to extraction XIX with the exception that 3 ml ethanol was employed in place of acetonitrile.

XXIV. NaCl/methylene chloride/ethanol. To the serum sample was added 1 ml pH 7 buffer, 0,72 g sodium chloride and 3 ml methylene chloride. The tubes were shaken, centrifuged and then the aqueous phase was removed by suction. 3 ml ethanol was added and the tubes were well mixed. They

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were then centrifuged and 4 ml of the supernatant was transferred to a tapered centrifuge tube.

(v) Quantitation and assessment of recovery:

The ratios of the peak heights of TMP and SMZ to that of the internal standard, sulphafurazole, were calculated for each sample injected. In order to calculate percentage recovery, these ratios were compared with those obtained from the injection of a standard solution in methanol which was prepared so as to contain pure drugs in concentrations corresponding to those expected if 100% recovery were to have occurred during the extraction procedure. Since identical dilution and concentration steps were applied to all extracts, these could be compared to a single standard solution. The same amount of internal standard was added to both extracted samples and the standard solution. Since the internal standard was not carried through the extraction procedure it was used to correct for volumetric errors in sample injection.

(vi) Background:

The nature and relative size of peaks appearing in the chromatogram which were due to serum components extracted by each of the procedures employed were investigated. This was carried out by applying these extractions to samples of serum known to be drug-free. The final extracts were reconstituted in 100 µl methanol before injection.

The appearance of the extracts in the tubes before and after reconstitution was also noted.

B. Results:

Typical chromatograms obtained after the injection of an extract of serum spiked with TMP and SMZ and of blank serum using extraction procedure I (acetonitrile/NaCl - see p.85) and a detector wavelength of 280 nm are shown in Figures 4.5 (a) and (b) respectively. The retention times under these conditions were found to be 5,8 minutes for TMP, 8,4 minutes for SMZ and 11,0 minutes for the internal standard, sulphafurazole.

It was found that, for all extraction procedures investigated, chromatograms of blank sera showed peaks at retention times similar to those depicted in Figure 4.5 (b). Thus, in all cases the majority of endogenous serum constituents or extraneous substances in the extracts elute at retention times of between 1,5 and 4,8 minutes and an additional small peak appears at 7,9 minutes. The relative sizes of these background peaks were, however, found to vary depending upon the extraction methodology used and variations were also encountered among sera obtained from different patients.

Table 4.3 lists the results obtained for the various extraction procedures carried out on serum samples spiked with TMP and SMZ.

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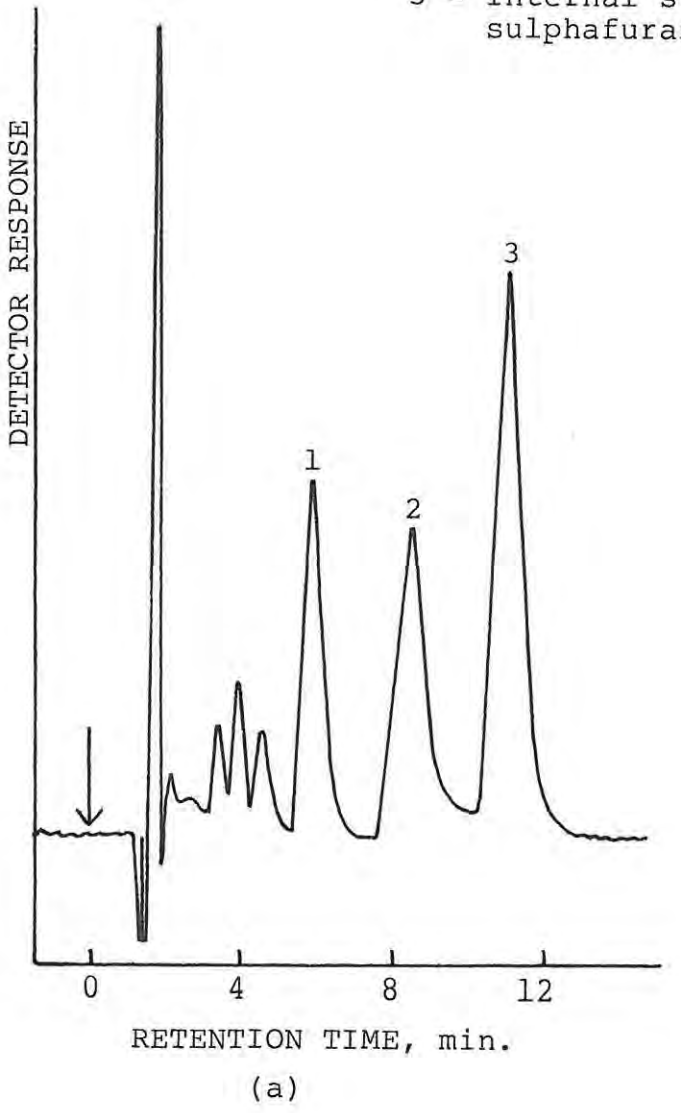
Fig. 4.5. Chromatograms of (a) an extract of serum containing 5,0 μg ml^{-1} TMP and 3,0 μg ml^{-1} SMZ and (b) a blank serum extract using extraction procedure I (acetonitrile/NaCl). Chromatographic conditions as in Section 4.3.1. The arrows denote times of sample injection (8 μl injected).

Peaks:

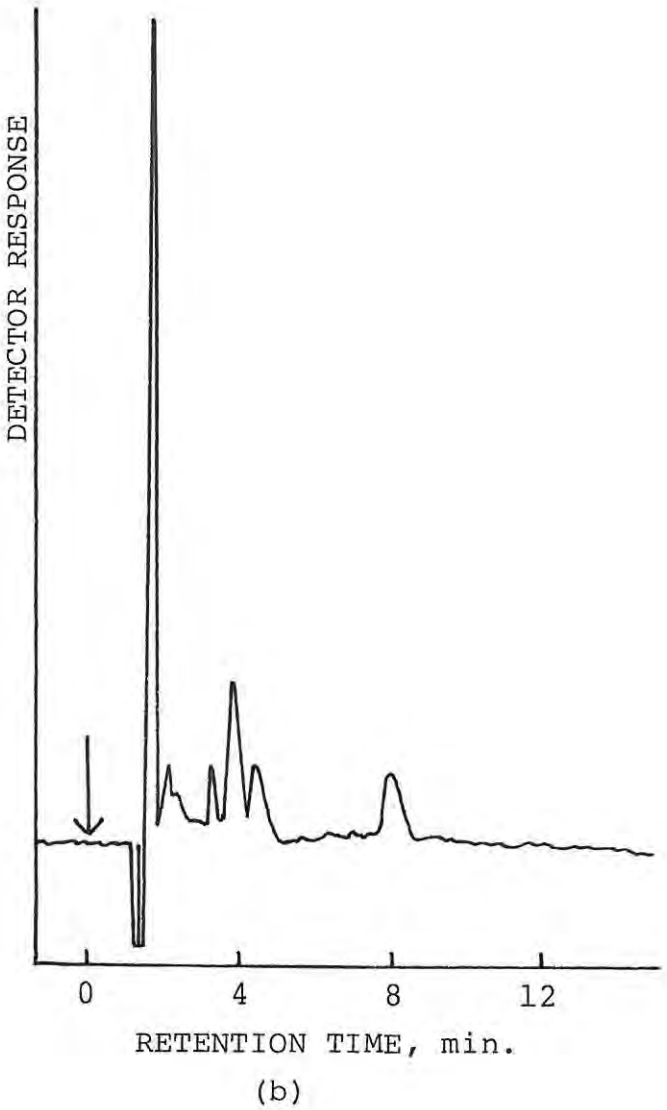
1 = TMP

2 = SMZ

3 = internal standard, sulphafurazole



(a)



(b)

TABLE 4.3:

EXTRACTION PROCEDURES CARRIED OUT ON SERUM SAMPLES[a]

EXTRACTION[b]	RECOVERY %		APPEARANCE OF EXTRACT		
	SMZ	TMP	DRY EXTRACT	EXTRACT AFTER RECONSTITUTION	PEAKS IN BLANK SERUM
I Acetonitrile/NaCl	55	115	Small salt deposit.	Clear, slightly yellow solution.	As in Fig. 4.5(b).
II Acetonitrile/$(NH_4)_2SO_4$	60	104	Slightly less salts deposited than after extraction I.	Clear, very slightly yellow solution.	Very large serum peak at 3,75 min. extracted.
III Acetonitrile/Na_2CO_3	85	100	Small salt deposit.	Clear, slightly yellow solution.	Very large peak at 3,79 min.
IV Acetonitrile/Na_2SO_4	80	95	Small salt deposit.	Clear, slightly yellow solution	Large serum peak at 3,81 min.
V Dilute/acetonitrile/NaCl	60	112	Small salt deposit.	Clear, slightly yellow solution.	As in Fig. 4.5(b).
VI NaCl/acetonitrile	50	105	Small salt deposit.	Clear, slightly yellow solution.	As in Fig. 4.5(b).
VII Pentane sulphonate sodium (0,005 M)/ acetonitrile/NaCl	35	112	Small salt deposit.	Clear, slightly yellow solution.	Serum peaks similar to those in Fig. 4.5(b).
VIII Pentane sulphonate sodium (1 g)/ acetonitrile/NaCl	43	102	Large salt deposit.	Clear extract but contained deposit of undissolved salts.	Serum peaks similar to those in Fig. 4.5(b)
IX Isopropanol/NaCl	80	102	Large salt deposit.	Slightly cloudy extract - deposit of undis= solved salts.	Very large serum peak at 3,77 min.
X Ethanol/NaCl	88	100	Large salt deposit.	Slightly cloudy extract - deposit of undis= solved salts.	Very large serum peak at 3,68 min.
XI Acetone/NaCl	60	98	Small salt deposit.	Clear, slightly yellow solution.	Large serum peak at 3,78 min.
XII Acetone-ethanol(1:1)/ NaCl	90	93	Large salt deposit.	Slightly cloudy extract containing deposit of undissolved salts.	Very large serum peak at 3,78 min.
XIII Trichloroacetic acid (14%)/acetonitrile/ NaCl	88	104	Large salt deposit.	Slightly cloudy extract with deposit of undissolved salts.	Very large serum peak at 3,80 min.
XIV Perchloric acid (2%)/ acetonitrile/NaCl	75	85	Small deposit.	Solution discoloured (green).	Serum peaks similar to those in Fig. 4.5(b).
XV Perchloric acid (5%)/ acetonitrile/NaCl	95	67	Small deposit.	Worse discolouration than that obtained in extraction XIV.	Serum peaks similar to those in Fig. 4.5(b).
XVI Na_2SO_4/sodium tungstate (10%)/sulphuric acid (0,1 N)/acetronitrile	87	114	Small salt deposit.	Clear, slightly yellow solution.	Very large serum peak at 3,95 min.
XVII NaCl/chloroform-ethyl acetate (85:15)	70	101	No deposit.	Clear extract.	Serum peaks slightly smaller than those in Fig. 4.5(b).
XVIII NaCl/chloroform-ethyl acetate-methanol (6:2:2)	50	109	No deposit.	Clear extract.	Serum peaks slightly smaller than those in Fig. 4.5(b).
XIX Acetonitrile/ethyl acetate(1:1)/NaCl	85	114	Very slight salt deposit.	Clear extract.	As in Fig. 4.5(b)
XX Acetonitrile/ethyl acetate (1:1)/Na_2CO_3	70	110	Very slight salt deposit.	Clear extract.	As in Fig. 4.5(b).
XXI Acetonitrile/ethyl acetate (1:1)/Na_2SO_4	60	100	Very slight salt deposit.	Clear extract.	Fairly large serum peak at 3,74 min.
XXII Isopropanol/ethyl acetate (1:1)/NaCl	85	113	Small salt deposit.	Slightly cloudy extract with small deposit of undissolved salts.	Large serum peak at 3,79 min.
XXIII Ethanol/ethyl acetate (1:1)/NaCl	92	113	Large salt deposit.	Slightly cloudy extract with deposit of undissolved salts.	Very large serum peak at 3,68 min.
XXIV NaCl/methylene chloride/ethanol	90	115	Large salt deposit.	Slightly cloudy solution with deposit of undissolved salts.	Very large serum peak at 3,75 min.

[a] Analysis using C_{18} reverse-phase column.

[b] For details of these extractions see text.

C. Discussion:

(i) Extraction procedures:

Proteins and other macromolecules in biological samples present the greatest potential problem in liquid chroma= tography. If not completely removed prior to injection onto the column, these will result in a rapid deterioration in column life. During this protein removal step, there must not however, be any loss of protein-bound portions of a drug. This is particularly important in the case of SMZ which is highly protein-bound[33] and the sample preparation procedure must hence ensure liberation of the bound moeity. In general, acidic drugs bind more strongly than bases[38b].

Numerous methods for protein removal are cited in the literature[38a,38b,91,102] and these have been widely used in drug analyses. Water-miscible organic solvents, e.g. acetone, acetonitrile, ethanol, isopropanol, can be utilized provided that an organic-to-aqueous phase ratio of not less than 2:1[38a] is employed. When added to serum, these solvents denature the protein, which is precipitated and can be removed by centrifugation. Inorganic salts, when present in sufficiently high concentrations, affect the miscibility of solvents[38c]. Hence, by the addition of salts such as sodium chloride, ammonium sulphate, sodium carbonate, etc., it is possible to separate even a water-miscible organic solvent from the aqueous phase. This so-called salt-solvent pair approach was utilized during these studies since the organic solvent could be removed and subsequently

concentrated to enable the detection of low concentrations of drugs, especially TMP, present in serum.

The use of acetonitrile as a deproteinization agent appeared to be superior to other water-miscible organic solvents in that the precipitated protein formed a "plug" which adhered to the bottom wall of the tube to yield a clear supernate after mild centrifugation, in contrast with other solvents which formed loose precipitates. This was also observed by Peng et al.,[109] who employed an acetonitrile deprotein= ization procedure for the determination of sulphisoxazole in plasma. In addition, when inorganic salts were added in an amount sufficient to saturate the aqueous (serum) phase, acetonitrile was found to separate readily as an upper layer. Upon subsequent evaporation of the acetonitrile layer to dryness, very small amounts of salts were observed in the dry residue. This facilitated reconstitution in small volumes of the appropriate solvent.

The use of acetonitrile in combination with a neutral salt e.g. sodium chloride (extraction I) was found to give inadequate recovery of SMZ. The addition of sodium chloride as a final step in the extraction procedure was found to yield slightly better recoveries for both drugs as compared with those obtained when the salt was added initially (extraction VI). This may be due to more thorough mixing of the acetonitrile with the serum sample which occurs if the salt is not present in the initial stages. Dissociation

of protein-bound sulphonamides (as well as other drugs) is favoured by dilution of the serum[42,96]. However, dilution of the sample resulted in only a very slight improvement in the recovery of SMZ (extraction V).

Improved SMZ recoveries were obtained by variation of the inorganic salt utilized in conjunction with acetonitrile. The use of ammonium sulphate, which has been employed in protein precipitation procedures[38b], yielded a slightly better recovery (extraction II), while use of anhydrous sodium carbonate and sodium sulphate (extractions III and IV) resulted in significant increases in recovery figures obtained. TMP recovery remained high for all of these extraction procedures. However, a very large peak at 3,8 minutes appeared in the chromatograms of blank sera when these salts were utilized for the extractions, although this peak did not interfere with the drugs of interest.

The use of ion-pairing reagents (as utilized in reverse-phase chromatography[117,118]) may be a possible approach to the simultaneous extraction of both a weak acid and a weak base. Ion-pair formation between ionized TMP molecules and alkyl sulphonate anions would result in the formation of an electrically neutral and non-polar complex and this would favour extraction into organic solvents. For SMZ, which is acidic, a quaternary amine, such as tetrabutyl= ammonium phosphate, could be used as the counter-ion at the appropriate pH. The use of pentane sulphonate sodium

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was investigated (extractions VII and VIII) as a possible aid in the extraction of TMP from serum. However, this approach was not found advantageous since TMP recovery was already adequate. Possibly, the use of ion-pairing reagents may be useful to improve SMZ recoveries, but these were not investigated here.

Recoveries obtained when other water-miscible organic solvents were utilized as protein-precipitants were also assessed. For these extractions, sodium chloride was utilized as the inorganic salt to enable separation of the organic phase. The use of acetone(extraction XI) did not greatly enhance SMZ recovery over that obtained with acetonitrile. Although isopropanol and, especially, ethanol and acetone-ethanol (1:1) combination[102] (extractions IX, X and XII) were found to displace the protein-bound sulphonamide and to yield good recoveries for both drugs, these solvents had a disadvantage in that they appeared to dissolve a large quantity of the inorganic salt so that a large deposit remained in the extract on drying. This hindered reconstitution in small volumes of solvents. Lower sulphonamide recoveries obtained when using acetonitrile may possibly be due to incomplete displace= ment of bound drug as a result of the formation of a "plug" of precipitated protein.

The use of acidic protein precipitants, e.g. trichloroacetic acid[119-121], perchloric acid[96,122] and tungstate followed by

sulphuric acid[102], has been described in the literature. An acidic pH will encourage the precipitation of protein[102]. To facilitate a concentration step, which is required for this analysis, the acidified sample, after protein precipitation, was extracted using an acetonitrile-inorganic salt combination. The acetonitrile layer was subsequently removed and concentrated.

Goehl et al.,[108] utilized a trichloroacetic acid protein precipitation procedure for the determination of Trisulpha= pyrimidines in serum and found the reagent to be superior to acetonitrile in that it resulted in more complete precipitation. Use of trichloroacetic acid for the present analysis (extraction XIII) resulted in good recoveries for both drugs (88% for SMZ and 104% for TMP). These results are in fairly good agreement with those obtained by Bury and Mashford[93] (81% and 95% recovery for SMZ and TMP respectively) who employed trichloroacetic acid without subsequent extraction for the determination of these compounds in plasma. However, the large salt deposit remaining in the extract after evaporation to dryness, which is due, possibly, to solubility of the trichloroacetic acid in the acetonitrile, limited the use of this procedure for the present determination.

Precipitation of serum proteins by perchloric acid followed by direct HPLC analysis of the acidic supernate was employed by Vree et al.,[96] who have described a method for the

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simultaneous determination of TMP and SMZ in biological fluids. They reported mean recoveries of 80% and 83% for SMZ and TMP respectively when using undiluted serum (prior dilution of the serum improved SMZ recovery). These values are fairly consistent with those obtained following the use of 2% perchloric acid plus subsequent acetonitrile extraction (extraction XIV; 75% recovery for SMZ and 85% for TMP). Increasing the perchloric acid concentration to 5% (extraction XV) yielded higher SMZ recoveries due, possibly, to more complete protein precipitation and hence greater displacement of bound drug. However TMP recovery was reduced since this drug will be essentially ionized at an acidic pH and hence will tend to remain in the aqueous phase. Use of perchloric acid, however, resulted in discolouration of the reconstituted extract which was considered a disadvantage since these pigments may have an adverse effect on column life.

The use of sodium tungstate followed by sulphuric acid and then extraction of the acidic sample with acetonitrile (extraction XVI) was found to yield good recoveries for both drugs without the disadvantages associated with trichloroacetic acid and perchloric acid. When using this procedure a large peak appeared in the blank extract at 3,95 minutes but this did not interfere with the drugs of interest.

Solvent extraction with a chloroform-ethyl acetate (85:15)

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mixture was previously found to be suitable for the extraction of TMP and SMZ from urine (see Section 4.2). When this procedure was applied to serum samples, it was necessary to saturate the aqueous phase, prior to extraction with sodium chloride to enable quantitative recovery. The final method utilized (extraction XVII) was found to give fairly good recoveries for both drugs. In addition, this approach provided a 'clean' extract for the analysis since the use of less polar, water-immiscible organic solvents results in the extraction of fewer polar impurities.

Since the addition of an alcohol to an organic solvent extractant has been found to reduce the likelihood of adsorption to glass or to protein[38c], the effect of combining a small quantity of methanol with a chloroform-ethyl acetate mixture (extraction XVIII) was investigated. This method did not, however, yield adequate SMZ recovery.

Extraction with methylene chloride (dichloromethane) followed by addition of ethanol[123] (extraction XXIV) was found to yield good recoveries for both TMP and SMZ. The presence of a large salt deposit in the dry residue, however, limited the use of this technique for the present analysis.

It was thought that combinations of ethyl acetate, which is commonly utilized as an extractant for SMZ[78], with water-miscible organic protein precipitants may yield a

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sample preparation system which would result in an adequate recovery of both drugs. Using sodium chloride as the inorganic salt to effect phase separation, good recoveries were obtained with acetonitrile-ethyl acetate, isopropanol-ethyl acetate and ethanol-ethyl acetate combinations (extractions XIX, XXII and XXIII respectively). Of these combinations, acetonitrile-ethyl acetate was considered most suitable since only a very small deposit of salts was present in the dry extract. Reconstitution in a small volume of solvent produced a clear solution for injection and peaks present in the chromatogram of a blank serum sample were not excessively large. The use of other inorganic salts, i.e. sodium carbonate and sodium sulphate, in conjunction with an acetonitrile-ethyl acetate mixture (extractions XX and XXI) yielded lower recoveries than those obtained when sodium chloride was employed. It was found that similar results were obtained whether samples were mixed using a vortex mixer or by shaking slowly on a mechanical reciprocating shaker.

Thus it was found that several of the sample preparation systems investigated would yield adequate recoveries of both TMP and SMZ from serum. Hence the selection of a suitable method depended to a large extent on the appearance of the extract before and after reconstitution, the relative size and nature of background peaks appearing in the chromatogram and the simplicity of the procedure. The final method selected for more thorough evaluation with

regard to its possible application for the TMP-SMZ analysis was that involving extraction with an acetonitrile-ethyl acetate (1:1) combination and employing sodium chloride as the inorganic salt to enable separation of the organic phase (extraction XIX). The procedure was slightly modified when applied to the final serum assay in that the extract was reconstituted in the mobile phase instead of in methanol. The method is relatively simple and rapid; simplicity of the extraction procedure tends to enhance its reproducibility. An analytically 'clean' extract is produced. When applied to blank serum samples, a chromatogram similar to that depicted in Figure 4.5(b) was obtained. Background peaks eluting between 1,5 and 4,8 minutes were fairly small and did not interfere with the analysis. The very small peak appearing at 7,9 minutes overlapped, however, with the SMZ peak. This peak was found to be present in the blank serum irrespective of the extraction methodology utilized or the wavelength employed for the detection. Several attempts to separate this peak from the other components by altering various chromatographic parameters and conditions proved unsuccessful.

Based upon this preliminary study, the percentage recovery of both drugs using the selected procedure was adequate. It must be borne in mind, however, that the quantities of SMZ and TMP incorporated into the 'spiked' serum in this instance were lower and higher respectively than those that would normally be encountered. These amounts were chosen so as to produce peaks of a suitable size at the wavelength

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used. The recovery of N^4-acetyl-SMZ was not evaluated during these preliminary studies.

The selected method, as well as several of the other methods investigated, indicate recovery values exceeding 100% for TMP. This may be due, possibly, to incomplete partitioning of the organic solvent from the aqueous phase. Thus, a small portion of the acetonitrile, or other water-miscible organic solvent, may remain dissolved in the aqueous layer thereby resulting in a lower volume of the organic phase than expected. This reduced volume would give rise to an apparently increased recovery when compared to the standard solution. Recoveries exceeding 100% are not encountered when water-immiscible organic solvents, such as chloroform-ethyl acetate (85:15), are utilized. In addition, salts which tend to dehydrate the aqueous layer, e.g. sodium sulphate, do not produce this effect. These observations tend to support the above explanation. The same amount of internal standard should be present in both extracted samples and standard solutions since this was added as a final step. However, an inadvertant small loss of the internal standard in the serum samples, due possibly to adsorption to unknown constituents in the extract, could also result in an apparently higher recovery for TMP when compared to the standard solution in methanol. The use of radioisotopically labelled compounds would be valuable for the determination of over-all recovery when developing and applying extraction methods.

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For a quantitative extraction, however, the most important factor is the reproducibility of the recoveries at all concentration levels. Losses as high as 50%[102] can be tolerated if reproducibility is good. The higher the recovery obtained, however, the more likely it is that reproducibility will be suitable.

(ii) Chromatographic conditions:

The selection of the mobile phase and other chromatographic conditions utilized are discussed in Section 4.3.2. The detector wavelengths employed for these preliminary investigations were 270 nm and 280 nm. These are not ideal in that, at the concentration levels normally encountered in serum, they will result in too large a sulphonamide peak in relation to the TMP peak. These wavelengths were, however, not used for the final assay, for the above reason.

4.3.2 Analysis in Serum:

A. Experimental:

(i) Standard solutions:

Trimethoprim stock solution. 6,00 mg TMP per 100 ml of methanol.

Sulphamethoxazole stock solution. 30,00 mg SMZ per 100 ml methanol.

N^4-acetylsulphamethoxazole stock solution. 15,00 mg N^4-acetyl-SMZ per 100 ml methanol.

Working internal standard solution. This solution consisted of 6 $\mu g\ ml^{-1}$ sulphafurazole (SFZ) in acetonitrile.

Internal standard solution for recovery studies. This solution contained 15,00 mg sulphafurazole per 50 ml of the mobile phase (1% acetic acid-methanol 80:20). The drug was dissolved with the aid of an ultrasonic bath.

All standard solutions used were freshly prepared. However, if stored at 4°C, solutions in methanol and acetonitrile were found to be stable for up to two months as determined by HPLC.

(ii) Preparation of "spiked" serum samples:

The method utilized for fortifying control serum with known amounts of pure drugs was carried out in the following manner.

Suitable aliquots of the TMP, SMZ and N^4-acetyl-SMZ stock solutions were transferred to a ground-glass stoppered flask. The final solution was then evaporated to dryness at 50°C under a gentle stream of nitrogen. Once dry, sufficient serum was added so as to yield the required concentration levels. The flask was then gently shaken for 4-5 hours at room temperature to ensure complete dissolution of all drugs in the serum. From this bulk serum pool dilutions could be made if required.

(iii) Chromatographic conditions and quantitation:

The following chromatographic conditions were utilized:

Column: C_{18} reverse-phase (μBondapak C_{18})

HPLC system: A (see Section 4.1.3.1)

Detection wavelength: 230 nm

Sensitivity: 0,020 A.U.F.S.

Chart speed: 12 inches hr^{-1}

Flow rate: 1,5 ml min^{-1}

Pressure: 1250 p.s.i.

Temperature: 30°C

Mobile phase: 1% acetic acid - methanol 80:20 (vol/vol).

Preparation of mobile phase:

The mobile phase was prepared as described in Section 4.3.1.

Quantitation:

Peak height ratios were calculated by dividing the peak height corresponding to the drug by the peak height

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corresponding to the internal standard. These ratios were quantitated by comparison with ratios obtained from the analysis of standards containing known concentrations of drugs.

(iv) Extraction procedure:

To 1 ml serum in a test tube (Vacutainer$^{(R)}$) was added 3 ml acetonitrile containing 6 µg sulphafurazole per ml as internal standard. The tube was then stoppered and thoroughly mixed on a vortex mixer for 30 seconds. The mixture was allowed to stand for 15 minutes to ensure complete protein precipitation. Three ml ethyl acetate was then added and the tube was sealed with Parafilm$^{(R)}$. It was then mixed on a vortex mixer set at low speed, to ensure that no liquid reached the Parafilm$^{(R)}$ surface, for 1 minute. The sample was allowed to stand for 30 minutes and then re-mixed. To the mixture was added an excess of sodium chloride (0,4 g) in order to saturate the aqueous layer. The tube was re-sealed with Parafilm$^{(R)}$, vortexed briefly and allowed to stand for 30 minutes with intermittent mixing, to ensure complete solution of the sodium chloride. Vigorous mixing at this stage may lead to slight emulsification and hence must be avoided. The mixture was then centrifuged at 3000 r.p.m. for 15 minutes. Five ml of the clear upper layer was 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.

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The dry extract was then reconstituted in 50 µl of the mobile phase and the centrifuge tube sealed as before. It was mixed thoroughly on a vortex mixer and placed in an ultrasonic bath for 1 minute to ensure complete solution of the drugs. The sample was then left to stand, with occasional mixing, for 15 minutes. Finally, it was centrifuged at 3000 r.p.m. for 15 minutes and 1-10 µl of the clear supernatant was injected onto the column.

(v) Calibration curves:

Control serum was fortified with pure drugs to yield five serum pools containing different concentrations of each drug. The range of serum concentrations produced varied between 0,25 and 3,00 µg ml^{-1} for TMP, between 5,00 and 60,00 µg ml^{-1} for SMZ and between 2,50 and 30,00 µg ml^{-1} for N^4-acetyl-SMZ. Each serum pool was assayed in triplicate as described above. Calibration curves were then constructed by plotting the ratios of the peak heights of each compound to that of the internal standard, SFZ, versus their respective concentrations. With all curves, a straight-line fit of the data was made by least-squares linear regression analysis.

(vi) Precision:

The within-run precision was assessed by spiking serum with TMP, SMZ and N^4-acetyl-SMZ to yield two different concentrations of each drug, corresponding approximately to the lower and upper limits of the therapeutic ranges

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expected. Six samples at each concentration level were than carried through the entire analytical procedure.

(vii) Extraction Efficiency:

The analytical recoveries of TMP, SMZ and N^4-acetyl-SMZ were evaluated at four different serum concentrations. At each concentration level, samples were assayed in triplicate. The analytical protocol utilized was as described above except that the following modifications were implemented for this particular study:

(a) All glassware was silanized prior to use with a 5% solution of dimethyldichlorosilane in toluene. Following this treatment, the glassware was rinsed first with toluene, then with methanol and was finally dried.

(b) The internal standard was omitted at the start of the extraction procedure; i.e. 3 ml pure acetonitrile was added to the serum sample.

(c) The internal standard was added as a final step prior to injection of the sample; i.e. the dry extract was reconstituted in 50 µl of a solution containing 15,00 mg SFZ per 50 ml of the mobile phase.

Aliquots of each sample were then chromatographed and the peak height ratios calculated for each component. In order to determine the percentage recovery, these ratios were compared with those obtained from the injection of standard solutions in the mobile phase which were prepared so as to contain pure drugs in concentrations corresponding exactly

to those expected if 100% recovery were to have occurred during the extraction procedure. These standard solutions were prepared by addition of the appropriate amounts of pure TMP, SMZ and N^4-acetyl-SMZ respectively in acetonitrile-ethyl acetate (1:1) solution to tapered centrifuge tubes in order to obtain the required final concentrations. These solutions were then evaporated to dryness under nitrogen and the residues were reconstituted in 50 µl of a solution containing 15,00 mg SFZ per 50 ml of the mobile phase.

Thus, the internal standard was present in identical quantities in both extracted samples and standard solutions. Since it was not carried through the extraction procedure, the value of the internal standard in this case was to account for variations in sample volumes injected.

Recovery of the internal standard, SFZ, during the extraction procedure was also ascertained. Pure samples were injected for comparison. In this case, TMP was employed as the correction standard by addition of a fixed amount (3 µg) as a final step to all samples.

B. Results and Discussion:

Chromatograms obtained following the injection of an extract of serum fortified with TMP, SMZ and N^4-acetyl-SMZ and of a blank serum extract are depicted in Figures 4.6(a) and (b) respectively. Under these conditions, the retention times were as follows:

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Fig. 4.6(a). Chromatogram of an extract of serum containing 1,07 μg ml^{-1} TMP, 19,48 μg ml^{-1} SMZ and 20,62 μg ml^{-1} N^4-acetyl-SMZ. Chromatographic conditions as in Section 4.3.2. The arrow denotes the time of sample injection (5 μl injected).

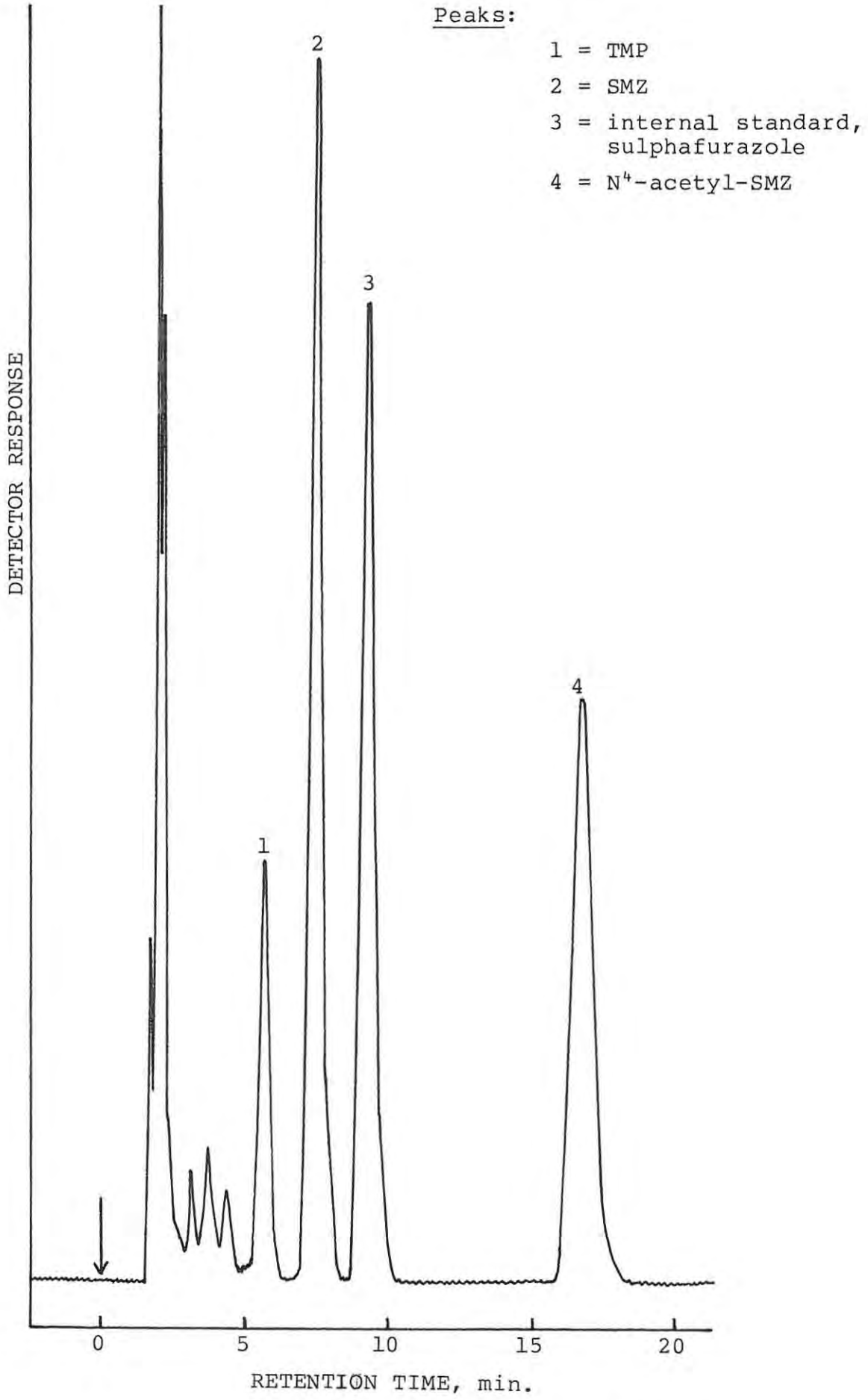

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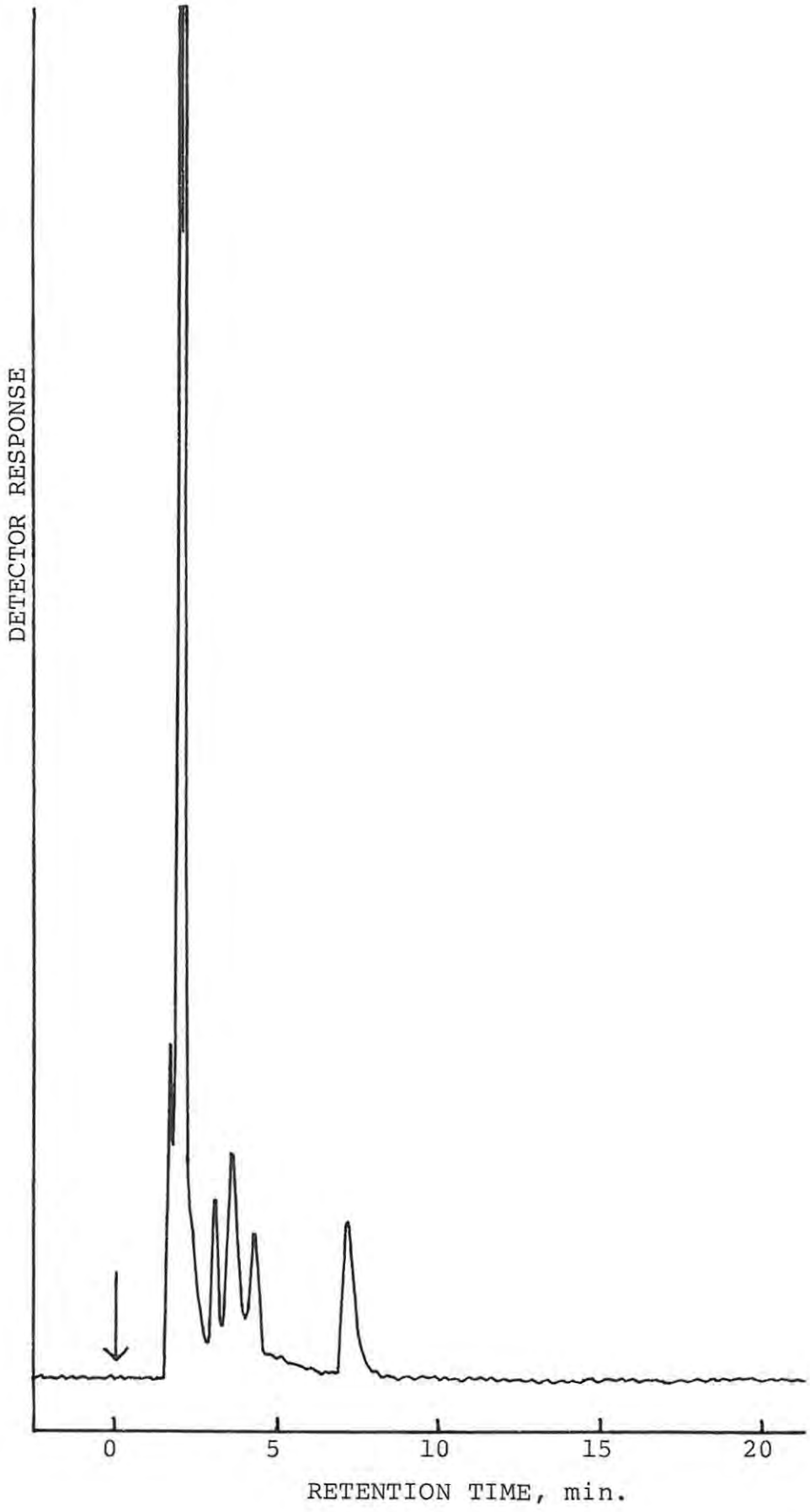
Fig. 4.6(b). Chromatogram of a blank serum extract. Chromatographic conditions as in Section 4.3.2. The arrow denotes the time of sample injection (7 µl injected).



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TMP	5,7 minutes
SMZ	7,5 minutes
Internal standard, SFZ	9,3 minutes
N^4-acetyl-SMZ	16,7 minutes

All peaks were well resolved and symmetrical.

When subjected to the analysis, serum samples obtained from volunteers after the ingestion of Co-trimoxazole tablets (each containing 80 mg TMP and 400 mg SMZ) were found to yield chromatograms identical to those produced when fortified serum pools were analysed. Thus no additional peaks, due perhaps to the other metabolites of these drugs, were evident.

(i) Linearity:

Calibration curves (see Fig. 4.7) for all compounds were found to be linear over the entire range of concentrations studied. Table 4.4 lists the constants of the respective linear regression lines.

TABLE 4.4:

LINEAR REGRESSION DATA FOR CALIBRATION CURVES IN SERUM[a].

Drug	Concentration Range, μg ml^{-1}	Slope	Y-intercept[b]	Correlation Coefficient
TMP	0,25 - 3,00	0,9426	-0,0631	0,9935
SMZ	5,00 - 60,00	0,1149	-0,2466	0,9988
N^4-acetyl-SMZ	2,50 - 30,00	0,0778	-0,1218	0,9904

a. Analysis using C_{18} reverse-phase column.
b. Peak height ratios are plotted on the y-axis and serum drug concentrations in μg ml^{-1} on the x-axis.

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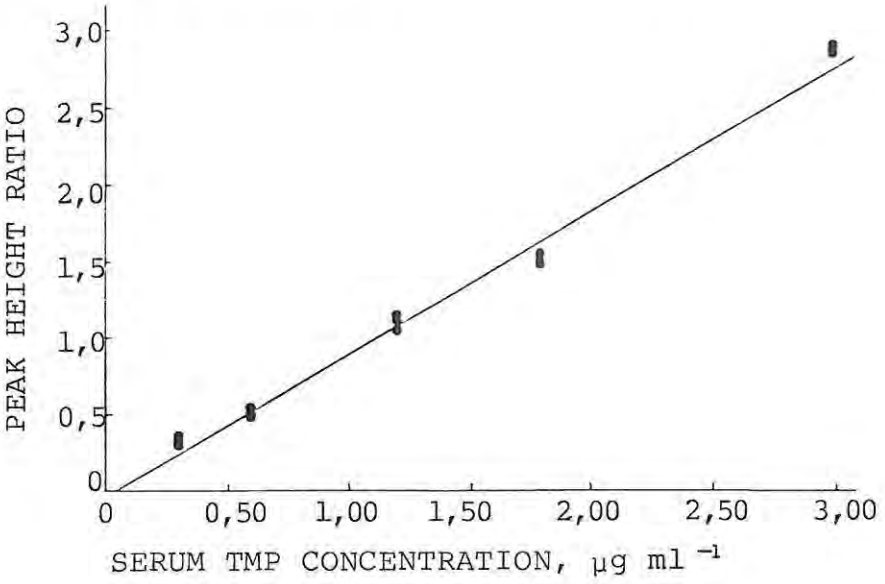
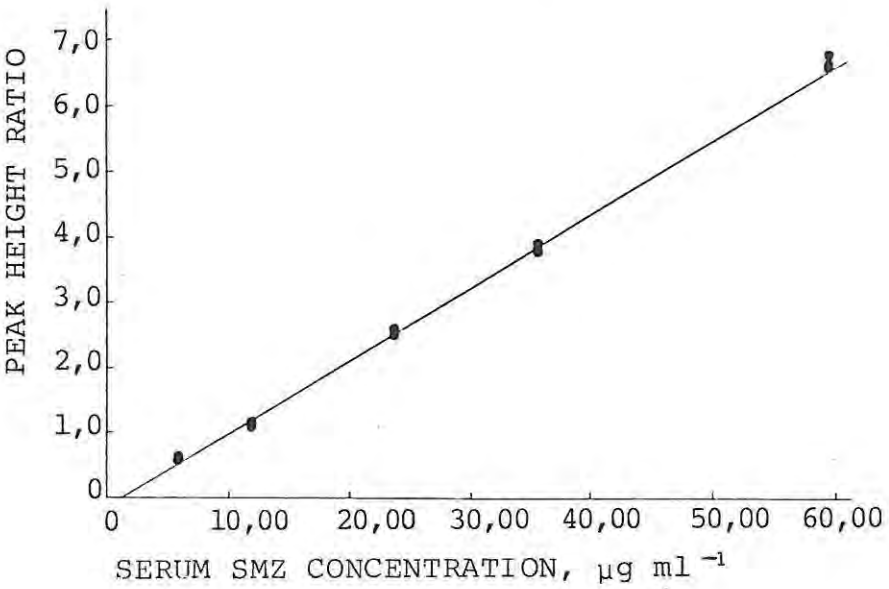
Fig. 4.7. Calibration curves for (a) TMP, (b) SMZ and (c) N^4-acetyl-SMZ in serum. Peak height ratio: ratio of peak height of indicated drug to that of the internal standard, sulphafurazole.

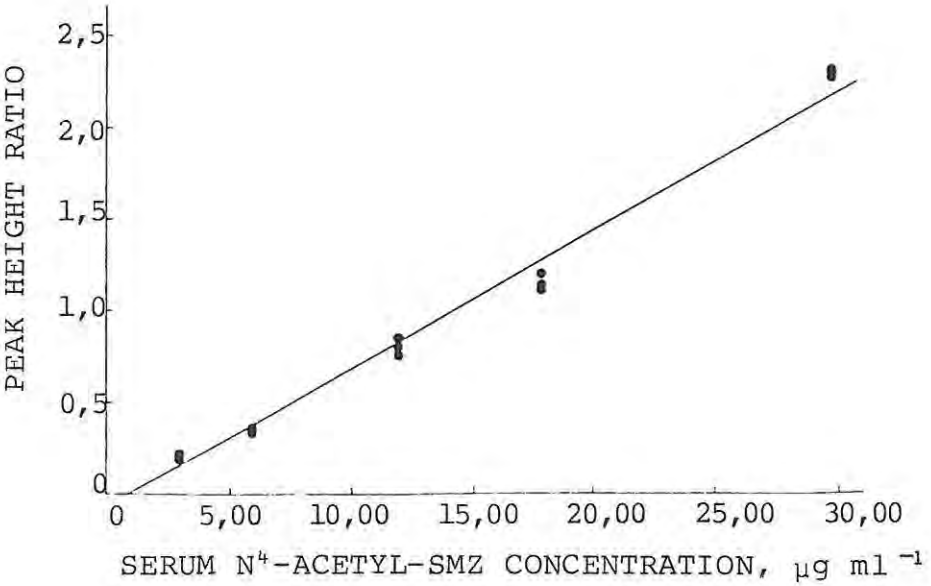
(a)



(b)



(c)



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Drug concentrations normally encountered in the serum during therapy fall within the linear range of the calibration curves.

(ii) Precision:

The results of the within-run precision studies carried out on fortified pools of serum are listed in Table 4.5.

TABLE 4.5:

WITHIN-RUN PRECISION OF ASSAY IN SERUM[a].

Sample No.	Concentration found, μg ml^{-1}					
	TMP		SMZ		N^4-acetyl-SMZ	
1	0,64	1,56	11,86	33,26	5,80	16,20
2	0,58	1,62	11,68	35,81	5,57	15,63
3	0,59	1,71	12,00	36,70	5,68	16,03
4	0,60	1,65	10,91	34,46	5,66	15,18
5	0,57	1,60	11,64	36,78	6,03	16,81
6	0,61	1,64	11,91	33,66	5,66	15,31
MEAN	0,60	1,63	11,67	35,11	5,73	15,86
Standard Deviation	0,023	0,046	0,361	1,400	0,149	0,557
Coefficient of variation, %	3,78	2,83	3,09	3,99	2,60	3,52

a. Analysis using C_{18} reverse-phase column.

These results indicate the method to be reproducible. Coefficients of variation for all drugs at all concentration levels studied are within the generally accepted limits for drug assays.

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(iii) Extraction Efficiency:

The analytical recoveries of TMP, SMZ and N^4-acetyl-SMZ from serum are depicted in Table 4.6.

TABLE 4.6:

ANALYTICAL RECOVERY OF DRUGS FROM FORTIFIED POOLS OF SERUM[a].

Drug	Serum Concentration, $\mu g\ ml^{-1}$	Recovery, %				
		Sample No.			Mean	Standard Deviation
		1	2	3		
TMP	1,23	107,9	100,6	98,3	102,3	4,1
	2,16	100,4	102,6	102,0	101,7	0,9
	3,08	102,8	99,6	94,2	98,9	3,5
SMZ	5,95	77,6	77,9	-	77,8	0,2
	23,79	72,6	71,5	75,8	73,3	1,8
	41,64	76,1	73,4	77,0	75,5	1,5
	59,48	77,4	78,5	79,3	78,4	0,8
N^4-acetyl-SMZ	3,09	78,4	73,8	-	76,1	2,3
	12,35	70,3	68,7	69,6	69,5	0,7
	21,62	69,7	72,0	72,2	71,3	1,1
	30,88	74,6	70,5	71,1	72,1	1,8

a. Analysis using C_{18} reverse-phase column.

These results indicate that the recovery of each drug is fairly consistent and quantitative at the concentration levels studied. Mean values obtained were 101,0%, 76,3% and 72,3% recovery for TMP, SMZ and N^4-acetyl-SMZ respectively. Prior dilution of the serum (by addition of an equal volume of distilled water) did not enhance the efficiency of extraction. Mean recovery of the internal standard, SFZ, was found to be 97,9%.

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Problems of variable and anomalous recoveries were experienced during preliminary studies. Unusually high values were obtained especially in the case of TMP. Apparent recoveries greatly in excess of 100% were recorded for this drug. It appeared that inadvertent losses of the internal standard in the extracted samples were largely responsible for these high values. Since the role of the internal standard for this determination was merely to check injection volumes, care must be taken to ensure that identical quantities are present in final samples of both extracted serum and standard solutions.

Adherence of the internal standard to unknown components in the final serum extract may represent one possible source of loss. The effect was not particularly pronounced if the internal standard was dissolved in the mobile phase and added to samples prior to injection. However, if the required amount of internal standard in methanol solution was added to both extracted samples and pure standards, followed by evaporation to dryness and then reconstitution in the mobile phase, particularly high and variable recoveries were observed.

Adsorption of drugs onto glass was found to be involved in the variability encountered during initial assessments of extraction efficiency. TMP adsorption has been reported[86]. Lanbeck and Lindström[144] have also suggested that variable recoveries of sulphapyridine and sulphamethazine observed

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when analyzing these drugs in plasma may be due to varying adsorption losses caused by the primary amino groups in these sulphonamides. It has been found that adsorption of drugs by various types of glassware[58,102,124] and even the source of glass tubes used in the extraction[124] can markedly affect the reproducibility and efficiency of the extraction. This problem can be overcome by silanization of the glass[58,102]. In this case, silanization appeared to rectify problems of high and variable recoveries and the results depicted in Table 4.6 were obtained. It is thus recommended that, for this determination, glassware should be inactivated by silanization.

Losses during the evaporation step may be responsible for variability in drug recovery, as suggested by Proelss, et al.,[125] in studies on tricyclic antidepressants. However, since the standard solutions were also subjected to an evaporation procedure, it was considered unlikely that variable losses were occurring at this stage.

It has been suggested[145] that recoveries of sulphonamides (sulphamethoxazole, sulphapyridine and acetylsulphapyridine) may be dependent on protein concentration of samples. The extent to which this may apply to the present analysis is not known.

(iv) Sensitivity and Detection Limit:

The principal sensitivity problem is associated with TMP since

relatively low serum concentration levels of this drug are encountered during Co-trimoxazole therapy. Under the conditions of this assay, the detection limit for TMP was established as 0,10 $\mu g\ ml^{-1}$ serum. This is adequate for pharmacokinetic studies involving TMP. In addition, the most sensitive of the various analytical methods currently available for the determination of TMP in biological fluids also report a minimum detectable quantity of 0,10 $\mu g\ ml^{-1}$ [70,86,92]. This lower limit of sensitivity is significantly better than that obtained by Vree et al.,[96] (0,75 $\mu g\ ml^{-1}$) who have recently described an HPLC method for the simultaneous determination of TMP, SMZ and N^4-acetyl-SMZ in biological fluids.

The detection limit for both SMZ and its N^4-acetylated metabolite under the described conditions was found to be 1,00 $\mu g\ ml^{-1}$. This provides adequate sensitivity for the determination of levels of these drugs normally encountered in the serum during treatment with Co-trimoxazole.

If very low levels of drugs are expected, it would be advantageous to incorporate less internal standard in order to retain useful peak height ratios. It may also be possible to increase accuracy when measuring very low concentrations of drugs, especially TMP, by addition of known amounts of the particular drug to the samples prior to extraction. Signal-to-noise ratios would thus be improved. The amount of drug present could then be determined by difference.

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It may be possible to reduce detection limits by increasing detector sensitivity specifically for each drug. Fluorescence detection is generally more sensitive[91] and, since both TMP[72] and SMZ[126] have been found to exhibit native fluorescence, this technique may be useful for the analysis. However, a recent HPLC method for the determination of TMP using fluorescence detection[92] reported a minimum detectable quantity similar to that obtained in the present analysis. Increased sensitivity for SMZ and N^4-acetyl-SMZ can be gained by monitoring these drugs at a higher wavelength (~260 nm) which is closer to the absorbance maxima for these compounds. Improved detection limits by the use of larger volumes of serum or greater concentration of the extracts is not feasable since the presence of large amounts of serum components would make it difficult to inject the required aliquot. In general, however, the proposed method is sufficiently sensitive for all clinical applications.

(v) Background and Interferences:

When processed by this procedure, blank sera obtained from several patients yielded chromatograms showing background peaks similar to those depicted in Fig. 4.6(b). The relative sizes of these peaks varied slightly among the different patients. Serum peaks eluting early in the chromatogram did not interfere with the analysis. However a small peak appeared at a retention time of 7,3 minutes in blank serum and this overlapped with the SMZ peak. The elution time of this peak was found to correspond to that

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of caffeine in serum. Carr, et al.,[58] have also reported the presence of a small peak in random plasma samples which has a retention time equal to that of caffeine. Caffeine-containing beverages and preparations should therefore be avoided in pharmacokinetic and therapeutic drug monitoring where this assay method is being employed for serum samples. Several attempts to separate this small peak from the other components by altering various chromatographic parameters and conditions proved unsuccessful. Possibly analyses should be conducted on blank patient sera to ensure that it does not constitute a significant interference.

Solutions of acetylsalicylic acid and salicylic acid were also tested for possible interference. Peaks for both compounds gave an identical retention time of 12,5 minutes which did not interfere with any of the compounds of interest.

(vi) Extraction Procedure:

The choice of the extraction procedure utilized for this determination is discussed in Section 4.3.1.

Reconstitution of the final dry extracts in the mobile phase as opposed to methanol markedly improved resolution and peak shapes obtained on injection of the samples. A chromatogram produced when a serum extract was dissolved in 50 µl methanol prior to injection is depicted in Fig. 4.8. When compared to the chromatogram shown in Fig. 4.6(a) which was obtained when the mobile phase was utilized for reconstitution, the

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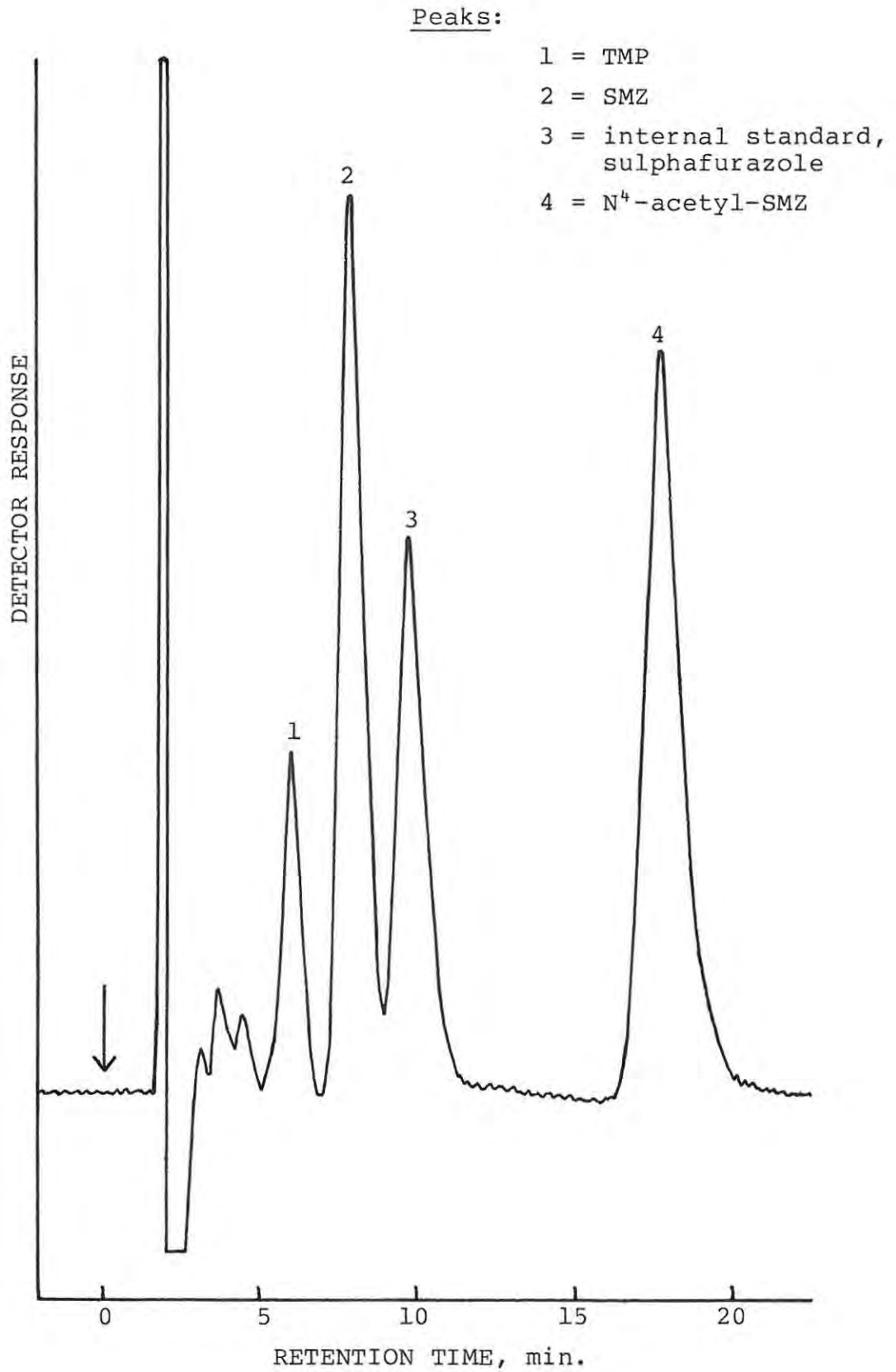
Fig. 4.8. Chromatogram obtained on reconstitution of the final serum extract in methanol prior to injection, in place of the mobile phase. Drug concentrations and chromatographic conditions as in Fig. 4.6(a). The arrow denotes the time of sample injection (7 µl injected).



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improvement is clearly apparent. Identical chromatographic conditions were employed for both samples. Reconstitution in the mobile phase also avoids the large methanol peak occurring initially in the chromatogram.

Hence, injection band broadening appears to occur when the extracts are reconstituted in methanol. This is probably due to a large extent to precipitation of components in the samples when they come into contact with the mobile phase. However, the use of methanol itself as the solvent for injected samples is also involved since resolution and peak shape obtained with pure drugs in methanol solution was not as good as that produced by similar samples in the predominantly aqueous mobile phase. The band spreading effect may have been enhanced if any area of dead volume was present within the injector system.

It was found that when serum samples were reconstituted in the mobile phase a precipitate appeared which had to be removed by centrifugation, whereas this did not occur when re-dissolving in methanol. Hence, reconstitution in the mobile phase is likely to prolong column life by removing these components prior to injection. The nature of the precipitate formed is not known although it may possibly be due to residual protein in the sample. Protein precipitation procedures are never 100% efficient[91].

A possible problem associated with the reconstitution of

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samples in the mobile phase is that the drugs may not readily dissolve in this solution. Sonication can be used to greatly enhance the rate at which compounds dissolve and was utilized for this study to ensure complete solution of all drugs. However, results obtained with and without sonication were identical. In addition, use of larger volumes of the mobile phase for reconstitution of samples yielded results identical to those obtained when a volume of 50 µl was employed. Thus, it appeared that all drugs were adequately soluble in the small volumes of solvent utilized for reconstitution.

The dry serum extracts before reconstitution were found to be stable for up to 5 days when kept at room temperature.

(vii) Chromatographic Conditions:

Mobile Phase:

(a) Reverse-Phase Chromatography:

Solutions of pure drugs in methanol were initially chromato=graphed to select a suitable mobile phase. Binary mixtures consisting of various proportions of methanol-water, methanol-acetate buffer and methanol-dilute acetic acid were tested as mobile phases in the preliminary studies. A solvent system comprising 1% acetic acid-methanol 70:30 (vol/vol) at a flow rate of 1,2 ml min^{-1} was found to yield satisfactory retention, resolution and symmetrical peak shapes. When applied to the chromatography of biological samples, however, it was found that the TMP peak overlapped

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with peaks of endogenous substances. Adjustment of the mobile phase composition to yield a 1% acetic acid-methanol 80:20 (vol/vol) mixture resulted in good separation of TMP from these serum peaks. Satisfactory resolution and efficiency was obtained with this mobile phase and it was selected for the analysis.

In reverse-phase HPLC the chromatographic behaviour is often predictable on the basis of pH of the eluent and the pKa and partition coefficient of the eluate[124]. Adjusting the pH on the acid side would suppress ionization of the acidic SMZ and result in increased retention of this compound on the column. This technique is termed ion suppression[117]. At this pH, TMP would be present in the predominantly ionic form and hence would elute more rapidly. This elution sequence is favourable as TMP tended to "tail" when retention on the column was prolonged. In addition, since TMP is usually present in a far lower concentration than SMZ, the sharper peak produced by early elution of this compound allows for more accurate quantitation.

(b) Ion-Pair Chromatography:

Weak acids and weak bases can also be simultaneously analysed by paired-ion chromatography[118]. Adequate resolution of TMP and SMZ was obtained with a mobile phase consisting of methanol-water 45:55 (vol/vol) which contained heptane sulphonate (5 mmol l^{-1}, PIC B-7: Waters Associates) as the counter-ion. The flow rate of the mobile phase (pH = 3,6)

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was set out 1,6 ml min^{-1} (1400 p.s.i.) and the separation was performed at ambient temperature. A chromatogram produced under these conditions is depicted in Fig. 4.9.

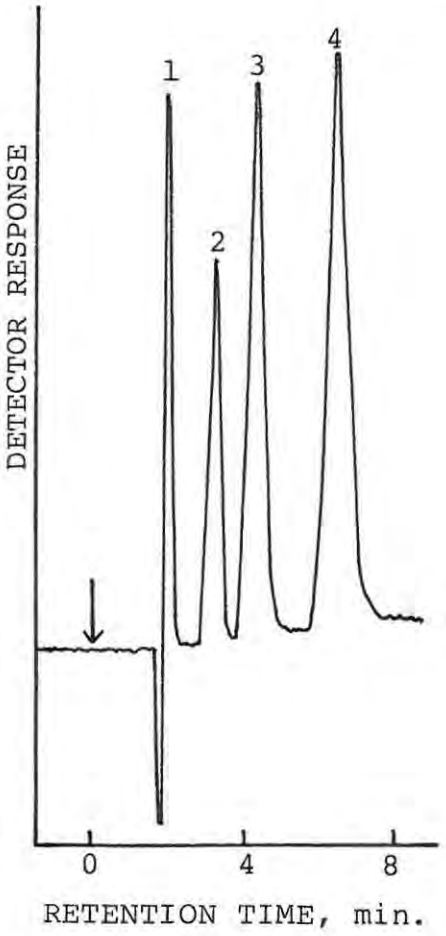

Fig. 4.9. Separation of TMP, SMZ and the internal standard, sulphaphenazole, on a μBondapak C_{18} column using methanol-water 45:55 containing PIC B-7 (heptane sulphonate, 5 mmol l^{-1}; Waters Associates) as the mobile phase at a flow rate of 1,6 ml min^{-1}. The arrow denotes the time of sample injection.

Peaks:

1 = solvent (methanol)
2 = SMZ
3 = internal standard, sulphaphenazole
4 = TMP

Sulphaphenazole could be employed as an internal standard. In this case, ion-pair formation between protonated TMP molecules and heptane sulphonate anions would result in a neutral and non-polar complex which shows increased retention on the reverse-phase column. This system could have been employed for the present analysis. Its application to biological fluids and the retention time of N^4-acetyl-SMZ were not, however, investigated here.

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Temperature and Flow Rate:

The primary reason for using elevated temperatures is to reduce the viscosity of the eluting solvent resulting, often, in improved resolution. This provides lower operating pressures and increased efficiency as a result of the greater solute diffusion and faster mass transfer experienced with low viscosity eluents. Reverse-phase separations often imply that some water is used in the eluting phase. Water has a relatively high viscosity. Thus elevated temperatures may frequently be advantageous for reverse-phase work.

At ambient temperature (~20°C), a flow rate of 2,2 ml min^{-1} (1950 p.s.i.) was required in order to provide conditions of maximum resolution with minimum analysis time for the assay of serum samples. Under these conditions, the retention time of the final peak in the chromatogram, N^4-acetyl-SMZ, was 22,4 minutes. Operation at 30°C enabled use of lower flow rates and pressures (1,5 ml min^{-1}; 1250 p.s.i.) and decreased analysis time (retention time of 16,7 minutes for N^4-acetyl-SMZ) whilst improving resolution and peak symmetry. Temperatures exceeding 30°C resulted in overlap between TMP and serum peaks and hence could not be used.

Use of elevated temperatures also avoids variations associated with fluctuations in the ambient temperature.

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Detection:

In view of the fact that the mean serum concentration ratio of SMZ to TMP is approximately 20:1, it was necessary to have a detection system that is considerably more sensitive for TMP in order to obtain responses of a similar magnitude for all components. Using a UV detection system, a wavelength of 230 nm proved to be optimal for this determination; at this wavelength the absorbance of TMP is five times that of SMZ on a weight basis (see Fig. 4.10). This enabled use of a single internal standard to quantitate both drugs, easy measurement of peak heights and a simultaneous analysis without the need to selectively attenuate certain peaks. When applied to the analysis of serum samples, the slightly lower recoveries obtained for SMZ will, in addition, further reduce discrepancies in relative peak heights.

At 230 nm, the molar response factors relative to the internal standard, sulphafurazole, were calculated by analyzing standard samples and were found to have the following values:

TMP	0,27
SMZ	1,13
N^4-acetyl-SMZ	0,69

A detection wavelength of 225 nm was employed by Vree et al.,[96] for the simultaneous determination of TMP and SMZ. Use of this wavelength will often result in too large a SMZ peak in relation to the TMP peak, making simultaneous determination from serum difficult.

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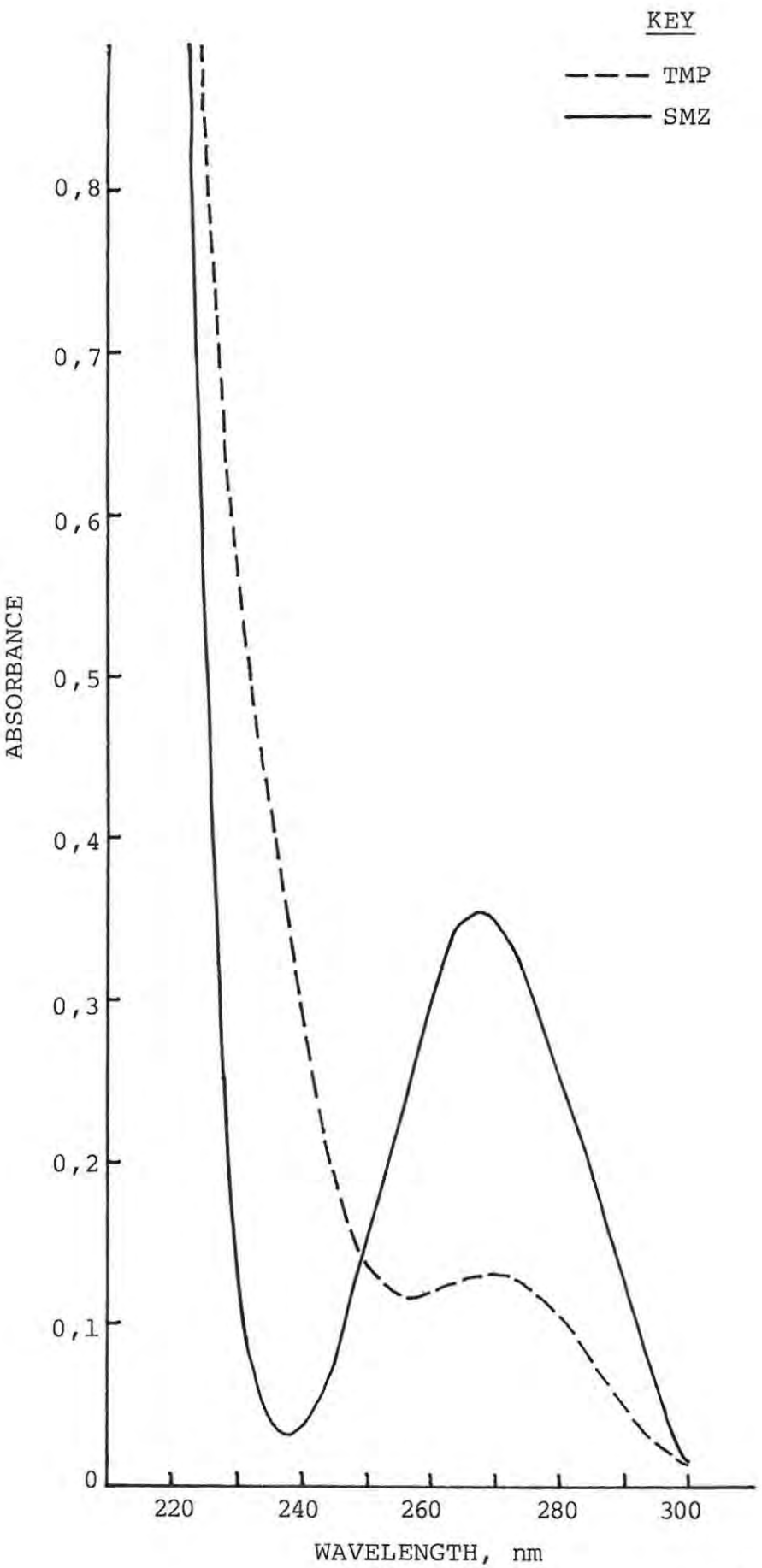
Fig. 4.10. Ultraviolet absorption spectra of 6 μg ml^{-1} TMP and SMZ in 1% acetic acid-methanol 80:20 (vol/vol).



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4.3.3 Analysis in Urine:

A. Experimental:

(i) Standard solutions:

TMP, SMZ and N^4-acetyl- SMZ stock solutions. Each stock solution contained 50,00 mg of the relevant drug per 50 ml of methanol.

Working internal standard solution. This solution consisted of 75 µg ml^{-1} sulphafurazole (SFZ) in acetonitrile.

Internal standard solution for recovery studies. This solution contained 75 mg SFZ per 50 ml of methanol.

All standard solutions were freshly prepared.

(ii) Preparation of "spiked" urine samples:

The procedure employed for fortifying control urine with known amounts of pure drugs was similar to that described for the preparation of "spiked" serum (Section 4.3.2).

(iii) Chromatographic conditions and quantitation:

The following chromatographic conditions were utilized:

Column: C_{18} reverse-phase (µBondapak C_{18})

HPLC system: A (see section 4.1.3.1)

Detection wavelength: 230 nm

Sensitivity: 0,080 A.U.F.S.

Chart speed: 12 inches hr^{-1}

Flow rate: 1,5 ml min^{-1}

Pressure: 1400 p.s.i.

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Temperature: Ambient (20 - 24°C)

Mobile phase: 1% acetic acid-methanol 80:20 (vol/vol).

Preparation of mobile phase:

The mobile phase was prepared as described in Section 4.3.1.

Quantitation:

Peak height ratios were used for the quantitation based on calibration curves established on the same day.

(iv) Extraction procedure:

To a 1 ml urine sample in a test tube (Vacutainer$^{(R)}$) was added an equal volume of pH 7,2 Sorensen's phosphate buffer[127]. Sodium hydroxide solution (0,1 N) was added as required in order to adjust the pH of the mixture to 7,2. Two ml acetonitrile containing 75 µg ml^{-1} SFZ as internal standard was then transferred to the tube. After sealing with Parafilm$^{(R)}$, the tube was thoroughly mixed on a vortex mixer for 30 seconds. The solution was left to stand for a period of 30 minutes and then re-mixed. Excess sodium chloride (1,0 g) was added in order to saturate the aqueous phase. The sample was left to stand, with intermittent mixing, for 30 minutes to ensure complete solution of the sodium chloride. It was subsequently centrifuged at 3000 r.p.m. for 15 minutes. One ml of the clear upper layer was transferred to a tapered centrifuge tube. It was then 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.

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The dry extract was reconstituted in 200 µl of the mobile phase. The tube was sealed as before, mixed thoroughly and placed in an ultrasonic bath for 1 minute to ensure complete solution of all drugs. The sample was then left to stand with occasional mixing for 15 minutes. Finally it was centrifuged at 3000 r.p.m. for 15 minutes and 1-10 µl was injected onto the column.

(v) Calibration curves and precision:

Linearity and precision of the method were assessed by procedures similar to those employed for the serum analysis (Section 4.3.2). The ranges of urinary drug concentrations employed for the calibration curves varied between 20,00 and 200,00 µg ml^{-1} for TMP, between 25,00 and 250,00 µg ml^{-1} for SMZ and between 40,00 and 400,00 µg ml^{-1} for N^4-acetyl-SMZ.

(vi) Extraction efficiency:

The analytical recoveries of TMP, SMZ and N^4-acetyl-SMZ from urine were evaluated at three different concentrations. Samples at each concentration level were analysed in triplicate. With the exception of the following modifications, the analytical procedure previously described was employed:

(a) The internal standard was omitted at the start of the extraction procedure; i.e. 2 ml pure acetonitrile was added to the urine sample.

(b) A fixed aliquot of the internal standard in methanol solution was added to all dry samples after extraction; i.e. 50 µl of a solution containing 75 mg SFZ per 50 ml methanol

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was added. The methanol was subsequently removed by evaporation at 50°C.

After reconstitution of the final samples in the mobile phase, aliquots of each were chromatographed. The results were compared with the peak height ratios obtained from the injection of standard solutions which contained pure drugs in concentrations equal to those expected if 100% recovery were to have occurred during the extraction. The same concentration of internal standard was present in both extracted samples and standard solutions.

B. Results and Discussion:

Chromatograms obtained following the injection of an extract of urine fortified with TMP, SMZ and N^4-acetyl-SMZ and of a blank urine extract are shown in Figures 4.11 (a) and (b) respectively. Under these conditions, the retention times were as follows:

TMP	6,7 minutes
SMZ	8,5 minutes
Internal standard, SFZ	10,8 minutes
N^4-acetyl-SMZ	20,1 minutes

Chromatograms of urine samples obtained from normal subjects after receiving Co-trimoxazole were identical to those obtained when "spiked" urine samples were assayed. No additional peaks or peaks due to other metabolites of these drugs were evident.

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Fig. 4.11(a). Chromatogram of an extract of urine containing 100,12 µg ml^{-1} TMP, 124,28 µg ml^{-1} SMZ and 199,40 µg ml^{-1} N^4-acetyl-SMZ. Chromatographic conditions as in Section 4.3.3. The arrow denotes the time of sample injection (3 µl injected).

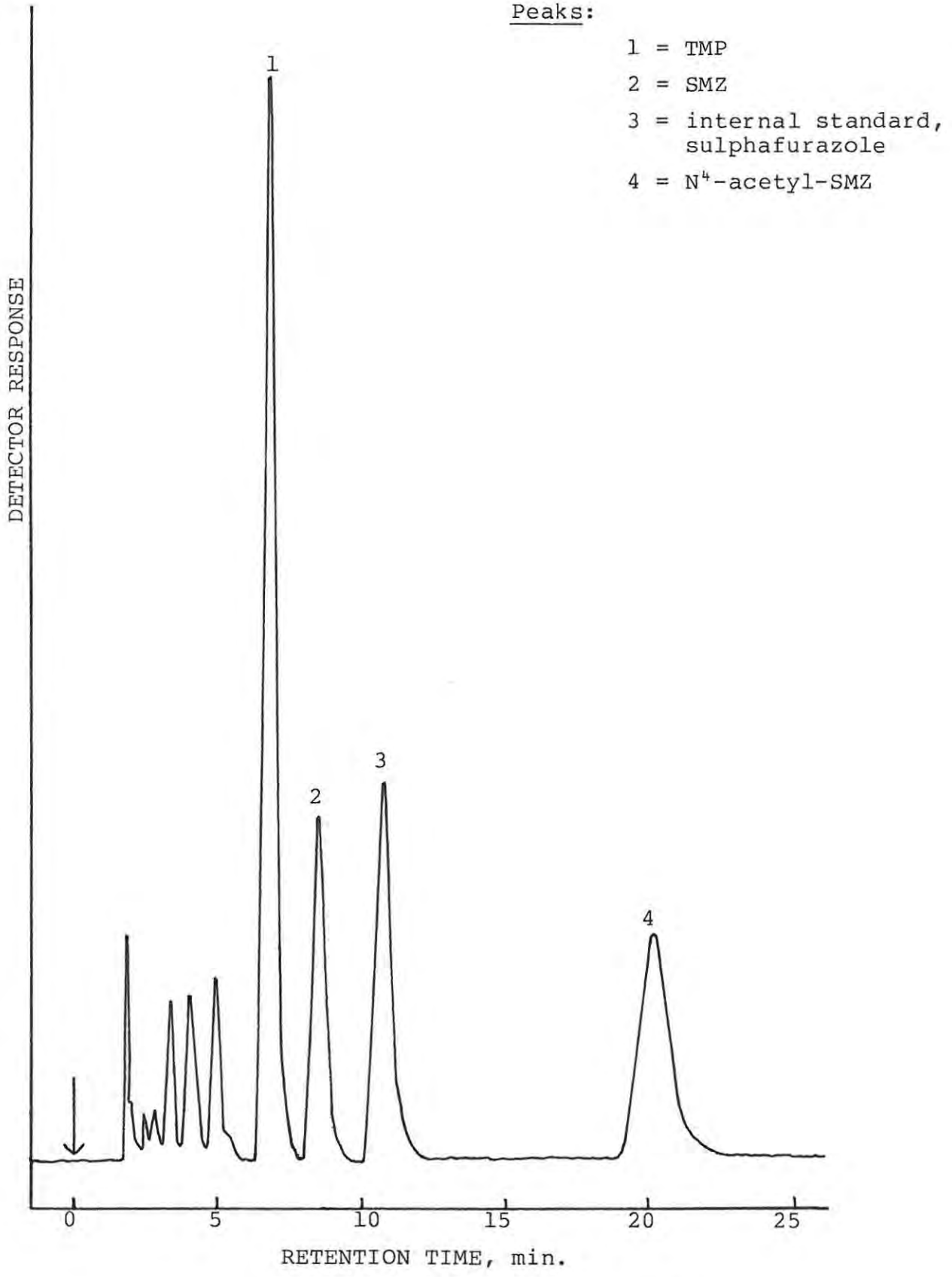

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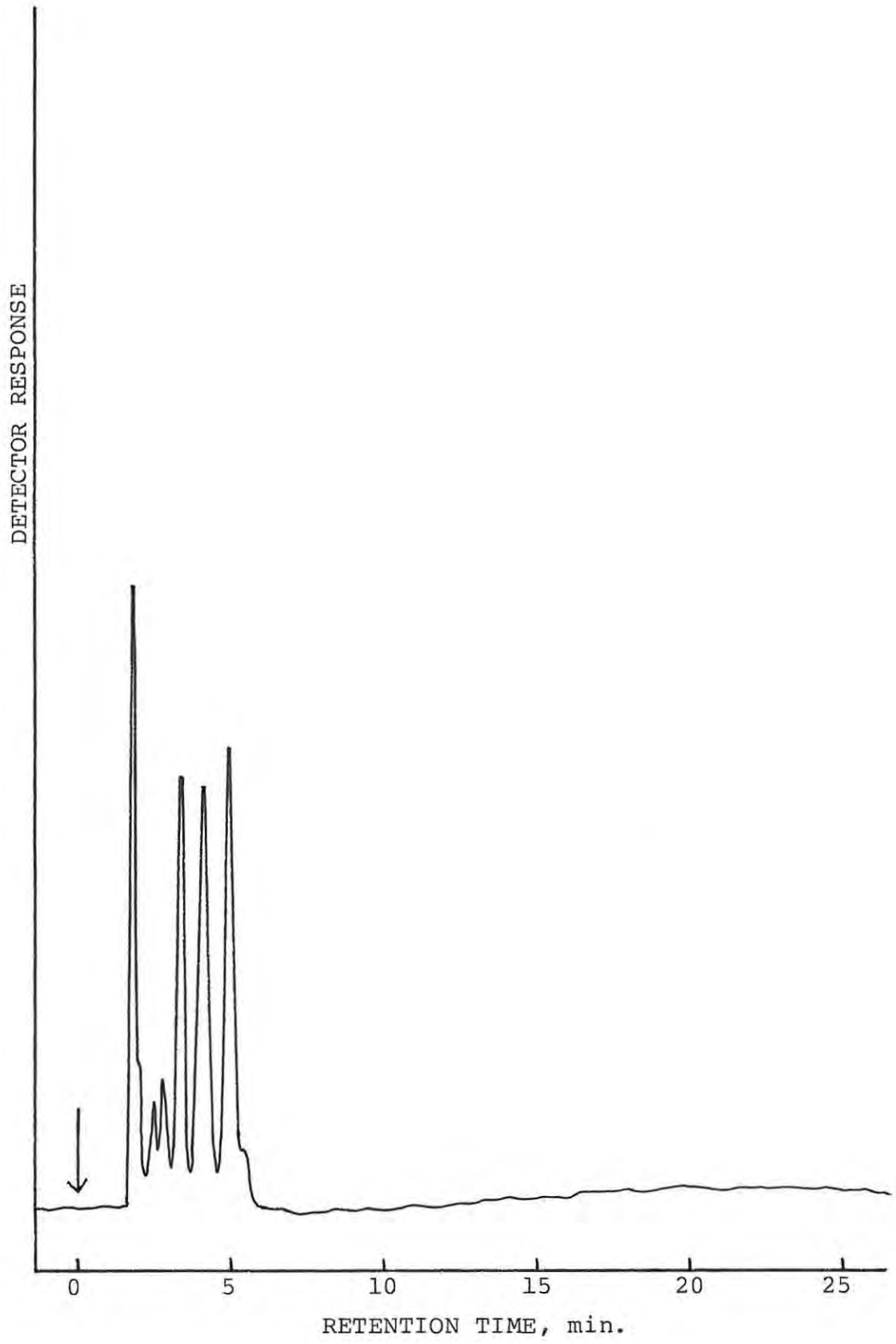
Fig. 4.11(b). Chromatogram of a blank urine extract. Chromatographic conditions as in Section 4.3.3. The arrow denotes the time of sample injection (10 µl injected).



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(i) Linearity:

Figure 4.12 depicts calibration curves obtained for TMP, SMZ and N^4-acetyl-SMZ in urine.

Linear regression data for the calibration curves (see Table 4.7) indicated a high degree of linearity for all drugs over the entire range of concentrations studied. Levels of these drugs normally found in the urine during therapy fall within the linear range of the calibration curves.

TABLE 4.7:

LINEAR REGRESSION DATA FOR CALIBRATION CURVES IN URINE[a].

Drug	Concentration Range, µg ml^{-1}	Slope	Y-Intercept[b]	Correlation Coefficient
TMP	20,00 - 200,00	0,0302	0,0397	0,9999
SMZ	25,00 - 250,00	0,0070	0,0091	1,0000
N^4-acetyl-SMZ	40,00 - 400,00	0,0026	-0,0058	0,9997

a. Analysis using C_{18} reverse-phase column.

b. Peak height ratios are plotted on the y-axis and urine drug concentrations in µg ml^{-1} on the x-axis.

(ii) Precision:

The results of the within-run precision studies carried out on fortified pools of urine are listed in Table 4.8.

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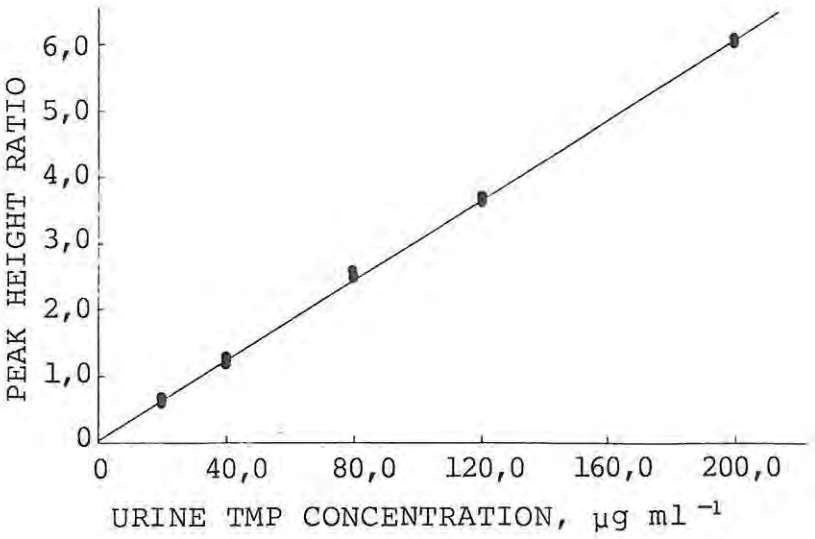
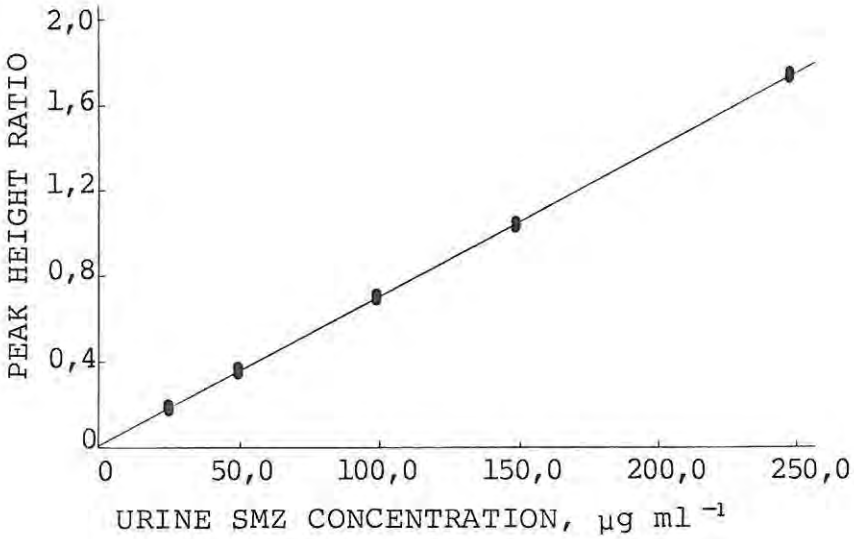
Fig. 4.12. Calibration curves for (a) TMP, (b) SMZ and (c) N^4-acetyl-SMZ in urine. Peak height ratio:ratio of peak height of indicated drug to that of the internal standard, sulphafurazole.

(a)



(b)



(c)

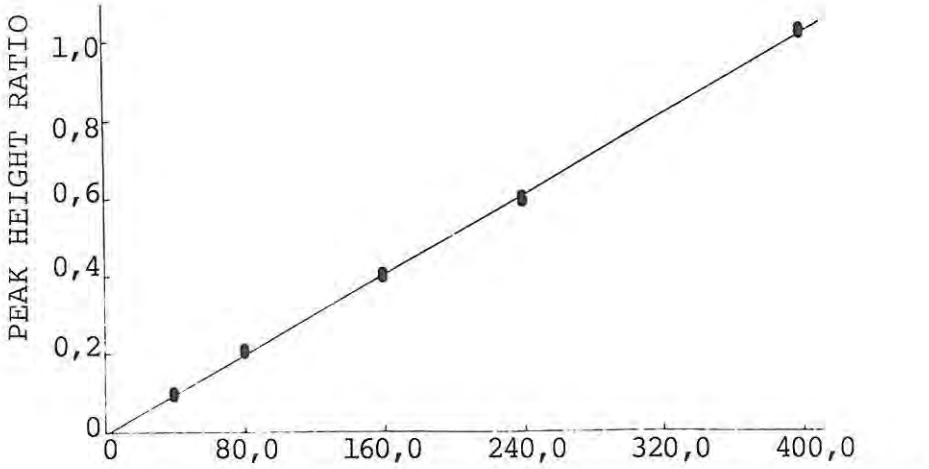


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TABLE 4.8:

WITHIN-RUN PRECISION OF ASSAY IN URINE[a].

Sample No.	Concentration Found, μg ml^{-1}					
	TMP		SMZ		N^4-acetyl-SMZ	
1	18,93	200,51	23,57	247,47	40,88	399,19
2	19,06	193,96	24,39	243,20	39,23	401,19
3	19,75	204,25	25,43	250,70	36,88	389,15
4	19,05	200,82	23,57	249,01	38,04	382,29
5	19,64	205,61	24,64	247,29	39,85	405,62
6	19,32	194,00	24,20	248,07	38,58	394,04
Mean	19,29	199,86	24,30	247,62	38,91	395,25
Standard Deviation	0,310	4,525	0,642	2,284	1,282	7,799
Coefficient of Variation, %	1,60	2,26	2,64	0,92	3,29	1,97

a. Analysis using C_{18} reverse-phase column.

These results indicate the method to be highly reproducible.

When assessing the precision associated with repeated injections of a single sample into the chromatograph, coefficients of variation of approximately 1,5% were obtained. This is extremely satisfactory since it probably represents variation inherent in the instrumentation utilized and slight inaccuracies in the measurement of peak heights. The coefficient of variation for sampling, instrumental and calculation errors has been found by other workers[119] to be approximately 2%.

(iii) Extraction efficiency:

The recoveries of TMP, SMZ and N^4-acetyl-SMZ from urine are given in Table 4.9. The values obtained were found to be fairly consistent and quantitative at all the concentration

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levels studied, with mean recoveries of 91,0%, 121,5% and 88,7% for TMP, SMZ and N^4-acetyl-SMZ respectively. Recovery of SMZ was found to increase slightly with increasing concentrations. This enhanced recovery of drugs as the concentration increases has been observed by others[125].

Recoveries exceeding 100% were found for SMZ. Failure to silanize the glassware in this determination may account for the high values obtained. Drug recoveries exceeding 100% have also been reported by other workers[123].

TABLE 4.9:

ANALYTICAL RECOVERY OF DRUGS FROM FORTIFIED POOLS OF URINE[a].

Drug	Urine Concentration, µg ml^{-1}	Recovery, %				
		Sample No.			Mean	S.D.
		1	2	3		
TMP	20,02	91,9	91,3	91,6	91,6	0,2
	100,12	90,0	91,8	91,6	91,1	0,8
	200,24	87,5	91,9	91,6	90,3	2,0
SMZ	24,86	114,6	119,1	120,0	117,9	2,4
	124,28	121,4	121,1	121,2	121,2	0,1
	248,55	126,3	126,3	123,7	125,4	1,2
N^4- acetyl-SMZ	39,88	86,7	88,5	87,5	87,6	0,7
	199,40	88,4[b]	92,4[b]	88,5	89,8[b]	1,9[b]
	398,80	84,6[b]	87,1[b]	-	85,9[b]	1,3[b]

a. Analysis using C_{18} reverse-phase column.

b. For these samples, urine extracts and standards, both evaporated to dryness, were kept at room temperature for 6 days before analysis. Instability was observed after this time since peak height ratios, particularly for the standard samples, were low and variable. These results were calculated by comparing values obtained for urine extracts at this concentration level with standards containing known amounts of pure drug at a lower concentration level (199,40 µg ml^{-1}).

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(iv) Sensitivity and detection limit:

Under the conditions of this assay, the detection limits for TMP, SMZ and N^4-acetyl-SMZ were established as 0,50; 5,00 and 10,00 $\mu g\ ml^{-1}$ respectively. This provides adequate sensitivity for all clinical applications.

(v) Background:

No peaks were evident in the urine control which could interfere with the analysis. Peaks due to endogenous urinary components elute early in the chromatogram and are well separated from the TMP peak. A marked reduction in the size of these peaks was observed if urine samples had been frozen, for storage purposes, prior to the analysis.

(vi) Extraction procedure:

A simple acetonitrile deproteinization procedure followed by separation of the organic phase by the addition of solid sodium chloride was found to yield adequate recoveries of all drugs from urine. Since sufficiently high drug concentrations are encountered in urine, concentration of the extract is not essential. However, this procedure was found to be advantageous in that (a) the size of background peaks in the chromatogram due to urinary constituents was reduced and (b) reconstitution of extracts in the mobile phase prior to injection resulted in improved peak symmetry and resolution.

The pH at which the extraction is carried out is important since fluctuations are likely to have a significant effect

on the extraction efficiency. Possibly, use of a buffer with a higher buffer capacity may obviate the need to adjust the pH of samples to the required value by the addition of sodium hydroxide.

The dry urine extracts before reconstitution were found to be stable for up to 5 days when kept at room temperature.

(vii) Chromatographic conditions:

When the optimal chromatographic conditions for the serum assay (Section 4.3.2) were applied to urine samples it was found that TMP was inadequately resolved from peaks due to endogenous urinary constituents. Separation of TMP from these background peaks was attained by reducing the operating temperature to ambient conditions (20-24°C). This change resulted in slightly longer retention times and somewhat broader peaks but this did not affect the quantitative aspects of the analysis.

Care should be taken to avoid variations that may be associated with fluctuations in ambient temperature during the course of an analysis. It was found that variations produced by changes in temperature between 20 and 24°C were insignificant. How= ever, temperature increases to 25°C and above resulted in a reduction in retention times. Close control over operating temperatures is especially important when peak heights are being utilized for the quantitation.

Detection:

Although the SMZ:TMP ratio in urine is approximately 1:1, detection at 230 nm, as employed for the serum assay, gave excellent results for the simultaneous analysis of these drugs in urine. Possibly the use of 225 nm would be more appropriate in view of the fact that TMP/SMZ responses are fairly similar at this wavelength. Background absorbance due to the acetic acid in the mobile phase was found, however, to be too high at this wavelength.

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4.3.4 General Discussion of the Method for the Simultaneous Determination of TMP, SMZ and N^4-acetyl-SMZ in Serum and Urine:

With the described method, TMP, SMZ and N^4-acetyl-SMZ were successfully quantitated simultaneously in serum and urine. The assay provides adequate sensitivity, specificity 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 dosage form of Co-trimoxazole for bioavailability assessment. It is well suited for routine application in the clinical laboratory because of the simplicity of the extraction procedure and the use of standard HPLC equipment. The final test of the method will, of course, be its application in the clinical situation and possibly correlation of the results with those obtained by conventional assay techniques.

Chromatographic conditions were chosen so as to provide the shortest analysis time with resolution of the drugs to be analyzed. Symmetrical and well-resolved peaks were obtained for all drugs. Signal-to-noise ratios are high despite use of the relatively low wavelength for the detection. Analysis time may possibly be reduced by incorporation of a gradient elution system to shorten the retention time of N^4-acetyl-SMZ. This, however, would no doubt result in baseline problems. In addition, re-equilibration time may be too long to warrant inclusion of a gradient system.

The proposed method offers considerable advantages over the only currently published HPLC method for the simultaneous determination of these compounds in biological fluids[96]. Detection limits for TMP are substantially lower. The incorporation of an internal standard, the advantages of which have been well established[128,129], represents an additional improvement. Despite the lack of chemical similarity between the internal standard employed, sulphafurazole, and TMP, the latter compound was successfully and accurately quantitated. The published method does not give any account of precision.

Peak heights were utilized for the quantitative determination of all compounds. It has been reported[130] that the use of peak heights for quantitative analyses gives the same degree of precision and accuracy as may be obtained with electronically measured peak areas. The same report also indicated that the precision of the peak height measurement was better than that of the product of the peak height and half-height width. However, peak height measurements are influenced by inadvertant small changes in the mobile phase composition and temperature effects and hence, for accurate quantitation, close control over these factors is essential. This assay technique could potentially be applied to the determination of these drugs in other biological fluids and tissues. Since TMP has a high volume of distribution (Section 1.4.3) its concentration in these tissues is likely to be sufficiently high to enable accurate quantitation. SMZ levels are usually

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readily detectable in most body fluids and tissues.

Column Stability:

The C_{18} reverse-phase column utilized for these studies was found to exhibit extremely high stability. A single column was employed for several hundred analyses of both biological samples and samples obtained during dissolution rate studies, without significant loss of resolution and efficiency. Retention times were found to be highly reproducible.

After some time, a slight loss of column efficiency became apparent. This was evident since a slight loss of resolution and shortened retention times resulted. Column back-pressure, however, remained low. Alteration of the mobile phase composition to yield a 0,4% acetic acid-methanol 78:22 (vol/vol) solvent mixture was found to improve resolution at this stage and permitted use of the column for further analyses.

After use, the column was rinsed and stored in a methanol-water 50:50 mixture. Occasionally it was flushed with water (150 ml), methanol (150 ml) and then methanol-water 50:50 in order to remove any accumulated impurities. An increase in column back-pressure occurred due to particles from injected samples blocking the column inlet filter. Filters were cleaned by sonication in a solution of concentrated nitric acid for 15 minutes, and also by sonication in water and methanol. This solved the problem

and operating pressures returned to normal. Rinsing the column with methanol was found on a few occasions to remove an unknown component which formed a white precipitate on contact with water. The nature of this precipitate is not known. Possibly it may have been due to impurities accumulating in the column as a result of the injection of biological samples. Loss of column packing material may also be involved although this was considered unlikely since resolution and efficiency remained unimpaired.

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CHAPTER 5

DISSOLUTION RATE STUDIES ON SOLID ORAL DOSAGE FORMS OF CO-TRIMOXAZOLE

The widespread use of the TMP-SMZ combination (Co-trimoxazole) has led to the development of a series of products with identical active ingredients. There is increasing evidence to indicate that therapeutic equivalence cannot necessarily be inferred from equivalence in the chemical constitution of different formulations of the same drug[4,146]. Hence, assessment of bioavailability, i.e. measurement of the rate and extent to which the active drug ingredient is absorbed and becomes available at the site of action[147], should, ideally, be carried out on all drug products. However, the standardized pharmacokinetic tests needed to ascertain bio-equivalence have a number of associated disadvantages[146,147]. In particular, these tests are very costly and time-consuming, requiring sophisticated analytical equipment, trained personnel and large numbers of volunteers. *In vivo* testing is an invasive technique since large numbers of blood samples are usually required. It is also difficult to predict whether pharmacokinetic parameters, such as blood levels, urine concentrations, etc. are directly related to drug product performance in terms of clinical effect. Practical problems concerning the measurement of the drug substance in biological fluids and the standardisation of the test in order to minimize inter- and intra-individual

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variation in pharmacokinetics are usually encountered. In the case of Co-trimoxazole products, strict control over urinary pH and flow would be required since these can profoundly influence excretion of the unchanged drugs (see Section 1.4.5). Determination of the acetylator status of volunteers may also be necessary.

Comparative bioavailability studies on a few Co-trimoxazole preparations have been conducted[31,131–134]. The bio= availability of TMP from single-component formulations[135] and from formulations with SMZ[62] has also been reported. With the exception of the last mentioned report, none of these studies made any provision for the control of urinary conditions.

It is important to search for suitable chemical/physical test procedures that can extend, supplement or perhaps even replace these <u>in vivo</u> bioavailability studies. In this regard, emphasis has been placed on determining the dissolution rates of active substances. The dissolution behaviour represents the speed-determining stage in the achievement of an optimal blood level, particularly in the case of therapeutically important active substances which are difficult to dissolve. Medical preparations with which such absorption problems can occur, and have been demonstrated, include SMZ tablets[136].

Dissolution rate studies have been developed as indicators

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of bioavailability[147]. However, well defined *in vitro*-*in vivo* correlations must be established before dissolution data can be accepted in lieu of *in vivo* bioequivalence data. Dissolution testing is extremely useful as a quality control parameter and as a tool for dosage form design. Moreover, it can be used to establish whether subsequent batches of a product will perform comparably to the batch on which bioavailability studies were originally conducted[147].

An extensive comparative trial on 15 Co-trimoxazole preparations was carried out by Steinigen and Bruene[136] by examinating their dissolution behaviour. They found that when artificial gastric juice was used as the dissolution medium, the TMP dissolved considerably more quickly than the SMZ. Thus, it was considered that measurement of the SMZ only, would give an adequate comparison. Simultaneous measurement of the comparative dissolution rates of TMP from these dosage forms was not carried out. Ghanem *et al.*,[137] have recently reported studies on the dissolution rates of different Co-trimoxazole dosage forms. They found release of the drugs to be pH-dependent.

At the time of this investigation, a total of eleven Co-trimoxazole preparations were commercially available in South Africa[138]. Since no extensive comparative dissolution rate data were available for these products, it was considered important to conduct a study on these preparations. HPLC

was utilized for the simultaneous determination of TMP and SMZ in the dissolution samples. (Difficulty was experienced in obtaining one of the products, Ultrasept (R) and hence it was omitted from the study.)

5.1 MATERIALS AND APPARATUS:

5.1.1 Reagents:

SMZ (Lot no. 3262) and TMP (Lot no. 3156) were supplied by Wellcome (Pty) Ltd., S.A. Sulphadiazine (Lot no. RFA 2298) was obtained from Maybaker (Pty) Ltd., S.A. All drugs complied with BP specifications.

All other solvents and reagents utilized were of analytical reagent grade and all were purchased from E. Merck, Darmstadt, with the exception of chloroform (Hopkin and Williams) and ammonium sulphamate (BDH Chemicals, Ltd., England). The water used was deionized and then glass-distilled.

5.1.2 Co-trimoxazole Products:

The names and batch numbers of the Co-trimoxazole preparations utilized in this study are given in Table 5.1. Six of the products studied contained 400 mg SMZ and 80 mg TMP per dose unit; these will be referred to as "standard" tablets or capsules. The remaining four preparations are "double-strength" tablets, i.e. they contain 800 mg SMZ and 160 mg TMP per dose unit. All dosage forms were compressed uncoated tablets with the exception of Co-Trim (R) which was a capsule.

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The individual samples were obtained either through a pharmacy or directly from the manufacturer. All the products were relatively fresh, none having been manufactured more than two years previously.

5.1.3 Apparatus:

(i) Beckman Model 25 U.V.-visible spectrophotometer.

(ii) Beckman Acta M VI U.V.-visible spectrophotometer.

(iii) Multiple spindle drive dissolution apparatus, Model QC72R-230B, Hanson Research Corp., Northridge, California, U.S.A.

(iv) HPLC system A (see Section 4.1.3.1)

(v) Pipetman(R) P and Finnpipette(R) continuously adjustable digital pipettes.

(vi) Wang 2200, Basic desk-top mini-computer.

5.2 EXPERIMENTAL:

A. Active Substance Content:

Each of the preparations studied was assayed according to the method specified in the British Pharmacopoeia 1973[7d]. Analyses were performed using 20 tablets or the contents of 20 capsules of each product. Each sample was assayed in triplicate.

The procedure for the determination of TMP content involved extraction of the active substance from alkaline solution with chloroform, re-extraction from the organic phase with dilute acetic acid, and subsequent measurement of the U.V.

absorption at 271 nm. A Beckman Acta MVI spectrophotometer was utilized for the absorbance measurements. The TMP content was calculated by comparison with a standard solution containing a known amount of pure drug.

A colorimetric procedure was employed for the assay of SMZ, which remains in the alkaline aqueous phase after chloroform extraction. The method involved diazotization with nitrous acid followed by coupling with N-(1-naphthyl)-ethylenediamine hydrochloride to form a coloured azo complex. The absorbance of the final solution was then measured at 538 nm using a Beckman Model 25 spectrophotometer. A standard solution containing a known quantity of SMZ was employed for comparison.

B. Dissolution Rates:

(i) Standard solutions:

Internal Standard Solutions. Sulphadiazine, SDZ, was utilized as the internal standard for these determinations. It was incorporated into the dissolution medium, which was 0,1N HCl (pH 1,1). The following stock solutions of SDZ were prepared:-

(a) 1,5000 g SDZ per 5000 ml 0,1N HCl. 100 ml aliquots of this stock solution were diluted to 1000 ml with 0,1N HCl. The final solutions, which contained 30,0 mg l^{-1} SDZ in 0,1N HCl, were employed as the dissolution fluids for the standard tablets and capsules.

(b) 3,0000 g SDZ per 5000 ml 0,1 N HCL. 100 ml aliquots of

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this stock solution were diluted to 1000 ml with 0,1N HCl. The resulting solutions, which contained 60,0 mg l^{-1} SDZ in 0,1N HCl were utilized as the dissolution fluids for the double-strength tablets.

Some difficulty was experienced in dissolving the SDZ but this was aided by application of gentle heat (< 40°C) and prolonged shaking. Use of an ultrasonic bath would greatly enhance solution of this compound.

TMP and SMZ Stock Solutions. Two stock solutions containing both TMP and SMZ were prepared as follows:
(a) 40,00 mg SMZ and 8,00 mg TMP were dissolved in 100 ml of the dissolution fluid utilized for standard tablets and capsules, i.e. 0,1N HCl containing 30,00 mg l^{-1} SDZ;
(b) 80,00 mg SMZ and 16,00 mg TMP were dissolved, with the aid of gentle heat, in 100 ml of the dissolution fluid employed for double-strength tablets, i.e. 0,1N HCl containing 60,00 mg l^{-1} SDZ.

By dilution of these stock solutions with the appropriate dissolution fluid, a series of calibration curve standards were prepared so as to cover the entire range of concentrations expected during the dissolution studies.

(ii) Chromatographic conditions:
Except for an alteration in the sensitivity setting, identical chromatographic conditions were utilized for all analyses. The following conditions were employed:
Column: μBondapak C_{18} (see Section 4.1.3.2)
HPLC System: A (see Section 4.1.3.1)
Detection Wavelength: 230 nm

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Sensitivity:

(a) For standard tablets, capsules and the appropriate calibration standards, the sensitivity setting was 0,020 A.U.F.S.

(b) For double-strength tablets and the appropriate calibration standards, a sensitivity of 0,040 A.U.F.S. was employed.

Chart Speed: 12 inches hr^{-1}

Flow Rate: 1,5 ml min^{-1}

Pressure: 2000 p.s.i. (Pressure was high during these determinations due to partial blockage of the column inlet filter caused by particles from previously analyzed biological samples.)

Temperature: 30°C

Mobile Phase: 1% acetic acid-methanol 80:20 (vol/vol).

Preparation of mobile phase:

The mobile phase was prepared as described in Section 4.3.1.

(iii) Dissolution methodology:

The dissolution rates of the Co-trimoxazole preparations were determined utilizing the U.S.P. XIX rotating basket method[139]. The test was conducted under the conditions indicated in Table 5.2. Six dosage units of each of the products studied were subjected to the test. The following methodology was applied to all dosage forms:

A single tablet or capsule was placed into each wire-mesh dissolution basket. The basket was then immersed in the

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TABLE 5.2:

CONDITIONS UTILIZED FOR DISSOLUTION RATE STUDIES

	Standard Tablets and Capsules	Double-Strength Tablets
Dissolution medium	0,1N HCl containing 30 mg l^{-1} SDZ	0,1N HCl containing 60 mg l^{-1} SDZ
Volume	1000 ml	1000 ml
Stirring rate	100 r.p.m.	100 r.p.m.
Temperature	37°C	37°C
No. of dosage units per test	1	1

dissolution fluid and stirring commenced immediately. One hundred microlitre samples of the dissolution medium were then withdrawn from a distance of 22 mm below the surface at 0,5; 1; 1,5; 2,5; 5; 10; 20; 30; 40; 60; and 90 minutes after immersion of the basket. These samples were removed using digital micro-pipettes equipped with disposable plastic tips. The tips were employed for a single sample only and were subsequently discarded. There was no replacement of the dissolution medium.

Once each sample had been withdrawn, it was placed into a clean, dry 1 ml amber ampoule. The neck of each ampoule had been shortened to facilitate delivery of the samples into the body of the ampoule. The ampoules were immediately sealed and kept in a cool place until analysis.

Six determinations could be conducted concurrently in the dissolution apparatus by appropriate staggering of the starting times.

(iv) Analysis of dissolution samples:

A 2 µl aliquot of each sample was injected onto the column under the specified HPLC conditions. If necessary, i.e. for samples removed during the initial stages of dissolution where drug concentrations were low, the injected volume was increased to 10 µl in order to more easily visualize the peaks.

It was found that a few samples had evaporated to dryness owing to improper sealing of the ampoules. These were reconstituted by addition of 100 µl of the mobile phase before injection.

(v) Calculation of results:

Calibration curves were constructed to cover the expected concentration ranges of TMP and SMZ in the dissolution media. In order to cover the range anticipated during the dissolution of standard tablets and capsules, standard solutions were prepared so as to contain five different concentrations of each drug varying between 80,00 and 400,00 mg l^{-1} for SMZ and between 16,00 and 80,00 mg l^{-1} for TMP. The appropriate dissolution fluid, i.e. 0,1N HCl containing 30 mg l^{-1} SDZ, was employed as the solvent for these standard solutions. Aliquots (2-10 µl) were then injected onto the column under

the conditions specified for standard preparations. Each sample was assayed in triplicate and the mean value obtained. Calibration curves were then constructed by plotting the ratios of the peak heights of each drug to that of the internal standard, sulphadiazine, versus their respective concentrations. Concentrations were expressed as a percentage of the labelled content of the particular drug.

Calibration curves to cover the concentration range for double-strength tablets were constructed in a similar manner. Solutions of pure drugs were prepared in 0,1N HCL containing 60 mg l^{-1} SDZ, and comprised concentration ranges of 160,00 - 800,00 mg l^{-1} SMZ and 32,00 - 160,00 mg l^{-1} TMP. These were analyzed under the sensitivity settings employed for double-strength tablets.

The ratios of the peak heights of TMP and SMZ to that of the internal standard, SDZ, were determined for each dissolution sample. Drug concentrations could thus be calculated by referring to the relevant calibration curve data.

5.3 RESULTS:

A. Active Substance Content:

The results of the assays conducted on the Co-trimoxazole preparations are indicated in Table 5.1. All products complied with the requirements of the British Pharmacopoeia 1973, i.e. they contained 92,5-107,5% of the stated amount of both TMP and SMZ.

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TABLE 5.1:

ASSAY OF CO-TRIMOXAZOLE PRODUCTS

NAME OF PREPARATION	BATCH NUMBER	Labelled Content, mg per dose unit		Assay, % of Labelled Content			
				TMP		SMZ	
		TMP	SMZ	MEAN[a]	S.D.	MEAN[a]	S.D.
Septran(R)	CBP/100/625	80	400	102,91	2,21	100,37	1,21
Bactrim(R)	B/N8	80	400	100,21	0,93	101,71	0,10
Thoxaprim(R)	110678	80	400	101,00	1,90	99,22	1,32
Purbac(R)	SW 1241	80	400	99,08	0,89	101,11	0,94
Mezenol(R)	432127	80	400	102,18	0,49	102,22	0,91
Co-Trim(R)	060978	80	400	103,19	0,38	101,06	0,32
Bactrim Double Strength(R)	J002A82P	160	800	99,75	0,15	99,00	0,73
Bactrim Double Strength Quiksolv(R)	J001A82E	160	800	101,88	0,16	104,49	1,83
Septran Double Strength(R)	-	160	800	103,31	2,64	101,19	0,65
Mezenol Double Strength(R)	-	160	800	101,31	0,15	99,41	0,32

a. Mean of 3 determinations.

B. Dissolution Rates:

(i) HPLC Analysis:

A typical chromatogram obtained from the HPLC assay of a dissolution medium sample is depicted in Figure 5.1. All peaks were well-resolved and symmetrical. Under these conditions, the retention times were as follows:

SDZ	3,1 minutes
TMP	4,8 minutes
SMZ	6,5 minutes

Calibration curves were linear over the entire range of concentrations tested. Table 5.3 lists the constants of the respective linear regression lines. Coefficients of correlation were 0,9999 in all cases. A single reference solution of known concentration could thus be used for quantitative determinations.

TABLE 5.3:

LINEAR REGRESSION DATA FOR CALIBRATION CURVES IN DISSOLUTION MEDIA.

Drug	Concentration Range, mg l^{-1}	Slope	Y-Intercept[c]	Correlation Coefficient
TMP[a]	16,00 - 80,00	0,0378	0,0070	0,9999
SMZ[a]	80,00 -400,00	0,0359	0,0086	0,9999
TMP[b]	32,00 -160,00	0,0373	0,0136	0,9999
SMZ[b]	160,00 -800,00	0,0354	0,0130	0,9999

a. Calibration curve for standard tablets and capsules.

b. Calibration curve for double-strength tablets.

c. Peak height ratios are plotted on the y-axis and drug concentrations as a percentage of the labelled content on the x-axis.

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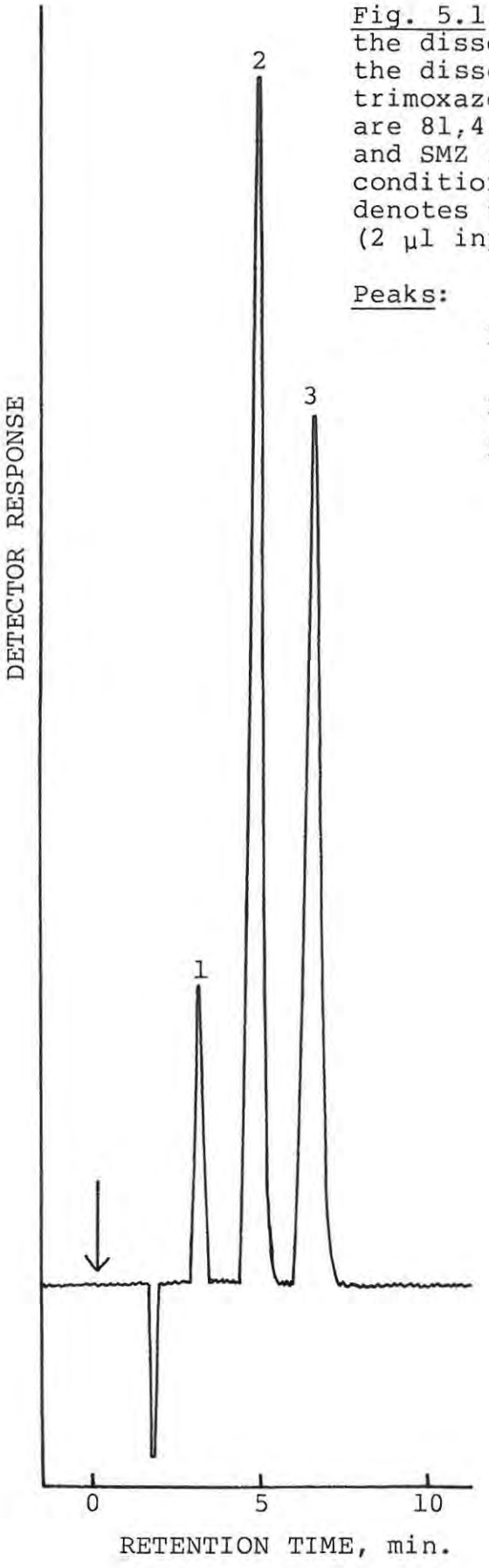

Fig. 5.1. Chromatogram of a sample of the dissolution medium obtained during the dissolution of a standard Co-trimoxazole tablet. Drug concentrations are 81,4 mg l^{-1} and 327,6 mg l^{-1} for TMP and SMZ respectively. Chromatographic conditions as in section 5.2. The arrow denotes the time of sample injection (2 µl injected).

Peaks:

1 = internal standard, sulphadiazine

2 = TMP

3 = SMZ

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(ii) Dissolution data:

The rates of dissolution of TMP and SMZ from the Co-trimoxazole formulations studied are depicted in Figures 5.2 - 5.5. These results represent the means of six individual determinations.

The N.F. XIV monograph on "sulphamethoxazole tablets"[10] specifies that at least 50,0% of the active substance must be dissolved within 20 minutes. For this measurement the rotating wire basket is specified, rotating at a speed of 100 r.p.m. in dilute hydrochloric acid medium. This specification was used by Steinigen and Bruene[136] as the standard for assessment of the dissolution time of SMZ in Co-trimoxazole tablets (standard strength). When applied to the present determinations, it was found that all standard tablets and capsules complied with the N.F. XIV specification as regards dissolution time of SMZ. The double-strength tablets, with the exception of Bactrim Double-Strength(R) (46,94% SMZ dissolved within 20 minutes) also met the N.F. requirements. However, the significance of this specification in the case of Co-trimoxazole formulations, especially double-strength preparations which have a high SMZ content, is not known.

No dissolution rate specifications are currently given in official Pharmacopoeias for either TMP or Co-trimoxazole tablets.

Fig. 5.2. Rate of dissolution of trimethoprim from standard Co-trimoxazole preparations. Each point represents the mean of 6 individual determinations.

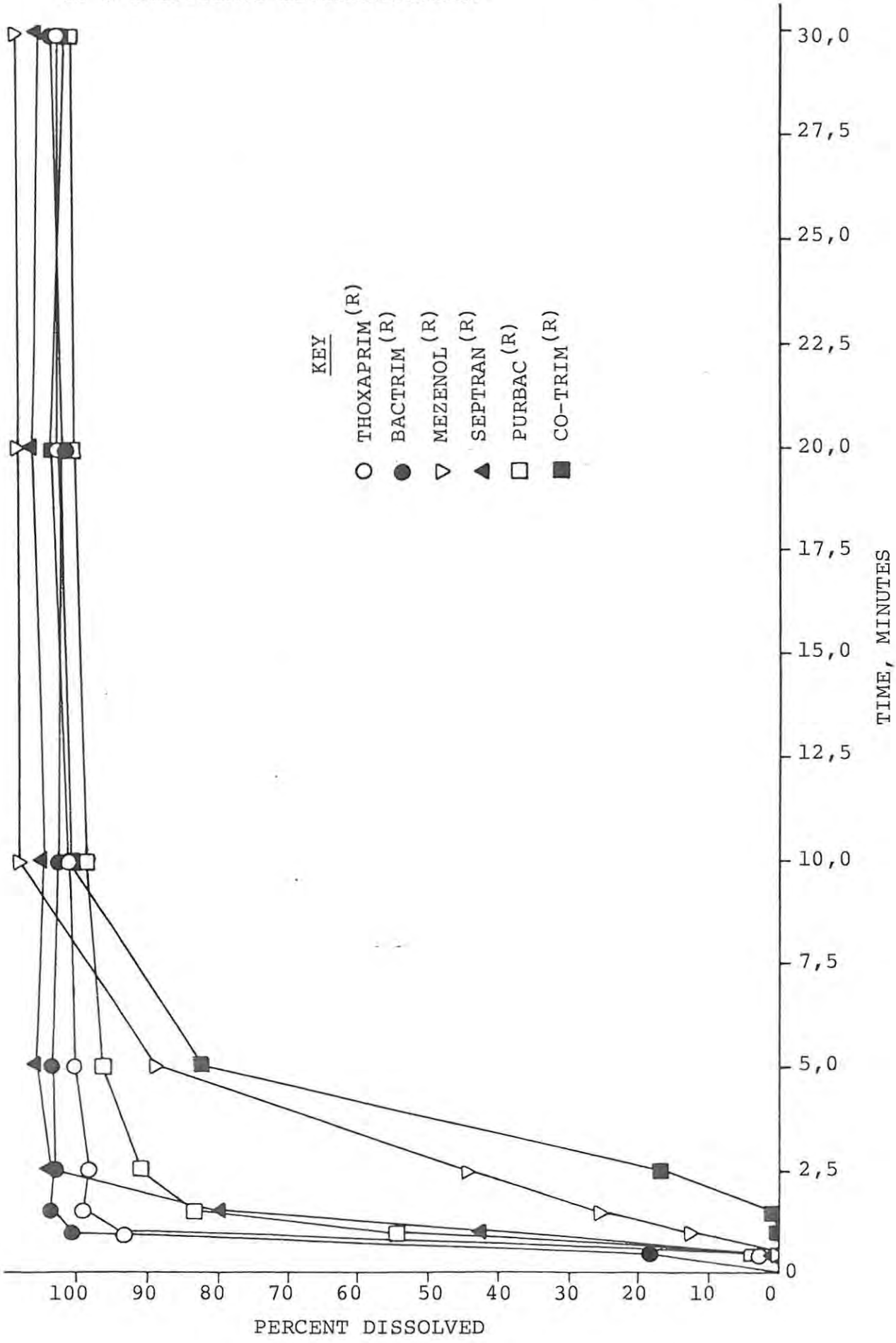

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Fig. 5.3. Rate of dissolution of sulphamethoxazole from standard Co-trimoxazole preparations. Each point represents the mean of 6 individual determinations. Readings for 0,5 minute samples not depicted.

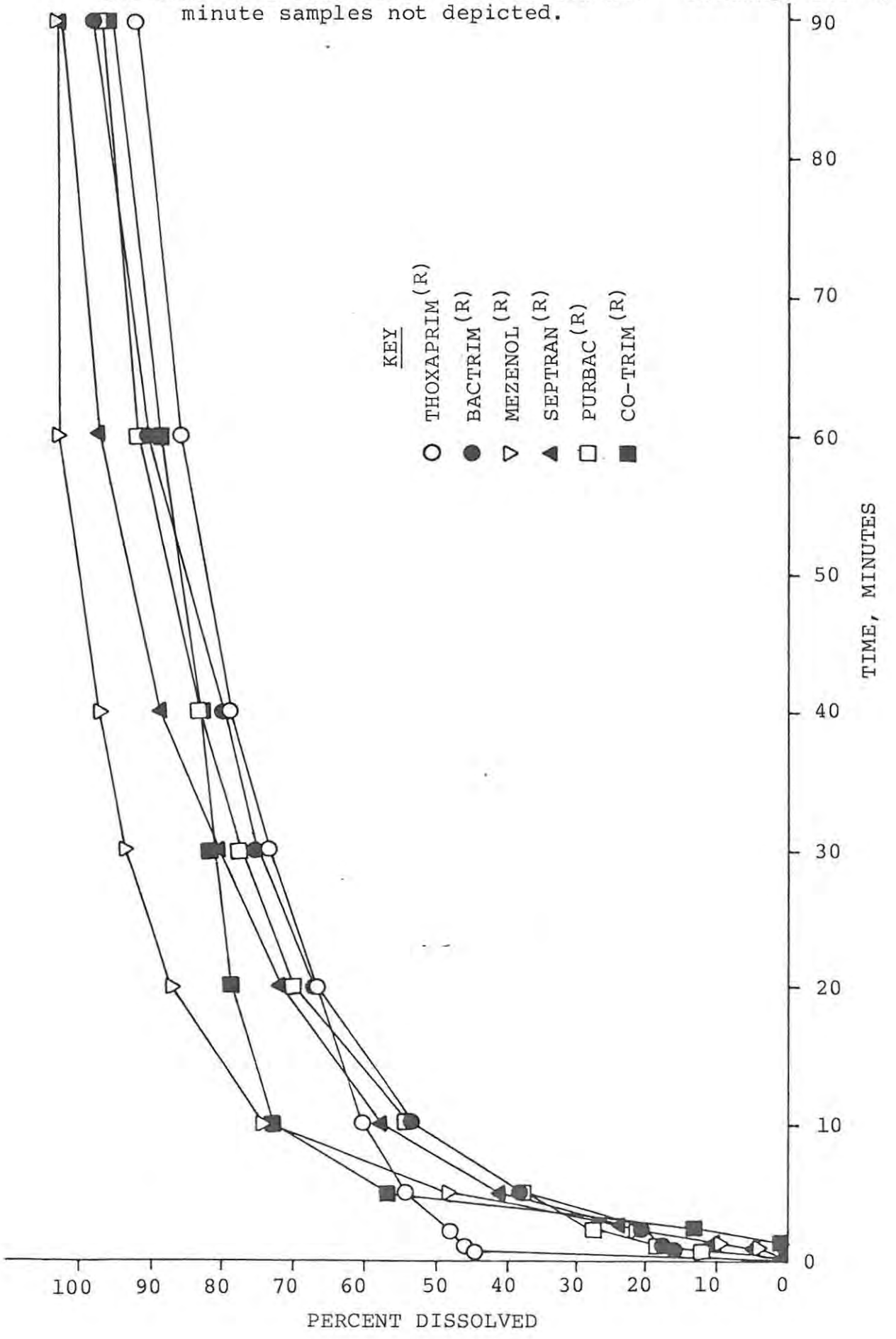

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Fig. 5.4. Rate of dissolution of trimethoprim from double-strength Co-trimoxazole preparations. Each point represents the mean of 6 individual determinations. Readings for 0,5 minute samples not depicted.

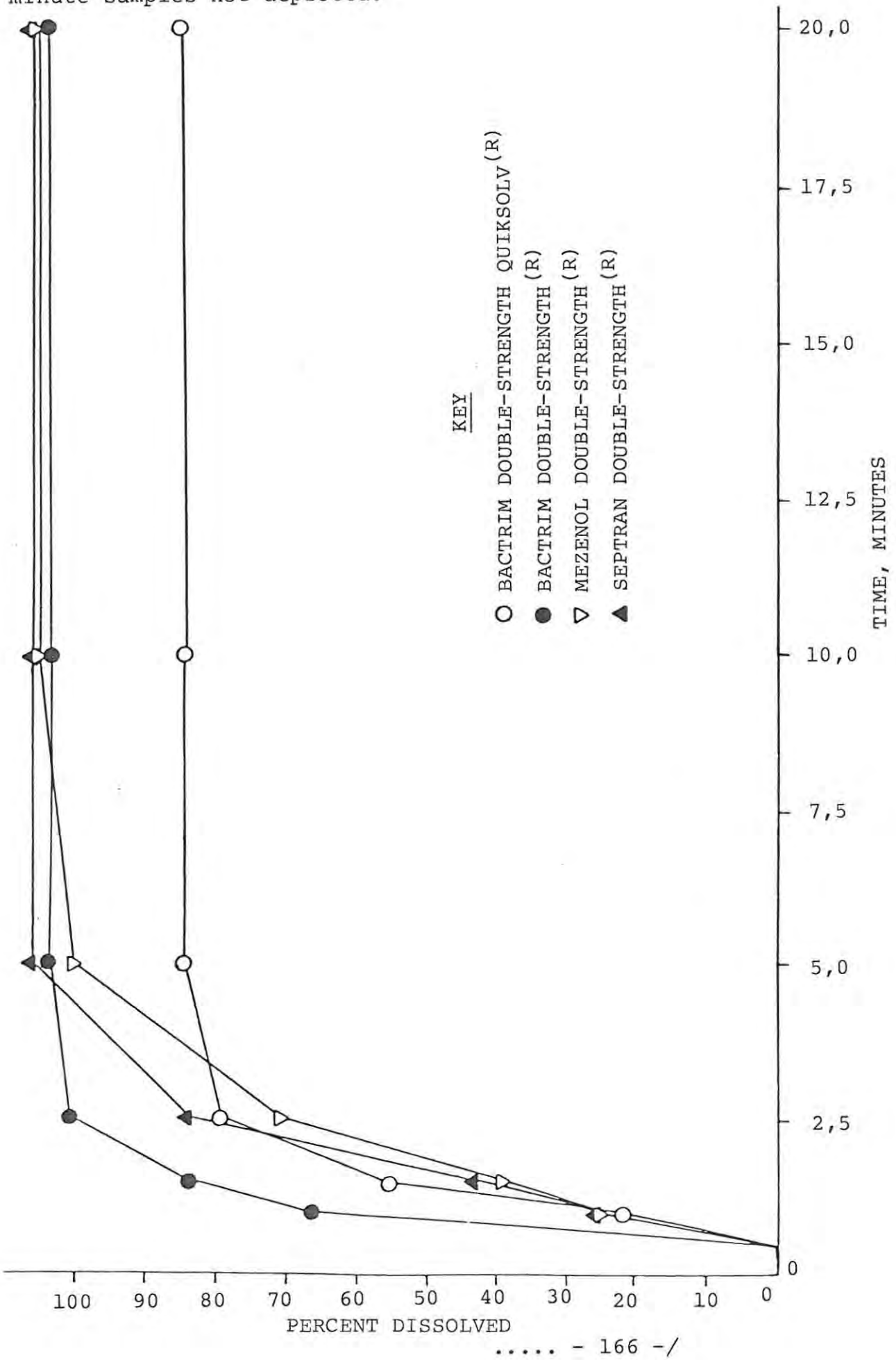

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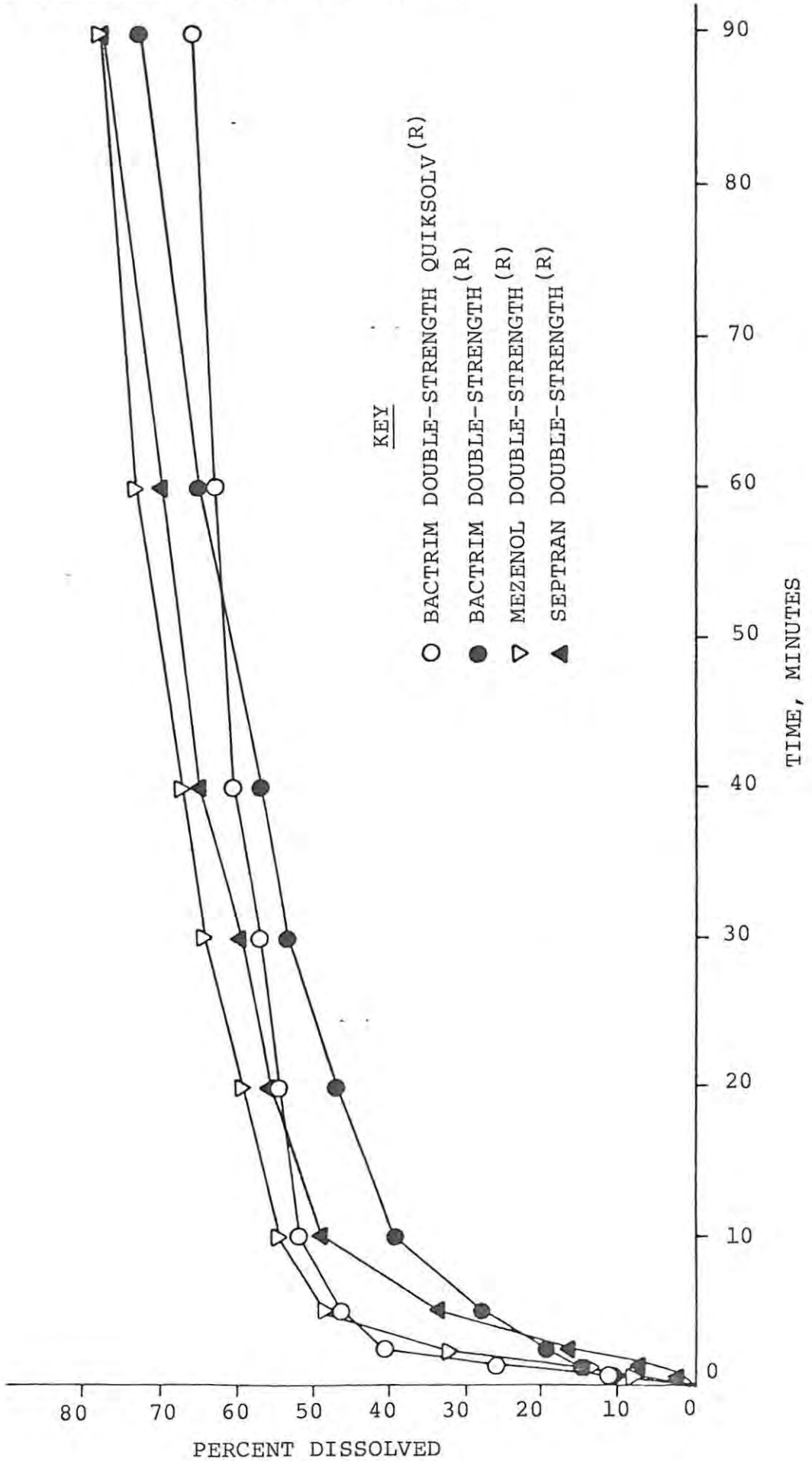
Fig. 5.5. Rate of dissolution of sulphamethoxazole from double-strength Co-trimoxazole preparations. Each point represents the mean of 6 individual determinations. Readings for 0,5 minute samples not depicted.



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Statistical Analysis of Dissolution Data:

Statistical methods were employed in order to ascertain whether significant differences existed between any of the products as regards their dissolution behaviour. Application of these methods would hence provide standardized parameters for comparison of the different products.

The percentages of TMP and SMZ dissolved from each dosage unit of (a) standard tablets and capsules and (b) double-strength tablets were compared at each sampling time. One-way analysis of variance was found most suitable for the analysis of this data. Whereas this technique takes into account variations in the values between individual tablets as well as variations among the means of the different products, it only considers variation within the group as a whole and does not, however, discriminate between individual products.

One-way analysis of variance of the data indicated statistically significant ($p < 0,01$[a]) differences among the products with respect to both TMP and SMZ at all time intervals with the exception of (a) the 0,5 minute samples and (b) samples obtained between 20 and 90 minutes with respect to the TMP component of standard tablets only.

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[a] p = probability. $p < 0,01$ implies that results are significant at the 1% level, i.e. the probability of a result not falling within the range is less than 1%.

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Further analysis of the data at those time intervals showing significant differences was required in order to distinguish the differing products. For this purpose, Scheffe's test was employed. This test was applied to the data in order to establish a range of values at each time interval within which variation between individual product means is not significantly different. Thus, the difference between the mean percentages of TMP and SMZ dissolved at each time was calculated for all possible pairs of products. If this difference was within the range determined using Scheffe's test, the product pair compared were not significantly different. A value outside the calculated range implied a statistically significant difference between the products compared at the particular time interval. The results obtained using Scheffe's test are summarized in Tables 5.4 and 5.5. (Full details of these results are listed in the Appendix.) The results indicate the time intervals at which a statistically significant ($p < 0,01$) difference between the relevant product pair was observed. However, they do not indicate for which product the higher value was obtained. This information can be obtained by inspection of the comparative dissolution profiles depicted in Figures 5.2 - 5.5. In some cases, one product may have been superior at certain time intervals while at other times the position was reversed.

TABLE 5.4:

STATISTICAL ANALYSIS OF DISSOLUTION DATA FOR STANDARD TABLETS AND CAPSULES

PRODUCTS COMPARED	Time Intervals (Minutes) at which Differences Between Products were Significant ($p < 0,01$)	
	TMP	SMZ
Bactrim(R)/Septran(R)	1; 1,5	1
Bactrim(R)/Mezenol(R)	1; 1,5; 2,5	1; 10; 20; 30; 40; 60
Bactrim(R)/Thoxaprim(R)	Nil	1; 1,5; 2,5
Bactrim(R)/Purbac(R)	1	Nil
Bactrim(R)/Co-Trim(R)	1; 1,5; 2;5	1; 1,5; 5; 10; 20
Septran(R)/Mezenol(R)	1; 1,5; 2;5	10; 20; 30
Septran(R)/Thoxaprim(R)	1	1; 1,5; 2,5; 60; 90
Septran(R)/Purbac(R)	Nil	Nil
Septran(R)/Co-Trim(R)	1; 1,5; 2,5; 5	5; 10
Mezenol(R)/Thoxaprim(R)	1; 1,5; 2,5	1; 1,5; 2,5; 10; 20; 30; 40; 60; 90
Mezenol(R)/Purbac(R)	1; 1,5; 2,5	10; 20; 30; 40; 60
Mezenol(R)/Co-Trim(R)	1,5; 2,5	30; 40; 60
Thoxaprim(R)/Purbac(R)	1	1; 1,5; 2,5
Thoxaprim(R)/Co-Trim(R)	1; 1,5; 2,5	1; 1,5; 2,5; 20
Purbac(R)/Co-Trim(R)	1; 1,5; 2,5	1; 1,5; 5; 10

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TABLE 5.5:

STATISTICAL ANALYSIS OF DISSOLUTION DATA FOR DOUBLE-STRENGTH TABLETS

PRODUCTS COMPARED[a]	Time Intervals (Minutes) at which Differences Between Products were Significant ($p < 0,01$)	
	TMP	SMZ
Bactrim D.S.(R)/Bactrim D.S. Quiksolv(R)	1; 1,5; 2,5; 5; 10; 20; 30; 40; 60; 90	2,5; 5; 10
Bactrim D.S.(R)/Septran D.S.(R)	1; 1,5	10; 40
Bactrim D.S.(R)/Mezenol D.S.(R)	1; 1,5; 2,5	2,5; 5; 10; 20; 30; 40; 60
Bactrim D.S. Quiksolv(R)/Septran D.S.(R)	5; 10; 20; 30; 40; 60; 90	1,5; 2,5; 5; 60; 90
Bactrim D.S. Quiksolv(R)/Mezenol D.S.(R)	5; 10; 20; 30; 40; 60; 90	30; 40; 60; 90
Septran D.S.(R)/Mezenol D.S.(R)	Nil	2,5; 5

[a] D.S.: Double-strength.

5.4 DISCUSSION:

(i) Analytical procedure:

HPLC was utilized for the simultaneous analysis of TMP and SMZ in samples of dissolution media obtained during the dissolution of various Co-trimoxazole formulations. The chromatographic conditions employed were similar to those selected for the assay of these drugs in serum (Section 4.3.2). Detection at 230 nm yielded responses of a similar magnitude for SMZ and TMP when these compounds were present in a 5:1 ratio by weight. Since this represents the ratio found in pharmaceutical preparations, the wavelength used is optimal for the analysis. The HPLC method was found to be specific, accurate and rapid. Calibration curves were highly reproducible which can be attributed to the stability of the column and the simplicity of the procedures.

Several advantages were associated with the use of HPLC for the analysis of dissolution samples. Firstly, since the volume of sample required for HPLC assay was very small, it was possible to remove 100 µl portions of the dissolution medium for analysis. Hence, the volume of medium removed during the entire dissolution procedure was 1,1 ml. This is a negligable quantity in a total volume of 1 litre. Replacement of the dissolution medium after each sampling was, thus, unnecessary and there was no need to adjust calculations to compensate for samples already removed. This methodology facilitated more frequent and rapid sampling

of the dissolution fluid.

Samples were removed from a distance of 22 mm below the surface of the dissolution medium. This was an arbitrary value utilized since it represented the maximum depth at which samples could be withdrawn using digital micro-pipettes. The latter devices made sampling simple and rapid. Representative samples appeared to be obtained in this manner since drug concentrations, in most cases, reached expected values, indicating complete dissolution of the active substances.

The use of HPLC also allowed for the direct analysis of dissolution medium samples without any pre-treatment. Despite the fact that the pH of the medium was low (pH 1,1) this direct injection had no apparent effect on column efficiency. At least 800 samples were injected with no adverse effects. Samples were of small volume and hence would be diluted to a level where column damage was highly unlikely. There was no evidence of any particulate matter or undissolved tablet material in the dissolution samples which could cause problems when injected. No increase in column back-pressure occurred. Calibration standards were injected throughout the procedure and these results indicated no change in column efficiency or separating ability.

Sulphadiazine was utilized as the internal standard for these determinations because of its favourable elution time.

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Incorporation of the internal standard into the dissolution medium had several advantages. Accuracy of the procedure was enhanced because the use of large volumes of internal standard solutions minimized errors. The same stock internal standard solutions were utilized for the preparation of all dissolution fluids for standard and double-strength tablets respectively. The internal standard compensated for sample volume changes that could occur as a result of temperature differences. It also accounted for inadvertant small losses in sample volume that may occur before or during sealing of the ampoules or due to improperly sealed ampoules. Inaccuracies associated with failure to remove precise sample volumes were avoided by inclusion of the internal standard into the dissolution fluid. In addition, this allowed for more rapid analysis since addition of the internal standard as a final step to the dissolution samples was unnecessary.

Inclusion of the internal standard into the dissolution medium did not interfere with the dissolution process. Preliminary tests were carried out on Co-trimoxazole preparations (Bactrim$^{(R)}$ and Septran$^{(R)}$) where 0,1 N HCl without additives was utilized as the dissolution fluid. The internal standard was then added to all samples and to standards prior to analysis. Dissolution profiles for SMZ obtained in this way (see Fig. 5.6) were identical to those obtained when the internal standard was included in the acid medium. TMP was found to be completely dissolved within 2,5 minutes for both products. This is also in agreement

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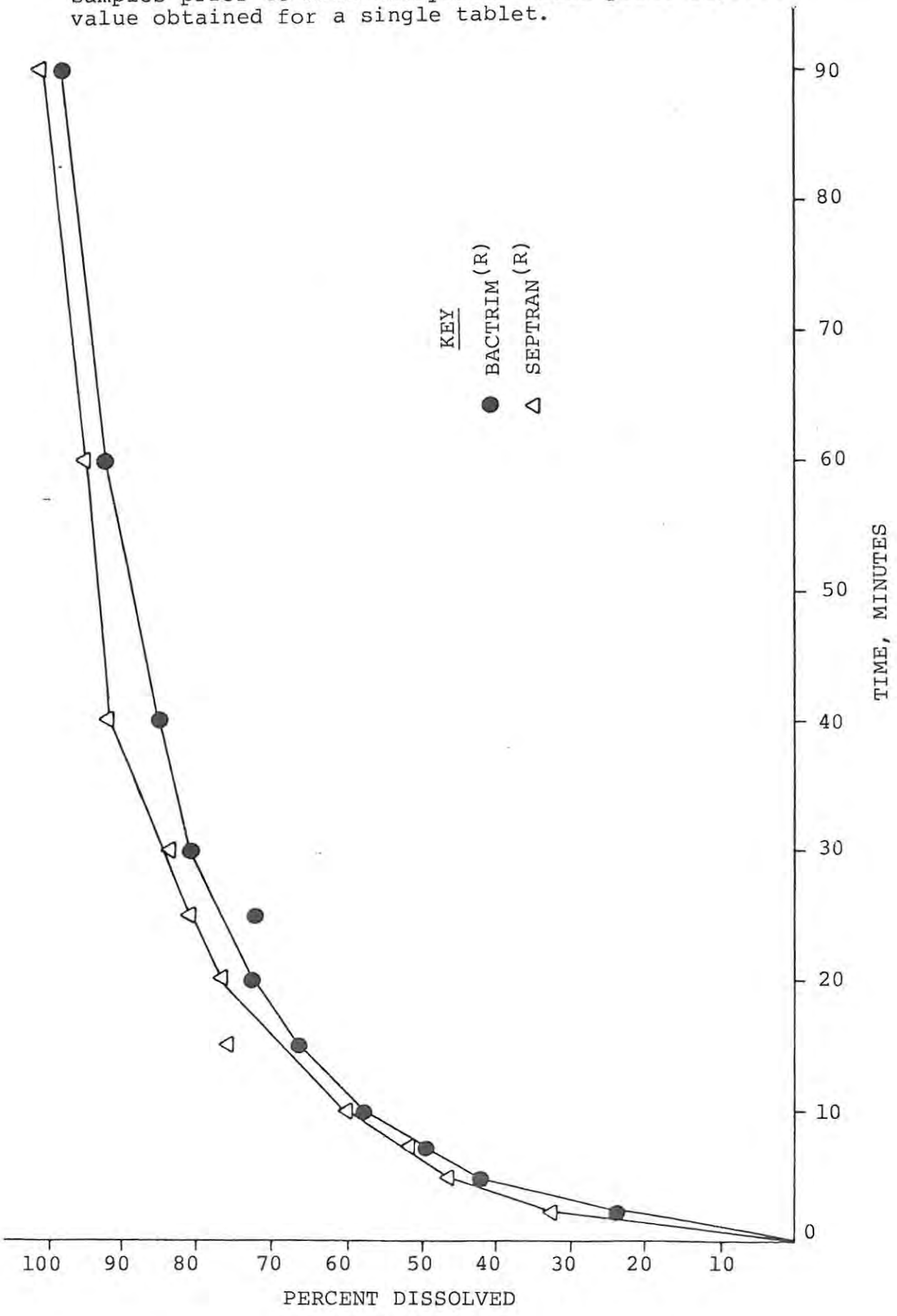
Fig. 5.6. Dissolution profiles of SMZ from standard Co-trimoxazole tablets obtained when the internal standard was not included in the dissolution medium but was added to samples prior to HPLC analysis. Each point represents the value obtained for a single tablet.



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with results obtained when the internal standard was incorporated into the dissolution fluid. In this case, 2,5 minutes represented the initial sampling time. Readings were found, however, to be less precise and occasional deviations from the dissolution curves were noticed.

It is likely that the HPLC method utilized during these studies for the analysis of dissolution samples could be adapted to enable the simultaneous determination of TMP and SMZ in combination in pharmaceuticals. The active substances could be extracted from the required aliquot of finely powdered tablets by shaking in the mobile phase or in methanol, with the possible use of sonication to ensure complete solution. SDZ could be added as internal standard. However, this application was not investigated during these studies. Methods for the simultaneous HPLC determination of TMP and SMZ in solid pharmaceutical dosage forms have, however, been reported in the literature[99,140]. In the former method, the separation was achieved on a reverse-phase column (LiChrosorb(R) RP-8,5 µm; E. Merck) at a temperature of 40°C, utilizing acetonitrile-0,05 M phosphoric acid (1:3) as the mobile phase and with spectrophotometric detection at 230 nm. The active ingredients were extracted into the mobile phase before injection. Quantitative determinations were carried out using external standardization. The latter method involved the use of an alkylnitrile bonded phase column (MicroPak(R) CN-10; Varian Associates). In this case, the mobile phase consisted of diethylamine-ethyl acetate-methanol-heptane

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(1:8:16:75) and a wavelength of 254 nm was utilized for the detection. The extraction solvent was 0,1 N HCl in methanol. Sulphanilylsulphanilamide was employed as an internal standard.

Stability of Samples:

Samples of the dissolution medium could be kept at room temperature and protected from light (amber ampoules) for twenty days before HPLC analysis without any evidence of degradation. However, in samples stored for longer periods, a slight "shoulder" was observed on the leading edge of the SMZ peak although this did not appear to significantly affect the measured peak height.

SMZ will hydrolyze partially in acidic or basic solutions[111]. In acidic solvents appearance of a white precipitate (5-methyl isoxazole ring), indicating hydrolysis of SMZ, has been reported[111]. Sulphanilamide would thus be formed at the same time and would remain in solution. Sulphanilic acid has also been reported to be a sulphonamide degradation product[140]. Analysis of samples by HPLC thus offers the additional advantage that sample decomposition would probably be clearly indicated in the chromatogram, i.e. the assay is "stability indicating".

(ii) Dissolution data:

Innumerable factors influence the dissolution characteristics of any drug or drug combination. Features of the production technique and the excipients employed are involved, as well

as physicochemical properties of the drug itself, e.g. particle size, surface area, solubility, polymorphism, differences in salt or ester form, etc. In addition, the apparatus[136] and conditions[141] utilized for the dissolution rate determination can have a profound effect on the results obtained. It cannot be assumed that in vitro dissolution rates are predictive of in vivo bioavailability. In vitro tests must be correlated on a quantitative basis with in vivo studies in order to establish a meaningful relationship.

When comparing the dissolution profiles obtained during this study, it was apparent that the TMP component of all preparations dissolved considerably more rapidly than the SMZ component. This feature is consistent with the solubility of the drugs in the dissolution fluid (0,1 N HCl). Complete dissolution of the TMP component occurred within 10 minutes for all products, with the exception of Bactrim Double-Strength Quiksolv(R) which appeared to yield a maximum of 85% of the labelled content of TMP. In the case of SMZ, however, 90 minutes was required for complete solution of the active ingredient in the majority of standard preparations. Double-strength tablets yielded only 65 - 77% of the total SMZ content within the same time interval. This may have been due to the limited solubility of the SMZ in the acidic medium and hence use of larger volumes of fluid or alteration of pH may provide more suitable conditions.

Discrepancies in the relative rates of release of SMZ and TMP

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were observed when comparing different formulations. For example, Bactrim(R) was found to release TMP more rapidly than Mezenol(R), but SMZ dissolved at a slower rate from the former preparation as compared with the latter. This feature did not, however, apply to all preparations studied. In addition, comparison of the percentage of active ingredient dissolved on completion of the dissolution process (i.e. the 90 minute sample) did not indicate relative differences among the products as regards the total amount of SMZ and TMP released.

Reasons for the discrepancies in the relative release rates of SMZ and TMP from Co-trimoxazole dosage forms are not known. Possibly this may be related to differences in the physico-chemical attributes of the particular drug or to formulation variables. The dissolution medium, which has been shown to markedly affect the dissolution behaviour of drugs[141], may not have been ideal. Selection of a single dissolution fluid to accommodate these drugs which have diverse characters and solubility properties may pose a problem. Possibly, comparison of results obtained using media of higher pH values may indicate whether this factor is involved. Ghanem et al.,[137] have reported an unexpected pH effect for SMZ when conducting dissolution rate studies on Co-trimoxazole formulations. Slow release profiles could also be accounted for by the fact that very small tablet particles, including perhaps undissolved SMZ, may pass through the mesh of the wire basket and fall to the bottom of the vessel where they largely escape the stirring

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action of the rotating basket[136].

Statistical Analysis:

Analysis of the dissolution data using statistical methods was essential in order to provide a basis for comparison of the different Co-trimoxazole preparations. Application of these methods to the data obtained for both standard tablets and capsules and double-strength tablets indicated large differences between several of the products.

In the case of standard preparations, significant differences between TMP dissolution profiles were encountered only at early time intervals. In view of this fact, it is probable that the products would not be considered different as regards dissolution of the TMP component under these conditions. SMZ dissolution curves for Mezenol(R) and Thoxaprim(R) were found to differ significantly at most time intervals. However, the latter product was superior during the initial stages whereas the former yielded better results at later time intervals. Mezenol(R) was also found to be significantly better than Bactrim(R) and Purbac(R) at several time intervals as regards dissolution of SMZ, although release rates of TMP from these preparations yielded contradictory results. For most other standard preparations dissolution profiles of SMZ were generally similar although significant differences were encountered at certain time intervals.

When comparing the double-strength tablets, it was found that

Bactrim double-strength Quiksolv(R) yielded a significantly lower percentage of the TMP component than the other products (viz. Bactrim double-strength(R), Mezenol double-strength(R) and Septran double-strength(R)). Differences in SMZ dissolution profiles were less marked and at early sampling times Bactrim double-strength Quiksolv(R) often yielded more favourable results. However, this product was found to yield a significantly lower amount of SMZ than either Septran double-strength(R) or Mezenol double-strength(R) when comparing samples removed at the end of the dissolution process (i.e. 60 and 90 minute samples). Significant differences in the percentage of SMZ released from Bactrim double-strength(R) and Mezenol double-strength(R) tablets were also observed at most time intervals. Discrepancies in the relative rates of release of TMP and SMZ from these products were, however, observed.

Thus, significant differences at several time intervals were encountered between the products studied. Often, however, evaluation of preparations was difficult since relative release rates of TMP and SMZ yielded contradictory results. In several instances, one product was found to yield better results than another during certain time intervals, whilst at other times the position was reversed. Under the conditions utilized, a meaningful comparison could not be obtained on the basis of either TMP or SMZ dissolution rates alone.

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Care should be taken in the use of arbitrary values, e.g. amount dissolved within 20 minutes, as these may not provide meaningful assessments of relative product performance. The data obtained after application of the various statistical analyses may facilitate selection of more suitable parameters for comparison of Co-trimoxazole products and, especially, may aid establishment of appropriate in vivo-in vitro correlations. Comparison of results at more frequent time intervals may provide more information concerning the dissolution process as a whole. It is therefore essential to optimize both apparatus and dissolution conditions in order to make a valid comparison and perhaps to attempt to establish further parameters which may be useful for comparative purposes to differentiate between generic products.

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CHAPTER 6

HIGH PRESSURE LIQUID CHROMATOGRAPHY OF SOME SULPHONAMIDES

Although they have largely been superceded by antibiotics in the treatment of infections, the sulphonamides continue to constitute a therapeutically important group of anti= microbial agents. Combinations of these drugs with TMP are becoming increasingly popular, particularly for the treatment of respiratory and urinary tract infections. Several studies have been conducted on methods of separation and quantitative evaluation of sulphonamides using HPLC[96,103-106,140].

During the present investigation, a number of sulphonamides were injected under varying chromatographic conditions. The chromatographic data are listed below.

6.1 REAGENTS:

All drugs utilized complied with BP standards. Solvents were of analytical reagent grade and were obtained from E. Merck, Darmstadt. The water used was deionized and then glass-distilled.

Predominantly aqueous mobile phases were filtered before use through a Millipore type HA (0,45 µm) membrane filter (Millipore Corp., Bedford, Mass.) while solutions that were

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mainly organic solvents were filtered through a type FH (0,5 μm) filter.

6.2 EXPERIMENTAL:

Solutions of pure sulphonamides were prepared in methanol so as to yield final concentrations of approximately 20 μg ml^{-1}. Ten μl aliquots of each solution were then injected into the liquid chromatograph under the appropriate conditions. TMP and other compounds were analysed in a similar manner. The following chromatographic systems were employed.

I. Column: Micropak$^{(R)}$ NH_2 - 10; 10 μm (see Section 4.1.3.2)
HPLC system: C (see Section 4.1.3.1)
Detection wavelength: 290 nm
Sensitivity: Vari-chrom 0,1A
CDS III 4
Recorder input 5 mV
Flow rate: 50 ml hr^{-1}
Pressure: 1000 p.s.i.
Temperature: Ambient (~20°C)
Mobile phase: Acetonitrile-methanol 80:20 (vol/vol).

The results of sulphonamide analyses conducted under these conditions are listed in Table 6.1.

II. Column: μBondapak C_{18}; 10 μm (see Section 4.1.3.2)
HPLC system: B (see Section 4.1.3.1)
Detection wavelength: 270 nm
Sensitivity: 0,040 A.U.F.S.

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Flow rate: 1,6 ml min^{-1}

Pressure: 1430 p.s.i.

Temperature: Ambient (~20°C)

Mobile phase: 1% Acetic acid-methanol 70:30 (vol/vol).

Retention times recorded for various sulphonamides and trimethoprim under these conditions are listed in Table 6.2.

III. Column: μBondapak C_{18}; 10 μm (see Section 4.1.3.2)

HPLC system: B (see Section 4.1.3.1)

Detection wavelength: 270 nm

Sensitivity: 0,040 A.U.F.S.

Flow rate: 1 ml min^{-1}

Pressure: 890 p.s.i.

Temperature: Ambient (~20°C)

Mobile Phase: Methanol-water 50:50 with PIC reagent B-7 (heptane sulphonate, 5 mmol l^{-1}, Waters Assoc.)

Table 6.3 lists the retention times obtained for several sulphonamides as well as TMP and pyrimethamine.

IV. Column: Spherisorb ODS: 5 μm (Spectra-Physics)

HPLC system: B (see Section 4.1.3.1)

Detection wavelength: 270 nm

Sensitivity: 0,040 A.U.F.S.

Flow rate: 0,8 ml min^{-1}

Pressure: 3400 p.s.i.

Temperature: Ambient (~20°C)

Mobile phases:

(i) Sodium acetate 0,05 M - methanol 50:50 (vol/vol).

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Sufficient glacial acetic acid was added to adjust the pH to 4,1.

(ii) Sodium acetate 0,05 M - methanol 55:45 (vol/vol). Sufficient glacial acetic acid was added to adjust the pH to 4,4.

(iii) Sodium acetate 0,05 M - methanol 55:45 (vol/vol). Sufficient glacial acetic acid was added to adjust the pH to 4,1.

Retention times obtained for several sulphonamides and other compounds under these conditions are listed in Table 6.4.

6.3 RESULTS AND DISCUSSION:

The majority of the sulphonamides investigated were found to elute fairly rapidly under all chromatographic conditions utilized. In general, no correlation was observed between pKa values and retention times. A number of factors, including partition coefficients, are also involved in determining the elution sequence. Sulphaguanidine and sulphanilamide, however, which have very high pKa values, appeared to elute early in the chromatograms. This was consistent for a number of the chromatographic systems investigated.

It was found that elution sequence could not necessarily be predicted by the nature of the stationary phase. When similar chromatographic conditions (chromatographic system IV, mobile phase (i)) were applied to two octadecylsilane

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TABLE 6.1:

RETENTION DATA FOR A RANGE OF SULPHONAMIDES AND TRIMETHOPRIM[a].

COMPOUND	RETENTION TIME, MINUTES	pKa[b]
Sulphanilamide	1,0[c]	10,4
Sulphaguanidine	1,4	12,0
Trimethoprim	1,4	7,2
Sulphadimidine	2,5	7,4
Sulphamethoxydiazine	2,5	6,8
Sulphamerazine	4,6	6,9
Sulphamoxole	5,8	7,4
Sulphadimethoxine	7,0	6,7
Sulphamethoxypyridazine	7,1	7,0
Sulphadiazine	7,7	6,4
Sulphamethoxazole	8,8	5,8
Sulphasomidine	15,4	7,5
Sulphathiazole	18,0	7,2
Sulphacetamide	18,0	1,8
Sulphafurazole	23,1	5,1

a. Chromatographic system I (Section 6.2).

b. Data from Francke[142] and Merck Index[143].

c. Eluted in void volume.

TABLE 6.2:

RETENTION DATA FOR A RANGE OF SULPHONAMIDES AND TRIMETHOPRIM[a].

COMPOUND	RETENTION TIME, MINUTES	pKa[b]
Sulphaguanidine	1,7[c]	12,0
Sulphanilamide	1,9[c]	10,4
Sulphasomidine	2,4	7,5
Sulphadiazine	2,6	6,4
Sulphacetamide	2,6	1,8
Sulphathiazole	2,7	7,2
Sulphapyridine	3,1	8,6
Sulphamerazine	3,3	6,8
Sulphamoxole	3,6	7,4
Trimethoprim	3,7	7,2
Sulphadimidine	4,4	7,4
Sulphamethoxydiazine	4,6	6,8
Sulphamethoxypyridazine	4,7	6,7
Sulphamethoxazole	5,8	5,7
Sulphafurazole	7,0	4,8
Sulphaphenazole	10,8	5,9
Sulphadimethoxine	15,5	6,0

a. Chromatographic system II (Section 6.2).

b. Data from Francke[142] and Merck Index[143].

c. Eluted in void volume.

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TABLE 6.3:

RETENTION DATA FOR SEVERAL SULPHONAMIDES, TRIMETHOPRIM AND PYRIMETHAMINE[a].

COMPOUND	RETENTION TIME, MINUTES	pKa[b]
Sulphaguanidine	2,9[c]	12,0
Sulphathiazole	3,2[c]	7,2
Sulphadiazine	3,4[d]	6,4
Sulphasomidine	3,5	7,5
Sulphamoxole	3,6	7,4
Sulphamethoxydiazine	3,7	6,8
Sulphamethoxazole	4,1	5,8
Sulphaphenazole	4,8	6,5
Sulphadimethoxine	5,9	6,7
Trimethoprim	6,6	7,2
Pyrimethamine	18,0	7,2

a. Chromatographic system III (Section 6.2).

b. Data from Francke[142] and Merck Index[143].

c. Eluted in void volume.

d. Not resolved from solvent (methanol) peak.

TABLE 6.4:

RETENTION DATA FOR SEVERAL SULPHONAMIDES AND OTHER COMPOUNDS[a].

MOBILE PHASE [a]	COMPOUND	RETENTION TIME, MINUTES	pKa [b]
(i)	Sulphapyridine	2,1[c]	8,4
	Dapsone	2,1[c]	1,0
	Sulphamethoxydiazine	2,2	6,8
	Sulphamethoxazole	2,3	5,8
	Sulphadimidine	2,3	7,4
	Salicylic acid	2,8	3,0
	Trimethoprim	5,4	7,2
(ii)	Sulphamethoxazole	2,5	5,8
	Sulphafurazole	2,6	5,1
	Sulphaphenazole	3,3	6,5
	Trimethoprim	7,4	7,2
	Pyrimethamine	19,3	7,2
(iii)	Sulphamethoxazole	2,4	5,8
	Sulphosalicylic acid	2,6	-
	Sulphadimethoxine	3,6	6,7
	Trimethoprim	6,7	7,2

a. Chromatographic system IV (Section 6.2).

b. Data from Francke[142] and Merck Index[143].

c. Not resolved from solvent (methanol) peak.

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reverse-phase columns, i.e. a μBondapak C_{18} column (10 μm particle size) and a Spherisorb ODS column (5 μm particle size), the observed elution order for TMP and SMZ differed. Whereas retention times under these conditions were 3,7 and 4,1 minutes for TMP and SMZ respectively using the former column, the values obtained using the latter column were 5,4 and 2,3 minutes respectively. Differences in column packing or particle size may account for these results. Thus, it cannot be assumed that columns obtained from different manufacturers will necessarily yield similar results despite having identical surface functionality.

The chromatographic data obtained in this study may possibly aid the development of suitable methods for the assay of single sulphonamides or sulphonamide mixtures, if adequate resolution can be attained. This data should also enable selection of a suitable internal standard. Recent interest in the clinical monitoring of sulphapyridine concentrations in inflammatory bowel disease patients receiving sulphasalazine has led to the development of HPLC methods for the analysis of this drug[144,145]. Possibly, a suitable system for the assay of sulphapyridine may be developed from data obtained in this study (system II). In addition, the chromatographic systems could be applied, possibly with slight modifications, to the assay of TMP-sulphonamide combinations, e.g. TMP-sulphadiazine (system II) and TMP-sulphamoxole (system III). A number of alternative systems for analysis of TMP-SMZ were also obtained.

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Reproducibility of retention times obtained using the amino-bonded column cannot, however, be assured since this column was found to be rather unstable during the present studies (Section 4.2).

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CHAPTER 7

SAMPLE INJECTORS - PROBLEMS OF DEAD VOLUME

The design of all parts of the chromatographic system from the injector, through the column and the detector must aim to reduce to an absolute minimum the void space within the components. It is also important to avoid badly flushed regions where sample molecules could be trapped and held back relative to the main sample plug. The presence of dead volume within the system will lead to a considerable broadening of the sample bands with an associated loss of resolution[148].

A number of different modes of sample introduction are utilized in HPLC[148,149]. These include both stop-flow and continuous-flow injectors. Diffusion in liquids is so small that the flow of the mobile phase can be stopped, the sample injected, and flow restarted without detriment to resolution. Sample introduction techniques may be divided into two main types:
(i) Direct injection with a microsyringe either onto the head of the column or into the liquid stream immediately ahead of the column. Samples can be injected through a septum with or without interruption of solvent flow. A septumless, stop-flow injector can also be employed.
(ii) A microsampling valve can be utilized for sample introduction. Small fixed-volume (four-port) valves or external-loop (six-port) valves are available. The sample

is introduced either into a cavity within the valve shaft or into an external sample loop. When the valve is actuated, the sample is diverted into the mobile phase stream immediately ahead of the column.

Problems associated with dead volume within the injector system were encountered during this study. These are discussed below.

A. Faulty Sample Loop in External-Loop Valve:

Chromatographic conditions:

Column: μBondapak C_{18} (see Section 4.1.3.2)

HPLC system: B (see Section 4.1.3.1)

Detection wavelength: 270 nm (Schoeffel detector)

Sensitivity: 0,040 A.U.F.S.

Chart speed: 5 mm min^{-1}

Flow rate: 1,6 ml min^{-1}

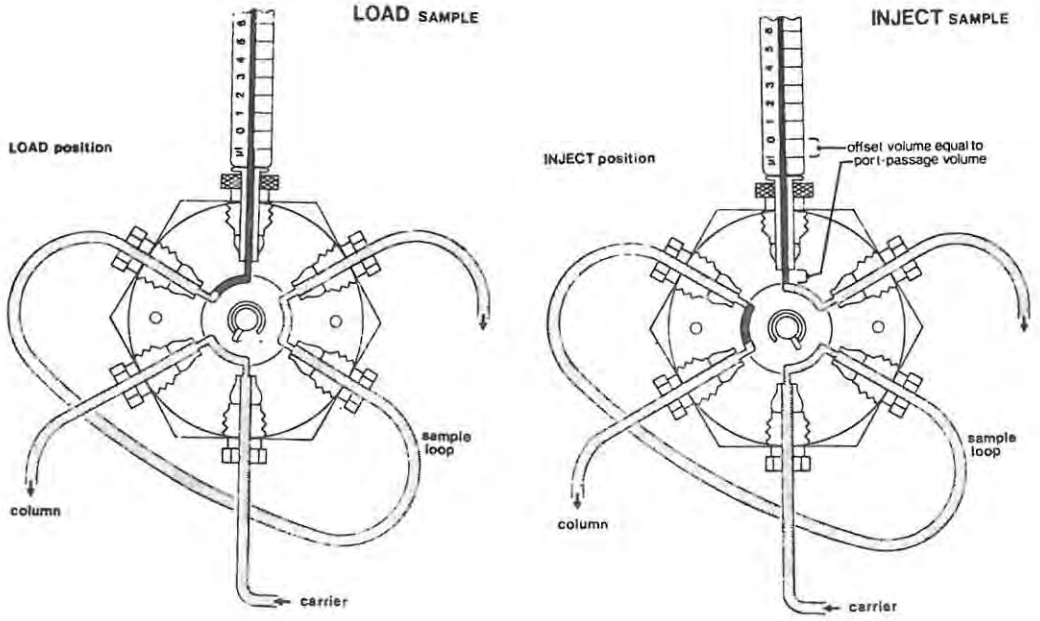
Pressure: 1430 p.s.i.

Temperature: Ambient (~20°C)

Mobile phase: 1% acetic acid-methanol 70:30 (vol/vol).

A Valco CV-6-UHPa sample injection valve equipped with a 10 μl loop was utilized for sample introduction. The basic design and mode of operation of this injector is depicted in Fig. 7.1.

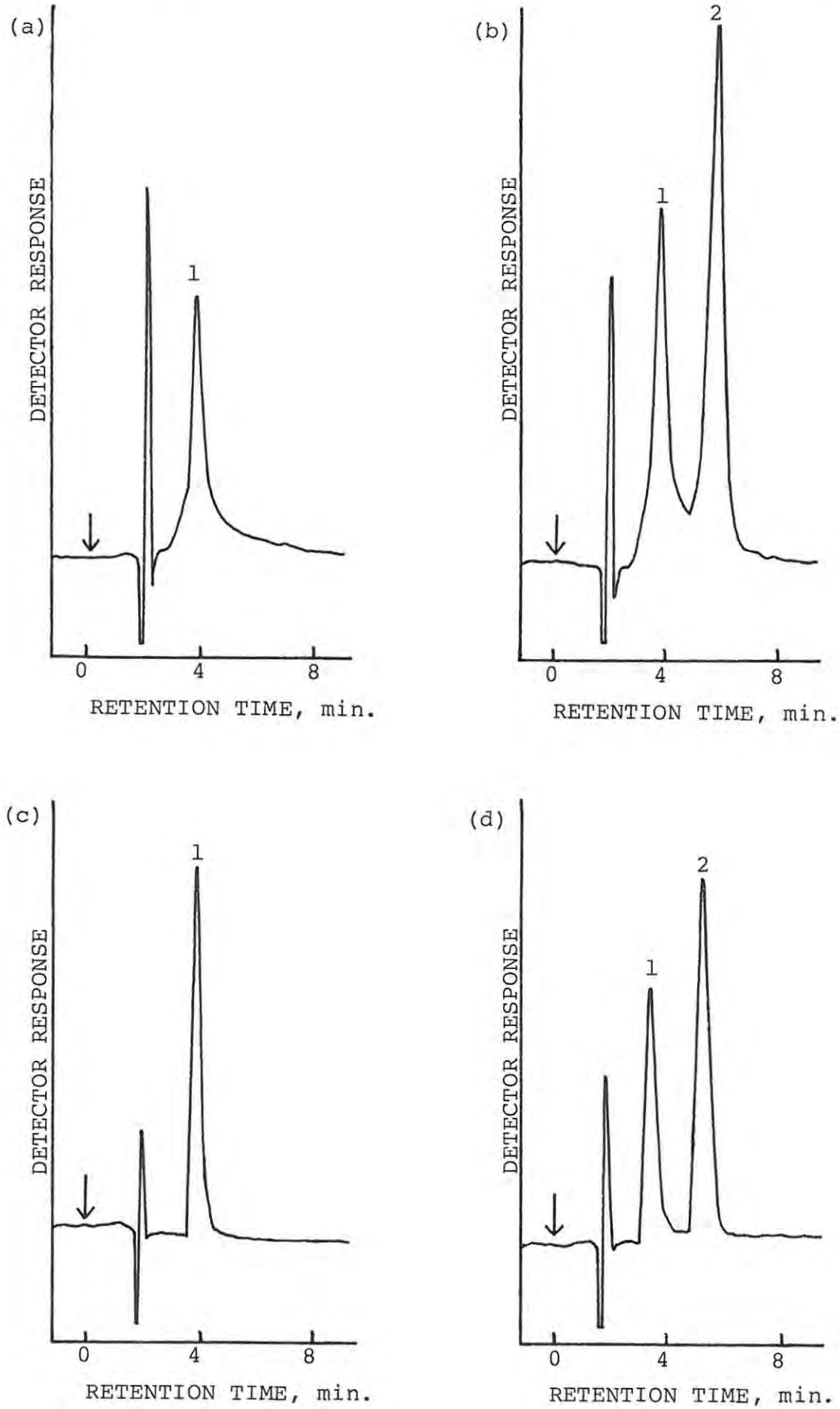
Fig. 7.1. Operation of external-loop valve.



Samples injected consisted of pure drugs in methanol solution; approximate concentrations were 10 mg per 100 ml and 2 mg per 100 ml for TMP and SMZ respectively. When the loop was completely filled with the solution to be injected (volumes in excess of 10 µl were loaded to ensure total filling), a considerable spreading of the sample bands with a resultant loss of resolution were observed (see Figures 7.2(a) and (b)). This did not occur if volumes of less than 10 µl were loaded into the sample loop. Chromatograms obtained when the loop was partially filled are depicted in Figures 7.2(c) and (d). Peaks in this case were sharp and well-resolved. At all times, the loop was rinsed with methanol between injections.

The defective sample loop was detached from the valve and was replaced with a second 10 µl loop. The valve itself was not changed. Chromatograms obtained using the new loop

Fig. 7.2. Chromatograms obtained using faulty sample loop. Chromatograms (a) and (b) were produced when the loop was completely filled with the sample solutions; (c) and (d) were obtained when the loop was partially filled. Peaks: 1 = TMP, 2 = SMZ. The arrows denote times of sample injection.



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were identical to those depicted in Figures 7.2(c) and (d). The same results were observed whether the loop was completely or partially filled. Comparison of results obtained with the two sample loops also indicated that the volume of the defective loop was actually slightly in excess of 10 µl.

The injector valve itself was not involved in the band spreading effects observed. Attachment of the faulty loop to a second valve did not alter results obtained, i.e. considerable broadening of sample bands continued to occur when this loop was completely filled with the sample solution. In addition, overloading of the column was not responsible for the effects seen. Sample volumes in excess of 10 µl were injected onto the column without resultant peak broadening when HPLC system A (Section 4.1.3.1) was utilized. The latter system was equipped with a Rheodyne Model 70-10 sample injection valve containing a 20 µl loop.

Thus, excessive dead volume may have been present within the sample loop which only became apparent when it was completely filled. Possibly badly flushed regions may have existed within the capillary tubing into which part of the sample could have been swept.

Examination of the loop indicated that the internal diameter was larger at one end of the tubing than at the other. However, no alteration in the chromatogram occurred when the positions of attachment of the loop to the valve were reversed.

The discrepancy in the internal capillary diameter may have been due to manufacturing errors, which could contribute to the adverse effects observed.

B. Dead Volume in Stop-Flow, Septumless Injector:

Serum samples spiked with TMP and SMZ were assayed according to the method described in Section 4.3.2 with the exception that chromatographic analysis was performed using HPLC system C (Section 4.1.3.1). A stop-flow, septumless injector was thus utilized in place of an external-loop valve for sample introduction. Chromatograms obtained under these conditions, however, depicted "split" peaks for all components (see Figure 7.3(a)). This effect did not occur when the same samples were analyzed using the HPLC system previously employed for serum assays (HPLC system A; see Figure 4.6(a)).

Splitting of the sample bands was also observed when the drugs were extracted from aqueous solutions using a procedure similar to that applied to serum samples (Figure 7.3(b)). The effect here, however, was less marked. In addition, chromatograms obtained on injection of pure drugs in the mobile phase depicted slight "shoulders" on all peaks produced (Figure 7.3(c)). When methanol was employed as the solvent for injected samples, these effects did not occur (Figure 7.3(d)).

The observed splitting of peaks was not due to the syringe utilized for sample injection. Several syringes were tested

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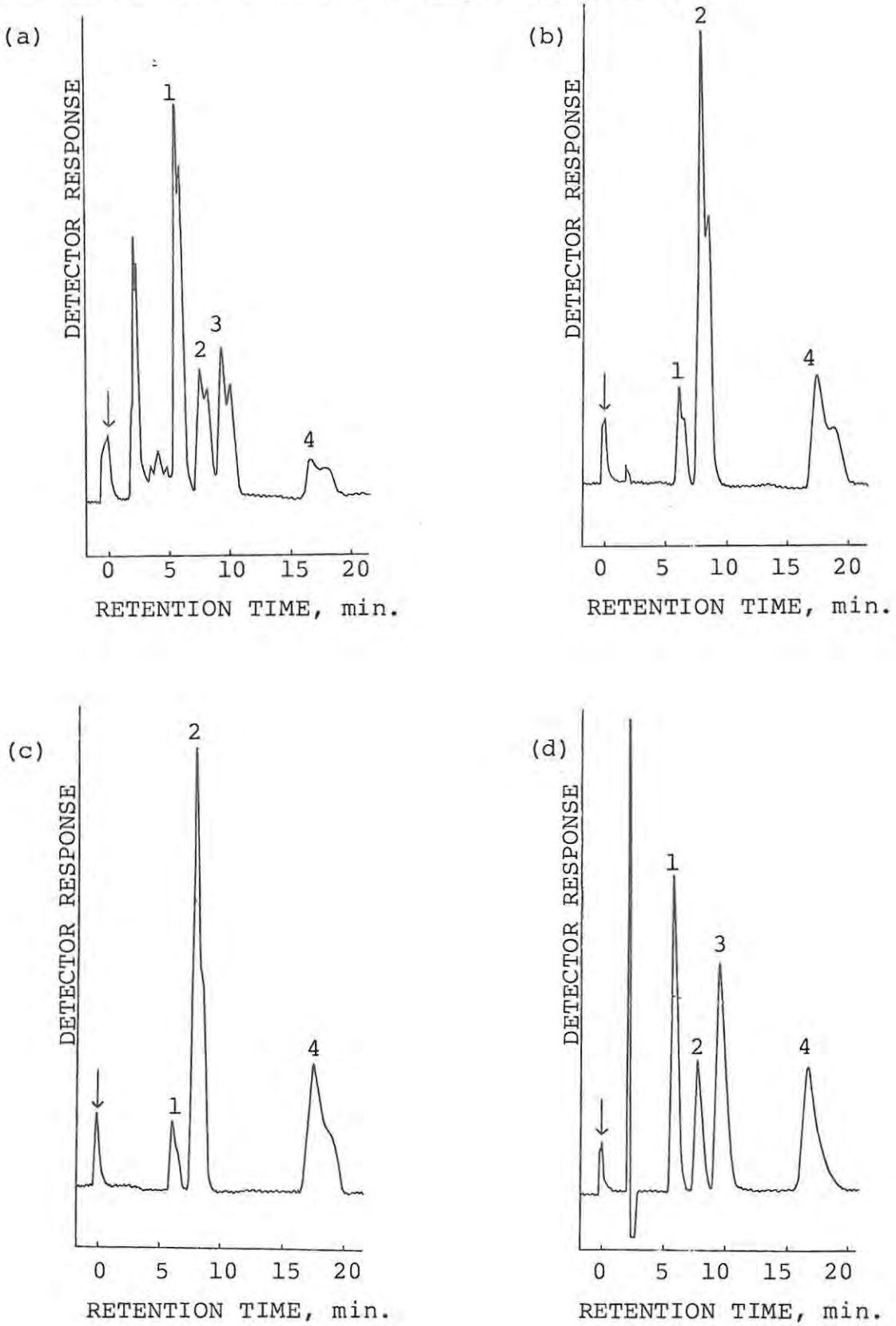
Fig. 7.3. Chromatograms obtained using stop-flow septumless injector. Samples injected were (a) drugs extracted from serum, (b) drugs extracted from an aqueous solution, (c) pure drugs in the mobile phase (1% acetic acid-methanol 80:20) and (d) pure drugs in methanol solution.
Peaks: 1 = TMP; 2 = SMZ; 3 = internal standard, sulphafurazole; 4 = N^4-acetyl-SMZ.
The arrows denote times of sample injection.



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without any effect on the resultant chromatogram.

It thus appeared that areas of dead volume or poorly flushed regions may have existed within the injector system, which resulted in failure to introduce samples as a single plug. Samples were not injected directly onto the head of the column although a zero dead-volume fitting was utilized to connect the injector to the column. Figure 7.4(a) depicts the type of fitting employed in order to connect the column to the stop-flow septumless injector. This special adaptor was required to attach the μBondapak C_{18} column (and any other standard columns) to the stop-flow septumless injector block of the Varian 8500 liquid chromatograph which is specially designed to only accommodate the unusual left-hand thread of Varian Micropak$^{(R)}$columns. Hence, the fitting differed from that employed when the external loop valve was utilized for sample introduction (see Figure 7.4(b)). In the latter case the peak splitting effect was not observed.

Thus the fitting utilized to connect the injector to the column may have contributed towards the adverse effects seen. With the stop-flow septumless injector system, it may be advantageous to inject samples directly onto the column packing to minimize injection band broadening caused by diffusion. Splitting of the sample bands was influenced by the nature of the sample and the solvent employed, since the effect was particularly marked when serum extracts were injected but was not observed when pure drugs in methanol

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solution were analyzed. The reason for this, as well as a complete explanation for the observed effects, is not, however, known.

Fig. 7.4. Zero dead-volume fittings utilized to connect the μBondapak C_{18} column to (a) the stop-flow septumless injector (Varian 8500 Liquid Chromatograph) and (b) the external-loop valve (Rheodyne Model 70-10).

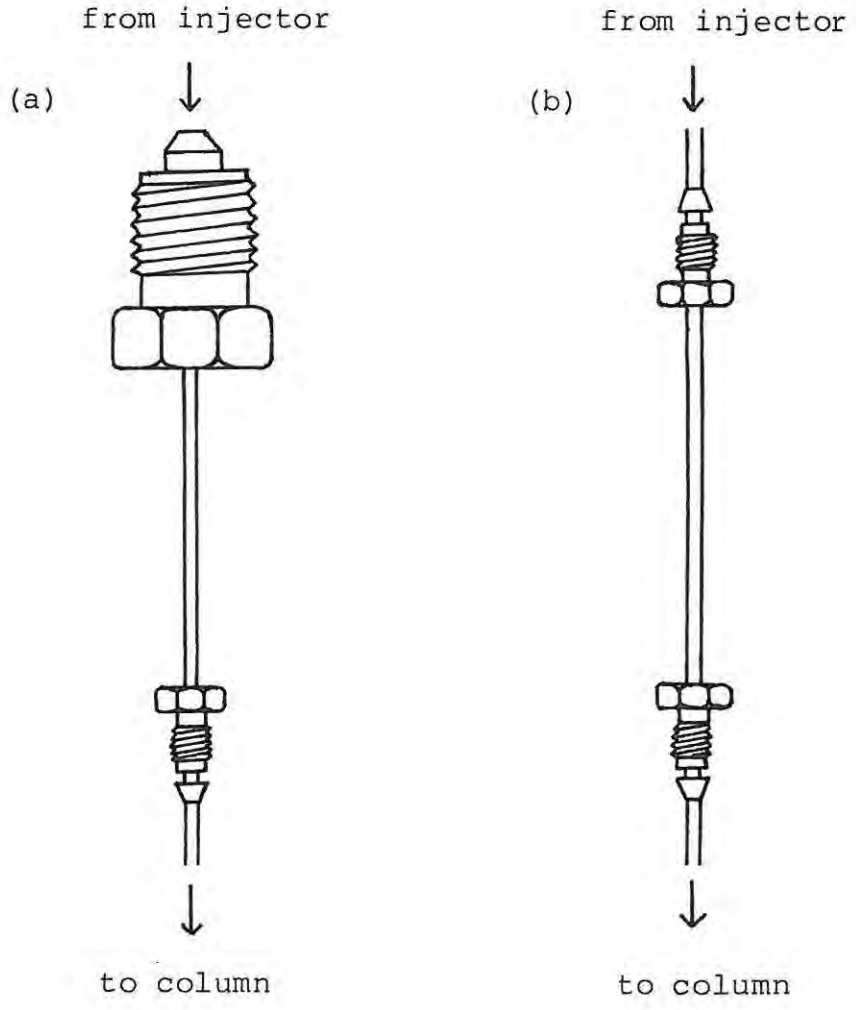

TABLE I.

ANALYSIS OF DISSOLUTION DATA FOR THE SMZ COMPONENT OF STANDARD TABLETS AND CAPSULES[a].

PRODUCTS COMPARED	Difference Between Mean Percent Dissolved at Each Time Interval									
	1	$1\frac{1}{2}$	$2\frac{1}{2}$	5	10	20	30	40	60	90
Bactrim(R)/Septran(R)	10,46*	6,77	2,30	1,34	5,00	4,63	5,21	8,86	6,12	4,54
Bactrim(R)/Mezenol(R)	11,50*	8,09	0,34	10,44	21,04*	20,04*	18,08*	17,2*	12,09*	4,68
Bactrim(R)/Thoxaprim(R)	28,49*	28,36*	26,62*	15,84	7,05	0,36	1,98	0,83	4,73	5,93
Bactrim(R)/Purbac(R)	4,15	1,09	6,91	0,25	0,79	3,18	2,16	3,22	1,35	1,09
Bactrim(R)/Co-Trim(R)	15,70*	16,43*	7,23	18,74*	19,76*	11,75*	5,39	2,65	1,88	2,61
Septran(R)/ Mezenol(R)	1,04	1,32	1,96	9,10	16,04*	15,41*	12,87*	8,34	5,97	0,14
Septran(R)/ Thoxaprim(R)	38,95*	35,13*	24,32*	14,50	2,05	4,99	7,19	9,69	10,85*	10,47*
Septran(R)/Purbac(R)	6,31	7,86	4,61	1,59	4,21	1,45	3,05	5,64	4,77	5,63
Septran(R)/Co-Trim(R)	5,24	9,66	9,53	17,40*	14,76*	7,12	0,18	6,21	8,00	7,15
Mezenol(R)/Thoxaprim(R)	39,99*	36,45*	26,28*	5,40	13,99*	20,40*	20,06*	18,03*	16,82*	10,61*
Mezenol(R)/Purbac(R)	7,35	9,18	6,57	10,69	20,25*	16,86*	15,92*	13,98*	10,74*	5,77
Mezenol(R)/Co-Trim(R)	4,20	8,34	7,57	8,30	1,28	8,29	12,69*	14,55*	13,97*	7,29
Thoxaprim(R)/Purbac(R)	36,64*	27,27*	19,71*	16,09	6,26	3,54	4,14	4,05	6,08	4,84
Thoxaprim(R)/Co-Trim(R)	44,19*	44,79*	33,85*	2,90	12,71	12,11*	7,37	3,48	2,85	3,32
Purbac(R)/Co-Trim(R)	11,55*	17,52*	14,14	18,99*	18,97*	8,57	3,23	0,57	3,23	1,52
RANGE ±	7,36	9,82	13,16	13,64	9,98	7,99	7,22	7,06	6,27	6,07

a. Scheffe's test.

*Statistically significant ($p < 0,01$) difference.

TABLE II.

ANALYSIS OF DISSOLUTION DATA FOR THE TMP COMPONENT OF STANDARD TABLETS AND CAPSULES[a].

PRODUCTS COMPARED	Difference Between Mean Percent Dissolved at Each Time Interval				
	1	1½	2½	5	10
Bactrim(R)/Septran(R)	55,88*	22,01*	1,59	2,42	2,18
Bactrim(R)/Mezenol(R)	87,56*	76,25*	57,77*	14,48	5,89
Bactrim(R)/Thoxaprim(R)	5,58	2,72	4,29	2,85	1,46
Bactrim(R)/Purbac(R)	45,77*	18,40	10,56	6,55	3,13
Bactrim(R)/Co-Trim(R)	100,04*	100,76*	85,87*	21,00	0,55
Septran(R)/Mezenol(R)	31,68*	54,24*	59,36*	16,90	3,71
Septran(R)/Thoxaprim(R)	50,30*	19,29	5,88	5,27	3,64
Septran(R)/Purbac(R)	10,11	3,61	12,15	8,97	5,31
Septran(R)/Co-Trim(R)	44,16*	78,75*	87,46*	23,42*	2,73
Mezenol(R)/Thoxaprim(R)	81,98*	73,53*	53,48*	11,63	7,35
Mezenol(R)/Purbac(R)	41,79*	57,85*	47,21*	7,93	9,02
Mezenol(R)/Co-Trim(R)	12,48	24,51*	28,10*	6,52	6,44
Thoxaprim(R)/Purbac(R)	40,19*	15,68	6,27	3,70	1,67
Thoxaprim(R)/Co-Trim(R)	94,46*	98,04*	81,58*	18,15	0,91
Purbac(R)/Co-Trim(R)	54,27*	82,36*	75,31*	14,45	2,58
RANGE ±	22,64	17,97	18,58	20,96	5,98

a. Scheffe's test.

*Statistically significant ($p < 0,01$) difference.

TABLE III.

ANALYSIS OF DISSOLUTION DATA FOR THE SMZ COMPONENT OF DOUBLE-STRENGTH TABLETS[a].

PRODUCTS COMPARED[b]	Difference Between Mean Percent Dissolved at Each Time Interval									
	1	$1\frac{1}{2}$	$2\frac{1}{2}$	5	10	20	30	40	60	90
Bactrim D.S.(R)/Bactrim D.S. Quiksolv(R)	0,98	10,98	21,94*	19,16*	10,83*	7,11	2,05	1,92	2,08	5,67
Bactrim D.S.(R)/Septran D.S.(R)	7,82	7,80	1,34	5,83	9,89*	8,80	6,48	8,50*	5,03	5,36
Bactrim D.S.(R)/Mezenol D.S.(R)	2,16	0,22	12,51*	20,70*	15,09*	12,31*	11,07*	10,24*	8,20*	5,37
Bactrim D.S. Quiksolv(R)/Septran D.S.(R)	8,80	18,78*	23,28*	13,33*	0,94	1,69	4,43	6,58	7,11*	11,03*
Bactrim D.S. Quiksolv(R)/Mezenol D.S.(R)	3,14	11,20	9,43	1,54	4,26	5,20	9,02*	8,32*	10,28*	11,04*
Septran D.S.(R)/Mezenol D.S.(R)	5,66	7,58	13,85*	14,87*	5,20	3,51	4,59	1,74	3,17	0,01
RANGE ±	7,67	10,27	7,17	8,30	4,33	4,11	4,38	5,63	3,62	3,71

a. Scheffe's test.

b. D.S.: double-strength.

*Statistically significant ($p < 0,01$) difference.

TABLE IV.

ANALYSIS OF DISSOLUTION DATA FOR THE TMP COMPONENT OF DOUBLE-STRENGTH TABLETS[a].

PRODUCTS COMPARED[b]	Difference Between Mean Percent Dissolved at Each Time Interval									
	1	1 ½	2 ½	5	10	20	30	40	60	90
Bactrim D.S.(R)/Bactrim D.S. Quiksolv(R)	44,85*	27,97*	21,65*	18,62*	19,17*	18,57*	20,06*	18,64*	17,86*	20,60*
Bactrim D.S.(R)/Septran D.S.(R)	39,85*	40,62*	16,92	2,35	1,82	1,36	0,57	2,21	1,69	0,41
Bactrim D.S.(R)/Mezenol D.S.(R)	41,59*	44,91*	30,08*	2,97	1,70	1,46	1,53	0,13	0,12	2,57
Bactrim D.S. Quiksolv(R)/Septran D.S.(R)	5,00	12,65	4,73	20,97*	20,99*	19,93*	20,63*	20,85*	19,55*	21,01*
Bactrim D.S. Quiksolv(R)/Mezenol D.S.(R)	3,26	16,94	8,43	15,65*	20,87*	20,03*	21,59*	18,77*	17,98*	18,03*
Septran D.S.(R)/Mezenol D.S.(R)	1,74	4,29	13,16	5,32	0,12	0,10	0,96	2,08	1,57	2,98
RANGE ±	25,42	23,45	15,06	6,28	3,13	2,94	3,84	3,72	3,99	5,41

a. Scheffe's test.

b. D.S.: double-strength.

*Statistically significant ($p < 0,01$) difference.

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