THE ASSAY AND PHARMACOKINETICS OF SOME CHEMOTHERAPEUTIC AGENTS

An Investigation of the Kinetics of Co-trifamole, Co-trimoxazole, Trimethoprim Alone and Clavulanate Potentiated Ticarcillin with Consideration of the Prediction of Plasma Drug Concentration.

submitted by

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α	Level of probability at which significance is reached. In chromatography, the separation factor (k_2'/k_1'), 1=no separation
[A]	Concentration of unionised acid
$[A^-]$	Concentration of ionised acid
ADP	Adenosine diphosphate
A_s	Assymetry, usually measured at 10% of peak height (measure of peak symmetry in chromatography)
AUC	Area under the plasma drug concentration-time curve (mg/l/h)
[B]	Concentration of unionised base
b.d.	twice a day
CAPD	Continuous ambulatory peritoneal dialysis
CLAV	Clavulanic acid
Cl	Clearance (l/h) usually of serum or plasma
Clr	Renal clearance (l/h)
Cls	Serum clearance (l/h)
C_{MAX}	Maximum observed serum drug concentration following a dose, usually a single dose (mg/l)
Cp	Plasma or serum drug concentration (mg/l)
C_{PRED}	Predicted serum drug concentration, usually following a single dose (mg/l)
CO_2	Carbon dioxide
CrCl	Creatinine clearance (ml/min)
Cs	Midpoint serum drug concentration used in the calculation of Clr (mg/l)
CSF	Cerebrospinal fluid
$C_{ss}AVE$	Observed average steady state serum drug concentration (mg/l) if PRED as a sub-script it is a predicted concentration
$C_{ss}MAX$	Maximum observed steady-state serum drug concentration (mg/l), if PRED as a sub-script it is the predicted concentration

$C_{ss}MIN$	Minimum observed steady-state serum drug concentrations (mg/l), if PRED as a sub-script it is the predicted concentration
C_uMAX	Maximum observed urine drug concentration (mg/l)
CV	co-efficient of variation (%)
δ	Hildebrand solubility parameter
δ_A	Proton-acceptor parameter
δ_B	Proton-donor parameter
Δ_{mse}	Relative mean squared prediction error (estimate of relative precision)
Δ_{me}	Relative mean prediction error (estimate of relative bias)
D	Dose (mg)
d.f.	Degrees of freedom
Dm	Maintenance dose (mg)
DHPS	Dihydropteroate synthetase
DNA	Deoxyribonucleic acid
F	Fraction of dose available
fe	Fraction of dose administered excreted unchanged
fp	Fraction of unbound drug in plasma
fs	Fraction of unbound drug in saliva
h	Reduced plate height (measure of chromatographic efficiency)
[HA]	Concentration of protonated acid
HD	Haemodialysis
HPLC	High performance liquid chromatography
iv	Intravenous
k'	Capacity ratio (measure of solute retention in chromatography)
ka	Absorption rate constant (h^-)
ke	Urinary excretion rate constant (h^-)

k_{el}	Elimination rate constant (h^{-1})
LC	Liquid chromatography (synonymous with HPLC)
LRTI	Lower respiratory tract infection
m-AAP	Meta-aminoacetophenone
me	mean prediction error (measure of absolute bias)
MIC	Minimum inhibitory concentration (mg/l)
mse	Mean squared prediction error (measure of absolute precision)
n	number of observations
o.d.	once daily
ODS	Octadecylsilane
OPT	Optimization (Bayesian pharmacokinetic programme)
P	(in S/P) Plasma drug concentration
PABA	Para-aminobutyric acid
pe	Prediction error
pH_p	Plasma pH
pH_s	Saliva pH
pKa	pH at which a compound is 50% ionised
psi	pounds per square inch
QC	Quality control
RBC	Red blood cell
R-factor	Resistance factor
rmse	root mean squared error
R_s	Resolution function (measure of resolution of two solutes in chromatography)
σ^2	variance
S	(in S/P) Saliva drug concentration
$se_{\Delta me}$	Standard error of me
$se_{\Delta mse}$	Standard error of mse

s.d.	Standard deviation
SDMO	Sulphamoxole
$se_{\bar{x}}$	Standard error of $\bar{x}$
S_m	Standard error of the gradient of x to y
SMZ	Sulphamethoxazole
$S\hat{y}$	Standard error of $\hat{y}$
τ	Frequency of dosing (h)
t	Time following dosing (h)
$t_{\frac{1}{2}}$	Half-life (may have sub-script relating to different phases (eg. $t_{\frac{1}{2}}\beta$) (h)
TB	Tuberculosis
TEM	Transferable enzyme material (c/f R-factor)
TIC	Ticarcillin
T_{MAX}	Time to C_{MAX} (h)
TMP	Trimethoprim
T_{ss}	Time to steady-state
T_uMAX	Time to C_uMAX (h)
UV	Ultraviolet
V	Average urine flow per collection period in calculation of Clr (l/h)
v	Volume by proportion (ml) for fluids
Vd	Volume of distribution (l). In some equations may be written as V. Units may be expressed as l/kg in which case it is referred to as normalised.
w	Weight by proportion (mol/l) for solids
WBC	White blood cell
x_i	the ith observation

Publications associated with the work in this thesis.

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SUMMARY

This thesis has examined aspects of the pharmco-kinetics and assay of some antimicrobial chemotherapeutic agents with particular reference to the kinetics of fixed dose combinations and methods of predicting plasma concentration.

An extracted diazotisation procedure for the assay of sulphonamides was evaluated and found to be satisfactory for estimation of both serum and urine concentrations.

An analytical procedure was devised for the liquid chromatographic determination of trimethoprim and was used to conduct pharmacokinetic studies of trimethoprim in serum, urine, saliva and sputum.

The disposition of trimethoprim and sulphonamide following the use of the fixed dose trimethoprim/ sulphonamide combinations co-trimoxazole and co-trifamole were determined; the kinetics of sulphamethoxazole and sulphamoxole, the constituent sulphonamides, and trimethoprim were compared; for sulphamoxole this was the first full charaterisation of its kinetics. From the ratios of trimethoprim to either of the sulph-omamides it was evident that the ratios achieved in urine were notably inferior to those found *in vitro* to have synergistic effect. Interpolation of these results with the clinical, bacteriological and pharmacokinetic findings of other workers strongly suggested that trimethoprim alone should be an effective

treatment in uncomplicated urinary tract infection.

A comparison of two trimethoprim formulations with different dosing schedules showed equivalent bioavailability and urine trimethoprim levels likely to be effective against susceptible organisms. A relationship between urinary trimethoprim concentrations and urine pH was noted. However serum concentrations were found to fall below the minimum inhibitory concentrations when the dosage schedule for 300 mg trimethoprim once daily was used; the 200 mg twice daily regimen was found to give rise to more acceptable plasma concentrations.

Saliva concentrations of trimethoprim were obtained simultaneously with serum concentrations to allow consideration of the relationship between drug concentrations in these fluids for a drug which is highly ionized at physiological serum and saliva pH. Trimethoprim was an ideal probe for such an investigation, association between saliva and serum concentrations was established and there was a non-linear correlation between saliva flow rate and hydrogen-ion concentration. The prediction of serum trimethoprim concentrations from saliva concentration read from the regression line with observed serum concentration, was compared with a predictive equation based upon the Henderson-Hasselbalch equation and was found to be superior.

In a double blind trial of trimethoprim and ampicillin in a general practice study of treatment

of exacerbations of chronic bronchitis, the levels of trimethoprim in sputum were compared with clinical and bacteriological cure. There was no clear relationship, but sputum trimethoprim levels well in excess of the minimum inhibitory concentration of sensitive organisms were shown to be acheived. The study demonstrated that trimethoprim was as effective as ampicillin in the treatment of exacerbations of chronic bronchitis.

A further examination of prediction of serum trimethoprim concentration, using the data from the bioequivalence study was undertaken, using a computer programme, OPT. The programme uses Bayesian forecasting with estimates of maximum likelihood. The precision and bias of forecasts of future serum concentration were acceptable provided care was exercised in interpretation; this would require a user having an appreciation of kinetic principles.

Liquid chromatographic assays were developed for the determination of clavulanic acid and the isomers of ticarcillin in serum and urine. These assays were applied to the investigation of the kinetics of the combination of these drugs in subjects with various degrees of renal failure and undergiong dialysis.

Both drugs showed notable renal clearance and their serum clearances decreased with increasing renal impairment, however the clearance of clavulanic acid in anephric subjects was greater than anticipated and it is postulated that this may be due to induction of

metabolism. Haemodialysis cleared both drugs more efficiently than continuous ambulatory peritoneal dialysis, the former was associated with significant rebound levels of ticarcillin on cessation of dialysis. From the observed kinetics a dosing scheme for the administration of the ticarcillin/clavulanic acid combination was proposed.

The limitations of current pharmacokinetic models and investigations are discussed and the relationship between antimicrobial and antineoplastic chemotherapy considered. It is anticipated that intracellular aspects of chemotherapy may be usefully examined.

Knowledge is of two kinds. We know a subject ourselves, or we know where we can find information upon it.

Samuel Johnson 1709-1784

Letter to J. McPherson

18th April 1775

CHAPTER 1

GENERAL INTRODUCTION

INTRODUCTION

1.1. PHARMACOKINETICS

1.1.1. History

From ancient times Man has made use of the pharmacological effects of natural products eg. for catharsis or to alter mood as part of a religious ceremony; although many formulations of supposed medicinal use were in reality ineffective, some did have an effect on the body. The pharmacological effect of a drug is termed its pharmacodynamics. Often on the basis of empirical experiment therapeutically effective dosing schedules were designed. The dose and frequency of dosing could be related to effect but natural products can so vary in their potency that a dose-effect relationship could be hard to establish.

With the chemical synthesis of drugs and their introduction to therapeutic practice, potency could be better controlled; with consistency of production the rate, extent and duration of effect of a drug were more dependent on how the body handled the drug i.e. its pharmacokinetics. A drug's disposition may be influenced in a number of ways: the dose given, the route of administration, the extent of its penetration to different sites within the body and the rate of removal of the active principle. Although this may seem intuitively obvious this aspect of therapeutics did not receive formal consideration until about forty years ago.

There is general consensus that theoretical consideration of pharmacokinetics originated with two papers by Teorell in 1937 entitled 'Kinetics of distribution of substances administered to the body! in which the mode and type of administration and the relationship of these to distribution and elimination were considered.

During the 1930's the relationship between drug concentration and response, either therapeutic or toxic, was studied eg. for sulphanilamide (Marshall, Emerson, and Cutting, 1937a). Examination of routes of administration and plasma concentration-time curves, (Marshall, Emerson and Cutting, 1937b: Fuller, 1937), the extent of drug distribution, expressed as a distribution ratio (Marshall, Emerson and Cutting, 1937c), consideration of disposition as related to dose, achievement of steady-state and the relevance of peak and trough levels (Marshall, Cutting and Emerson, 1938: Stewart Rourke and Allen, 1938) laid the observational foundation of modern pharmacokinetics, indeed one of the fundamental reasons stated for performing therapeutic drug monitoring on drugs with a low therapeutic index was made by Stewart et al (1938) viz. "Another point of practical significance is the variability of blood level in different patients resulting from similar doses of the drug per pound. The need for doing frequent blood determinations in patients under intensive therapy is clear." Within ten years terminology and concepts relating to half-life of elimination , elimination rate constant and volume of distribution had been developed

(Boxer, et al, 1948). Establishment of these principles did not mean they were always heeded - phenobarbitone was introduced in slow release form to allow once daily dosing, a pharmacokinetic study of these and the 'ordinary' preparation, showed that no advantage in terms of blood level could be demonstrated (Butler, Mahaffee and Waddell, 1954) as the authors state for a drug with such a long half-life- "Of all the drugs in current use, phenobarbital is one of the most inappropriate for this ingenious method of administration".

Growth in pharmacokinetics was relatively slow, the first review was not published until 1961 (Nelson, 1961), but the second was published only seven years later (Wagner, 1968), in the intervening period a number of limitations in the early models had been demonstrated and alternatives proposed, including: articles on the need for a re-appraisal of methods of calculation of volume of distribution (Wagner and Northam, 1967; Riegelman, Loo and Rowland, 1968a, Riegelman, Loo and Rowland 1968b), consideration of absorption rates (Wagner and Nelson, 1964; Wagner, 1967; Wagner and Metzler, 1967; Riegelman et al, 1968a; Loo and Riegelman, 1968) and elimination rates (Wagner, 1967; Wagner and Metzler, 1967; Riegelman et al 1968). Determination of these parameters was also possible using an iterative computer least squares regression fitting program (Wagner, 1967).

Although the mathematical descriptions were adequate and could be complex, the early observations often relied

upon inaccurate and/or imprecise assays or were based on studies with radio-labelled drugs. The investigative impetus was enhanced by methodological developments such as gas-liquid chromatography and radio-immunoassay which permitted direct, accurate and precise determination of the drug and its metabolites.

1.1.2. Simple Concepts

A brief outline of the principles of pharmacokinetics is given below, more detailed information may be obtained from a number of sources (eg. Rowland and Tozer, 1980; Gibaldi and Perrier, 1982; Benet, Massoud and Gambertoglio, 1984). The period of drug duration *in vivo* is a balance of the factors considered below.

i) Administration

If a drug is not administered directly into the blood stream it must be absorbed from its site of administration eg. gut, muscle etc. the extent and rate of absorption depends on a number of factors including the drugs pKa , blood flow to the site of administration, the drug stability and the pharmaceutical presentation.

ii) Distribution

Once in the circulation, or more accurately in the plasma water, the drug is distributed to various sites in the body and will accumulate preferentially depending on its lipophilicity at physiological pH, organ blood flow and tissue protein binding.

iii) Elimination

Removal of active drugs is usually effected by renal excretion or hepatic metabolism followed by excretion of metabolites in the urine and possibly bile. Hepatic removal is by metabolic modification of the original nucleus and this may be considered in two phases with first an oxidation product formed often, but not exclusively, by cytochrome P450 and then with subsequent conjugation with a highly polar moiety eg. glucuronide. Reductive metabolism may also occur. There are a large number of possible routes of drug metabolism and a drug may be metabolised by several routes, some of these routes are not mutually exclusive.

Polar drugs and metabolites are usually readily excreted renally, the extent of clearance is dependant on a number of factors including percent and avidity of plasma protein binding, rate of delivery and hence filtration, tubular secretion and tubular reabsorption.

As elimination proceeds the whole body drug load declines, the plasma water drug concentration declines, reflecting a decrease in abundance at receptor level and hence a decline in pharmacodynamic effect.

iv) Multiple dosing

Few drugs are taken as a single dose for short-term effect and it is more common for drug use to be required over an extended period. The

only method of maintaining a steady plasma concentration once steady-state had been reached, would be to use continuous infusion. Regular i.v. bolus or oral dosing, at appropriate intervals and doses, may achieve the average steady-state plasma levels seen with continuous infusion due to the intermittance of the dosing there will be peaks just after dosing and troughs just prior to dosing, this 'sawtooth' plasma profile is often referred to as 'steady-state'. Dosing at the frequency equivalent to the drug half-life will lead to accumulation of plasma levels towards steady-state, which for practical purposes, can be considered complete after such dosing has been continued for a period equivalent to five half-lives.

1.1.3. Uses of Pharmacokinetics

Knowledge of the rate constants of drug absorption and elimination and extent of distribution can be the basis of designing a rational drug regimen for the population in whom the data has been determined. Generally such information is widely applicable, however disease states may alter pharmacokinetics, there may be population sub-sets with kinetics markedly different from the general population. Even within the population there is a wide spread of clearance and distribution and hence blood levels following a standard dose, thus individualization

of therapy is required.

The pharmacokinetics of most drugs are usually well documented, and antimicrobial agents are no exception; where this class differs from others is that there is often a strong rationale for using such agents in combination; this may be to extend antimicrobial cover, but in some situations one agent potentiates the action of another, enhancing its effect. Potentiation of action may be an option that can be reserved until required with the addition of the appropriate drug to the regimen, however an alternative is to use the agents in fixed combination in pre-formulated presentations. Usually such preparations require the constituents to be formulated in a fixed ratio to ensure appropriate levels or ratio for action in vivo in the majority of the population in which it is to be used; from the rationale of these formulations it follows that achievement of inappropriate levels due to incorrect formulation, effect of disease etc means that the fixed combination is inappropriate; if the drugs present do not parallel each other in their deviation it becomes more difficult to design appropriate alternative dosage schemes. The pharmacokinetics of such combinations, and prediction of plasma drug concentration, required more study than they had received hitherto.

1.2. ANTIMICROBIAL AGENTS

Antimicrobial agents are structurally diverse and range from simple inorganic molecules eg. Arsenic Oxide to complex proteins (bacteriocins); the majority are intermediate in size comprising a group of structurally unrelated molecules with molecular weights of between about 150 and1200 daltons.

Their mechanisms of action reflect their structural diversity and no facet of microbial activity remains unchallenged by antimicrobial agents (Fig 1.1). Such agents are most frequently produced by other micro-organisms in the natural environment and exert evolutionary pressure through competition and natural selection. Similar mechanisms have also resulted in the development of resistance.

Man has harnessed a small proportion of these compounds to combat a range of infectious diseases; our current understanding of the mechanisms of action of, and development of resistance to, effective antimicrobial agents are largely based on the investigation of these compounds.

The range of their mechanisms of action inevitably implies that antimicrobial agents exhibit variability in their action against different species. If they are effective against a wide range of organisms i.e. both Gram positive and Gram negative bacteria then they are termed broad spectrum agents.

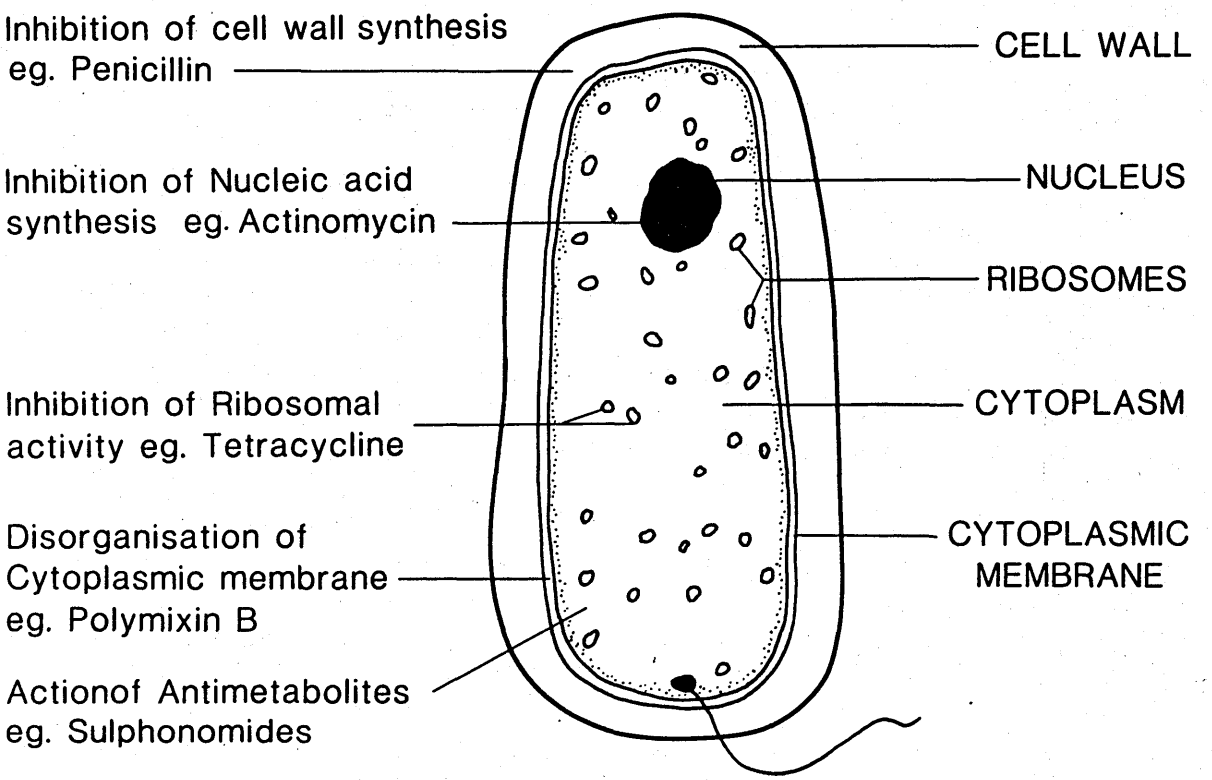


FIGURE 1.1. Sites of action of antimicrobial agents in bacteria

1.2.1. History

Use of natural antimicrobial agents was reputedly practised in antiquity, Selwyn, Lacey and Bakhtiar (1980) note that the line "purge me with hyssop and I shall be clean" occurs in Psalm 51 verse 7 and that Penicillium notatum was first isolated from the hyssop plant. In mediaevel times many apparently strange medications were used; Phaire(1560) stated that "For quinsy and swellynge under the eares. Take the musherim that groweth vpon an elder tre, called in englysshe iewes eares (for it is in dede crōcled and flat, mouch lyke an eare) heate it agaist the fyer, and put it hote in any drynke, the same drynke is good and holsome for the quynsy." Selwyn et al (1980) suggest that the symptoms were consistent with haemolytic Streptococcal infection and the "mushroom" has been found to be infected with Penicillium.

In the late 19th Century Lord Lister (1875) noted the effects of Penicillium glaucum on bacteria during the course of studies on the Germ Theory; he has been reported as utilising the effect clinically in 1888 (Selwyn et al, 1980).

The modern age of chemotherapy began with the work of Ehrlich on organic arsenicals, resulting in the production of Salvarsan which was remarkably effective against spirochaetal infections, particularly syphilis. From this and earlier work Ehrlich developed the concept of: "a medicament ..... (that) .....would .....strike the parasites with full force and, in this sense, correspond to the immune substances which, in the manner of

magic bullets, seek out the enemy." (Ehrlich, 1913).

The rationale for the magic bullet concept was that as there was differential uptake of dyes by bacteria eg. Gram's stain, then this discriminatory uptake could be linked to a toxic substance which would selectively kill the bacteria i.e. selective toxicity.

Pursuit of this approach resulted in the discovery of Prontosil (sulphonamido-chryosidin) in 1932, and successful clinical trials were reported in 1935 by Domagk. The necessity for the Chyrosidin part of the compound was questioned by Trefouel et al (1935) who suggested that para-aminobenzene sulphonamide was released and was the antimicrobially active moeity. Fuller (1937) confirmed that para-aminobenzene sulphonamide (sulphanilamide) was formed from Prontosil and was the active component (Fig 1.2). Ironically sulphanilamide had been synthesised thirty years earlier (Gelmo,1908) during a series of investigations on azo dyes but its antimicrobial properties passed unnoticed.

The discovery of the sulphonamides therefore has a direct link with the research initiated by Ehrlich in his search for 'magic bullets'.

The description of penicillin by Fleming (1929) pre dated the discovery of Prontosil by five years. Although Fleming recognised the clinical possibilities of penicillin the emphasis in his original report was on its use for typing bacteria; that he did not pursue the clinical aspects of penicillin was most probably

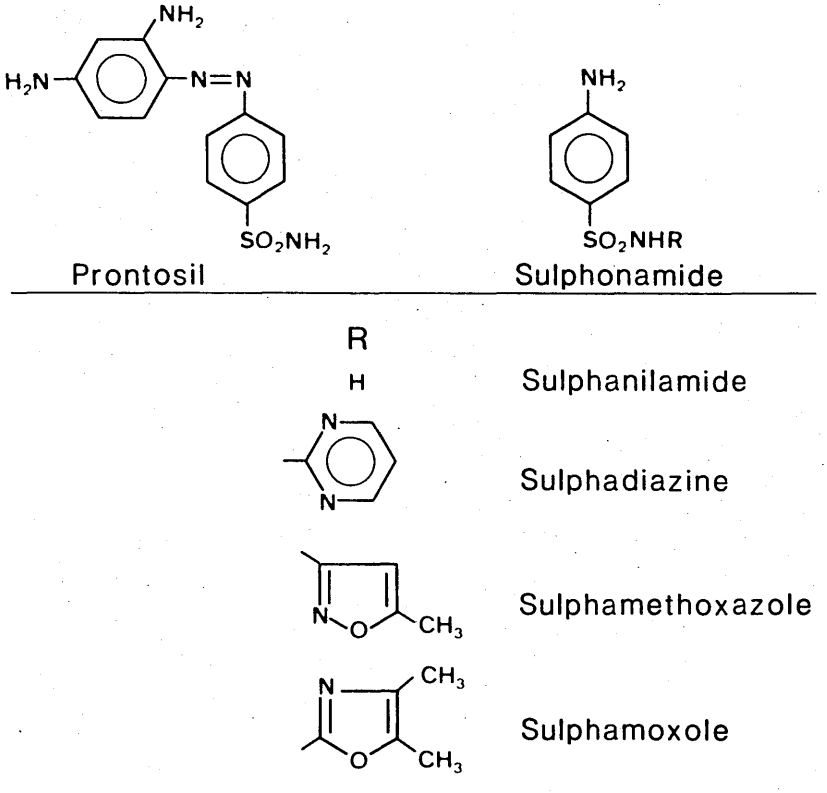


FIGURE 1.2. Structure of Prontosil, sulphonamide, sulphanilamide, sulphamethoxazole and sulphamoxole.

due to the difficulties of isolating therapeutically useful quantities. It was not until Florey and Chain published their observations on the clinical efficacy of penicillin that its true potential was realised (Chain et al, 1940; Abraham et al, 1941).

Due to the military requirements of World War II British and American teams investigated penicillin in an attempt to improve the yield of product following culture of the Penicillium mould and to modify, and thus enhance, the antimicrobial effect. Large scale deep-fermentation techniques provided an adequate supply of the most active penicillin, penicillin G; however due to the instability of the molecule it proved very difficult to determine the exact molecular structure (Sheehan, 1982). It was possible by changing the precursor to phenoxy-acetic acid to produce penicillin V. The discovery of 6-amino-penicillanic acid (Batchelor et al, 1959) in the fermentation media led to the use of this product for subsequent substituent group modification, allowing a relatively cheap and flexible approach to modification of the properties of penicillins and gave rise to the semi-synthetic penicillins. Although penicillin has been totally synthesised chemically (Sheehan, 1982) this is an expensive method of production.

Both sulphonamides and penicillin principally affect Gram-positive organisms. As the sulphonamides were found to competitively inhibit para-aminobenzoate, the principle of antimetabolite chemotherapy was extensively investigated in the search for new antimicrobials,

but proved fruitless due to the fact that the metabolism of eukaryotic and prokaryotic cells is very similar and thus most antimetabolites are toxic to Man.

Penicillin however indicated that micro-organisms could inhibit each others growth by the secretion of metabolites. Study of saprophytes for such compounds, led to the discovery of the first useful antibiotic streptomycin, for use in infections by Gram negative organisms (Schatz, Bugie and Waksman, 1944).

Thus by the end of the Second World War the principle of modern antimicrobial chemotherapy had become established.

1.2.2. Resistance

Even as the therapeutic successes of penicillin were being described there were reports of resistant bacteria (Abraham and Chain, 1940). From this early recognition of the phenomenon of resistance it rapidly grew to become a major problem.

Resistance to antimicrobial agents may arise in a number of ways:

i) Antimicrobial inhibition of the bacterial enzyme is no longer effective due to structural modification; the normal physiological role of the enzyme continues but may be impaired and possibly be reflected in a more fastidious organism. Such modification is the mechanism of pneumococcal resistance to sulphonamides.

ii) The target area of the agent can be bypassed either by supplying the product lost due to the inhibition

or by synthesis of the product through an alternative pathway; the net effect is the same, avoidance of the effects of the antibiotic eg.Penicillin resistant Gram-negative species may be able to survive a reduced peptidoglycan component in their wall structure.

iii) One of the principle causes of antimicrobial insensitivity in Gram negative bacteria is the difficulty of penetration of antibiotics through the cell wall. Modification of permeability can confer resistance, and is the basis for low level resistance to many unrelated antimicrobial agents.

iv) Inactivation of an antibiotic by metabolic degradation is a common source of resistance; a classic example being the inactivation of penicillins and cephalosporins by β-lactamases which cleave the β-lactam ring to produce the microbiologically inactive acids.

The acquisition of resistance is genetically mediated; any evolution that is advantageous will be phenotypically expressed, and in the presence of an antibiotic expression as resistance, although it might normally be physiologically disadvantageous, will be important in terms of survival value. In eukaryotic cells genetic material is invariably chromosomal, however prokaryotic cells are able to transfer genes through exchange of genetic material. Resistance can thus be transferred or may be intrinsic to the species.

Resistance to antibiotics is encoded by the bacterial chromosome or carried on a plasmid (extrachromosomal DNA),

both are self-replicating although some plasmids require vectors. Chromosomal and plasmid DNA are not inter-dependent and on cell division they will replicate and be present in each daughter cell. However while anti-biotic resistance will only be a small part of the information carried on the chromosome it can be a dominant part of the plasmid information.

In certain bacteria it is not uncommon for the plasmid to integrate with the chromosome, this process may be irreversible, however of major significance is the fact that the plasmid is of a sufficient size to be readily transferred between cells and is able to express its resistance.

Plasmid transfer is achieved either through a bacteriophage vector (transduction) or by direct cellular contact between two cells (conjugation). There may be more than one plasmid in a cell and resistance factors (R-factors) may be whole or part of a plasmid and may or may not be self replicating. Each R-factor may have several resistance markers on it, although the rate of plasmid loss on transfer is such that this information may be lost if the pressure of selection is no longer present.

The transmissibility of resistance information has resulted in bacterial populations rapidly adapting to new antibiotics, this is particularly so in the hospital environment. Transfer of resistance is important, however once established an antibiotic may well be faced

with an initial and continuing resistant bacterial population.

Microbial drug sensitivity is only determinable *in vitro*. Discs impregnated with antimicrobial agents are placed on agar plates innoculated with a lawn of isolated organism. Subsequent diffusion of the agent into the medium results in the production of a circular zone of inhibition if the organism is sensitive. Microbial sensitivity to antimicrobial agents is quantitativley characterised by determination of the minimum inhibitory concentration (MIC) which is the concentration required to produce a bacteriostatic, or inhibitory, effect on growth *in vitro*, this allows an empirical correlation with efficacy. MIC determinations can be adversly affected by a number of factors such as the size of the inoculum, media used and pH of the medium.

MIC's can be used in general terms to describe the concentration at which sensitive organisms will be susceptible; higher figures will apply to strains with some resistance and will be relevant to particular isolates, but therapeutic concentrations can usually be achieved, very resistant strains, characterised by high MIC's will remain unaffected by the drug at the concentrations achievable *in vivo*, and an alternative antimicrobial should be considered.

Selection of resistant strains is caused by the inhibition of sensitive organisms and the subsequent proliferation of resistant variants within the original population. Uncritical use of antimicrobial agents

selects for resistance, by rational use of agents it would be possible to decrease the trend of increasing resistance.

In addition to microbial resistance there may be failures in treatment i.e.'therapeutic' resistance. Examples of therapeutic resistance include; delay in commencement of therapy such that institution is too late to significantly affect the course of the infection, inability to achieve and maintain therapeutic concentrations from whatever cause eg. malabsorption of an oral dose, or difficulty of penetration to the infected site due to eg.ischaemia, there may be a drug interaction or perhaps adverse conditions at the site of action eg. pus, that antagonise the antibiotic's action.

The use of antibiotics in combination may also reduce the emergence of resistant strains (Jawetz, 1968). One of the commercially most succesful antimicrobial combination has been that of sulphamethoxazole (SMZ) and trimethoprim (TMP) i.e. co-trimoxazole; more recently clavulanate potentiated penicillin combinations have been introduced.

This thesis is concerned with the pharmacokinetics of these combinations as measured using chemical methods of assay.

1.3. BETA-LACTAMS

There are two main classes of antibiotic in this group, penicillins and cephalosporins, plus other compounds containing the β-lactam ring eg. clavulanic acid.

This thesis is concerned with a penicillin and clavulanic acid, it is therefore proposed to confine discussion to these groups.

1.3.1. Penicillins

a) Mechanism of Action

Penicillin (Structure Fig. 1.3) inhibits the formation of peptidoglycan from UDP N-acetylmuramic acid pentapeptide forming a peptide bond with the N-acetyl-glucosamine portion of a second mucopeptide with elimination of D-alanine. It is thought that penicillin's main role is as a structural analogue for the terminal D-alanyl-D-alanine of the pentapeptide and this is the reason for its interference in the final transpeptidation. There is also evidence that it acts as a carboxypeptidase, there are seven penicillin binding proteins (Spratt,1983).

b) Microbial Resistance and Spectrum of Activity

Resistance to penicillins is most commonly caused by destruction of the penicillin by bacterial β lactamase which cleaves the β lactam ring at the position shown in Fig. 1.3. The widespread resistance of hospital staphylococci is due to the prevalence of β lactamase in this species.

Beta-lactamase production may be chromosomally mediated or R-plasmid mediated, the latter produce the greatest amount of enzyme and result in a higher level of resistance. Of the β lactamases,types II-V act on penicillins, types II and IV are chromosomal and III and V plasmid mediated (Sykes and Matthew, 1976).

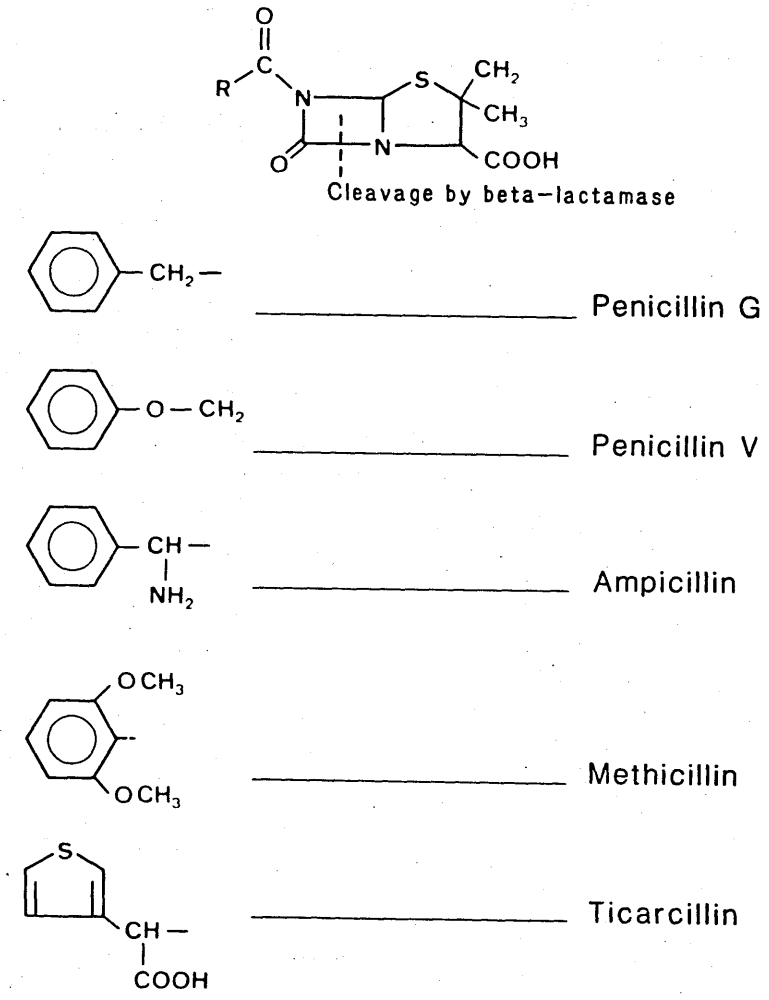


FIGURE 1.3. Structure of some penicillins, including ticarcillin showing the site of beta-lactamase cleavage.

Penicillin G is active against most Gram positive organisms, in these bacteria the β-lactamase diffuses from the cell into the bacteria's surroundings and thus attacks β lactam antibiotics, obviously a number of similar bacteria would be required to ensure survival. As access and egress to the cell is uncomplicated the penetration of an antibiotic is unhindered.

In Gram-negative bacteria the cell wall structure is more sophisticated than in Gram positive organisms, this wall acts as a barrier to penetration by antibiotics and is a cause of microbial insensitivity to antibiotics, changes in permeability can cause resistance to agents that could previously penetrate. In addition β lactamases are present as wall bound enzymes and can therefore inactivate susceptible β lactam antibiotics. Modification of Penicillin G to give Ampicillin (Fig. 1.3) increases the spectrum of susceptible Gram negative bacteria from Neisseria to include eg. enteric Escherichia coli, further modification of the side-chain eg. ticarcillin will include Pseudomonas aeruginosa.

Some penicillins are β-lactamase stable, but this depends on the type of enzyme encountered.

Penicillins are not effective against cells with minimal metabolic activity.

c) Pharmacokinetics

Penicillin G is acid labile; following an oral dose, less than 30% of the dose will be absorbed (Weinstein 1975 a) this proportion is not increased for the acid

stable Penicillin V. Absorption is dependent on the particular salt used, the rate of tablet dissolution and the presence or absence of food in the stomach. The semi-synthetic penicillins (eg. amoxycillin) are more acid stable and are well absorbed after oral administration. Other semi-synthetic penicillins are penicillinase resistant, this is of much greater significance than acid resistance although the available MIC data suggests that they are less potent. (Garrod et al, 1981). The degree of protein binding determines the amount of 'free' penicillin available and thus the achievable in vivo concentration available for antimicrobial action. The isoxazolyl penicillins eg. flucloxacillin are both acid resistant and β-lactamase resistant whereas methicillin is acid labile and requires intramuscular or intravenous administration.

Of particular relevance to this study are the antipseudomonal 'broad-spectrum' penicillins carbenicillin and ticarcillin. They are acid labile and β lactamase sensitive, they are more susceptible to TEM type enzymes than some of the chromosomal enzymes; the usual route of administration is i.v.

Ticarcillin has a rapid distribution phase with a volume of distribution of around 13-15 litres (Davies et al, 1982; Gouyette et al, 1982) and the high concentrations achievable ensure the maintenance of effective anti-pseudomonal concentrations.

Active renal tubular secretion is the principal

route of penicillin elimination. The carrier system is common for a variety of organic acids and they compete with each other for transport, the binding affinity for the carrier protein determining the proportion of the compound excreted. Probenecid blocks penicillin excretion by this mechanism; it has a high affinity for the carrier protein and its activity is further potentiated by tubular reabsorption (Weiner, Washington and Mudge, 1960) As a result penicillins with short elimination half-lives have delayed excretion, which maintains the blood level and improves efficacy.

d) Toxicity and Adverse Reactions

A great number of adverse reactions to penicillins have been reported, ranging from the minor to the fatal. The most common adverse effect is hypersensitivity occurring at a rate between 0.7 and 10% (Idsøe et al, 1968). Antipenicillin antibodies can be detected in patients who have received the drug and in some individuals who have not been knowingly exposed (Klaus and Fellner, 1973).

Dermatological reactions to penicillins are fairly common and moderate or mild reactions often spontaneously resolve whilst therapy continues. Penicillin rash may be seen during an initial course of treatment but is not noted on a subsequent course. However rapid, life-threatening anaphylactic reactions can occur on the first known exposure to even small doses of the drug. Between 0.015 and 0.04% of penicillin treated patients have

anaphylactic reactions and about 0.002% of all such patients die (Idsøe et al, 1968). Sensitivity to one penicillin greatly increases the risk of a reaction to another as does a family history of severe reaction.

Penicillins may cause pain following i.v. injections and it was for this reason that procaine-penicillin became available. One of the major side-effects of penicillin therapy is electrolyte imbalance. Some penicillins for injection are sodium salts, to improve their solubility, however the large doses of penicillin that can be given can result in sodium overload, the resultant hypervolaemia is particularly dangerous for patients with cardiac failure or hypertension. A further problem is occasional cases of hypokalaemia, the additional sodium load may contribute to the urinary loss of potassium and it has been suggested that penicillin can act as an anionic ion-pair for potassium in the renal tubule (Brunner and Frick, 1968), blockage of ticarcillin excretion with probenecid resulted in decreased urinary potassium losses but did not reverse the hypokalaemia (Mittal, Pierce and Priestley, 1980). This lends support to the suggestion that the alkalosis induced increase in intracellular potassium may also be compounded by a direct, but undefined, effect of the penicillin on the sodium/potassium pump; redistribution of potassium from the extracellular compartment is significant (Tattersall, Battersby and Spiers, 1972)

Finally there have been reports that carbenicillin

causes defects in platelet function, related to impaired ADP-induced platelet aggregation (Brown et al, 1974).

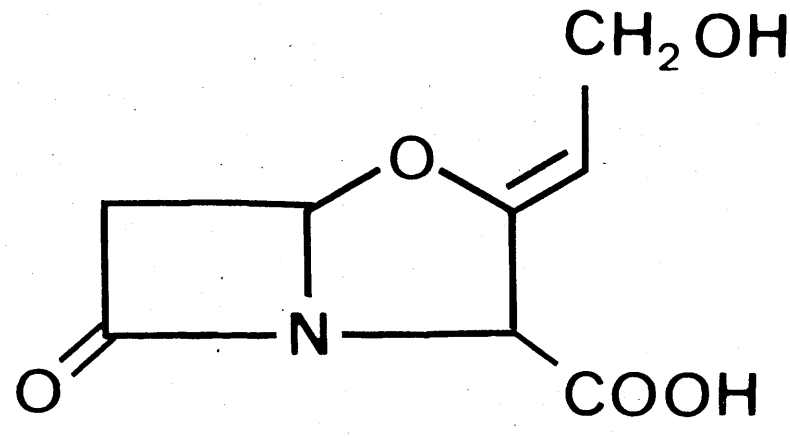
1.3.2. Clavulanic Acid

Although it is possible to obtain penicillins which have either a broad spectrum or are β-lactamase resistant, it has not yet proved possible to produce a potent β-lactamase resistant broad spectrum penicillin.

Clavulanic acid is structurally similar to the core of the penicillins (Fig 1.4) although at the concentrations achievable *in vivo* using current therapy it has little if any direct antimicrobial action; at much higher concentrations it does exhibit notable antimicrobial effect. Its major importance is as a potent inhibitor of most bacterial β-lactamases (Reading and Cole, 1977); thus the combination of clavulanic acid with a broad-spectrum penicillin results in the protection of the penicillin from the action of β-lactamase thereby potentiating its activity. This occurs both *in vitro* and *in vivo*.

a) Mechanism of Action and Penicillin Potentiation

Destruction of the lactam ring in a penicillin by β-lactamase renders the antibiotic ineffective. β-lactamase acts by cleavage of the penam ring, a nucleophilic attack on the lactam ring resulting in a penicilloyl-enzyme complex with hydrolysis to the penicilloic acid and regenerated enzyme (Virden, Bristow and Pain; 1975). Clavulanic acid undergoes the same nucleophilic attack, however the resultant enzyme/substrate complex does



Clavulanic acid

FIGURE 1.4. Structure of clavulanic acid.

not readily dissociate (Cherry and Newall, 1982), initially the reaction is readily reversible but later it tends to be irreversible or slowly reversible (Rolinson, 1980).

Clavulanic acid was first reported by Howarth, Brown and King, (1976) and was found to have significant penicillinase-inhibiting properties (Reading and Cole; 1977). The similarity in structure between the penam and clavam ring is immediately noticeable, and it was already known that potentiation ofβ-lactamase sensitive antibiotic could be achieved in the presence of a penicillinase resistant antibiotic (Hamilton-Miller, Smith and Knox, 1964; Sutherland and Batchelor, 1964)

There are a number of different types of β -lactamase and these can occur simultaneously in the same organism (eg. see Sykes and Matthew, 1976). As summarised by Cherry and Newall (1982) some enzymes are irreversibly bound by clavulanic acid, some may slowly recover while others are not inhibited at all. The ratio of inhibitor to enzyme can vary markedly depending on the source of the enzyme; one mole of clavulanic acid inhibits one mole of S.Aureus penicillinase, while 115 moles of clavulanic acid are required to inactivate one mole of E. Coli RTEM-2 enzyme (Sykes and Bush, 1982). Of the β-lactamases only chromosomal cephalosporinase is readily resistant to the effects of clavulanic acid (Slocombe, 1980).

Clavulanic acid is therapeutically relevant only if used in conjunction with a β-lactamase-sensitive broad spectrum antibiotic. Clavulanate in combination with amoxycillin has been succesfully launched, and early

reports of the combinations efficacy were published (Ninane, et al, 1978; Symposium, 1980).

b) Pharmacokinetics and Toxicology

Clavulanic acid can be administered orally or intravenously, the choice of route depending on the acid stability of its co-penicillin. It is poorly protein bound and is rapidly excreted mainly by renal elimination and is found unchanged in the urine (Jackson et al 1980). These authors also reported no significant toxicological problems, although Leigh et al (1980) found 8% of their patients suffered diarrhoea.

1.4. FOLATE ANTAGONISTS

1.4.1. Sulphonamides

Soon after the identification of sulphanilamide as the active component of Prontosil, a second sulphonamide, sulphapyridine, was introduced into clinical practice. It had a broader spectrum of activity and was effective against Streptococcus pneumoniae.

The early sulphonamides were poorly water soluble and their acetylated metabolites even less so which led to deposition of crystals within the urinary tract causing renal failure; the more modern sulphonamides do not commonly cause significant renal damage.

a) Mechanism of Action

Sulphonamides are anti-metabolites which act by inhibiting bacterial folate synthesis, for unlike eukaryotic cells bacteria cannot utilise exogenous folate (Fig 1.5), the reduction in available folate causes a failure of DNA synthesis; (Woods, 1940; Fildes, 1940). Sulphonamides

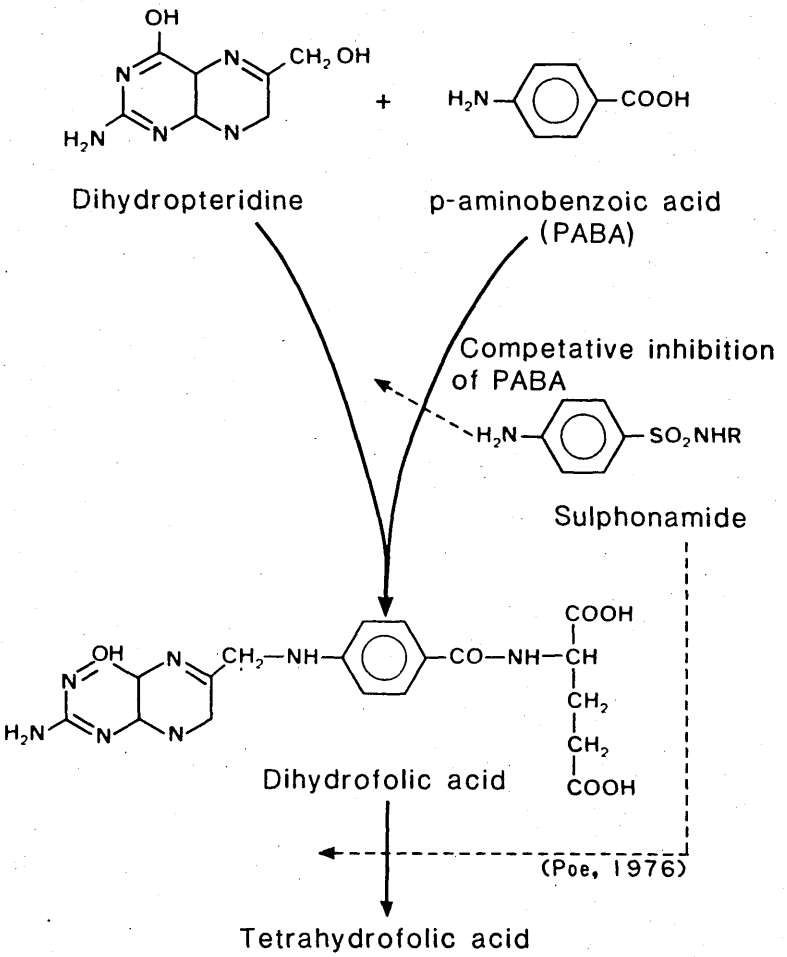


FIGURE 1.5. Mode of action of sulphonamide inhibition of bacterial folate synthesis

act by competitively inhibiting the conversion of para-amino-benzoic acid (PABA) to dihydrofolic acid. However the affinity of dihydropteroate synthetase (DHPS) for PABA is one thousand times that for sulphonamide and thus high doses of sulphonamides are required to inhibit enzyme activity. There is a delay between the onset of therapy and the appearance of evidence of inhibition which results from the utilisation of the intracellular folate pool (Struller, 1968). Only when this has been depleted does the antimicrobial effect of the drug become apparent.

More recently evidence has been accumulating that sulphonamides may also inhibit dihydrofolate reductase (Poe, 1976; Golde, Bersch and Quan, 1978; Lacey, 1979; Lacey, 1982), Golde et al (1978) found there was inhibition of haemopoiesis in humans, such a finding is only of clinical significance in folate deficient individuals since pre-existing folate stores and the low affinity of the human enzyme usually protect against these effects during sulphonamide therapy.

b) Spectrum of Activity

Sulphonamides exhibit a broad spectrum of activity although the development of resistance has limited their application. The site of infection determines whether one chooses highly soluble compounds for use in acidic urine to avoid renal precipitation eg. sulphafurazole or poorly absorbed compounds for use against gut flora eg. succinylsulphathiazole. A wide choice of halflives

is available eg. sulphamethoxazole with a moderately long half-life requires to be given twice per day, sulphadimethoxime with a longer half-life requires only one dose per day and others with very long half lives require dosing only weekly eg. sulphametopyrazine.

For best compliance dosing frequency should be kept to a minimum, the longer half-life drug requiring to be taken less frequently.

The sulphonamides with the best antimicrobial activity are sulphadiazine, sulphafurazole and sulphamethoxazole (Garrod et al, 1973) the last is of particular note as it is available in combination with trimethoprim as is sulphadiazine; other sulphonamides have been considered on the basis of their activity and pharmacokinetics for combination with trimethoprim (Ortengren, Fellner and Bergan, 1979a).

c) Microbial Resistance

Although there are a great variety of sulphonamides, organisms resistant to one are resistant in some degree to all others i.e. cross-resistance. As the sulphonamides have been in continual clinical use for nearly fifty years it is perhaps not surprising that resistance, although usually slow in developing, is widespread. The introduction of the antibiotics superceded the use of sulphonamides in some instances and some previously resistant microbes, eg. gonococci, are sensitive once again.

Resistance may arise from modification of the active site of the enzyme with consequent reduced affinity for the sulphonamide, or by increased uptake of endogenous PABA, thereby diminishing the competitive effect. These

result from chromosomal mutation whereas much of this resistance is specified by plasmids which encode an enzyme (DHPS) which has virtually zero affinity for the sulphonamides.

d) Pharmacokinetics

Most sulphonamides are well absorbed following an oral dose. Protein binding is variable within the class and there are a range of pKa's (Struller, 1968), the distribution within the body is variable. The routes of metabolism of all sulphonamides include acetylation and oxidation; the metabolites are inactive and in the case of the acetylsulphonamides, are less soluble in urine than the parent drug. The acetylator phenotype for some sulphonamides eg. sulphadimidine is bimodal (Van Outdshoorn and Potgieter, 1971) and the percentage of fast to slow acetylators has been shown to vary between races (Curry 1980a: Utrecht and Woosley, 1981). Renal clearance of sulphonamides is significant; filtration plays a major part with varying influences of secretion and reabsorption: it is possible to model these changes (Hori, Sunayashiki and Kamiya, 1976).

As the different sulphonamides undergo different rates of metabolism, renal secretion and reabsorption the excretion of the unchanged, active sulphonamides also varies. The effectiveness of the drug is related to the concentrations achieved at the site of action, the intrinsic potency of the sulphonamide and the susceptibility of the organism.

e) Toxicity and Adverse Reactions

There are many side effects of sulphonamides, the

most serious of which may be fatal.

Of the haematological reactions, the most common is an acute haemolytic anaemia in subjects with glucose-6- phosphate dehydrogenase deficiency i.e.favism. The most severe haemolytic episodes are rare but may have a fatal outcome.

Although the modern sulphonamides are more water soluble than those used originally, there is still some risk from crystallisation in the renal tubules; Buchanan (1978) reported several patients with renal failure following treatment with co-trimoxazole (sulphamethoxazole and trimethoprim) although such reactions may arise due to hypersensitivity.

Anorexia, nausea and vomiting are most common and occur in 1-2% of subjects, (Weistein, 1975b), these effects are reversed on withdrawal.

Stevens - Johnson syndrome may be a rare and occasionally fatal complication.

1.4.2. Trimethoprim

The antimetabolite action of the sulphonamides may be improved by combined blockade of the bacterial folate pathway with the agent trimethoprim. It was the dramatic *in vitro* synergy claimed by Bushby and Hitchings (1968) that led to the great interest in this combination of drugs.

a) Mechanism of Action and Potentiation

Trimethoprim (TMP) was first synthesised in 1961 by Hitchings and Bushby; TMP was found to inhibit the

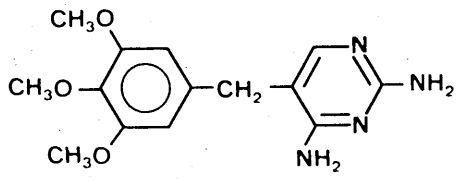
enzyme dihydrofolate reductase (DHFR) which is responsible for the production of tetrahydrofolate from dihydrofolate, whereas the sulphonamides inhibit the formation of dihydro folate (Fig 1.6) although it has been suggested that sulphonamides may inhibit both sites (Poe, 1976). The concept of combining TMP with a sulphonamide was clearly attractive and TMP was found to be a potent potentiator of sulphonamide action (Bushby and Hitchings, 1968). From pharmacokinetic considerations it was evident that the particular sulphonamide chosen should have a similar rate of elimination to TMP. Sulphamethoxazole (SMZ) was chosen and the combination of TMP/SMZ, known as co-trimoxazole, was marketed as Septrin by Burroughs Wellcome, the manufacturers of trimethoprim and, Bactrim by Roche, the manufacturers of sulphamethoxazole.

The opportunity to market TMP alone was not taken despite the evidence in Bushby and Hitchings work (1968) of the much greater potency of TMP. This approach was not changed until 1979, although experience in Finland, where TMP alone was available from 1973 for the treatment of urinary tract infection, indicated that TMP alone was at least as effective, as well as being less expensive and less toxic than the combination (Kasanen et al 1978).

b) Pharmacokinetics

Trimethoprim is well absorbed after oral administration. It has a volume of distribution (Vd) of the order of 11/kg which is about six times the Vd of SMZ, and taking into account the relative potency and ideal ratios for

STRUCTURE



Trimethoprim

MECHANISM of ACTION

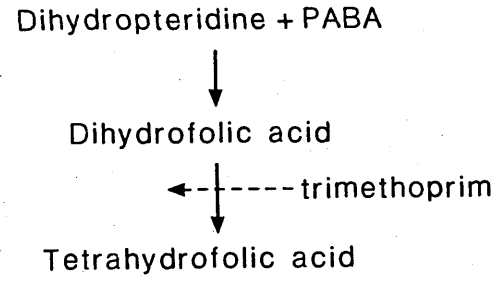


FIGURE 1.6. Structure and mode of action of trimethoprim by inhibition of bacterial folate synthesis.

synergy, a dosing ratio of 5:1, SMZ/TMP, is used. Elimination of TMP is partly through metabolism to hydroxylated/conjugated inactive metabolites and partly by renal excretion of unchanged drug; the latter accounts for approximately half of the drug eliminated (Kasanen et al 1978) although the rate of renal elimination of TMP is very sensitive to changes in urinary pH (Sharpstone, 1969).

c)Toxicity and Adverse Reactions

As TMP is a folate antagonist it is reasonable to expect that in depleted individuals there will be cases of acute folate deficiency. However TMP has an affinity 50,000 times less for mammalian DHFR than the bacterial enzyme (Burchall and Hitchings, 1965) and severe toxicity is rare. Surveys of co-trimoxazole therapy have shown that the commonest adverse reactions are skin rashes and nausea with an incidence rate of about 3.5% (Lawson and Jick, 1978), the majority of side effects noted in a double-blind trial of TMP and co-trimoxazole could be attributed to the sulphonamide component (Lacey et al 1980).

1.5. FIXED DOSE COMBINATION OF ANTIMICROBIAL AGENTS

The activity of antimicrobial agents in combination may be: a) antagonistic, for example when a bacteriostatic and bacteriocidal drug are used together. b)additive, as in the combination of two bacteriostatic agents or c) synergistic when two bacteriocidal agents, usually with different modes of action are combined

(Jawetz et al, 1951; Jawetz and Gunnison, 1952).

The simultaneous administration of two or more antimicrobial agents was suggested by Weinstein (1958) as being useful on four circumstances: 1) In the treatment of mixed bacterial infections, 2) to delay the emergence of bacterial resistance, 3) to enhance therapeutic activity and 4) In treatment of severe infections where the cause is not yet established.

In mixed infection it is uncommon that one drug has optimal therapy against two (or more) organisms, and therefore two (or more) antibiotics with high specific activity against one of the organisms is used; a similar argument holds for 4 above where a combination of drugs covers the wide spectrum required most appropriately. Delay in emergence of resistance and enhanced activity are essentially different facets of synergy. Comprehensive coverage such as is required in the treatment of bacterial endocarditis where it is essential to kill all infecting organisms in a poorly vascularised site is a further facet. Triple therapy in the treatment of tuberculosis is an example of the efficacy of combined therapy.

There are a number of disadvantages to combination therapy (Cohen, 1975), the most significant of which are: adverse drug reactions, superinfection and cost.

1.6. TECHNIQUES

1.6.1. Methods of Assay

Traditionally assay of antimicrobial agents has been performed microbiologically using an indicator organism. With care the precision and accuracy of these

assays can be good and they are useful for the processing of large numbers of samples.

The confirmation of achievement of inhibitory or bacteriocidal concentration *in vivo* during therapy or monitoring of compliance should prove more rapid using chemical assay.

Chemical assays, including chromatography can also be accurate and precise; in the work described herein only chemical assays have been used and this allows a comparison to be made with the pharmacokinetics obtained by other workers using microbiological assay.

1.6.2. Pharmacokinetics

From knowledge of the disposition of antimicrobial agents it is possible to assess their therapeutic effect and determine appropriate dosages. In fixed dose combinations the assumption is inherent that an individual will handle the drugs in combination in a predictable fashion allowing the benefits of the combined product to be effected. Examination of the pharmacokinetics of 'fixed-dose' combinations is necessary to ascertain how efficiently the rationale applies in practice and this will be considered in this thesis.

In addition the methods and effectiveness of pharmacokinetic prediction techniques will be considered.

1.7. AIMS and OBJECTIVES

The intention of this thesis is to describe:

1. The development and validation of chemical methods of assay for the antimicrobial agents under study in serum, urine and other biological fluids. The drugs are: trimethoprim, the sulphonamides sulphamethoxazole and sulphamoxole, ticarcillin and clavulanic acid.
2. To examine the kinetics of the following:
   a) the fixed dose combinations co-trimoxazole (trimethoprim and sulphamethoxazole) and co-trifamole (trimethoprim and sulphamoxole) in normal volunteers.
   b) two preparations of trimethoprim alone in normal volunteers.
   c) the fixed dose combination of ticarcillin and clavulanic acid in individuals with varying degrees of renal failure, during haemodialysis and during continuous ambulatory peritoneal dialysis.

   The implications for therapy from the results of these disposition studies for the above antimicrobial agents will be considered.
3. To examine the penetration of trimethoprim in chronic obstructive airways disease.
4. To consider the validity of pharmacokinetic predictions for trimethoprim using:
   a) Salivary data
   b) Minimal individual data and population kinetics with Bayesian estimates.

I pass with relief from the tossing sea of Cause and Theory to the firm ground of Result and Fact.

Winston S. Churchill 1874-1964

The Story of the Malakand Field Force 1898

CHAPTER 2

ASSAY OF SULPHONAMIDES AND TRIMETHOPRIM IN BIOLOGICAL FLUIDS

2.1. SULPHONAMIDES

Sulphanilamide was the first effective sulphonamide (Fuller, 1937) however the early sulphonamides were poorly soluble in water and metabolism by acetylation rendered them both bacteriologically inert and even less soluble; high rates of urine flow were necessary to avoid precipitation of both drug and metabolite in the renal tubules (Struller, 1968).

2.1.1. Spectrophotometric Assays

Investigation and control of these phenomena was facilitated by the development of a simple colorimetric procedure for sulphonamides by Bratton and Marshall (1939).

All sulphonamide drugs are derivatives of sulphanilamide (p-aminosulphonamide); the p-amino group is essential for antibacterial action. Acetylation occurs at the N^4-amino group; this group is also diazotised in the assay of Bratton and Marshall (1939) with subsequent coupling with N(1-napthyl)ethylenediamine dihydrochloride; the reaction is outlined in Fig 2.1.

It was originally believed that the acetyl derivative was the only metabolite, thus assay of an acid-hydrolysed sample gave 'total' sulphonamide whereas assay of an untreated sample provided an estimate of 'active' sulphonamide; the amount of acetyl metabolite was then obtained by difference.

There have been a great number of modifications to

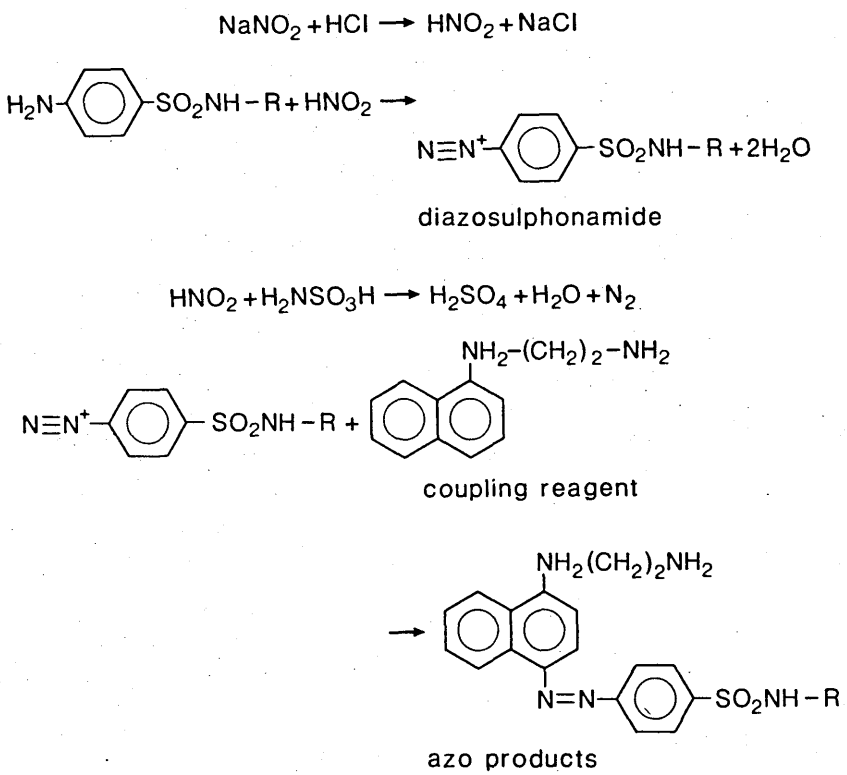


FIGURE 2.1. The Bratton-Marshall reaction: diazotisation of sulphonamide and coupling to naphthyletheylenediamine.

the original assay, most of which relate to the reagent composition. The assay quoted by Varley, Gowenlock and Bell (1976) is fairly typical.

It is now known that sulphonamides undergo N^1acetylation, N^1glucuronidation, ring-N-glucuronidation and to a lesser extent N-N^4-diacetyl formation and 3-hydroxylation.

The N^4glucuronide is unstable under the acid conditions required for diazotisation, thus all of the above metabolites with a free N^4 primary amine react in the Bratton-Marshall reaction.

The non-specificity of the Bratton-Marshall reaction is particularly important because it results in an overestimate of the apparent 'active' sulphonamide. Apparently effective concentrations could in reality be sub-therapeutic with respect to the active drug and thus select for resistance. The probability of misinterpretation is greater in the urine than serum as inactive metabolites predominate.

It was originally shown by Koechlin , Kern and Engelberg (1959) that solvent extraction could improve the assay specificity as the metabolites are not extracted. Subsequently Rieder (1972) described an assay with an ethyl acetate extraction following which a Bratton-Marshall reaction was performed in the organic solvent. Thus the polar, conjugated, acetylated metabolites were excluded.

The assay is much more specific than the procedures

previously in use and is claimed to give a good estimate of the bacteriologically active fraction in urine. With the addition of an acid hydrolysis step, the 'total' sulphonamide can also be measured.

Although this procedure is much more complex and time consuming than the simpler aqueous assays it has been claimed to have a precision of better than 5% and to be suitable for routine analysis.

Other reagents that react with the primary amine group can be used e.g. o-phthalaldehyde with fluorimetry of the final product (Amano and Mizukami 1965).

The pairing of SMZ with TMP post-dated most of this work; subsequently a spectrophotometric assay for the simultaneous measurement of both TMP and SMZ in pharmaceutical preparation has been described (Ghanem, Meshali and Foda, 1979). A spectrophotofluorimetric procedure for serum TMP and SMZ levels (Lichtenwalner, et al, 1979) employs differential extraction of TMP and SMZ at alkaline and acid pH's into chloroform and n-butyl chloride respectively followed by separate measurements of each fraction.

The assay has limited accuracy and there was significant, although predictable, interference from salicylate. Despite this a precision of better than 5% was claimed for the assay of both components, however the complexity and lack of adequate characterisation of the method make it unsuitable for routine use.

2.1.2. Microbiological Assays

Although the usual method for the routine determination of sulphonamides in biological fluids is by the diazo-coupling reaction discussed above, quantitative determination can be performed microbiologically.

There are a number of options including agar diffusion, dilution and turbidimetric assays; of these only the first is sufficiently flexible to be routinely used in the determination of antimicrobial agents in serum and plasma.

In agar diffusion analyses an indicator organism is incorporated into the agar, wells are cut into the agar and calibrators, controls and test samples are placed in the wells.

The degree of inhibition of growth following incubation is proportional to the concentration of antibiotic in the well. To perform these determinations succesfully a trained, experienced analyst is required. The indicator organism should ideally be sensitive only to the antibiotic of interest, although this specificity may be achieved, it is usually dependent on the concentration of the potentially interfering antibiotic.

Manoeuvres to destroy or inactivate interfering substances, including antagonism , enzymic removal or chromatographic purification may be used (Holt and Reeves, 1978), electrophoretic separation followed by microbiological determination has been claimed to improve specificity (Dornbusch, 1974).

In view of the potential difficulties inherent in the technique it is not surprising that national quality control surveys, for gentamicin, have indicated significant error rates (Reeves and Bywater, 1975)

As sulphonamides, particularly SMZ, are used in combination with TMP the indicator strain must be susceptible only to the component of interest (Dornbusch, 1974). The available microbiological plate assay may prove to have problems of interference that may be insoluble. (Reeves, Bywater and Holt, 1978).

2.1.3. Chromatographic Assays

Thin layer chromatography has been used to separate sulphonamides and their metabolites (Sigel et al, 1974); following separation the absorbance of the SMZ zone was determined by reflectance densitometry.

Gas-liquid chromatography with electron capture detection has been used (Gyllenhaal and Ehrsson, 1975) but was not applied to biological samples. However HPLC separation of a mixture of sulphonamides was reported as early as 1972 by Kram.

Cobb and Hill (1976) using normal phase chromatography reported on the chromatographic behaviour of a number of sulphonamides and a more sophisticated study involving the use of normal phase ion-pairing and counter-ions was reported by Su, Hartkopf and Karger (1976). The determination of sulfamethazine in bovine tissue was reported in 1975 (Johnson, Jeter and Claiborne,1975) and SMZ and its N^4 acetyl metabolite were measured in blood by Harzer, (1978).

Most investigations of SMZ and other sulphonamides used in combination with TMP have reported the simultaneous analysis of both drugs. Helboe and Thomson (1977) reported an assay for pharmaceutical preparations but did not obtain the sensitivity necessary for biological fluids. A reverse-phase procedure allowing simultaneous determination of SMZ, N^4acetyl SMZ and TMP in serum and urine with a simple dilution and protein precipitation procedure was described (Vree et al, 1978) and a similar type of method was reported by Bury and Mashford (1979).

Gochin, Kanfer and Haigh (1981) reported a technique which gave excellent resolution, however a small peak attributed to caffeine co-eluted with SMZ. As caffeine is widely available in drinks and proprietary medicines the method is not suitable for routine use. In addition the extraction procedure, although simple was lengthy.

The above procedures used reverse phase chromatography, Ascalone (1981) used normal phase chromatography for the separation of TMP and sulfadiazine and its N^4acetyl metabolite and the procedure may be used for the assay of TMP and SMZ and its N^4acetyl metabolite. The analysis is simple, precise and sensitive, however no information is given on accuracy. The eluant used is a mixture of a halo-hydrocarbon, alcohol and base and is very similar to that used for the determination of tricyclic structured drugs by normal phase chromatography (Watson and Stewart, 1977a, 1977b). Drugs of this type, particularly the phenothiazines

have numerous metabolites and since such solvents have been noted as being suitable for the retention of basic compounds (Watson, 1979; Watson et al 1983), it is unlikely that the procedure would be interference-free when used for specimens from hospital patients, particularly as only a single stage extraction is used.

Due to the simplicity of the Bratton-Marshall assay either in its aqueous form or as modified by Rieder (1972) there has been no great incentive for the introduction of chromatographic procedures for sulphonamides.

2.2. TRIMETHOPRIM

2.2.1. Photometric Assays

TMP has been measured by UV spectrophotometry (Bushby and Hitchings, 1968) but this technique lacks the sensitivity needed for the determination of plasma concentrations although it is suitable for pharmaceutical preparations (Ghanem et al 1979). Sensitive measurement of TMP in plasma can be attained using ^{14}C TMP (Schwartz and Zeigler, 1969) with detection by scintillation spectrophotometry but this technique is of limited application.

The first succesful reported 'routine' assay for TMP was the spectrofluorimetric method of Schwartz, Koechlin and Weinfeld, (1969), the principle of which was the extraction of TMP at a basic pH into chloroform, back extraction into acid, oxidation to trimethoxy-benzoic acid by potassium permanganate, removal of excess permanganate, extraction of trimethoxy-benzoic acid into chloroform and measurement of the fluorescence.

The wavelengths for excitation and emission were 275nm and 350nm respectively. It is a fairly demanding technique although a precision of 6.2% was claimed, the throughput was low and a skilled analyst was necessary; TMP-N-oxide can interfere (Rieder, 1973) but the other known metabolites do not. An alternative procedure was reported which used a single stage extraction for TMP and a second for SMZ (Lichtenwalner, et al 1979), although good comparability was claimed with the technique of Schwartz et al, (1969) on the basis of a regression line, the accuracy of the assay was not fully investigated and lacked sensitivity, allowing the detection only of concentrations greater than 0.5 mg/l.

2.2.2 Microbiological Assays

The principle outlined earlier for sulphonamides may be applied to the determination of TMP. The first microbiological assay was described by Bushby and Hitchings (1968), other authors have modified the procedure (Dornbusch,1974). Separation from SMZ followed by diffusion testing can be used (Dornbusch,1974). A microbiological assay was compared to the spectrofluorimetric method of Schwartz et al (1969) and found to compare well (Kasanen et al, 1978); however after showing this the authors then chose to use the fluorimetric procedure in their kinetic studies. The microbiological method for TMP is prone to interference problems (Allan and Nimmo-Smith, 1978).

2.2.3. Chromatographic Assays

In an attempt to improve the specificity of the

TMP assay and also quantitate the metabolites, a fluorimetric thin layer chromatographic procedure was developed (Sigel and Grace, 1973), however the unidentified fluorescent product was produced only after exposure to light for 24 hours or more which is a limitation; absorbance appeared to be as sensitive and immediately measurable (Sigel, et al, 1974). More recently a procedure that requires a single stage extraction followed by detection of a fluorescent derivative produced by heating the plate has been described (Schlobe and Thijssen, 1982). This method appears to be rapid, sensitive and precise.

Gas chromatography with a nitrogen-phosphorus detector has been used for TMP analysis (Land, Dean and Bye, 1978); although very sensitive, the use of a brominated analogue which is not readily available as an internal standard, must be considered an important limitation. The authors found the use of an internal standard necessary to compensate for analytical losses.

There are a number of liquid chromatographic assays for TMP, either alone or in combination with a sulphonamide, usually SMZ. The analysis of Helboe and Thomsen (1977) for sulphonamides and TMP in pharmaceutical preparations lacked the required sensitivity and did not yield chromatographically efficient peaks for TMP (Watson et al, 1980). Vree et al (1978) claimed to have an assay with sufficient sensitivity for the determination of plasma TMP levels. Unfortunately two levels of sensitivity were quoted, 0.1 mg/l and 0.75 mg/l (equivalent to on column

weights of 1.8 ng and 13.6 ng respectively) the former figure is unlikely with the UV detectors then available, the latter limit is probably correct, however such a concentration is too high for satisfactory pharmacokinetic profiles to be obtained. Bury and Mashford (1979) described an LC procedure for TMP but the peak was poorly resolved from early eluting material. The reverse phase method of Gochin et al, (1981) had satisfactory performance characteristics for TMP, but suffered from a lengthy extraction procedure. Reverse-phase ion-pair chromatography has been used succesfully (Watson et al, 1980).

All of the above authors employed reverse-phase chromatography. Two assays for TMP using normal phase chromatography have also been described. The procedure of Weinfeld and Macasieb (1979) required a large serum sample (2 ml) but used a relatively straightforward extraction procedure. The performance of the method was not reported. The eluant consisted of a mixture of chloroform, methanol, water and ammonium hydroxide. Another technique had a similar solvent composition (Ascalone, 1981) using dichloromethane in place of chloroform; a different extraction procedure was used and again there was no check on accuracy. As noted earlier these eluants are similar to those used for tricyclic structured drugs (Watson, 1979) although at the wavelength used (280 nm) their response would be attenuated.

Although TMP has been determined by differential pulse polarography (Brooks, de Silva and D'Arconte, 1973)

and lacked effective sensitivity, there is potential for the use of this technique as an HPLC detector; recently an electrochemical procedure for TMP has been described (Nordholm and Dalgaard, 1984), however the reported sensitivity of the electrochemical detector was no better than a UV detector under the conditions used.

2.2.4. Assays for TMP in Other Biological Fluids

TMP has been analysed in cerebrospinal fluid (Lichtenwalner et al, 1979), saliva (Eatman et al,1979; Sardi et al 1981) and in sputum (Hansen et al, 1973a,b; McIntosh et al 1983). Assay of the first two named fluids is simple as neither has any significant protein content and is not viscous, however sputum is viscous and is difficult to manipulate if too thick and liquefaction is necessary in order to improve its handling (Li, Lee and Baker, 1980; McIntosh et al 1983).

2.3. SIMULTANEOUS ASSAY OF SULPHONAMIDES AND TMP

Simultaneous determination of SMZ and TMP in biological fluids can only be succesfully achieved using chromatography, the spectrophotofluorimetric technique of Lichtenwalner et al (1979) is a two stage extraction of a single sample but requires further validation. The liquid chromatographic procedures for sulphonamides and TMP are all suitable for SMZ and TMP (Ascalone, 1981; Gochin et al , 1981; Weber et al 1983) to date there has been no report of a liquid chromatographic assay for SDMO with or without TMP.

MATERIALS AND METHODS

2.4. SULPHONAMIDE

2.4.1. Bratton-Marshall Assay.

i) Materials

The procedure used was based on that given by Varley et al (1976).

The following reagents were prepared in deionised water from Analar grade reagents. (British Drug Houses, Poole, Dorset, U.K.).

(a) Trichloroacetic acid (200 g/l)

(b) Sodium nitrite (1 g/l)

(c) Ammonium sulphamate (5 g/l)

(d) N(1-naphthyl)ethylenediamine dihydrochloride (0.5 g/l)

The sodium nitrite and ammonium sulphamate were prepared weekly and the N(1-naphthyl)ethylenediamine dihydrochloride was freshly prepared prior to each assay.

ii) Equipment

A Pye Unicam 1800 spectrophotometer (Pye Unicam, Cambridge, England) with 1 cm glass cuvettes was used.

2.4.2. Rieder (1972) Assay.

i) Materials

The following reagents were prepared as described below:

(a) McIlvain buffer (pH 5.5)

0.2 M citric acid and 0.4 M disodium hydrogen phosphate were prepared in deionised water from Analar grade reagents (BDH). One litre of buffer was prepared by mixing 430 ml of 0.2 M citric acid with 570 ml of

0.4 M disodium hydrogen phosphate and adjusted to pH 5.5 if necessary.

(b) 8 M hydrochloric acid

The following reagents were prepared prior to each assay as described below

(c) 2 M acetonic hydrochloric acid: 1 volume of aqueous 8 M hydrochloric acid was mixed with 3 volumes of acetone.

(d) Sodium nitrite (1 g/l) in acetone:water (3:1)

(e) Sulphamic acid (50 g/l) in acetone:water (3:1)

(f) N(1-naphthyl)ethylenediamine hydrochloride (1 g/l) in acetone:water (3:1)

Calibrators for both procedures were prepared from a stock solution of 2 g/l SMZ or SDMO in 0.5 M hydrochloric acid. SMZ was supplied by Wellcome Medical Division, Crewe Hall, Crewe, Cheshire) and SDMO was supplied by Berk Pharmaceuticals Limited, (St. Leonards Road, Eastbourne, Sussex). Calibrators covering the range 0-100 mg/l were prepared in Horse Serum No. 5 (Wellcome, Northwich, England) and urine calibrators over the range 0-750 mg/l were prepared in pooled drug-free human urine.

Quality control materials were independently prepared in the same matrix at concentrations of 50 mg/l and 150 mg/l SMZ for serum and urine respectively. All calibrators and quality control material were stored at -20^{o}C and thawed immediately prior to analysis.

ii)Sample handling

Serum was separated by centrifugation, and stored

at -20°C prior to analysis. Urine volumes were noted and 10ml aliquots were stored at -20°C prior to analysis

2.4.3. Procedures

i) Bratton-Marshall assay.

To 3 ml of deionised water in a Z10 glass tube (Brunswick, Ballymoney, Northern Ireland) was added 0.1 ml of sample. Proteins were precipitated by addition of 0.9 ml of 200 g/l trichloroacetic acid, the tubes were mixed and centrifuged at 2000 rpm for 5 minutes. Two ml of the supernatant solution were transferred to a fresh tube and the sulphonamide diazotised with 0.2 ml of 1 g/l sodium nitrite, the tubes were mixed and allowed to stand for 3 minutes. Excess acid was eliminated by the addition of 0.2 ml of 5 g/l ammonium sulphamate, the tubes were mixed and allowed to stand for a further 3 mins. Then 0.2 ml of 0.5 g/l N(1-naphthyl)ethylene-diamine dihydrochloride was added, the tubes were mixed and allowed to stand for 10 minutes.

The optical densities were read on an SP 1800 spectrophotometer (Pye Unicam) at 540 nm.

For urine, 0.1 ml of sample was added to 1.5ml of water and 0.5 ml trichloroacetic acid to which was then added sodium nitrite, ammonium sulphamate and N(1-napthyl) ethylenediamine dihydrochloride as described for serum; on completion of the reaction 5 ml of water was added and the optical density read at 540 nm.

ii) Rieder assay

To 0.2 ml sample diluted in 1 ml of McIlvain buffer in an MF 24/1/5 Q and Q tube (Corning Ltd., Staffs.)

was added 5 ml ethyl acetate. The tubes were stoppered and shaken for 10 minutes on a lateral shaker and then centrifuged for 5 minutes at 2000 rpm. 3 ml of the organic extract was transferred to a second tube and 0.5 ml of acetonic 2 M hydrochloric acid added; the tubes were mixed and 0.5 ml of 0.1% acetonic sodium nitrite added and the contents again mixed and left for 6 minutes 0.5 ml of 5% acetonic sulphamic acid was added and after mixing and the release of the gas bubbles produced, the tubes were left for 3 minutes and 0.5 ml of 0.1% acetonic N(1-napthyl)ethylenediamine dihydrochloride added; due to phase separation, 0.5 ml of methanol was added as co-solvent and the tubes mixed until the contents were homogeneous. The tubes were stoppered and incubated at room temperature for 30 minutes.

The absorbances were read on a Pye Unicam 1800 instrument against distilled water at 545 nm and corrected for serum blank.

Urine was diluted 0.1 ml to 1 ml in McIlvain buffer and extracted with 5 ml ethyl acetate and then the prodedure for serum was followed.

2.4.4. Experimental Design

i) Accuracy

The assay of Rieder (1972) is claimed to have higher specificity than procedures based on the method of Bratton and Marshall (1939). All urine and serum samples for SMZ and SDMO were therefore analysed by both assays and the results compared.

ii) Linearity

The linearity of the method was established using standards prepared as described earlier over the range 0-250 mg/l for serum and 0-1000 mg/l for urine. Serum and aqueous calibrators were prepared of both SMZ and SDMO and the results compared to establish if there were any differences in the intensity of the absorbance at 540 nm.

iii) Precision

The Rieder assay is an established assay and precision was confirmed using 12 serum samples with an added concentration of 50 mg/l and 30 urines randomly selected and re-assayed. The precision of the modified Bratton-Marshall assay was checked by re-assaying a randomly selected 30% of serum and urine samples.

iv) Sensitivity

Sensitivity was taken as twice the absorbance for the serum blank when read against a reagent blank.

2.5. ACETYLATOR PHENOTYPING

Subjects participating in the co-trifamole/co-trimoxazole study had their acetylator phenotype determined by a modification of the procedure of Schroder (1972)

2.5.1. Procedure

Subjects were given 10 mg/kg sulphadimidine by mouth and urine passed between the 5th and 6th hours post-dose was collected.

2.5.2. Assay

To 10 ul of urine was added 1 ml of 6 M hydrochloric acid and this was heated for 5 minutes at 90^{o}C (Test A).

To 50 ul of urine was added 1 ml of 6 M hydrochloric acid (Test B).

Test A is for total sulphadimidine and test B for non-N^4acetylsulphadimidine.

To both tests 1 ml of 0.1% sodium nitrite was added and after 2 minutes, this was followed by 1 ml of 0.5% ammonium sulphamate, left for 1 minute and then 1 ml of freshly prepared 0.05% N(1-naphthyl)ethylenediamine dihydrochloride was added. After 5 minutes at room temperature the tests were read at 540 nm versus a water blank.

The percent sulphadimidine was calculated as:

$$(1-B/A.5)100 = \% \text{ acetylated} \qquad \text{Equation 2.1.}$$

Fast acetylators are defined as those with > 80% acetylation and slow acetylators those with <70% acetylation. Those falling between 70-80% were equivocal.

In view of the suspect accuracy of non-extracted Bratton-Marshall procedures a study of ten individuals was performed using the procedure above and a procedure based on the Rieder technique as detailed below.

2.5.3. Extracted Procedure

10 ul urine was heated with 1 ml of 6 M hydrochloric acid for 5 minutes at 90°C then cooled (Test A).

50 ul urine had 1 ml of 6 M hydrochloric acid added (Test B).

Sufficient 5 M sodium hydroxide (approximately 1.2 ml) was added to obtain a pH of 5.5. To both tests was added

1 ml of pH 5.5 McIlvain buffer and 5 ml ethyl acetate. These were shaken for 10 minutes and centrifuged at 1500 rpm for 5 minutes. 3 ml of supernatant were transferred and 0.5 ml of acetone:8M hydrochloric acid (3:1) added, mixed and 0.5 ml 0.1% sodium nitrite in acetone:water (3:1)was added, mixed, the tubes were left for 6 minutes and then 0.5 ml 5% sulphamic acid in acetone:water (3:1) was added, mixed and following the release of all the gas bubbles, left for 3 minutes; 0.5 ml 0.05% N(1-naphthyl) ethylenediamine dihydrochloride in acetone:water (3:1) was added and 0.5 ml of methanol mixed in until the solution was homogeneous the tubes were stoppered and incubated for 45 minutes at room temperature and read at 545 nm versus a water blank.

The percentage acetylated was calculated using equation 2.1.

2.6. TRIMETHOPRIM

This was an LC assay.

2.6.1. Materials

The eluant initially used was methanol:sodium lauryl sulphate 6 g/l in 0.05M phosphoric acid (70:30) this was subsequently modified by the substitution of hexane sulphonic acid (Fison's Loughborough, England) for the sodium lauryl sulphate. When TMP was assayed using an autosampler and a Varian 8500 pump it was necessary to decrease retention by the addition of sodium nitrate; in these circumstances the eluant was methanol: hexane sulphonic acid 1 g/l in 0.05M phosphoric acid and 2 g/l sodium nitrate (30:70). 2M sodium hydroxide

was prepared in deionised water. Diazepam 250ug/l, (Roche Products Ltd., Welwyn Garden City, Herts, U.K.) or m-amino acetaphenone 300 ug/l (Aldrich Chemical Co. Ltd. Gillingham, Dorset U.K.) were prepared in dichloromethane and used as an internal standard for the serum assays only. All reagents were Analar grade from British Drug Houses, Poole, Dorset, England) unless otherwise stated.

Serum calibrators were prepared by weighing TMP (Berk Pharmaceuticals, Eastbourne, England) into horse serum No. 5 (Wellcome Reagents Ltd., Beckenham, England) over the concentration range 0-6 mg/l; urine calibrators were aqueous based and were prepared over the range 0-600 mg/l. Quality control materials were produced independently of the calibrators in horse serum No. 5 (Wellcome) and urine from a drug-free subject at levels of 1.5 mg/l for serum and 100 mg/l for urine.

Calibrators and QC material were stored at -20^{o}C until required and proved stable for at least one year.

2.6.2. Equipment

Assays for TMP were performed over an extended period of time and a number of items of equipment were used for liquid chromatography. In chronological order the following HPLC systems were used:

1. A Varian 8500 pump (Varian Associates Ltd., Walton on Thames, England) with a Varian stop-flow slide injector or latterly a Rheodyne 7120 loop injector (Rheodyne, Berkeley, California, U.S.A.) with a Varichrom (Varian) variable wavelength UV detector latterly

temperature controlled with a circulating water bath at 25°C to improve stability and a Chessel (Worthing, Sussex, England) single pen flat-bed recorder.

The stop flow injector originally used allowed purging of the pump, this is not possible with a Rheodyne injection valve and a'3 way-2 to pressure' valve was fitted for this purpose. One of the ports is a high pressure inlet, there is a high pressure outlet to the column and a low pressure outlet to waste. Pump flow had to be stopped prior to purging to avoid 'spurting' of eluant from the drain port.

Initially a 25 cm x 4.6 mm internal diameter Partisil 10/ODS-2 column (Whatman Ltd., Maidstone, England), a 10u C_{18} column was used, but this was soon replaced with a 5u C_{18} column of the same dimensions,an S5ODS column (Hi-Chrom Woodley, Berks, England).

2. The second configuration consisted of the above equipment with the addition of a Varian 8050 liquid autosampler and an Infotronics 304-40 integrator (LDC, Co. Clare, Eire) and the replacement of the above columns with 10 cm x 5 mmid columns packed in the laboratory with Hypersil 5u ODS (Shandon Southern Ltd., Runcorn, Cheshire England) using a balanced density slurry technique in an upward packing mode.

Initially a line from a Varian 8500 pump was fitted with a Whitey high pressure needle valve (Whitey Forge Co., London). Columns with Shandon fittings were attached to a slurry reservoir (Shandon Southern, Runcorn) and packed in the downward mode at 8500 psi using 1% sodium acetate

in 50% methanol as the balanced density solvent with methanol as follower solvent. Subsequently a Shandon column packer was used and packing in the upward mode at 10,000 psi was adopted as a standard technique, the balanced density solvent was changed to propan-2-ol with methanol as the follower solvent.

Column performance was checked using a test mix of 1 mg/l of phenol, o-cresol and acetone in water, the eluant was methanol:water (50:50) at a flow rate of 1 ml/min with detection at 254 nm. Efficiency in theoretical plates and peak asymmetry at 10% peak height were calculated; columns with efficiencies of less than 6500 plates (h=>3.0) for o-cresol (the longest retained peak) and asymmetry outwith the range 0.9-1.3 were rejected.

In use column performance could deteriorate and removal of the top 5 mm-1 cm of packing material and replacement with fresh, methanol-damped, material and tamping into place restored column performance.

3. The last configuration consisted of the items noted in 2. above with the following: A Pye Unicam LCX5 pump (Pye Unicam, Cambridge, England), a Rheodyne 7125 loop injector (Rheodyne) for manual injections, a Pye Unicam LC-UV variable wavelength UV detector and a Philips 8501 single pen recorder.

In addition other items that were required were a Pye Unicam 1800 spectrophotometer, a Pye Unicam 8000 scanning spectrophotometer and an Astrup pH meter (Radiometer, Copenhagen, Denmark) for the measurement of

salivary pH.

2.7. DEVELOPMENT OF A LIQUID CHROMATOGRAPHIC ASSAY FOR TRIMETHOPRIM IN SERUM AND URINE

The original intent was to develop a reverse phase LC assay for SMZ, SDMO and TMP. Due to the limitations of the procedures of Bury and Mashford (1979) and Vree et al (1978) for TMP, the procedure of Helboe and Thomsen (1977) appeared the most amenable to modification; when it proved impossible to reproduce their chromatography it was decided to develop a new method, as the assay of Weinfeld and Macasieb (1979) was the only other alternative and normal phase LC is more difficult to operate reliably for any extended period. The development of the procedure had two aspects: i) optimisation of the chromatography and ii) optimisation of the extraction.

Therefore systematic changes in methanol/water ratios were investigated followed by investigation of the effect of alkali or acid as a controller of TMP ionisation. Finally an anionic ion pairing agent was examined.

The sample preparation approach chosen was liquid/liquid extraction. Initial examination of the solvents: diethyl ether, chloroform, hexane, dichloromethane, ethyl acetate and toluene was performed using 1 M sodium hydroxide for extraction of aqueous 100 mg/l TMP standard. Ionisation control was examined by using 1 ml of 100 mg/l TMP standard and 5 ml dichloromethane plus one of: 100 ul ammonia solution (rel. dens 0.88), 100 ul of 1 M hydrochloric acid, 1 ml 0.1 M borate buffer pH 9.0, 1 ml of

0.3 M sodium carbonate, 1 ml of 1 M sodium hydroxide, 1 ml sodium bicarbonate; in addition ion pair extraction was attempted using sodium lauryl sulphate (0.1%) plus 100 ul 0.1 M HCl. The extract residues were examined spectroscopically by scanning from 210 nm-290 nm at 10 nm/sec.

Once the final solvent and alkali were chosen further experiments on recovery etc. were performed using LC and are explained in more detail below.

2.7.1.Linearity

To determine linearity as a function of on-column sample weight, 10 ul injection of aqueous TMP solutions over the concentration range 0-1000 mg/l (0-10 µg on column weight) were used.

2.7.2.Recovery

Serum trimethoprim standards covering the range 0-6 mg/l were analysed; the difference in detector response between these and directly injected aqueous standards represented the absolute percentage loss. Relative analytical recovery was assessed from the ratio of directly injected TMP/internal standard solution to comparable assayed serum standards at 0.5 mg/l and 1.25 mg/l with diazepam or 1 mg/l with m-aminoacetophenone as internal standard. Urine recovery is absolute, no internal standard is required.

2.7.3.Precision

The within-batch precision was assessed from alternate analyses of trimethoprim controls at two concentrations: serum at 0.45 and 1.25 mg/l with diazepam as internal

standard and 1 mg/l with m-aminoacetophenone as internal standard and for urine 100 and 200 mg/l; the values were assigned on the basis of preparation from a stock solution of TMP at 1 g/l (weighed in value) and prepared by dilution.

2.7.4. Accuracy

Accuracy may be judged from the results of the recovery studies. Potential interference was examined for a number of drugs in aqueous solution at 1 mg/ml (10 ug on column): acepifycline,, amitriptyline, amylobarbital, butriptyline, carbamazepine, chlordiazepoxide, clomipramine, chlormethiazole, chlorpromazine, clonazepam dapsone, desipramine, dextropropoxyphine, dothiepin, doxepin, fluphenazine, gentamicin, imipramine, lorazepam, nialamide, medazepam, methotrexate, nitrazepam, nortriptyline, oxyphenbutazone, paracetamol, penicillin, pericyazine, phenobarbital, prazepam, primaquine, primidone, promazine, promethazine, prothiaden, proxiphylline, salicylate, sulphadiazine, SMZ, SDMO, temazepam and trimipramine.

Thirdly the relative error for both serum and urine procedures were estimated using the formula:

$$\text{relative error} = t = \frac{\text{observed concentration} - \text{expected concentration (added)}}{\text{standard error}}$$

Equation 2.2.

and applied to the 0.45 mg/l serum standard and the 100 mg/l urine standard.

In addition a number of drug free samples were run.

2.7.5. Quantitation

Serum: Once linearity was established and found to be reproducible unknown serum concentrations were calculated as follows

$$T = \frac{R_T . S}{R_S} \qquad \text{Equation 2.3.}$$

where T = TMP concentration of unknown

S = TMP concentration of calibrator

R = $\frac{\text{TMP peak height}}{\text{internal standard peak height}}$

for calibrator (S) and test (T) respectively (sub-script).

Urine: External standardisation was used; concentrations ranged from 20-400 mg/l; to allow for any within batch chromatographic variation a 100 mg/l calibrator was injected after every fourth sample; the values obtained from this calibrator were referred to the value of the 100 mg/l standard in the calibration line, this was the 'target' value. Any variation in a drift standard was corrected and the correction applied to the two unknowns on either side of that calibrator.

2.7.6. Optimised Procedure

i) Serum

To 1 ml of serum (or less) in a Quickfit MF 24/1/5 tube (Corning Ltd.,Stone, Staffs) was added 1 ml of 2 M sodium hydroxide and 5 ml of internal standard solution. The tubes were tightly stoppered and shaken for 5 minutes

on a lateral shaker (Gallenkamp, London) then centrifuged at 3000 rpm for 5 minutes; the organic layer was transferred to a conical tube and the solvent evaporated in a water bath at 40°C under a stream of air. The residue was dissolved in 25 ul LC eluant and 10 ul injected via the sampling loop.

ii) Urine

A 10 ul sampling loop was used and 25 ul of sample or calibrator was injected.

2.7.7. Chromatographic Conditions

The eluant in the developed assay was pumped at 2 ml/min and detection performed at 230 nm at 0.2 AUFS for serum extracts and 1.0 AUFS for urine extracts.

2.7.8. Modified TMP Assay

Hexane sulphonic acid could be desorbed from the C_{18} phase using acetone, sodium lauryl sulphate could not; the 5 u Hypersil columns were shorter and the eluant flow rate was decreased to 1 ml/min. The selectivity of the Hypersil columns was very different from Spherisorb and retention was too great. Sodium nitrate was therefore added as counter-ion. The eluant for the modified assay was a 70:30 mixture of methanol and 0.05 M phosphoric acid containing 0.1% hexanesulphonic acid and 0.2% sodium nitrate. Detection was at 235 nm.

The substitution of hexane sulphonic acid for sodium dodecyl sulphate in the eluant and the addition of sodium nitrate quite clearly could be anticipated as having an effect on analytical performance. In addition Hypersil 5 u ODS is a more completely end-capped material.

For these reasons it was necessary to re-evaluate certain aspects of the assay performance; in particular accuracy, precision and sensitivity.

2.7.9. Modification of the Varian 8050 Autosampler

A Varian 8050 sampler operates under gas pressure which is used to force the analyte from the sample vial to the automatic loop, the sample also being used to flush the line (Fig 2.2) resulting in a minimum sample volume requirement of the order of 150 ul for a 10 ul loop (i.e. 7.5% loaded). In pharmacokinetic studies it is useful to maintain as good a sensitivity limit as possible, the operation was therefore modified.

The recommended inlet gas pressure to conform to the above sampling volumes is 20 psi; by placing a pressure gauge in the inlet line and continually monitoring the pressure to ensure that an inlet pressure of 7 psi is maintained it became possible to inject 10 ul from 70 ul i.e. 14.3% of available analyte.

To assess whether there was an adverse effect on high to low sample carryover sera containing 250 ug/l and 2.5 mg/l were assayed in consecutive pairs with a water wash vial between each sample, there were ten such pairs of each serum. Low to high sample carryover was also checked.

2.7.10. Accuracy, Precision and Sensitivity

Precision, accuracy and sensitivity were assessed as described previously.

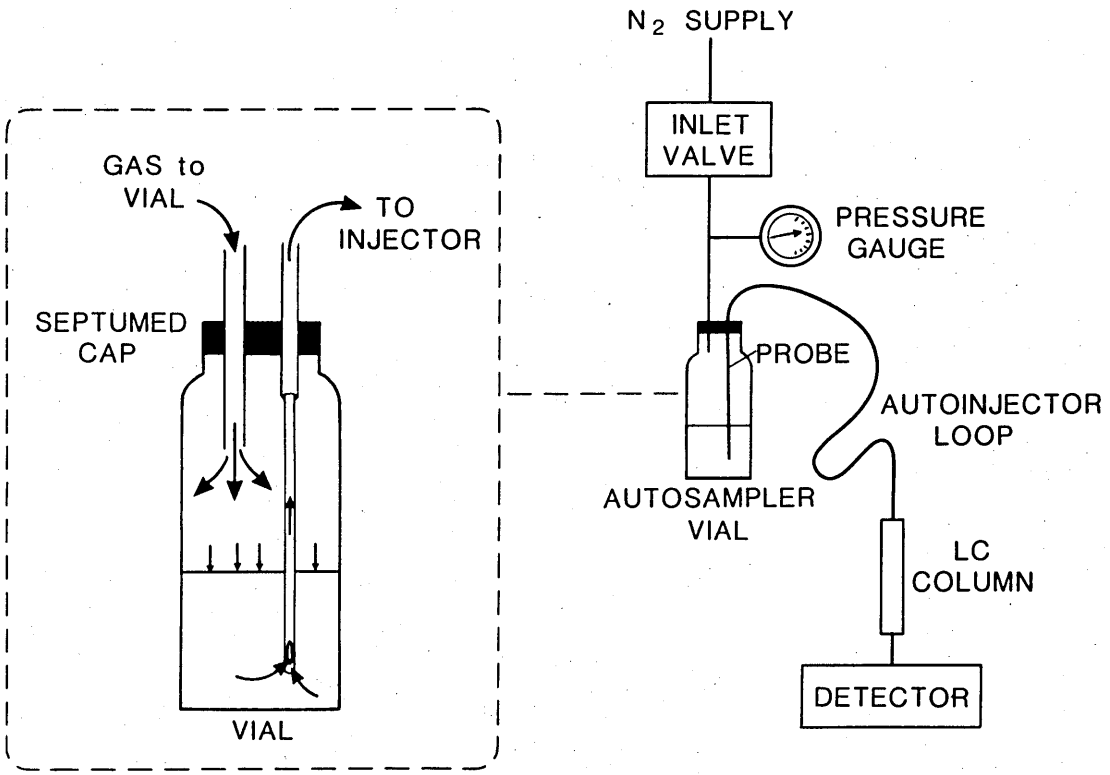


FIGURE 2.2. Principle of pneumatic sample feed of Varian 8050 sampler with in line pressure monitoring.

2.8. ASSAY FOR TMP IN SALIVA AND SPUTUM

2.8.1. Saliva

i) Sample Handling

Saliva flow was stimulated by mastication of a washed elastic band, the mixed saliva produced over the first two minutes was discarded and then saliva was collected into pre-weighed vials for five minutes or until 5 ml had been produced and the time noted, whichever was the shorter. The vials were weighed and the weight of saliva noted; The specific gravity of saliva is virtually 1.0 (Documenta Geigy, 1975). Immediately the collection was completed the saliva pH was measured using an Astrup micro pH meter (V.A.Howe, London) and the value recorded.

Samples were stored at -20^{o}C prior to analysis. Freezing prior to analysis also served to clarify the saliva and decreased its viscocity by precipitation of the mucopolysaccharides which facilitated subsequent handling.

2.8.2. Sputum

Sputum is a thick mucoid product secreted in response to bronchial irritation and is continuously produced in COAD and alters during exacerbations

i) Sample Collection

Expectorated sputum samples were collected in vials. These were labelled with time of collection and then split, one half being sent for bacteriological study, the other for assay of TMP. Following receipt, samples were stored frozen at - 20^{o}C prior to analysis.

ii) Sample Handling

The high viscocity of sputum requires that liquefaction be performed prior to analysis. The technique, developed in conjunction with Dr. D. Platt, Dept. Bacteriology, Glasgow Royal Infirmary was as follows: to a volume of sputum (1-5 ml) was added 2,3 dithiothreitol (diluted 1:10 in water) in the ratio 10:1. This was then incubated for 30-45 minutes at 60°C with occasional shaking. The viscosity was assessed by inspection and if the treatment was inadequate the lysis was repeated until a satisfactory result was achieved. This procedure also served to 'pasteurise' the specimen thereby excluding the possible dissemination of TB. Analysis was then as for serum.

iii) Analysis of Saliva and Sputum Samples

The modified TMP assay described above with the modified autosampler were used.

iv) Precision and Recovery

Pools of drug free saliva and sputum were obtained and spiked at TMP concentrations of 1.5 mg/l and 1 mg/l respectively

v) Calibration

Calibrators over the range 0-10 mg/l were prepared in pooled saliva and sputum respectively.

2.8.3. Ultrafiltration of Serum

To enable comparison of 'free' TMP and saliva, ultrafiltration of serum was performed using AMICON Centriflo CF25 membranes centrifuged at 3000 rpm for 30 minutes at 4°C. The ultrafiltrate was collected and stored at -20°C prior to assay.

RESULTS

2.9. SULPHONAMIDE ASSAYS

2.9.1. Linearity

The colour produced by both the modified Bratton-Marshall procedure and that of Reider was shown to obey Beer's Law from 0-250 mg/l in serum and 0-1000 mg/l for urine.

The relationship between concentration and absorbance for SDMO calibrators and from SMX calibrators were superimposable.

2.9.2. Sensitivity

The sensitivity of the assays was better than 5 mg/l but variation in the blank reading and poor discrimination of absorbance at this level made precise determination impossible. In use concentrations of less than 10 mg/l were not quantified.

2.9.3. Precision

The precision of the Bratton-Marshall procedure was 1.4% (n=114) and 4.6% (n=41) for serum and urine respectively and for the Reider procedure were 3.2% (n=12) and 7.6% (n=30) respectively.

2.9.4. Accuracy

i) Serum

There were no authenticated metabolites of either SDMO or SMZ available to allow assessment of their interference in the assays. The correlation of serum SDMO and SMZ levels assayed by both the modified Bratton-Marshall and Reider procedures is shown in Fig. 2.3 (r=0.984, gradient 1.04, intercept +3.55, n=61). In

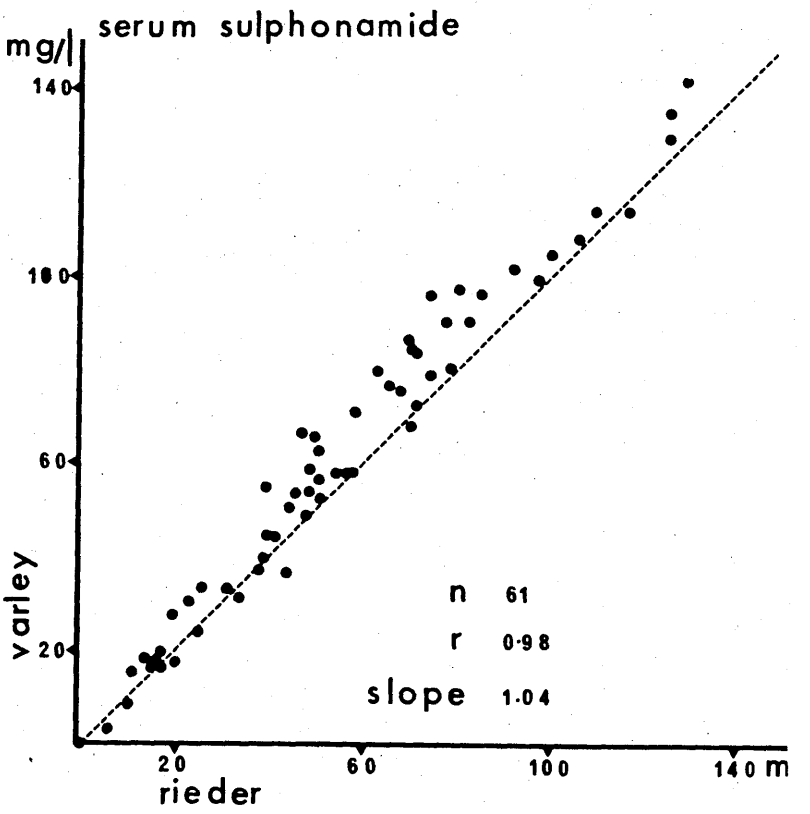


FIGURE 2.3. Association of serum sulphonamide using extracted (Rieder) versus unextracted (Varley) assays.

practical terms there is no difference in serum sulphonamide levels measured by either assay.

ii) Urine

Comparison of urine sulphonamide concentrations from subjects taking SDMO and SMZ showed statistical association (r=0.78, gradient 1.62, intercept 152.7, n=118). The gradient (Fig.2.4) shows quite clearly that there is a large overestimate of sulphonamide by the Bratton-Marshall technique and that the procedure of Rieder is more specific.

2.9.5. Acetylator Phenotyping Accuracy

Ten individuals 5 male and 5 female of between 18 and 30 years of age participated; none had any detectable physical, or biochemical abnormality. The phenotyping is given in Table 2.1. The procedure using the Reider adaptation was more clearcut in the group studied however it is interesting how for two subjects the percentage acetylated has risen significantly; both subjects KR and DD could have fallen into the equivocal category.

2.10. TRIMETHOPRIM

2.10.1. Original Assay

i) Development of the chromatography

There was no retention of TMP using the solvent of Helboe and Thomsen (1977); methanol/water gradients were used but were chromatographically inefficient. As TMP has a pKa of 7.3 and the packing material is only stable to a pH of 7.5 it was not possible to ensure that TMP could be kept in the free base form; by addition of acid to a pH of 5 or less the amino groups will be protonated.

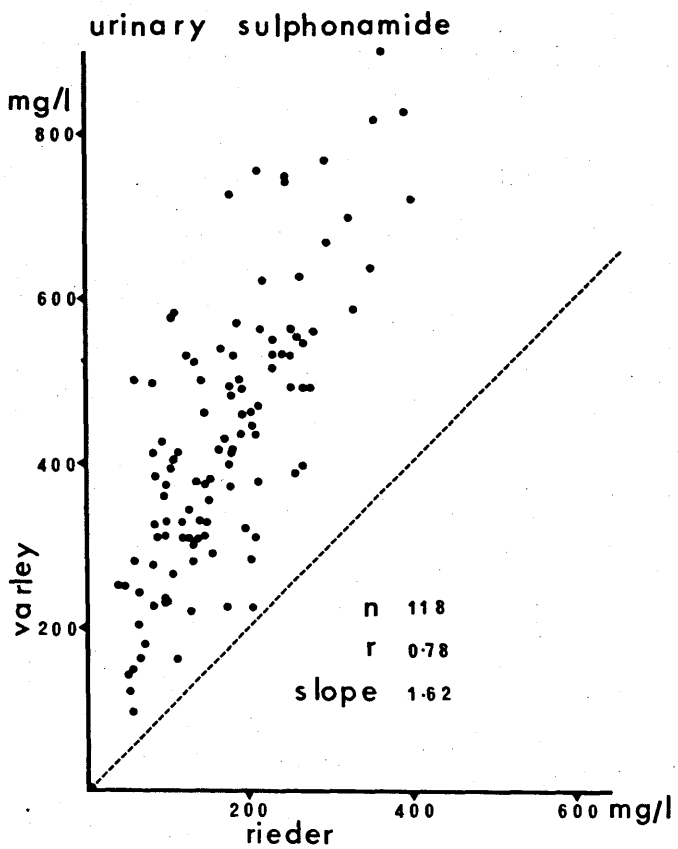


FIGURE 2.4. Association of urine sulphonamides using extracted (Rieder) versus unextracted (Varley) assays.

TABLE 2.1

Comparison of sulphadimidine phenotyping by extracted and non-extracted Bratton-Marshall procedures.

		NON-EXTRACTED		EXTRACTED	
Subject	Sex	% Acetylated	Phenotype	% Acetylated	Phenotype
K.C.	F	90	Fast	92	Fast
C.C.	F	89	Fast	88	Fast
K.R.	F	48	Slow	68	Slow
A.McL.	F	55	Slow	56	Slow
I.H.	F	89	Fast	85	Fast
D.D	M	54	Slow	70	Slow
I.S	M	89	Fast	88	Fast
G.H.	M	53	Slow	46	Slow
D.McM.	M	90	Fast	91	Fast
R.S.	M	78	Equivocal	58	Slow

$\geq$80% Fast, $\leq$70% Slow, 70-80% Equivocal

The chromatography obtained using methanol:water (90:10) is shown in Fig. 2.5.

At an acid pH the cationic state of TMP can be utilised to form ion-pairs with an anionic surfactant resulting in ion-pair chromatography. Various acetonitrile: 0.05 M phosphoric acid: sodium lauryl sulphate eluants were tried, a typical chromatogram with eluant ratios of 60:40:0.1(v/v/w) is shown in Fig2.6, a very similar pattern but with better resolution was seen with methanol:0.05 M phosphoric acid:sodium lauryl sulphate (15:85:0.2)(v/v/w). (Fig. 2.7).

Typically the k' of the sulphonamides was <1 and α for SDMO and SMZ tended to 1. The primary interest therefore was in the development of an assay for TMP. Although useable peaks were obtained in neutral, basic and acidic solvents the best chromatographic efficiency was obtained with ion-pairing; a comparison of this and other parameters is given in Table 2.2. Neutral and basic solvents were the least efficient; controlling the pH and chromatographing TMP in its protonated form doubled the efficiency but retention was poor with minimal resolution from the sulphonamides. The addition of an anionic ion pair improved efficiency by a further threefold (sixfold in all), improved retention and hence resolution from the sulphonamides, and is clearly the eluant of choice.

ii) Development of the extraction

The results of single stage basic extraction into a variety of solvents is summarised in table 2.3; the chlorinated solvents gave best recoveries but recovery

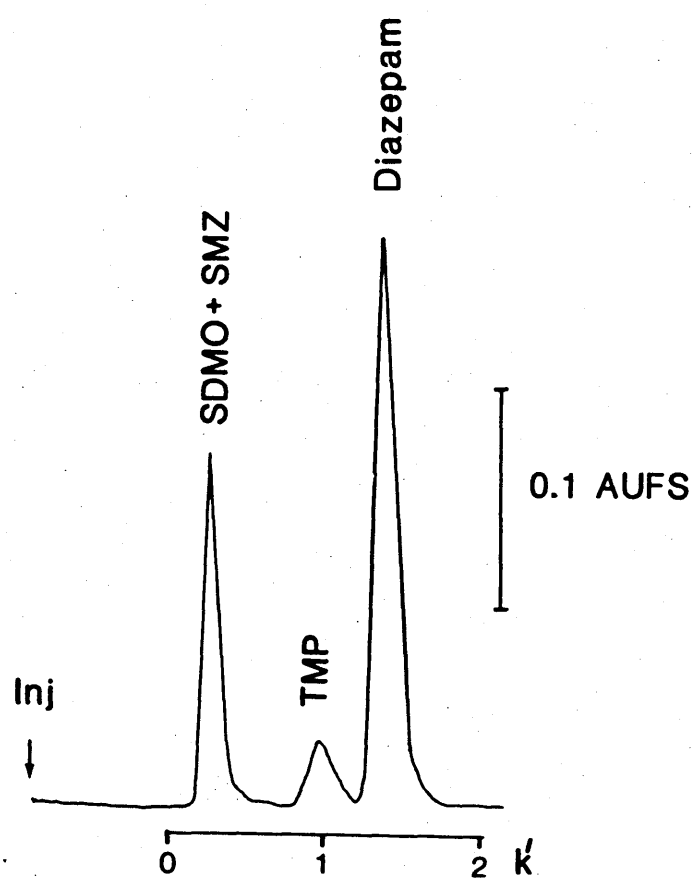


FIGURE 2.5. LC of TMP, SDMO and SMZ with methanol/water (90:10) eluant. Diazepam as internal standard.

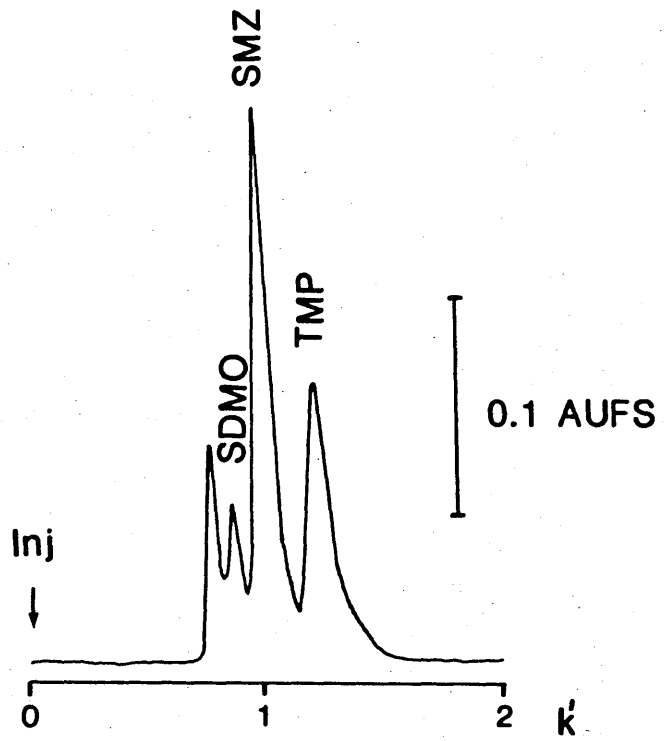


FIGURE 2.6. LC of TMP, SDMO and SMZ with acetonitrile/0.05M phosphoric acid/sodium lauryl sulphate (60:40:0.1) (v/v/w), no internal standard.

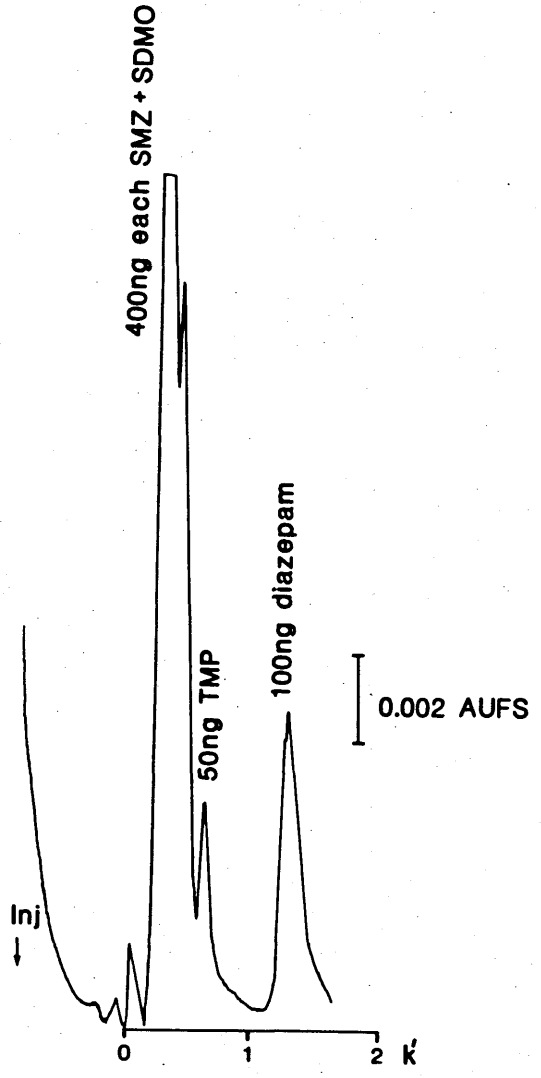


FIGURE 2.7. LC of TMP, SDMO and SMZ with methanol/0.05M phosphoric acid/sodium lauryl sulphate (15:85:0.2) (v/v/w). Diazepam internal standard.

TABLE 2.2

Comparison of capacity ratio (k'), reduced plate height (h) and resolution function (Rs) in neutral, acidic, basic and ion pair eluants

Eluant Composition	k'	h	Rs
Methanol:Water (90:10)(v/v)	1.1	30	1.5
Acetonitrile:0.05 M phosphoric acid(25:75)(v/v)	1.3	15	0.3
Methanol:Water:Ammonia(rel. dens. 0.88)(90:10:0.1)(v/v/v)	1.1	30	1.5
Methanol:0.05 M phosphoric acid:Sodium lauryl sulphate(70:30:0.2)(v/v/w)	3.5	5	3.0

TABLE 2.3

Solvent extraction of TMP : 1M sodium hydroxide as base and various solvents (ranked in order of increasing polarity) from Snyder and Kirkland (1974)

Solvent	δ	δ_A	δ_B	Approximate Absolute Recovery(%)
Hexane	7.3	0	0	0
Diethyl ether	7.4	2.0	0	20
Ethyl acetate	8.6	2.0	0	55
Toluene	8.9	0.5	0	0
Chloroform	9.1	0.5	3.0	80
Dichloromethane	9.6	0.5	0	80
Dichloromethane/Isopropanol(98:2)	9.7	0.6	0.1	80

δ Hildebrand solubility parameter

δ_A proton-acceptor parameter

δ_B proton-donor parameter

did not seem related to the proton donor effects of the solvents used. The use of alcohol to reduce adsorption or the use of silanisation both resulted in recoveries of around 80%.

Spectrophotometry of the aqueous phase confirmed that approximately 20% of TMP was still present.

As TMP is a base, a basic extract into the organic solvent should be effective. A number of choices were investigated including ion-pairing from acid conditions. The results are summarised in table 2.4. Sodium hydroxide solution gave the best recovery. The ion-pairing result indicates the potential of this technique.

As suggested by the results in tables 2.3 and 2.4 a dichloromethane extract with 1 M sodium hydroxide was chosen for further investigation. The absolute recovery for TMP in this system was around 80%. Initially diazepam was used as internal standard, but latterly m-amino-acetophenone was also used, both gave absolute recoveries of around 80%.

Using manual extraction, recovery of TMP had been variable and usually significantly lower than the figures quoted above. However it had been realised early on that the use of a mechanical shaker improved recovery and reproducibility, and this was used in all the work reported here.

The assay was then investigated with regard to its applicability to serum. Direct injection of urine did not result in any significant peaks in the region of TMP and

TABLE 2.4

Solvent extraction of TMP : dichloromethane as solvent and various reagents for pH control (ranked in order of decreasing pH)

Reagent	Approx pH of Aqueous Phase After Addition	Approximate Absolute Recovery(%)
Sodium Hydroxide	12.9	75
Sodium Carbonate	11.5	35
Ammonia Water	11.3	20
Borate Buffer	9.0	10
Sodium Bicarbonate	8.3	50
Hydrochloric Acid	1.0	0
Hydrochloric Acid + Sodium Lauryl Sulphate	1.0	40

thus direct injection was pursued for urine analysis of TMP. The performance characteristics of the serum extraction and urine injection methods are described below.

iii) Linearity

Linearity was demonstrable as a function of on-column weight over the range 0-6 ug. As the assay was originally constituted this represented a linear concentration range of 0-18 mg/l for serum and 0-600 mg/l for urine.

iv) Recovery

a) serum

Absolute recovery was dependent on extraction efficiency and the amount of solvent transferred, this was found to be $80\pm10\%$. Recovery relative to diazepam as internal standard at two concentrations 0.5 mg/l and 1.25 mg/l was $97.5\pm9.6\%$ and $98\pm9.2\%$ respectively. With respect to m-aminoacetophenone at 1 mg/l recovery was $101.6\pm7.7\%$. Each mean above is the result of thirty determinations.

b) urine

Absolute recovery was $100\pm1.0\%$ (n=10).

v) Sensitivity

Serum and Urine

Sensitivity, defined as twice the signal to noise ratio, was 8 ng on-column weight using 5u ODS material and 30 ng on-column weight for 10u material. These were equivalent to 25 ug/l and 90 ug/l respectively for serum

and 800 ug/l and 3 mg/l for urine.

vi) Precision

a) serum

With diazepam as internal standard the mean value for the 0.45 mg/l material was 0.442 mg/l (CV=6.1%) and for the 1.25 mg/l the mean was 1.229 mg/l (CV=4.6%) for within-batch precision the equivalent data for m-amino-acetophenone for 1.0 mg/l was a mean of 1.022 mg/l (CV=7.9%).

The corresponding between batch data for the 0.45 and 1.25 mg/l samples were: mean 0.443 mg/l (CV=6.6%) 1.229 mg/l (CV=6.3%) and for m-aminoacetophenone at 1.0mg/l 1.01 mg/l (CV=9.0%).

Each mean quoted above was based on 30 determinations. In addition to the above, one-third of all serum and urine analyses in the co-trifomole study were repeated on randomly selected samples. The precision found for serum and urine assays were 4.8% (n=121) and 4.6% (n=40) respectively. The precision of the m-aminoacetophenone internal standard was less satisfactory and diazepam was routinely used as internal standard.

b) urine

The within-batch precision for the urine assay at 100 and 200 mg/l was 98.3 mg/l (CV=3.3%) and 205.2 mg/l (CV=1.5%)(n=30 in both cases). A further thirty injections at each level for between batch precision gave means of 98.9 mg/l (CV=3.6) and 205.0 mg/l (CV=2.0%) respectivley.

vii)Accuracy

Of the drugs chromatographed, the sulphonamides were poorly extracted, but some basic drugs were extracted,

only carbamazepine had a k' near that of TMP on the 10u ODS column (k'TMP=2.7, carbamazepine 2.1 - Rs=0.8). With a more efficient 5u ODS column these were separated. If m-aminacetophenone is used as an internal standard, it was found that even on a 5u ODS column neither lorazepam nor nitrazepam were completely resolved. (Rs= 1.0). No interference was noted from endogenous components in the serum assay. In the urine assay a small endogenous peak equivalent to 5 mg/l could not be resolved from TMP using 10u ODS (Rs=0.3, h=20), however resolution was obtained using 5u ODS (Rs=1.25, h=5). Several hundred assays have been performed with no interference noted.

The calculated relative error for a serum concentration of 0.45 mg/l and a urine concentration of 100 mg/l gave t values of 1.4 and 1.65 respectively, these were not significant at the 5% level.

viii) Separation

A chromatogram obtained from an extract of serum from a subject taking trimethoprim, with diazepam as internal standard is shown in Fig 2.8a; an extract from a subject prior to medication is shown in Fig 2.8b; the differences in internal standard peak height are due to differences in injected volume (a, 10ul; b, 20ul). A chromatogram of a serum TMP extract with m-aminoacetophenone is shown in Fig 2.9.

The chromatogram obtained from the urine of a subject taking TMP is shown in Fig 2.10a and the chromatogram obtained prior to dosing is shown in Fig 2.10b.

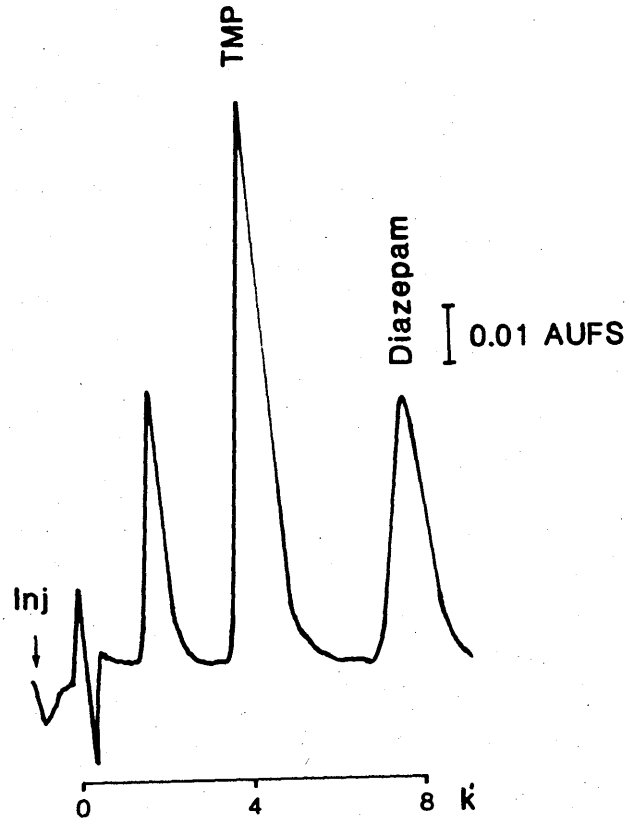


FIGURE 2.8a. LC of serum extract from subject dosed with TMP. Diazepam as internal standard

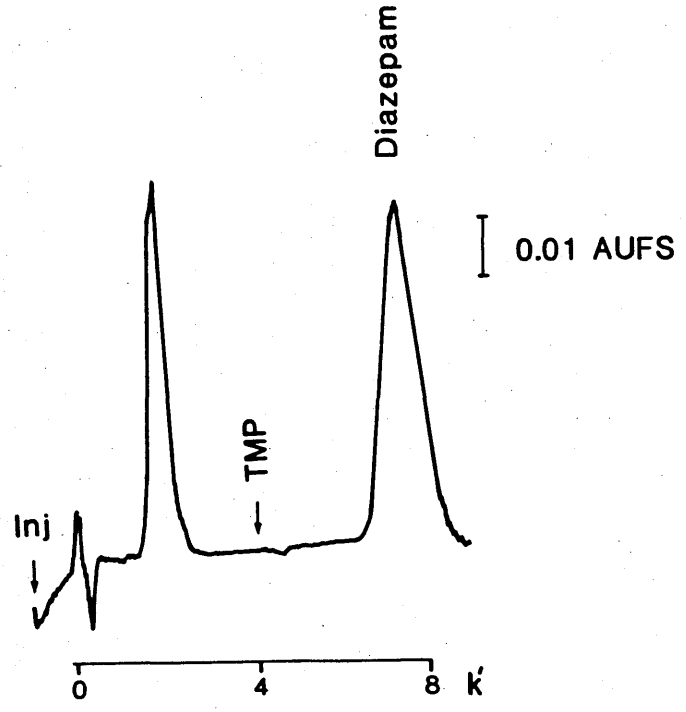


FIGURE 2.8b. LC of serum extract from subject prior to dosing with TMP. Diazepam as internal standard.

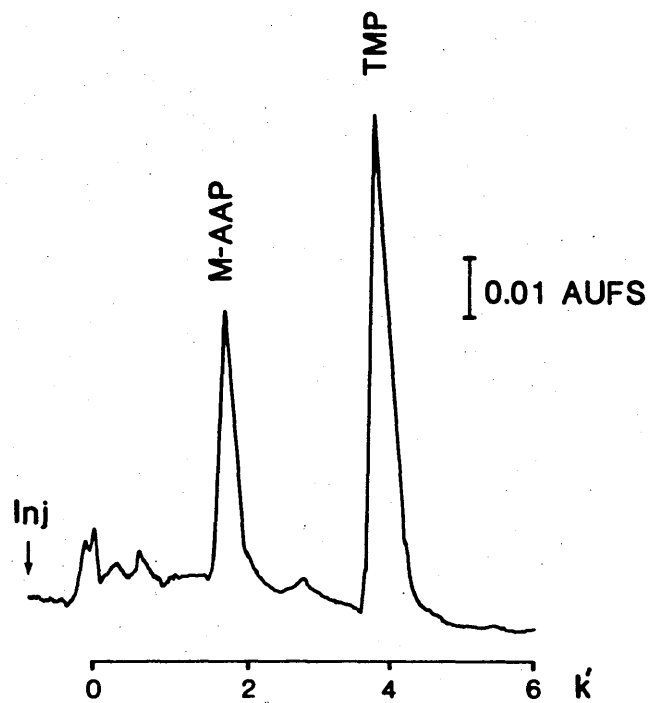


FIGURE 2.9. LC of serum extract from subject dosed with TMP. m-aminoacetophenone as internal standard.

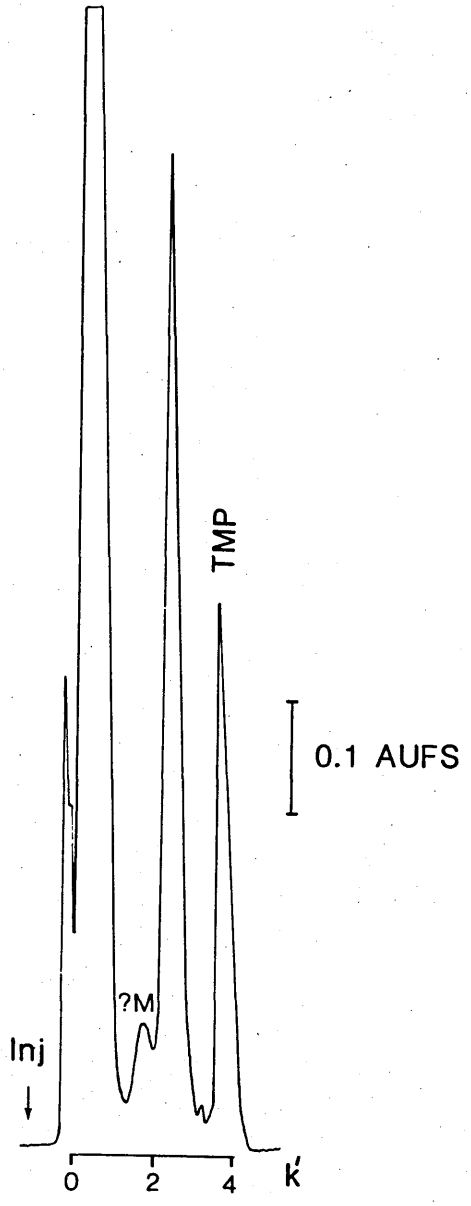


FIGURE 2.10a. LC of direct injection of urine from subject dosed with TMP. ?M = ? metabolite

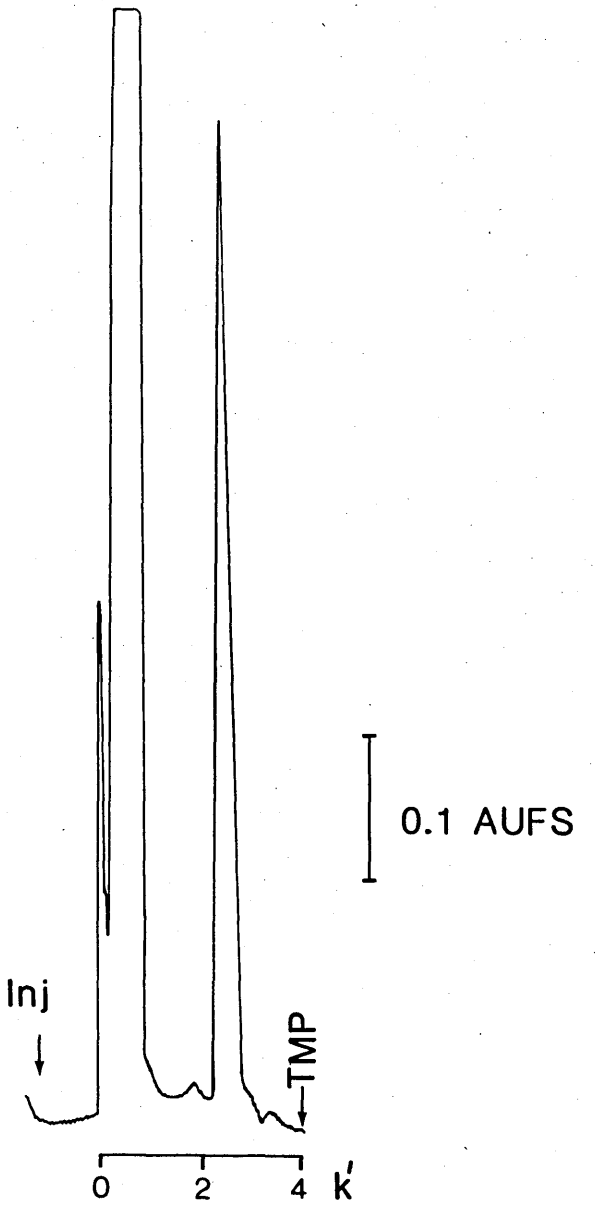


FIGURE 2.10b. LC of direct injection of urine from subject prior to dosing with TMP.

2.10.2 Modified TMP Assay

i) Separation

The change to 10 cm x 5 mm id 5u Hypersil ODS columns and the substitution of 0.1% hexane sulphonic acid for 0.2% sodium lauryl sulphate resulted in increased retention (TMPk'=5.7, diazepam k'=9.7) with a total chromatogram time of 17 minutes (Fig 2.11). Addition of 0.2% sodium nitrate decreased retention (TMPk'=1.9, diazepam k'=3.6) with a total chromatogram time of 7 minutes (Fig 2.12).

ii) Accuracy

The accuracy of the assay was evaluated in the same manner as described above, carbamazepine did not interfere with the estimation of TMP and the endogenous peak did not interfere with TMP. Diazepam was the internal standard of choice and interference with m-aminoacetophenone was not checked. No new interferences were noted.

iii) Linearity and Sensitivity

The linearity and sensitivity as a function of on-column weight remained unchanged, however when the modified assay was used in conjunction with the autosampler the concentration range of linearity for serum and urine was 0-50 mg/l and 0-600 mg/l respectivley.

The sensitivity limits for serum and urine calculated from the percent of analyte presented are 85 ug/l and 800 ug/l respectively; however if nitrate is used as counter-ion it absorbs at 230 nm. Offsetting to 235 nm decreases the signal to noise ratio but is still larger than that previously noted and the measured sensitivity

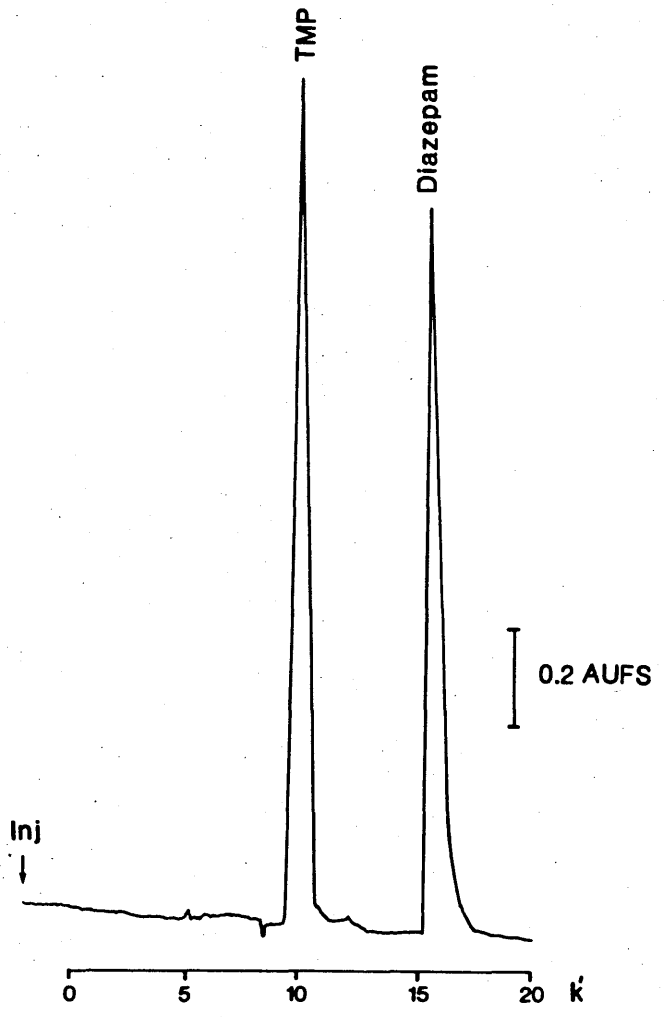


FIGURE 2.11. Modified LC eluant for TMP, containing hexanesulphonic acid in place of sodium lauryl sulphate. Column packing material changed to 5μ Hypersil ODS, flow rate also reduced.

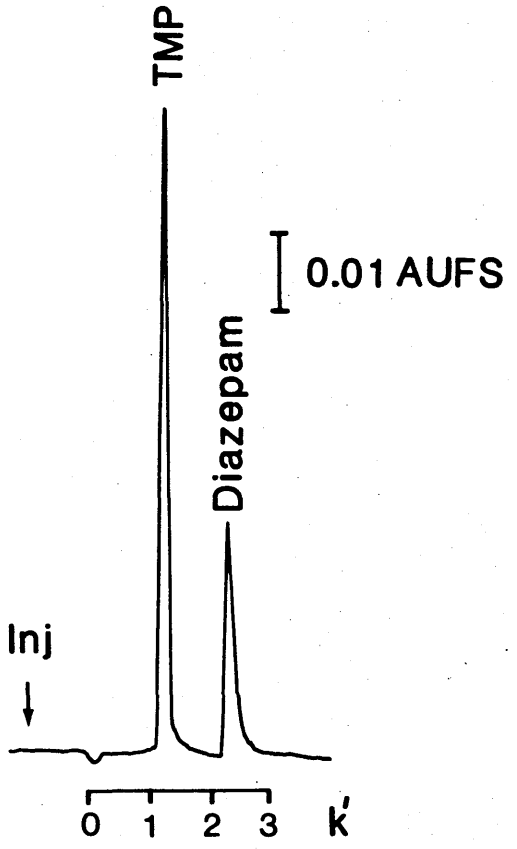


FIGURE 2.12. Conditions as for FIG 2.11 but eluant includes sodium nitrate as a counter-ion.

limits are 200 ug/l and2.0 mg/l respectively for serum and urine. By reverting to the manual injection procedure, but with nitrate in the eluant the detection limits were 70 ug/l and 2 mg/l i.e. a decrease in sensitivity of approximately 2.5 for both assays. These limits are well below those that were expected to be encountered.

iv) Precision

The precision was checked initially and was 2.9% (n=17) at 2 mg/l TMP further validation is given in the assay for saliva and sputum.

2.10.3 Autosampler Modification

As the autosampler used the injected sample to flush the system of the previous sample there is a need for an intermediate wash vial. Reducing the pressure decreases the flush volume and can therefore lead to increased carryover. Sampling a serum of 250 ug/l followed by a water wash vial and then by a serum of 2.5 mg/l there was no observable decrement in the observed concentration of the second vial i.e. carryover is 0% (n=10 pairs) i.e. there is no 'negative carryover'. Sampling a serum of 2.5 mg/l followed by a water wash vial and then a serum of 250 ug/l led to an increment in the observed concentration of the second vial of 2% (n=10 pairs) i.e. equivalent to 5 ug/l. There is therefore 'positive carryover' but this is insignificant.

2.10.4. Assay for TMP in Saliva and Sputum

i) Recovery

Absolute recovery of TMP added to saliva and sputum was 91±8% (n=10) and 84±9% (n=10); recovery

relative to the internal standard was 100±3% and 104±4% respectively.

ii) Precision

The precision for TMP saliva analysis was 10.9% (n=21) for saliva samples assayed in duplicate. For sputum the precision was found to be 5.6% (n=16) for samples run in series. There was no apparent reason for the differences in precision.

iii) Accuracy

The components of 2,3 dithiothreitol eluted prior to TMP. No interferences other than those previously noted were found.

iv) Separation

Chromatograms from saliva and sputum containing TMP are shown in Figs. 2.13 and 2.14 respectively.

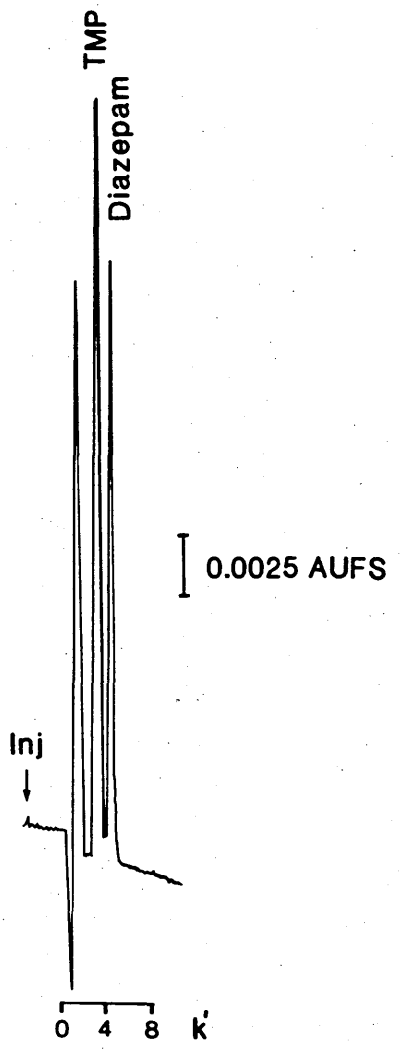


FIGURE 2.13. Condition as for FIG 2.12; LC of saliva extract from subject dosed with TMP.

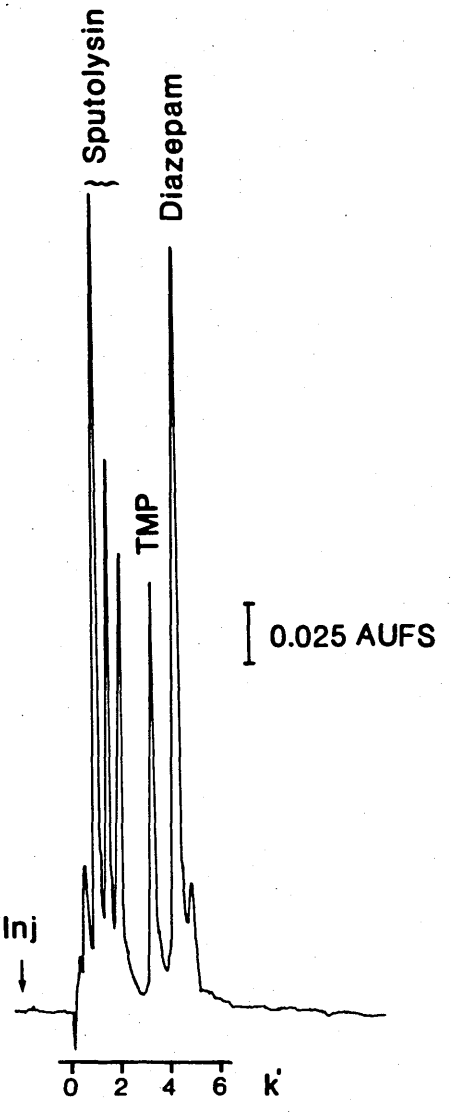


FIGURE 2.14. Conditions as for 2.12: LC of liquefied/pasteurised sputum extract from subject dosed with TMP. Sputolysin is the liquefying agent.

DISCUSSION

2.11 SULPHONAMIDE ASSAY

The assay of Rieder (1972) for sulphonamides is well established as the procedure of choice in pharmacokinetic studies (Bergan and Brodwall, 1972; Wilfert, 1973; Nolte and Buttner, 1974; Liedtke and Haase, 1979; Broughall et al, 1979; Ortengnen, et al 1979a; Trottier, Bergeron and Lessard, 1980; Singlas et al, 1982) and despite the amount of manual manipulation, acceptable precision was obtained. As this assay gave lower levels for serum sulphonamide than the standard non-extracted assay this implies greater specificity although in practical terms there is minimal difference. With extracted serum sulphonamides there is minimal return for the extra effort and the non-extracted assay tends to be more convenient.

The marked differences seen for the urine sulphonamide concentrations supports the contention of Rieder (1972) that the extraction step yields a more specific assay and there is no evidence to indicate that any significant interference occurs from metabolites in this method. Rieder did not perform a comparison as described here and it is surprising that there is an apparent absence of reports in the literature. In addition to its accuracy the procedure had good linearity, sensitivity and precision and is amenable to batch analysis.

In view of the large number of pharmacokinetic papers that have used the method of Rieder for sulphonamide analysis it is remarkable that the technique has

not been applied to the determination of acetylator phenotyping using sulphadimidine. A number of analytical approaches have been adopted ranging from qualitative assessment (Schroder 1972) to fairly sophisticated models (Lee and Lee 1982).

Of the individuals assessed in the phenotyping experiment none of them changed classification although an equivocal typing using the Bratton-Marshall procedure was typed as a slow acetylator with the extracted procedure. The percent acetylated differed markedly in three individuals one of which was the previously equivocal phenotyping. For subject K.R. there was an increase of 20% and for subject D.D. an increase of 16% using the modified assay; as the percent acetylated is calculated by difference then overestimation of the unmetabolised sulphonamide will lead to underestimation of the percent acetylated. These individuals must apparently have a reactive metabolite eg. N^1acetylsulphadimidine which is not estimated using the extraction procedure. In both of these cases clear 'slow' metabolisers have come very close to the 'equivocal' range and it is probable that this range would no longer be applicable. The distribution of fast and slow metabolisers are consistent with the distribution to be expected in a Caucasian population (Curry, 1980).

2.12 TRIMETHOPRIM ASSAY

The lack of retention using the eluant of Helboe and Thomsen (1977) may be attributable to different column selectivity, although the columns initially used were

not notable for being well end-capped. In the investigation of methanol/water eluants there was minimal retention of SDMO and SMZ, both co-eluted with a k' of <0.5; the TMP peak was asymmetric (As=1.8). It had been suggested by Helboe and Thomsen (1977) that the addition of alkali would increase the k', this appeared to have no notable effect although the peak shape improved (As=1.5), such an effect would suggest that the mechanism was normal phase chromatography and would tend to be confirmed by the use of ammonia in normal phase assays for TMP (Weinfeld and Macasieb, 1979; Ascalone 1981). All reverse phase assays for TMP use an acidic eluant with a pH <5.0 i.e. at least two pH units below the pKa of TMP (7.3), the change from neutral and alkaline eluants was associated with an improvement in peak shape, it has been suggested that strong acids should be used to effect this improvement (Helboe and Thomsen, 1977).

In all the assays described to date (Helboe and Thomsen, 1977, Vree et al 1978, Weinfeld and Macasieb, 1979; Bury and Mashford, 1979; Ascalone, 1981; and Gochin et al 1981) the order of elution is TMP followed by SMZ despite the fact that the procedures of Weinfeld and Macasieb (1979) and Ascalone (1981) were normal phase procedures whilst the others are reverse phase assays. In all conditions observed in this work including the solvent of Helboe and Thomsen (1977) on a variety of columns, SDMO andSMX eluted before TMP. There are two possible explanations either the column selectivity used in this work is different or the solvent selectivity is

different. The former possibility seems unlikely as Bury and Mashford (1979) also used a Spherisorb ODS column, and the second explanation appears equally unlikely as Gochin et al (1981) used a methanol:acetic acid eluant.

0.05 M phosphoric acid has a pH of 2.2, the dissociation will be attenuated by organic solvent as will the dissociation of the drugs, SMZ having a pKa of 5.6 and SDMO of 7.4. Thus at pH2.2 the primary amino groups on TMP, SMZ and SDMO will be protonated; the addition of an anionic ion-pair resulted in an increase in retention of TMP but not the sulphonamides, the k' of which remained at ≈0.3 thus any protonation of the sulphonamide primary amino group is not utilisable due to the inductive acidity of the substituted sulphonic acid.

In normal phase chromatography when ammonia is used in the eluant this serves to reduce the apparent acidity of the silanol groups which behaves as an ion-pair to anionic species and as a counter-ion to cationic species. The presence of alcohol in the eluant in addition to acting as a co-solvent and liquid layer to 'protect' the silica will act as a partition phase for hydrophilic compounds. A fuller account of the mechanisms is given elsewhere (Watson, 1977 ; Watson, 1979). If the above is true then anionic species should have increased retention and cationic species decreased retention i.e. TMP should elute before SMZ. In a reverse phase system that is totally free of normal phase effects then to achieve such an order of elution a lipophilic cation

should be used. It would be predicted therefore that a lipophilic anion will decrease SMZ k' and increase TMP k' which is what is observed. This does not explain the finding of this elution order prior to the addition of the ion-pairing agent.

From simple partition theory in normal phase chromatography the least polar compound should elute first followed by thosewith increasing polarity, such elution orders may not be those intuitively expected, but can be predicted (Watson and Stewart 1977a and b), in reverse phase systems the reverse is true i.e. the most polar compound is eluted first and this is followed by those with decreasing polarity. TMP is weakly basic whereas SMZ is more strongly acidic; TMP is less polar than SMZ, therefore in normal phase systems TMP should elute before SMZ and in the reverse order in reverse phase systems unless the eluant so modified one or other of the molecules, eg. acetate ion-paired to the primary amine group in the separation of Bury and Mashford (1979) and Gochin et al (1981) this would increase the apparent polarity of TMP, but not SMZ and cause earlier elution. The incomparability of the k''s obtained in this work and that of Helboe and Thomsen (1977) cannot be explained by this last mechanism as their eluant was acetonitrile/phosphoric acid. As the only difference was the column, and the results obtained in this work accord with theory, it is possible that what they observed was a mixed normal/reverse phase partition with the selectivity of the former dominating. As additional support for this contention Cobb and Hill

(1976) using normal-phase chromatography, report the elution order of the sulphonamides as SMZ followed by SDMO, implying that SDMO is more polar than SMZ, although it may be sterically hindered. If the former explanation is correct then the elution order on reverse phase should be SDMO followed by SMZ; in the early stages of the TMP assay development, this was the order observed, it therefore is most probable that reverse phase chromatography predominated in this assay.

In the assay described here a lipophilic long chain (C_{12}) anion was used to ion-pair the protonated TMP. The advantages of this technique have been known for some time (eg. Knox and Jurand, 1976) and the expected improvment in efficiency and selectivity was found. It seems probable that the predominant mechanisms operating in this separation are: ion-pair retention of cations, solute counter-ion elution of anionic solutes and reverse phase partition.

Preliminary studies had shown that with a 10u ODS column with a limit of sensitivity of 35 ng on-column weight, reproducible quantitation was possible at twice this level. If 100 ul each of serum and protein precipitant with injection of 50 ul of the supernatant were performed this would give a sensitivity limit of around 275 ug/l. Bury and Mashford (1979) used a 20:1 ratio of sample:precipitant and 100 ul injection and a sensitivity limit of around 200 ug/l. To improve assay specificity, sensitivity and minimise the column deterioration frequently found with such protein precipitation

techniques, liquid/liquid extraction was used.

Partition of analytes into the organic phase is most readily achieved when the analyte is in its most lipophilic i.e. unionised form, for TMP this means a pH >9.3; and this lipophilic form must be soluble in the solvent, essentially reflecting its polarity. The most suitable solvent was chosen from a range of medium to non-polar solvents, the chlorohydrocarbons investigated were found to give the best recoveries. Dichloromethane was chosen as it has a much lower boiling point than chloroform,thus facilitating the evaporation stage, and was also less toxic. Recovery was not consistently related to any solvent solubility parameter.

Generally the more basic the aqueous phase the better the absolute recovery with sodium hydroxide yielding the best result, the recoveries using sodium carbonate or ammonia water seem somewhat low. At a pH of 1.0 a recovery of 0% would be expected as protonated TMP is hydrophilic, however the addition of an anionic ion-pair reagent to the organic phase gave a recovery of 40%. The anionic ion-pair was the same as that used in the chromatography and the mechanism of partition is the same i.e. the ion-pairing agent enhances the lipophilicity of TMP increasing its partition into the organic phase.

Absolute recoveries in the dichloromethane/sodium hydroxide system tended to be of the order of 80%, spectrophotometry of the aqueous phase suggested that 10-20% of TMP remained in this phase following the first

extraction although there was some loss of organic phase during the removal of aqueous phase; absolute recovery could have been enhanced by a second extraction step which would have improved absolute recovery up to 96%, however the extra complexity involved was unnecessary as recovery was fairly consistent and, more importantly, variations were compensated for using on internal standard. Internal standards should exhibit physicochemical properties very similar to the analyte, thus they should partition to the same extent on extraction, have the same stability and separate from the analyte on chromatography without interfering with the analysis or resulting in excessive assay times. The ideal internal standard is an analogue of the analyte, unfortunately these can prove very difficult to obtain and thus some other compound must be chosen.

Initially diazepam was chosen as an internal standard which extracted and chromatographed well, in the studies in which the assay to be used other medication, including diazepam were excluded. If the assay were to be of use in a more general population the common use of diazepam could cause inaccuracy therefore an alternative internal standard was sought; m-aminoacetophenone chromatographed before TMP and thereby helped to cut assay times but was less precise, m-aminoacetophenone has a boiling point of 298^{o}C but some losses, presumably due to decomposition occurred and care in controlling the temperature was required; when using this internal standard an evaporation temperature of 35^{o}C was more reliable.

The above highlights some of the difficulties of choosing an internal standard, particularly in the case of m-aminoacetophenone in which instance there was imprecision introduced into the assay due to the inconsistent performance of the internal standard. This type of behaviour has been reported before (Curry and Whelpton, 1978). With care this problem could be avoided but resulted in a technically more demanding procedure. The potential for underestimating TMP with the diazepam internal standard is there, however the extent of the underestimation is difficult to judge, on the therapeutic doses which most individuals take typical peak levels are 200 ug/l with trough levels of around 100 ug/l; as 1250 ng of diazepam is added as internal standard then an error of around 10-20% could be expected, this is entirely unsatisfactory therefore both diazepam and m-aminoacetophenone were latterly included in a composite internal standard.

As a matter of course quality control procedures were performed on all assays and this served to check on analytical credibility, and particularly of m-aminoacetophenone performance.

The limits of linearity and sensitivity were adequate for the performance of meaningful pharmacokinetic studies, the precision figures were acceptable. No interferences have ever been noted in the assay either during volunteer studies or during the analysis of samples from the general population. The assay proved

robust and reliable during operation and was technically simple.

The performance characteristics of the modified assay may appear to be slightly worse than the original assay, due to the lower amount of substance presented by the autosampler and the use of nitrate causing a larger noise signal. The assay times were shorter and compatability between the autosampler's capacity and the limited solvent supply of the Varian 8500 pump was achieved (this pump is of the syringe-limited reservoir type). The lack of sensitivity resulting from the lower column loading by the autosampler was improved by reverting to manual injection, removal of nitrate and use of less ion-pairing agent could recover all the original sensitivity. As sensitivity was not a problem time was not invested in such optimisation.

The classical procedure for the liquifaction of sputum is homogenisation but this could lead to problems of carryover and equipment sterility. It has been shown that N-acetylcysteine is a satisfactory liquefactant (Li, et al, 1980), however we chose to use 2,3 dithiothreitol (McIntosh, et al, 1983) which has the same net effect, the conditions we chose are also suitable for pasteurisation which would remove any danger from tuberculosis which is still fairly prevalent in the West of Scotland (SHHD Annual Report, 1983). The assays for both saliva and sputum have been found to perform satisfactorily.

2.13. CONCLUSION

In conclusion all the assays used in this study have been validated and found to perform well with the exception of the unextracted Bratton-Marshall procedure for urinary sulphonamides.

The great tragedy of Science - the slaying of a beautiful hypothesis by an ugly fact.

Thomas H. Huxley 1825-1895

CHAPTER 3

COMPARATIVE PHARMACOKINETICS OF CO-TRIFAMOLE AND CO-TRIMOXAZOLE TO 'STEADY-STATE' IN NORMAL SUBJECTS

INTRODUCTION

3.1. TRIMETHOPRIM/SULPHONAMIDE SYNERGY

As sulphonamides and TMP both blockade the pathway of bacterial folate synthesis it was anticipated that the emergence of bacterial resistance would be significantly diminished by the use of a combination product.

To ensure the success of such a combination it would be desirable to maintain an inhibitory concentration of both drugs. Sulphamethoxazole (SMZ) had an elimination half-life that closely matched TMP and was more potent than other similar sulphonamides. Thus these two compounds were formulated as co-trimoxazole. It has been shown that the synergy between TMP and SMZ is significant (Bushby and Hitchings, 1968), that it is maximal at a ratio of around 1:20 for most organisms (Bushby, 1973) and probably extends to the range 1:10 to 1:40 for SMZ and other sulphonamides (Brumfitt, Hamilton-Miller and Kosmidis, 1973; Ekstrom et al, 1979); the ratio reflects the relative potency of the drugs. As the apparent volume of distribution of TMP is of the order of 1 l/kg and that of SMZ approximately 0.2 l/kg (Schwartz and Rieder, 1970), to achieve the optimum ratio (i.e. 1:20) in serum a fixed dose combination in the ratio 1:5 (TMP/sulphonamide) is required.

3.2. CLINICAL USE OF CO-TRIMOXAZOLE

Co-trimoxazole has been used succesfully in the treatment of a wide variety of infections principally urinary tract infection (Reeves et al, 1969; Brumfitt

et al, 1969; Brumfitt and Hamilton-Miller, 1979; Trimethoprim Study Group, 1981) and respiratory tract infections (Hughes et al 1969; Jordan et al, 1975). It has also been reported as effective against a number of other infections eg. typhoid (Snyder et al, 1973; Butler et al 1977); some protozoal infections, for instance those caused by Pneumocystis carnii. (Winston et al, 1980); otitis media (Japan Co-operative Bacteriological Study Group for Co-trimoxazole, 1973) and gonorrhoea (Csonka, 1969; Lawrence, Phillips and Nicol, 1973).

3.2.1. Alternative Sulphonamide/Trimethoprim Combinations

If it is assumed that the effectiveness in vivo of the combination is due to the synergy between TMP and SMZ then other sulphonamides should be acceptable substitutes for SMZ provided that they are equipotent with SMZ and their pharmacokinetics are similar.

On the basis of published half-lives Ekstrom et al (1979) compiled a list of seventeen sulphonamides with elimination half lives ranging from 7-27 hours that would be suitable for pairing with TMP. From the known kinetics and potency this group examined the following sulphonamides in a multiple cross-over study: sulphachloropyridazine, sulphadiazine, sulphaisodimidine, sulphamerazine and sulphamethoxazole (Ortengren et al, 1979a); from consideration of elimination half-life and serum non-protein bound fraction sulphadiazine compared well with sulphamethoxazole with a

longer half-life (10.2±4.1 hours as against 7.0±1.7 hours, n=7). Due to the higher concentrations achieved in urine, these authors have suggested that sulphadiazine would be a more suitable partner for TMP than SMZ in urinary tract infections which they confirmed subsequently in a comparative pharmacokinetic study (Ortengren, Magni and Bergan, 1979). These findings confirm the work of Andreason et al, (1978). Considering that sulphadiazine was introduced some twenty years prior to SMZ (Struller, 1969) it is perhaps surprising that SMZ was originally chosen in preference although Reeves and Wilkinson (1979) suggest this may have been due to a misunderstanding of sulphadiazine kinetics leading to an inappropriately high dosage regimen.

Although Ortengren et al (1979a) dismiss sulphamoxole (SDMO) as having too low an intrinsic activity to be of interest and Struller (1968) states that SDMO is five times less active than SMZ, Seydel (1980) determined that there was significant synergy between TMP and SDMO in vitro and that such levels were obtainable in vivo. Bohni (1976) found the in vitro action of SDMO/TMP to be sub-bacteriostatic and that organism regrowth could occur, however the experiments of Kuhne et al (1976) and Broughall et al (1979) on the inhibitory effect of urine from subjects taking the combination support the view that bacteriocidal action will occur; on the basis of pharmacokinetic arguments Gladtke (1980) suggests this action is due to synergy; however Reeves et al

(1980a) found that SDMO had a shorter elimination half-life than SMZ and would not compare favourably with the half-life of TMP.

3.3. DOSING SCHEDULES FOR TMP/SULPHONAMIDE COMBINATIONS

The manufacturers' recommended daily dose of the TMP/sulphonamide combinations currently available is as follows: TMP/SMZ (co-trimoxazole), two tablets twice daily i.e. TMP 320 mg/SMZ 1600 mg per day; TMP/sulphadiazine (co-trimazine), one tablet twice daily i.e. TMP 180 mg/sulphadiazine 820 mg per day and TMP/SDMO (co-trifamole) 2 tablet loading dose with one tablet twice daily thereafter i.e. the maintenance dose is TMP 160 mg/SDMO 800 mg per day. The lower doses decrease the risk of dose related side effects from either component. The fact that co-trifamole is given with a loading dose ensures rapid achievement of steady-state conditions. With co-trimoxazole therapy the larger doses used soon swamp any differences in SDMO kinetics, but the plasma and urine concentrations obtained using co-trimazine would not be expected to compare favourably with those of co-trifamole for at least the first 2 days.

3.4. CO-TRIFAMOLE

3.4.1. Clinical Use

Co-trifamole has been claimed to be effective in a variety of infections including those of the urinary tract (Eckstein, Etzel and Wesenberg, 1976; Peters, Nuri and Popa, 1977; Kaldeway, 1978; Leonetti, Crimi and Russo, 1980); the respiratory tract (Etzel, Neuhof and

Wesenberg, 1976; Knothe et al 1978; Burkhardt and Undeutsh, 1978; Wettengel et al 1980) and the gastro-intestinal tract (Etzel et al, 1976b).

One of the arguments advanced for the dosing regimen for co-trifamole was the prospect of a decrease in side-effects. In a study of 1371 subjects taking co-trifamole, 4.1% exhibited side effects (Etzel and Wesenberg, 1976), whereas in a survey of 649 patients taking co-trimoxazole a rate of 8.0% was found (Lawson and Jick, 1978). These figures support the regimen adopted for co-trifamole.

3.4.2. Pharmacokinetics

There is a paucity of data concerning SDMO kinetics, in the pharmacokinetic studies performed on co-trifamole. Kuhne et al (1976) did not calculate any parameters, Seydel (1980) quoted a half life of 11±3 hours and Reeves et al (1980a) found a range of 7.2-9.8 hours. On the basis of pKa and consequently lipid solubility, Seydel (1980) anticipated that SDMO would exhibit better penetration than SMZ and have a larger apparent volume of distribution than that of SMZ which has been found to be approximately 12 l (Schwartz and Rieder, 1970: Welling et al, 1973). Reeves and Wilkinson (1979) subsequently quoted a value of 21 l for the Vd of SDMO.

A comparative pharmacokinetic study of co-trifamole and co-trimoxazole was undertaken to investigate the implications for therapy of the disposition of the

components of the combinations and to determine the pharmacokinetic parameters for SDMO.

METHODS

3.5 CLINICAL

3.5.1. Subjects

Five male and five non-pregnant female subjects aged 18-25 years and of acceptable body weight (mean 66.2 kg, range 58-80 kg) participated in a two-way crossover study of co-trimoxazole and co-trifamole. All were normotensive; and their haematological (full blood count, haemoglobin and packed cell volumes) and biochemical (urea and electrolytes and liver function tests) profiles were within normal limits. Informed consent and ethical approval were obtained.

3.5.2. Study Design

The co-trimoxazole dosage regimen used was the standard treatment suggested by the manufacturers i.e. two tablets (each containing 80 mg TMP and 400 mg SMZ) orally and for a further nine doses every 12 hours. Co-trifamole was also administered according to the manufacturers recommended protocol i.e. a loading dose of two tablets (each containing 80 mg TMP and 400 mg SDMO) followed by a maintenance dose of one tablet every twelve hours for nine doses.

This was a two-way crossover study with a three week washout period between study legs; the order of administration of regimens was randomized and balanced.

No concomitant medication was allowed one week prior to and during the study, although the minor analgesics aspirin and paracetamol were permitted up to

48 hours prior to commencement. Alcohol was not permitted 48 hours prior to or during the dosing period. The subjects were required tc fast for 12 hours prior to each part of the study and for 4 hours following; the initial doses were taken with 100 ml of water, and volunteers were requested to restrict fluids (to water or fruit juices) for 12 hours prior to each leg.

3.5.3. Serum Collection

On the first day only,indwelling venous catheters were inserted into the cephalic vein and 10 ml blood samples drawn prior to the initial dose and thereafter at 0.5, 1, 2, 3, 4, 8 and 12 hours. Subsequently blood was obtained by venepuncture immediately prior to the morning dose and 2 hours after that dose for the remaining days of the study. The blood was allowed to clot and was centrifuged for 10 minutes at 2000 rpm. The serum was removed and stored in two 1.5 ml aliquots at -20^{o}C for subsequent analysis.

3.5.4. Urine Collections

The bladder was emptied prior to medication and a 50 ml aliquot kept at -20^{o}C. After dosing, urine was collected between 0-12 hr and 12-24 hr on the first day and 24 hourly on subsequent days; the volumes of the collections were noted and two 25 ml aliquots taken and stored at -20^{o}C for subsequent analysis. The creatinine concentrations were measured in order to detect major errors in urine collection.

3.6. ANALYTICAL

3.6.1. Assays

TMP and sulphonamide analysis were performed as described in section 2.7.6.i and 2.4.3.ii. A manual injection procedure was used. Sodium lauryl sulphate was added as ion-pairing agent as detailed in section 2.10.1.i.

3.6.2. Acetylator Status

The acetylator status of each subject was determined using the procedures described in section 2.5.1.

3.6.3. Pharmacokinetics

Over the initial period (0-12 hours) the observed serum concentrations and corresponding times to peak were taken as representing peak serum concentration and time to peak respectively. The area under the serum concentration time curve (AUC) was calculated from the 12 hour data, extrapolating to the sensitivity of the assay (70 µg/l) using the trapezoidal rule.

The derived pharmacokinetic parameters were obtained by least squares linear regression analysis of the logarithm of serum concentrations against time from peak levels to 12 hours after the first dose, extrapolated to the sensitivity of the assay. First order one-compartment kinetics and complete absorption were assumed. The absorption rate constant was obtained by the method of residuals. The following equations were used to calculate serum pharmacokinetic parameters:

$$t_{\frac{1}{2}\beta} = \frac{0.693}{k_{el}} \qquad \text{Equation 3.1}$$

$$Vd = \frac{D}{AUC.k_{el}}$$ Equation 3.2.

$$Cl_s = \frac{D}{AUC}$$ Equation 3.3.

where $t_{\frac{1}{2}\beta}$ = elimination half-life (h)

k_{el} = elimination rate constant (h^{-1})

Vd = apparent volume of distribution (l)

D = dose (mg)

AUC = total area under the serum drug concentration time curve (mg.hour/l)

Cl_s = total clearance of drug from serum (l/h)

The mean achieved serum concentrations were compared with those predicted using equations 3.4-3.7 and assumptions noted below.

$$C_{PRED} = \frac{k_a.F.D.}{V(k_a-k_{el})} \quad (e^{-k_{el}.t} - e^{-k_a.t})$$ Equation 3.4.

$$C_{ss}^{MAX}{}_{PRED} = \frac{F.D_m}{V(1-e^{-k_{el}\cdot\tau})}$$ Equation 3.5.

$$C_{ss}^{AVE}{}_{PRED} = \frac{1.44.F.D_m.t_{\frac{1}{2}}}{V\tau}$$ Equation 3.6.

$$C_{ss}^{MIN}{}_{PRED} = C_{ss}^{MAX}{}_{PRED}.e^{-k_{el}\cdot\tau}$$ Equation 3.7.

where ka = absorption rate constant (h^{-1})

F = fraction of dose absorbed

D = dose (mg)

t = time following dose (h)

V = volume of distribution (l)

D_m = maintenance dose (mg)

τ = dosage interval (h)

assuming: complete absorption for TMP, SMZ, and SDMO.

Following multiple dosing the time to 'steady state was taken as the time at which the average of the 'peak' and 'trough' serum values for a 24 hour period were within 10% of the average for one or more subsequent 24 hour periods (using levels at 24 and 26, 48 and 50, 72 and 74 and 96 and 98 hours).

RESULTS

3.7. SERUM

3.7.1. Serum Drugs Concentrations

The predicted and mean observed serum concentrations for TMP and SDMO following co-trimoxazole are given in tables 3.1 and 3.2 and for TMP and SMZ following co-trimoxazole given in tables 3.3 and 3.4.

3.7.2. Initial Dose

For the initial dose the predicted concentrations for all drugs are consistently higher, but within 1 sd, of the observed concentrations; the mean concentrations for both preparations are graphed in Fig. 3.1

3.7.3. Steady State

The mean steady-state nadir levels compare well for TMP but the predicted peak levels are higher than the observed for SDMO, SMZ and TMP following co-trifamole.

Although the predicted levels following a single dose compare well with the observed for both SDMO, SMZ and TMP at steady state these were underestimates, but were within 2 sd of the observed levels.

The MIC for TMP and sulphonamides acting independently are 1.5-2.5 mg/l and 45-55 mg/l (Reeves and Wilkinson, 1979)

Steady state conditions were achieved for both components of co-trifamole earlier than for co-trimoxazole, median 24 hours and 48 hours respectively, individual times are noted in appendix A. As would be predicted from the dosing regimens, the peak serum TMP

TABLE 3.1.

Observed (mean) and predicted TMP concentrations following co-trifamole therapy (n=10)

Time	CONCENTRATION (mg/l)				
	mean	sd	range		predicted
			low	high	
0	0	0	0	0	0
0.5	0.97	0.69	0.08	2.15	1.43
1	1.48	0.50	0.61	2.33	1.79
2	1.57	0.24	1.25	1.90	1.82
3	1.58	0.29	1.16	1.98	1.71
4	1.35	0.30	0.79	1.68	1.59
8	1.12	0.20	0.77	1.40	1.21
12	0.86	0.12	0.65	1.02	0.91
24	0.91	0.20	0.60	1.19	
26	1.73	0.34	0.99	2.00	$C_{ss}MIN = 0.78$
48	0.85	0.18	0.51	1.11	$C_{ss}AVE = 1.29$
50	1.50	0.27	1.10	1.80	$C_{ss}MAX = 1.81$
72	0.83	0.26	0.44	1.26	
74	1.51	0.39	0.71	1.91	
96	0.81	0.24	0.46	1.03	
98	1.40	0.39	0.78	2.02	

TABLE 3.2.

Observed (mean) and predicted SDMO concentrations following co-trifamole therapy (n=10).

Time (h)	CONCENTRATION (mg/l)				
	mean	sd	range		predicted
			low	high	
0	0	0	0	0	0
0.5	46	27	2	97	57
1	69	26	17	105	72
2	80	26	38	132	72
3	72	15	48	88	66
4	67	13	43	82	60
8	49	12	28	57	40
12	34	13	18	49	27
24	38	17	16	71	
26	76	24	27	111	$C_{ss}MIN = 18$
48	42	19	18	80	$C_{ss}AVE = 41$
50	75	27	38	134	$C_{ss}MAX = 61$
72	42	21	15	77	
74	75	31	29	136	
96	41	20	16	82	
98	72	24	34	119	

TABLE 3.3.

Observed (mean) and predicted serum TMP concentrations following co-trimoxazole therapy

Time (h)	CONCENTRATION (mg/l)				
	mean	sd	range		predicted
			low	high	
0	0	0	0	0	0
0.5	1.07	0.33	0.64	1.80	1.57
1	1.55	0.35	0.95	2.33	1.97
2	1.76	0.29	1.10	2.14	2.00
3	1.64	0.26	1.13	2.03	1.88
4	1.52	0.29	1.02	1.98	1.75
8	1.21	0.24	0.93	1.60	1.33
12	0.90	0.21	0.69	1.20	1.00
24	1.37	0.41	0.83	2.05	
26	3.34	0.76	1.77	4.05	$C_{ss}MIN = 1.71$
48	1.93	0.45	1.42	2.85	$C_{ss}AVE = 2.97$
50	3.58	0.49	2.53	4.23	$C_{ss}MAX = 3.97$
72	2.05	0.65	1.10	3.12	
74	3.83	0.95	2.10	5.23	
96	2.05	0.49	1.13	2.95	
98	3.66	0.94	2.00	5.55	

TABLE 3.4.

Observed (mean) and predicted serum SMZ concentrations following co-trimoxazole therapy (n=10)

Time (h)	CONCENTRATION (mg/l)				
	mean	sd	range		predicted
			low	high	
0	0	0	0	0	0
0.5	23	12	9	45	33
1	45	12	26	59	48
2	65	13	43	91	56
3	62	10	42	72	55
4	58	8	46	68	51
8	43	4	37	49	37
12	31	7	18	44	27
24	48	7	37	56	
26	79	21	52	102	$C_{ss}MIN = 41$
48	65	12	39	81	$C_{ss}AVE = 72$
50	104	20	71	110	$C_{ss}MAX = 108$
72	70	16	39	97	
74	113	26	71	143	
96	70	13	39	86	
98	110	29	66	140	

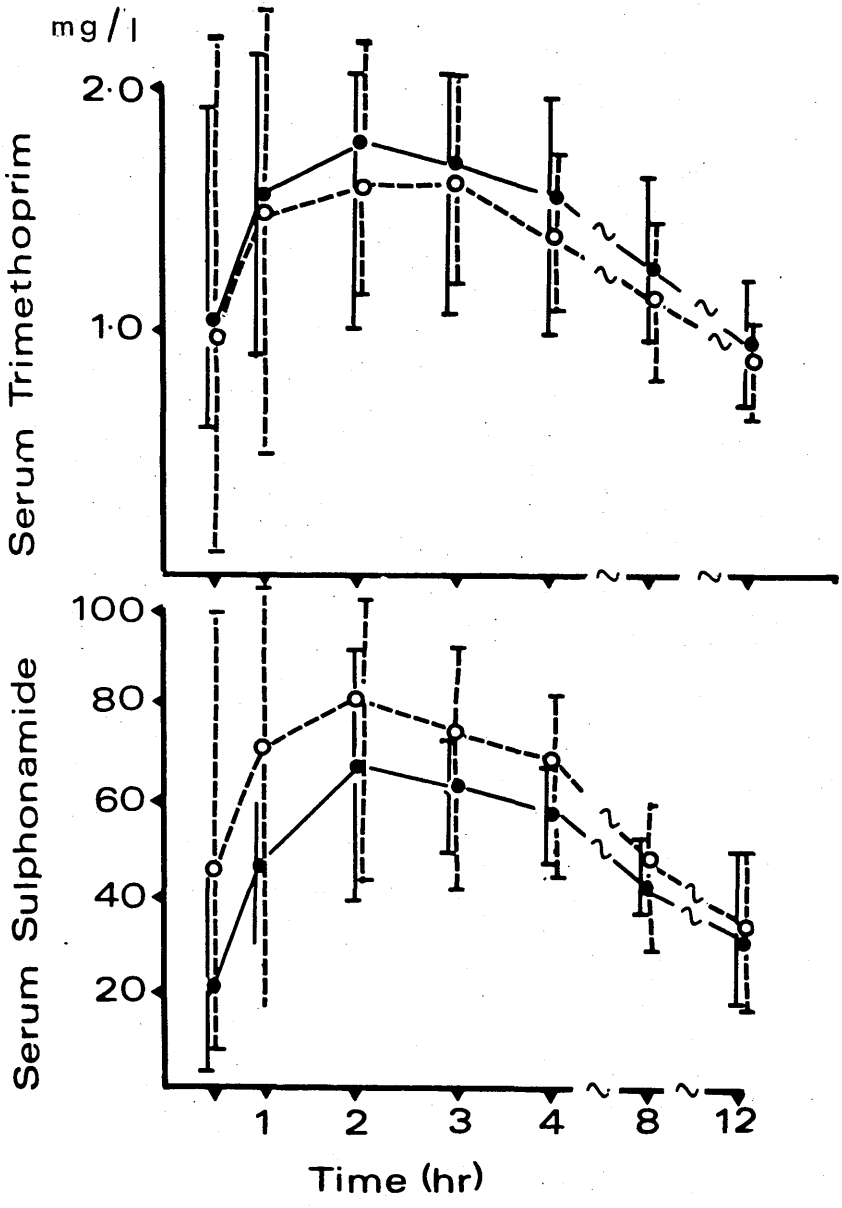


FIGURE 3.1. Time v serum concentration curves following co-trifamole and co-trimoxazole for SDMO, SMZ and TMP.

● = co-trimoxazole

o = co-trifamole

and sulphonamide concentrations obtained from 24 h onwards during multiple dosing with co-trifamole were significantly lower than for co-trimoxazole ($p<0.05$, t test) except at 24 h and 26 h.

3.7.4. Serum Concentration Ratios

Using the mean data and the predicted values the serum ratios for co-trifamole and co-trimoxazole have been calculated and are presented in tables 3.5 and 3.6.

Assuming the synergistic range to be 1:10 to 1:40, the predicted ratios for co-trifamole are initially at the extremes, however the mean observed ratios are consistently greater than40:1 with a great deal of scatter over the observed range, the scatter is inter-individual rather than intra-individual. For co-trimoxazole the predicted and observed ratios tend to fall within the synergistic range and there is much less variation, this situation continued throughout the period of the study.

Statistically significant differences between treatments were noted at 26, 50, 74 and 98 hours ($p<0.05$, t test).

3.7.5. Kinetics (Single Oral Dose)

Due to the small number of observations obtained the absorption rate constant was calculated from averaged data; the k_a of TMP was found to be 2.5 h^{-} (for both preparations) and the k_a for SDMO and SMZ to be 2.3 and 1.4 h^{-1} respectively The mean ($\pm$sd) data on the serum pharmaco-

TABLE 3.5.

Serum ratio of SDMO:TMP following co-trifamole therapy

Time (h)	mean	sd	RATIO range	predicted
0.5	84.3	138.2	25-477	40
1	47.7	17.1	27-85	40
2	51.0	14.9	28-82	40
3	46.2	10.4	29-68	39
4	52.1	19.5	37-104	38
8	44.4	13.9	31-78	33
12	39.7	14.1	18-63	30
24	41.7	20.1	27-92	
26	43.1	9.3	27-56	at C_{ss}MIN 23
48	49.5	20.9	17-96	at C_{ss}AVE 32
50	51.3	23.6	36-116	at C_{ss}MAX 34
72	53.6	28.9	12-113	
74	50.9	22.1	27-104	
96	49.9	14.5	22-80	
98	52.6	15.9	29-91	

TABLE 3.6.

Serum ratio of SMZ:TMP following co-trimoxazole therapy

	RATIO			
Time (h)	mean	sd	range	predicted
0.5	22.4	12.6	9-45	21
1	31.3	13.5	17-60	24
2	38.1	9.4	23-51	28
3	38.3	6.3	27-51	29
4	38.8	5.3	28-49	29
8	35.9	4.4	28-41	28
12	34.9	7.1	25-47	27
24	36.7	8.8	23-54	
26	24.3	5.7	17-30	at C_{ss}MIN 24
48	35.1	9.6	21-50	at C_{ss}AVE 24
50	29.2	4.6	20-36	at C_{ss}MAX 27
72	37.2	13.2	19-64	
74	30.6	6.9	17-41	
96	35.6	10.9	20-58	
98	30.7	7.2	19-41	

kinetics are reported in table 3.7, the individual data for each subject are reported in appendix A.

The area under the serum drug concentration-time curve was greater for SDMO than SMZ ($p<0.05$) There was no significant difference in the AUC for TMP using the two formulations although there were individual instances where this general observation did not hold true. In particular there were larger differences than anticipated between formulations for TMP in subjects 1, 3, 7 and 9 and for sulphonamides in subject 5. The observed mean peak serum concentrations reflect the differences found for AUC between SDMO and SMZ and the similarities for TMP. There was no difference in time to peak between individuals.

The mean elimination half-life for SDMO is shorter than SMZ and is at the level of significance ($0.1>p>0.05$, t test), there was no statistically significant difference between the TMP elimination half lives although there was intra-individual variation. This and other estimates of elimination are noted in table 3.7.

There was no statistically significant difference between the serum clearance of TMP following the two preparations neither was there any difference between the clearance of SDMO and SMZ.

The apparent volume of distribution for SDMO was less than for SMZ ($p<0.05$, t test); in two subjects (5 and 9) Vd's were comparable. There was no statistically significant difference between the Vd's for

TABLE 3.7.

Mean observed and derived pharmacokinetic parameters for TMP, SMZ and SDMO following dosing with co-trimoxazole and co-trifamole

Drug	Preparation	t_{MAX} (h)		C_{MAX} (mg/l)	k_{el} (h^{-1})	$t_{\frac{1}{2}}$ (h)	AUC (mg/l/h)	Vd (l)	Cl_s (l/h)
TMP	Co-trifamole	median = 2	mean ±	1.73	0.067	10.5	31.8	77.8	5.1
		range 0.5-3.0	sd	0.35	0.007	1.0	4.8	16.6	0.8
	Co-trimoxazole	median = 2	mean ±	1.86	0.071	11.0	37.1	71.3	5.0
		range 0.5-4.0	sd	0.28	0.024	4.2	16.1	15.6	1.7
SDMO	Co-trifamole	median = 2	mean ±	88	0.096	8.0	1030	9.4	0.9
		range 1.0-4.0	sd	21	0.031	2.8	393	2.0	0.4
SMZ	Co-trimoxazole	median = 2	mean ±	68	0.082	9.1	874	12.0	1.0
		range 2.0-4.0	sd	11	0.027	2.6	210	2.3	0.2

TMP between the two formulations; the inter-individual variation was more marked than the intra-individual variation.

3.7.6. Acetylator Phenotyping

Using sulphadimidine, four of the nine subjects were found to be fast acetylators and five were slow acetylators; the acetylator status of one individual could not be determined as the subject was no longer available. For the fast acetylator group the mean (range) half-life of SDMO and SMZ were 7.3 h (4.9-13.2 h) and 8.7 h (4.7-12.6 h) respectively and for the slow acetylator group 9.0 h (6.6-11.0 h) and 9.7 h (7.6-13.9 h) respectively. Thus there was no correlation between acetylator status and SDMO or SMZ half-life.

3.8 URINE

3.8.1. Urine Concentrations

Disregarding synergy, the MIC's for sulphonamide and trimethoprim in urine may be considered to be 200 mg/l (Brumfitt et al, 1973) and 8 mg/l (Huovinen, Mantyjarvi and Toivanen, 1982) respectively; the mean urinary data are given in table 3.8. However following co-trifamole therapy no individual had a urinary SDMO concentration that consistently exceeded 200 mg/l; with the higher doses used in co-trimoxazole therapy this level was achieved, but only one subject (subject 3) consistently exceeded this figure. The individual data are reported in appendix A.

In all urine collections the TMP concentration

TABLE 3.8.

Mean urinary pharmacokinetics of TMP, SDMO and SMZ

Drug	Preparation		Urinary concentration (mg/l) 0-12 h	12-24 h	24-48 h	48-72 h	72-96 h	96-108 h
SDMO	co-trifamole	mean	155	151	134	145	136	123
		± sd	47	62	75	81	77	61
SMZ	co-trimoxazole	mean	179	185	188	175	205	201
		± sd	110	89	75	84	104	92
TMP	co-trifamole	mean	66	102	80	74	70	66
		± sd	20	28	15	15	17	23
TMP	co-trimoxazole	mean	73	162	134	155	135	112
		± sd	27	49	37	45	32	26

were in excess of 2 mg/l; the lowest concentration following co-trifamole therapy was 34 mg/l and following co-trimoxazole therapy 30 mg/l.

Urinary TMP and sulphonamide concentrations achieved during the first 12 hours following the initial dose were not significantly different for the two preparations. However, TMP concentrations achieved during the 12 hour to 108 hour period were significantly lower following co-trifamole therapy, as was expected; similarly SDMO concentrations were lower than SMZ over the same period ($p<0.05$, t test).

3.8.2. Urine Concentration Ratios

The mean urinary sulphonamide:TMP ratios for the different collection periods during co-trimoxazole and co- trifamole therapy are given in table 3.9.

Urinary sulphonamide to TMP concentration ratios for both co-trimoxazole and co-trifamole were similar with mean ratios ranging from 1.2 to 2.4. The highest individual ratio was 4.1.

3.8.3. Cumulative Urinary Excretion

The mean cumulative excretion of the sulphonamides from 0-108 hours following co-trifamole and co-trimoxazole therapy were 791.5 mg (393.2-1296.2 mg) and 1046.9 mg (619.8-1545.0 mg) i.e. 18.0% (8.9-29.5%) and 13.1% (7.7-19.3%) respectively; the corresponding figures for TMP were 425.7 mg (305.7-504.4 mg) and 750.4 mg (579.9-944.1 mg) i.e. 48.4% (34.7-57.3%) and 46.9% (36.2-59.0%) respectively. The differences in amount excreted but not percentage excretion was statistically significant ($p<0.05$).

TABLE 3.9.

Urinary SDMO:TMP and SMZ:TMP ratios during therapy

Time(hrs)	0-12	12-24	24-48	48-72	72-96	96-108
SDMO:TMP						
mean	2.4	1.5	1.6	2.0	2.0	2.0
sd	0.5	0.6	0.7	1.1	1.1	1.2
range	1.4-3.1	0.5-2.4	0.7-3.1	0.7-3.7	0.8-3.9	0.8-4.1
SMZ:TMP						
mean	2.2	1.3	1.5	1.2	1.6	1.8
sd	0.8	0.8	0.7	0.5	0.9	0.9
range	1.4-3.4	0.4-2.5	0.6-3.0	0.7-2.0	0.6-3.8	1.0-3.5

DISCUSSION

3.9. SERUM

3.9.1. Serum Drug Concentrations

As would be anticipated there was good agreement of TMP concentrations following the initial dose. SDMO levels were higher than those of SMZ and may reflect differences in the completeness of absorption but more probably the difference in volume of distribution.

3.9.2. Initial Dose

The predicted single dose concentrations for TMP are within one sd of those observed.

The predicted sulphonamide concentrations following the initial dose of co-trifamole or co-trimoxazole are lower following mean time to peak although always within one sd of those observed.

3.9.3. Steady State

The predicted $C_{ss}MIN$ and $C_{ss}MAX$ for TMP were within 1 sd of those observed for TMP. The difference in predicted TMP levels found during subsequent doses is due to the use of the different mean Vd's found for each preparation, although the differences were not statistically significant for both SMZ and SDMO. $C_{ss}MAX_{PRED}$ was also within one sd of the observed mean, however $C_{ss}MIN_{PRED}$ for both SDMO and SMZ are notably lower than the mean trough levels although within two sd. Thus use of a one compartment open model with first order kinetics provides an adequate approximation for the observed data.

The observed steady state SMZ peak and trough levels of 109 mg/l and 70 mg/l respectively compare with levels found by others using specific liquid chromatographic procedures having made appropriate adjustments for dosage as required with mean peak and trough levels of 110 mg/l and 62 mg/l (Vree et al, 1978; Siber et al, 1982; Spicehandler, et al 1982). Ortengren et al, (1979a,b), using the Rieder assay, found peak and trough levels of 106 mg/l and 60 mg/l which again compare with the current study but are in marked contrast to the peak and trough levels of 84 mg/l and 50 mg/l reported by Reeves et al (1980). One report (Nolte and Buttner, 1974) found SMZ trough levels ranging from 30-50 mg/l as did Reeves and Wilkinson, (1979) and Bergan and Brodwall (1972).

It is difficult to explain these differences as the assays used by all workers appear to be accurate with minimal interference. There may have been differences in the purity of material used in the preparation of calibrators but this supposition cannot be substantiated.

The differences noted above are important as the study of Reeves et al (1980a) is one of only two detailed papers on the comparative kinetics of SDMO and SMZ, the other report derives from this work (Watson et al, 1982). The mean peak and trough SDMO levels reported herein were 75 mg/l and 42 mg/l and in the study of Reeves et al (1980a) 50 mg/l and 23 mg/l respectively. In support of the higher levels reported here, trough SDMO levels of 46 mg/l were found by Kuhne et al (1976)

If the MIC values of 1.5-2.5 mg/l for TMP and 45-55 mg/l for sulphonamide (Reeves and Wilkinson, 1979) are relevant in serum then the trough levels of SDMO found in this study are inadequate while the levels of TMP achieved throughout the period of co-trifamole therapy are also inadequate if the drugs were to act independently, indeed while the SMZ levels are acceptable, TMP levels following co-trimoxazole therapy are barely adequate; considering that such levels can easily be predicted it may seem surprising that the lower dose regimen of co-trifamole was considered. Consequently there is an increased reliance on synergy for adequate effect.

3.9.4. Serum Concentration Ratios

Co-trimoxazole is formulated in the ratio TMP:SMZ (1:5) to attain ratios between 1:10 and 1:40 *in vivo*, the limits of optimal synergy (Schwartz andRieder, 1970; Brumfitt et al, 1973). This was achieved in nearly all individuals for co-trimoxazole; however the greater relative "availability" of SDMO due to its poorer distribution means that higher concentrations are achieved during co-trifamole therapy and consequently the majority of subjects had ratios well in excess of the optimum for synergy, with considerable inter-individual scatter. The use of the 1:5 formulation may seem inappropriate for co-trifamole however the percentages of free i.e. unbound TMP, SDMO and SMZ in serum are 55, 10 and 20% respectively (Seydel, 1980), therefore for co-trifamole and co-trimoxazole during treatment the respective mean serum ratios

achieved are between 7 and 15 which is the lower range for optimal synergy in vitro. Synergy in serum is not usually relevant to treatment of the infected site as the degree and rate of penetration from the plasma water for the drugs may well differ and is reflected in reported TMP:sulphonamide ratios for sputum of 1:1.25 (Reeves and Wilkinson, 1979) and cerebrospinal fluid of 1:1.6 (Bach, Gold and Finland, 1973).

The anticipated dose-dependent differences in concentration are seen following multiple dosing. The expected rates of achievement of steady state for the two preparations were found.

3.9.5. Kinetics (Single Oral Dose)

i) Absorption

Although there are a large number of studies on the pharmacokinetics of TMP and SMZ they contain minimal derived pharmacokinetic parameters, thus comparison with other studies can be difficult particularly if any discrepancy exists.

Due to the small number of data points on each individual, k_a could not be reliably calculated, however the use of meaned values enabled estimates of k_a to be made using the method of residuals. The k_a for SMZ was found to be 1.4 h^{-1} which compares very well with the mean value obtained by Ortengren of 1.36 h^{-1} (Ortengren, et al, 1979a) and 1.29 h^{-1} (Ortengren, et al 1979b). Reeves et al (1980a)obtained an "invasion rate" of 0.4 h, assuming that this phrase refers to the

absorption half-life then a k_a of 1.7 h^{-1} is obtained. The k_a for TMP of 2.5 h^{-1} found in this work agrees with the value of Kasanen et al (1978) of 2.54 h^{-1} rather than the value of 1.79 h^{-1} found by Ortengren et al (1979b). The k_a for SDMO of 2.3 h^{-1} is lower than the value of 3.5 h^{-1} derived from the "invasion rate" of Reeves et al (1980a); thus absorption is rapid for all of the drugs.

ii) Volume of distribution

The Vd obtained for SMZ of 12.0±2.3 l compares well with the values of 12.3, 10.8, 12.5±2.1 and 15.4 ; obtained by other workers (Schwartz and Rieder 1970; Welling et al, 1973; Ortengren et al, 1979a and Ortengren et al, 1979b).

There are some discrepancies in the literature as to the Vd of TMP; the mean values obtained in this study were 71.3 and 77.8 l which compares with Vd's of 71.7±4.9 l found by Kasanen et al (1978) and 78±11.3 l (derived) found by Ortengren et al (1979b). This is in contrast to the higher values reported by other workers of 94 l (Schwartz and Rieder, 1970), 100 l (Nolte and Buttner, 1973; Naber, Vergin and Weigard, 1981), 126 l (Liedtke and Haase, 1979 and 143 l (Singlas et al, 1982). The large Vd of TMP reflects its uptake into the tissue compartment and is affected by body weight. It is possible that some of these differences could be resolved by the use of normalised data. The lower estimates of Vd tend to have been determined on a larger number of individuals

and thus a more statistically representative sample of the general population is likely to be found. Wilkinson and Reeves (1979) quote a range of 69-130 l.

The Vd of SDMO has been quoted as 21 l (Wilkinson and Reeves, 1979), although there is no evidence presented for this. Using data from Reeves et al (1980a) the Vd may be calculated as 11 l which is much closer to the value found in this study.

iii) Elimination

Elimination half-life for SMZ was found to be 9.1±2.6 hours which compares with previous reports of 9.0 (Schwartz and Rieder, 1970), 8.7 hours (Welling et al, 1973) 9.5 hours (Kaplan et al, 1973), 9.6-11.2 hours (Reeves et al, 1980a)and 7.7-10.7 hours (Ortengren, 1979b), the report of Ortengren et al, (1979a) quotes a slightly shorter half-life of 7.0±1.7 hours. Serum clearance of 1±0.2 lh^{-1} is similar to that of Ortengren et al (1979a; 1979b) of 1.13±0.3 and 1.33±0.45 respectively.

There has been less consistency in the quoted elimination half-life of TMP. The mean half-lives for TMP in this study were 10.5 and 11.0 hours, this compares with 8.8±0.8 hours (Kasanen et al, 1978), 8.6 hours (Schwartz and Rieder, 1970), 9.4 hours (Nolte and Buttner 1973) 9.6±2.7 hours (Ortengren, 1979b) and somewhat higher, 14.5 hours (Kaplan et al, 1973). The serum clearance of 5.0±1.7 lh^{-1} compares with 4.5 lh^{-1} found by Ortengren et al (1979b) and 5.76 lh^{-1} found by Kasanen et al (1978).

A half-life of 11±3 hours for SDMO was given by Seydel, (1980) although the derivation of this not clear. In the current study an elimination half-life of 8±2.8 h was found and compares with the half-life of 7.2-9.8 h found by Reeves et al (1980a). As these elimination half-lives are lower than those commonly found for TMP, SDMO is thus no better a match than SMZ on "kinetic" grounds for combination therapy; in addition SDMO is five times less active than SMZ (Struller, 1968) and with a Vd similar to SMZ there is no advantage in the substitution of SDMO for SMZ.

3.9.6. Acetylator Phenotyping

Acetylator phenotyping was performed to ascertain if there was polymorphic metabolism of SDMO, as this had not previously been investigated. There was no evidence that polymorphism existed in this small group of subjects.

The time to steady state reflected the dosing regimens, as stated in the introduction the use of a loading dose is not necessary in co-trimoxazole therapy but as the levels attainable with the co-trifamole regimen are lower it is necessary to achieve steady state as soon as possible.

3.10. URINE

3.10.1. Cumulative Urinary Excretion

The mean fraction of SMZ excreted was 13.1% which compares with other reports (Ortengren et al, 1979b) although the TMP recovery of 48% was somewhat lower

than the 58% found by the same author, Kasanen et al, (1978) obtained 51-60% recovery. As TMP excretion in urine is pH dependant (Sharpstone, 1969) there may have been some differences in the groups. There are no directly comparable data available for SDMO.

3.10.2. Urine Concentrations

There is little doubt that co-trimoxazole is effective in the treatment of urinary tract infection (Reeves et al, 1969; Acar et al, 1979; Ekstrom et al, 1979), co-trifamole has been found to be as effective as co-trimoxazole (Eckstein et al, 1976; Helwig et al, 1976; Peters, et al, 1977) and TMP has been found to be as effective as co-trimoxazole (Brumfitt and Pursell, 1972; Kasanen et al, 1978; Lacey et al, 1980; Kasanen et al, 1983) or even better (Brumfitt et al, 1982). Obviously the efficacy may be considered as being comparable.

The lower maintenance dose of co-trifamole inevitably results in lower concentrations of sulphonamide and trimethoprim in the urine. If synergy occurs in urine then sensitive organisms should be eradicated, if there were no synergy then the organisms would be controlled by the independent actions of the component drugs. Although the MIC in urine for sensitive organisms has been quoted as approximately 200 mg/l for sulphonamides and 2 mg/l for TMP (Brumfitt et al, 1973, an MIC of 8 mg/l has been suggested for TMP in urine by Huovinen et al, (1982). The urinary SDMO levels, and to lesser

extent urinary SMZ levels, are intermittently below the MIC in all subjects, except subject 3 when on co-trimoxazole, whereas even the higher MIC for TMP is exceeded by all subjects by at least a factor of 4 and usually much more at all points. In addition to this must be considered the ratios of TMP/sulphonamide achieved.

3.10.3. Urine Concentration Ratios

In vitro the range of optimal synergy for TMP/SMZ is 1:10 to 1:40 (Ekstrom et al, 1979), although synergy may occur outwith this range it would be expected to be of a lesser order of magnitude as indeed was found by Bushby and Hitchings (1968). The highest ratio for co-trimoxazole was 1:3.8 and for co-trifamole 1:4.1, substantially below optimum synergy. In addition SDMO is intrinsically about five times less potent than SMZ although this effect is not maintained on in vitro testing in combination with TMP (Reeves, Bywater and Holt, 1980) which is in contrast to the results of Bohni (1976). Rieder et al (1974) after noting the attainment of sub-optimal ratios suggest that the high levels of TMP will compensate for this. A further factor that requires consideration is that although TMP/SMZ was initially claimed to be bacteriocidal in urine, it has subsequently been shown to bacteriostatic (Lewis, Anderson and Lacey, 1974; Anderson, et al, 1974). The reason for this anomaly may be that the sulphonamide component was either swamped by the action of TMP (Greenwood and O'Grady, 1976) or that the action of TMP was antagonised by the presence

of SMZ in the urine (Anderson, Sellin and Lacey, 1973; Anderson et al, 1974) and indeed any bacteriocidal action was due to the TMP component (Lewis et, 1974). It is probable that antagonism is the cause of the result, reported by Kasanen et al (1983),that TMP alone had a higher cure rate than co-trimoxazole; they also noted fewer side effects for TMP alone which reflects the findings of Lacey et al (1980); and Brumfitt et al (1982).

It therefore seems reasonable to anticipate that the explanation of the comparable efficacy of co-trimoxazole, co-trifamole and TMP alone in urinary tract infection is due to the reliance of the combination products principally on the effect of TMP.

It is of interest to note that one of the first papers on co-trimoxazole therapy in urinary tract infection (Reeves et al, 1969) noted that effectiveness could be related to the organisms susceptibility to TMP.

3.11. CONCLUSION

The argument for TMP alone in urinary tract and other infections is very strong. There is agreement amongst a number of authors who have reached this conclusion on the basis of bacteriological findings *in vitro* (Anderson et al, 1973; Lewis et al, 1974), clinical cure rates (Kasanen et al, 1978; Brumfitt and Hamilton-Miller, 1979; Lacey et al 1980; Brumfitt and Hamilton-Miller, 1980; Trimethoprim Study Group, 1981; Brumfitt and Hamilton-Miller, 1982; Brumfitt et al 1982; Kasanen et al, 1983) and on pharmacokinetic grounds.

(Reeves and Wilkinson, 1979; Wilkinson and Reeves, 1979; Watson et al, 1981; Watson et al, 1982).

New things are made familiar,
and familiar things are made new.

Samuel Johnson 1709-1784
Lives of the English Poets

CHAPTER 4

THERAPY WITH TRIMETHOPRIM ALONE: PHARMACOKINETICS OF TRIMETHOPRIM AT TWO DOSAGE LEVELS

INTRODUCTION

4.1. THE CHANGING RATIONAL OF THERAPY WITH TMP AND ITS RELATIONSHIP TO BACTERIAL RESISTANCE

4.1.1. Trimethoprim Resistance

When TMP was first marketed it was available only in combination with SMZ, as it was anticipated that the mechanism of sequential blockade would afford protection against the emergence of resistance, particularly to TMP (Darrell, Garrod and Waterworth, 1968)

Sequential blockade is however unlikely to be synergistic (Lacey, 1979; Lacey, 1982), the synergism observed is more probably attributable to the simultaneous blockade of dihydrofolate reductase by both TMP and sulphonamides (Poe, 1976), thus sulphonamides would have two modes of action and may not therefore protect against the emergence of TMP resistance *in vivo* (Lacey, 1982).

TMP resistance is R factor mediated (Fleming, Datta and Grüneberg, 1972) and is a high level resistance. The antibacterial action of TMP is due to thymineless death (Then and Angehrn, 1973; Then and Angehrn, 1974), thus bacteria able to utilise thymine or other sources for the production of thymidylate, a cofactor in biochemical reactions, will be resistant to the action of TMP (and sulphonamides) (Then and Angehrn, 1974).
TMP resistance may also be due to insensitive dihydrofolate reductase (Then and Angehrn, 1979) presumably due to deviations from the normal conformational state; pteridine dihydrofolate reductase inhibitors had similar potency to TMP which is in keeping with the finding that dihydro-

folate reductase bound TMP is very similar in conformation to dihydrofolate reductase bound methotrexate (Roberts et al, 1981).

TMP sensitivity in general practice and hospital populations was virtually unchanged during the period 1971-1978 (Grünberg, 1980), while TMP was available only in combination with a sulphonamide, about 5% of urinary E.Coli were found to be resistant. In Finland TMP alone was available for use from 1973, and initially there was a low incidence (<1%) of resistant strains (Kasanen et al 1978), however the level of resistance in another Finnish study was found to be 20.3% in outpatients and 39.8% from in-patients (Huovinen and Toivanen, 1980). The same authors found lower levels of resistance in two acute hospitals in the same city of 12.2% and 8.6%, (Huovinen, et al, 1982). A comparative study during 1977-1978 between resistance in long-stay hospitals in Finland of 49% and resistance in Sweden of 3.6% (Dornbusch and Toivanen,1981) was said to reflect the level of usage of TMP alone in the Finnish hospital.

In 1973 TMP resistance in the U.K. was 11.5% (Hamilton-Miller, Gooding and Brumfitt, 1981) and in 1979 was 12.4% (Brumfitt, Hamilton-Miller and Gooding, 1980) however in 1979 resistance in certain genera showed increases especially for E.Coli and Proteus. During this period TMP was only available with SMZ in the U.K. Covering a slightly later period an increase in high level plasmid or transposon mediated resistance was noted for E.Coli (Kraft, Platt and Timbury, 1984).

Currently the extent of use of TMP alone is too low, and the period of its use in this was may be too short to allow clear conclusions to be drawn as to the probability of increased resistance to TMP alone; however in 1981 the incidence of TMP resistance was levelling off (Brumfitt, Hamilton-Miller and Wood, 1983). The level of TMP resistance found during the period prior to its being available as a single drug formulation indicates that the sulphonamide component did not protect against the emergence of resistance to TMP, particularly as the incidence of sulphonamide resistance was higher during this period, of the order of 30% or higher depending on the strains (Huovinen and Toivenen, 1980; Grüneberg, 1980; Dornbusch and Toivanen, 1981), and thus the use of TMP alone may not be disadvantageous, restriction of its use only being necessary in certain clearly defined circumstances (Kasanen et al, 1983). Additionally Lacey et al (1980) concluded that TMP rarely selected for resistance, although epidermal staphylococci did show an increase in resistance.

4.1.2. Use of Trimethoprim Alone

Opinion is moving towards the use of TMP alone in certain circumstances (Reeves, 1982; Brumfitt and Hamilton-Miller, 1982; Salter, 1982; Lacey, 1982) and its breadth of application and frequency of use is likely to increase. It is particularly appropriate in the treatment of uncomplicated urinary tract infection (Kasanen et al, 1978, Lacey et al, 1980, Trimethoprim Study Group, 1981 Neu, 1982).

4.1.3. Pharmacokinetics

TMP as a single drug formulation was introduced in the U.K. in 1981 for use in urinary tract and other infections, but two different dosing schedules were available. Brumfitt and Hamilton-Miller (1980) have observed that detailed pharmacokinetic studies are necessary to decide upon appropriate dosage regimens for use of TMP alone and also to ensure that there was no variation between different brands, a problem previously encountered with nitrofurantoins (Di Santo et al, 1976).

A study was therefore undertaken of serum and urinary TMP concentration in normal subjects taking two different TMP preparations according to the manufacturers recommended regimens (200 mg twice daily or 300 mg once daily); the pharmacokinetics and the relative bioavailability of the two preparations were examined.

4.2. CLINICAL

4.2.1. Subjects

Volunteers were admitted to the study subject to being of acceptable weight (mean 72.1 kg) (within 25% of desirable weight for height, weight and sex), with normal blood pressure, normal haematological profile (differential white count, ESR and platelets) and normal biochemical profile (urea and electrolytes and liver function tests). The individual sex, age and weights are given in appendix B.

4.2.2. Dosage Regimen

This was a two way crossover trial in which the order of administration of treatment was randomised and balanced. There was a four-week wash-out period between each leg of the trial.

Administration was of either 300 mg TMP every 24 hours for 5 doses or 200 mg TMP every 12 hours for 10 doses. Mean doses were 5.95 and 4.47 mg/kg/day respectively.

No alcohol was permitted for 24 hours prior to, and during the administration of TMP, and no concomitant medication was allowed prior to or during the study period. Subjects were fasted for 12 hours prior to and 4 hours after the initial dose; no fluid was allowed 2 hours prior to the initial dose, thereafter there were no fluid or dietary restrictions. The medication was taken with 100ml of water on each occasion; following the initial dose 100ml water was taken every hour for 4 hours to ensure adequate urinary production.

4.2.3. Serum Collection

On the first day of each leg an indwelling catheter was inserted into the cephalic vein and 10 ml samples of blood drawn pre dose and at 0.5, 1, 2, 4, 7, and 12 hours after the initial dose. Thereafter samples were obtained by venepuncture immediately prior to the morning dose (0900) and 2 hours afterwards. The blood was allowed to clot, centrifuged at 1500g for 10 minutes, the serum removed and stored in 1.5 ml aliquots at -20°C prior to subsequent analysis.

4.2.4. Urine collection

The bladder was emptied prior to the first dose and 2x20 ml aliquots of urine collected. After the initial dose complete urine collections were made for the following times: 0-2, 2-4, 4-6, 6-8, 8-12, and 12-24 hours. Following completion of a collection period the urine volume was recorded, the pH measured and 2x20 ml aliquots retained and stored at -20°C for subsequent assay.

4.2.5. Adverse Reactions

Three subjects complained once each of headache during treatment with the 300 mg tablet.

4.3. ANALYTICAL

4.3.1. Trimethoprim Estimation

Concentrations of unmetabolised TMP were determined using the LC assay described in section 2.7.6; the instrumentation configuration was the grouping described in section 2.6.2.(2) and the assay calibrated and quality controlled as previously detailed (section 2.6.1.).

4.3.2. Pharmacokinetic Parameters

i) Serum

The criteria for peak serum concentration, time to peak, derived and predicted pharmcokinetic parameters following the initial dose and the criteria for achievement of steady state were as described section 3.6.3.

ii) Urine

The highest observed urinary TMP concentration and the time at which this occurred relative to dose are referred to as peak urinary concentration ($C_u MAX$) and time to peak ($T_u MAX$) respectively. The observed peak urinary TMP excretion rate is $C_u MAX.V$.

Renal clearance (Cl_r) for a given collection period was calculated from the following equation:

$$Cl_r = \frac{C_u.V}{C_s} \qquad \text{Equation 4.1.}$$

where C_u is the average urine concentration per collection period, V is the average urine flow per collection period and C_s the mid-point serum concentration.

4.3.3. Statistical Analysis of Data

Appropriate parametric and non-parametric statistical tests were applied to the data as indicated in the method section. Where the results for the two preparations are compared, the correlation co-efficient, r, was calculated using a least squared regression programme. The limit of statistical significance was $p < 0.05$.

4.3.4. Clinically Effective Trimethoprim Levels

TMP levels considered desirable i.e. in excess of the MIC of common sensitive pathogens were taken as 2 and 8 mg/l for serum and urine respectively.

RESULTS

4.4. SERUM KINETICS

4.4.1. Single Dose Pharmacokinetics

i) Predicted and observed concentration

The predicted and mean (± s.d.) and range of observed serum concentrations for both preparations at the different sampling points are presented in table 4.1. Following the initial dose the predictions are higher than the observed mean serum concentrations although within one s.d. The s.d. on 200 mg b.d 0.5 hr and 300 mg o.d. 0.5, 1, and 2 hours are large and the observed mean values are notably lower than the predicted values. Subject 5 had unusual absorption characteristics; omitting this subject from 300 mg o.d., (0.5, 1 and 2 hours) resulted in mean values of 2.0, 3.1, and 2.8 mg/l respectively and omission of subjects 5 and 8 from 200 mg b.d. (0.5 hr) resulted in a mean concentration of 2.2 mg/l.

The predicted and observed mean trough serum concentrations agree well for both regimes; predicted $C_{ss}MAX$ is higher than the observed concentrations 2 hours following dose at steady state.

ii) Absorption

The lag times of absorption for both preparations was the same at 0.2 hours, the k_a for each preparation was calculated from mean serum concentration to minimise error and were found to be 3.01 h^{-1} for the 200 mg b.d. preparation and 2.77 h^{-1} for the 300 mg o.d. preparation. The k_a for subject 5 following the 300 mg dose is notably

TABLE 4.1

Mean, s.d. and range of serum TMP levels following 200 mg b.d. and 300 mg o.d. (mg/l).

Preparation	200mg b.d.				300mg o.d.			
Time	$\bar{x}$	s.d	range	Predicted	$\bar{x}$	s.d	range	Predicted
0	0	0	0	0	0	0	0	0
0.5	1.2	0.7	<0.07-2.1	1.8	1.8	1.0	<0.07-2.9	2.7
1	2.1	0.6	0.8-2.6	2.3	2.9	1.1	0.9-4.6	3.4
2	2.1	0.4	1.4-2.8	2.1	2.6	0.8	1.0-3.3	3.4
4	1.8	0.3	1.3-2.4	2.0	2.6	0.5	1.7-3.1	3.0
7	1.4	0.3	1.0-1.9	1.6	2.1	0.3	1.5-2.4	2.4
10	1.2	0.3	0.9-1.7	1.3	1.8	0.4	1.3-2.2	2.0
12	1.0	0.2	0.8-1.3	1.1	1.5	0.4	0.9-1.9	1.7
24	1.5	0.3	1.0-1.8	-	0.8	0.3	0.5-1.3	0.7
26	3.4	0.8	2.2-4.6		3.3	1.0	1.6-4.5	
48	2.1	0.6	1.1-3.2		1.1	0.3	0.8-1.7	
50	3.8	1.0	2.3-5.3		4.0	1.0	2.6-5.5	
72	2.2	0.6	1.4-3.1		1.0	0.4	0.1-1.3	
74	4.0	0.9	2.3-4.9		3.8	0.8	2.3-4.8	
96	2.1	0.7	1.1-2.9		1.3	0.6	0.5-2.3	
98	4.0	0.8	2.7-5.1		3.8	0.6	2.4-4.4	
C_{ss}MIN	-	-	-	2.0	-	-	-	0.9
C_{ss}AVE	3.1	0.7	-	3.3	2.5	0.5	-	2.5
C_{ss}MAX	-	-	-	4.5	-	-	-	4.7

slower than this at 0.7 h^{-1}; it was not possible to calculate the k_a for this individual during the 200 mg b.d. regimen as C_{MAX} was observed at 1 hour.

The mean serum pharmacokinetics are presented in table 4.2. The individual data are contained in appendix B. The median time to maximum concentration was identical and the ratio of C_{MAX} means was 0.688, the expected ratio is 0.67.

iii) AUC

As expected the AUC for the 300 mg o.d. dose is significantly different from that for the 200 mg b.d. dose ($p<0.001$, paired t test); the predicted AUC is higher than the mean in both cases as would be anticipated from the calculated values in table 4.1.

The AUC ratio for the 200:300 mg dose is 0.64 showing equivalent bioavailability (predicted ratio is 0.67).

iv) Volume of distribution

The mean volumes of distribution are comparable and are in agreement with the values obtained in Chapter 3. The inter-individual variation was greater than intra-individual variation and this was confirmed on examination of normalised Vd, this approach minimised differences due to weight and indicated good agreement.

v) Elimination

The elimination half-life, elimination rate constant and serum clearance are in good agreement with the mean values between the preparations and those previously

TABLE 4.2

Mean observed and derived serum pharmacokinetics for 200 mg b.d. and 300 mg o.d. TMP preparations

Preparation		Single Oral Dose										Steady State			
		T_{MAX} (h)		C_{MAX} (mg/l)	AUC (mg/l/h)	Vd (l)	Vd (l/kg)	$t_{\frac{1}{2}\beta}$ (h)	k_{el} (h^{-1})	Cl_s (h^{-1})		T_{ss}		C_{ss} PEAK mg/l	C_{ss} TROUGH mg/l
200 mg	median	1.0	mean ±	2.2	40.0 (Predicted 56.5)	75.4	1.08	10.2	0.072	5.4	Median	48	mean ±	3.9	2.2
			sd	0.4	12.4	14.7	0.12	2.6	0.020	1.3			sd	0.9	0.6
300 mg	median	1.0	mean ±	3.2	62.2 (Predicted 86.5)	74.9	1.07	10.3	0.072	5.4	median	48	mean ±	3.8	1.1
			sd	0.7	20.5	16.6	0.14	2.7	0.020	2.2			sd	0.8	0.3
	co-trifamole					77.8 ± 16.6		10.5 ± 1.0	0.067 ± 0.007	5.1 ± 0.8					
	co-trimoxazole					71.3 ± 15.6		11.0 ± 4.2	0.071 ± 0.024	5.0 ± 1.7					

reported in chapter 3. There was however marked inter and intra subject variation, particularly the elimination half life found for subject 4.

4.4.2. Steady State

There was no significant difference in time to steady state and C_{ss} peak between treatments ($p<0.05$), there was a significant difference between treatments for C_{ss} trough ($p<0.0001$, t test).

Comparable bioequivalence was again confirmed as the observed ratio for C_{ss}MIN (200 mg b.d./300 mg o.d.) was 2.0 and the predicted ratio was 2.2.

Throughout the 300 mg o.d. treatment no subject attained a C_{ss} trough of 2 mg/l, on 200 mg b.d. three subjects (3, 4 and 5) did not exceed 2 mg/l.

The mean observed C_{ss}AVE for 300 mg o.d. and 200 mg b.d. were 2.5±0.5 mg/l and 3.1±0.7 mg/l respectively; the predicted C_{ss}AVE levels (table 4.1) were 2.5 and 3.3mg/l respectively.

4.5. URINE KINETICS

4.5.1. Cumulative Excretion

The mean cumulative excretion of TMP over 96 hours for the 200 mg preparation was 575 mg (range 491-640 mg) i.e. 35.9% (30.7-40.0%) and 300 mg was 497 mg (range 402-581 mg) i.e. 41.4% (33.5-48.4%) of the dose.

4.5.2. Urinary Concentrations

The mean and range of observed urinary TMP for each collection period during the first 24 hours for both regimes (table 4.3) shows that for both preparations

TABLE 4.3

Mean and range of urinary TMP concentrations for each collection period (0-24hrs) for both regimens mg/l

Time (h)	0-2	2-4	4-6	6-8	8-12	12-24
200 mg						
mean	59	82	89	58	49	116
range	10-140	20-155	50-160	35-105	20-110	44-235
300 mg						
mean	76	77	112	95	85	74
range	34-160	26-124	86-148	60-180	42-176	28-192

a concentration of 8mg/l is exceeded in all subjects; as would be anticipated higher initial concentrations are achieved for the 300 mg preparation, these reached statistical significance at 6-8 and 8-12 hr ($p<0.05$). The lowest individual urine concentrations for each treatment were for 200 mg, 10 mg/l (subject 4, 0-2hrs) and 300 mg, 26 mg/l (subject 8, 2-4 hrs).

The urine concentrations achieved following multiple dosing reflect the dosing regimen (table 4.4). The lowest individual concentrations achieved after 24 hours were 40 mg/l for 200 mg b.d. (subject 8, 60-72 hours) and 24 mg/l for 300 mg o.d. (subject 3, 36-48 hours). The mean individual urinary concentrations achieved are noted in table 4.5; subject 4 had the lowest mean urine TMP levels.

Statistically significant differences between the preparations were found at 12-24 hours, 60-72 hours and 72-84 hours only ($p<0.05$, t test).

4.5.3. Excretion Rates

For the 0-12 hour period the mean maximum excretion rate for the 200 mg and 300 mg doses were 5.1± 1.4 mg/h and 7.0±1.1 mg/h respectively; the corresponding rates over the total period of the study were 9.2±1.9 mg/h and 8.0±1.4 mg/h. The peak excretion rates were significantly different for the 0-12 hour period, but did not reach significance over the period of the study.

4.5.4. Relationship of Renal Clearance of Trimethoprim to Other Parameters

The associations between renal clearance at 8-12 hours (i.e. after absorption and distribution are complete) and

TABLE 4.4.

Mean and range of urinary TMP concentrations for each collection period for both regimens mg/l

Time	0-12	12-24	24-36	36-48	48-60	60-72	72-84	84-96
Dose								
200 mg b.d.								
mean	54	116	90	75	117	113	129	149
range	22-69	44-235	46-110	50-104	58-224	40-210	50-215	42-380
300 mg o.d.								
mean	77	74	89	65	103	76	80	89
range	43-151	28-192	36-150	24-106	56-245	40-130	45-115	42-180

TABLE 4.5.

Mean and range of individual urine concentrations for both preparations (48-96 hours) mg/l

Subject	1	2	3	4	5	6	7	8
200 mg b.d.								
mean	146	98	159	65	185	185	91	88
range	114-224	80-115	120-215	50-86	58-380	135-240	40-170	42-168
300 mg o.d.								
mean	137	103	72	63	106	79	61	75
range	72-245	80-130	54-88	40-85	45-180	42-100	44-80	50-115

serum clearance, urinary flow, urine pH and mid-point serum TMP concentration were compared. The mean parameters for both dosage regimens are listed in table 4.6, they were not statistically significantly different between treatments. There was no correlation between renal clearance and either urinary flow or mid-point serum TMP concentration. There was statistically significant negative association with urine pH $r=-0.493$ (200 d.f.) ($p<0.01$) and also an association between renal and serum clearance $r=0.603$, (15 d.f. $p<0.01$)

The fraction of drug excreted unchanged (fe) for the 200 mg b.d. and 300 mg o.d. regimens respectively was 0.29 and 0.33; k_e the renal elimination rate constant was thus 0.021 and 0.024h^{-1} respectively ($k_e=fe.k_{el}$). The estimated metabolic clearance was approximately 2.9 1/h for both preparations.

TABLE 4.6

Mean renal clearance, serum clearance, urinary flow, urine pH and mid-point serum TMP concentration (10^h) for the period 8-12 hours post initial dose for both regimens

Preparation		Cl_r (l/h)	Cl_s (l/h)	V (l/h)	pH	C_p (mg/l)
200 mg	mean ± sd	2.49 ± 1.39	5.35 ± 1.34	0.074 ± 0.035	6.3 ± 0.4	1.22 ± 0.3
	range	1.3-5.5	3.1-6.9	0.023-0.125	5.6-6.7	-
300 mg	mean ± sd	2.60 ± 0.84	5.43 ± 2.19	0.068 ± 0.034	6.1 ± 0.6	1.78 ± 0.36
	range	1.4-4.2	3.6-9.3	0.013-0.123	5.3-6.8	-

DISCUSSION

4.6. SERUM KINETICS

4.6.1. Single Dose Pharmacokinetics

The absorption, elimination and distribution kinetics of the two TMP preparations were found to be similar; the principle area of difference being in the concentrations achieved, which was dose-related. As in chapter 3 the predicted serum TMP concentrations were in good agreement with those found following the initial dose although unusual individual absorption kinetics were noted.

4.6.2. Steady State Pharmacokinetics

In steady-state there was good agreement between predicted and observed $C_{ss}MIN$ and $C_{ss}AVE$. The predicted $C_{ss}MAX$ was higher than the mean 2 hour post morning dose samples which might be anticipated as this sampling point does not coincide with true $C_{ss}MAX$, following the initial dose median C_{MAX} was at one hour. The observed $C_{ss}MIN$ and $C_{ss}AVE$ were significantly different between treatments however $C_{ss}MAX$ was not; but substituting mean $C_{ss}MIN$ values (table 4.1) in the equation, with V=75_1

$$C_{ss}MAX = \frac{F.D}{V} + C_{ss}MIN \qquad \text{Equation 4.2}$$

then the predicted $C_{ss}MAX$ values for the 200 mg b.d. and 300 mg o.d. regimens are 4.9 mg/l and 5.1 mg/l

respectively; thus no difference at or near $C_{ss}MAX$ would be anticipated.

4.6.3. Bioequivalence

Bioequivalence was demonstrated by the finding of the anticipated ratios for C_{MAX} and AUC and the comparability between the ratio anticipated for $C_{ss}MIN$.

4.6.4. Intraindividual Differences

Although there was variation in the absorption of TMP between individuals sometimes markedly so, the finding was not reproducible between study legs; assuming that the individual(s) concerned obeyed the manufacturers instructions as to dosage and timings, no explanation can be offered for the anomalies.

4.6.5. Effect of Sulphonamide on Trimethoprim Kinetics

The pharmacokinetic parameters calculated for both dosage regimens are comparable with those calculated for TMP in chapter 3 for co-trimoxazole and co-trifamole; therefore the presence of sulphonamides has no detectable effect on the kinetics of TMP, a finding which confirms the work of others (Nolte and Buttner, 1973; Welling et al, 1973)

4.6.6. Half-life of Elimination and Dose

Although there are no great differences in the TMP dosages studied it is of interest to note that TMP exhibits linear disposition kinetics; in a report of an overdose a half-life of 11.9 hours was found following an ingested dose of around 3.2 g (Hoppu,Partanen and Koskela 1980)

particularly as large single oral doses of co-trimoxazole have been used (Yoshikawa and Guze, 1976); it is possible that such an approach could be used for TMP alone.

4.6.7. Serum: Clinical Implications of the Dosage Regimens

The MIC for species sensitive to TMP is 2 mg/l (Brumfitt and Hamilton-Miller, 1980). The serum trough concentrations obtained using the 300 mg dose may therefore be regarded as unsatisfactory, and the level achieved in subject 3 on 200 mg b.d. was also unsatisfactory. However TMP serum concentrations are not as relevant as the concentrations achieved at the site of the infection.

4.7. URINE KINETICS

4.7.1. Urine: Clinical Implications of the Dosage Regimens

At the time this work was performed the 300 mg o.d. preparation was intended only for treatment of urinary tract infections whereas the 200 mg b.d. regimen was also to be used in respiratory tract infection. In urine the MIC of TMP should be considered as 8 mg/l (Huovinen and Toivanen, 1980; Huovinen et al, 1982); the lowest urinary TMP concentration on repeated dosing exceeded this value by a factor of 3 and usually more. Comparing mean urine TMP levels from the achievement of steady state on showed that the lowest mean TMP level exceeded the MIC by a factor of more than 7. Therefore from a pharmacokinetic standpoint either preparation would be acceptable for the treatment of urinary tract infection.

4.7.2. Relationship of Renal Clearance of Trimethoprim to Other Parameters

Renal clearance correlated with pH (negative correlation) and serum clearance (positive correlation), such findings are consistent with previous reports (Sharpstone, 1969; Bergan and Brodwall, 1972) the mean Cl_r/Cl_s ratio is of the order of 0.5, somewhat lower than the 0.68 reported by Andreason et al,(1978). There was no association between renal clearance and urine flow, which is contrary to the findings of Andreason et al (1978), but in agreement with the findings of Sharpstone (1969).

The relationship between urinary pH and renal clearance is not surprising in view of the pKa of the drug and the physiological range of urinary pH, over this range TMP will to varying extents be protonated. The evidence suggests that renal tubular reabsorption of TMP is pH dependant (Sharpstone, 1969, Friesen, Hekster and Vree, 1981). Thus at the pH of blood, TMP is 50% ionised i.e. 50% of the non-protein bound fraction is unionised and able to passively diffuse across the renal tubular cells, TMP will also undergo filtration. As the hydrogen-ion concentration of the urine increases, TMP is increasingly protonated, as an ionised molecule it is difficult to back diffuse, hence the concentration of TMP is greater in situations where pH dependant accumulation can occur.
The renal clearance of TMP would be expected to be

variable due to the influence of pH. The renal clearances from this study compare with values of 1.1-8.9 l/h (mean 3.3 l/h) (Sharpstone, 1969), 3.1-8.3 l/h (Bergan and Brodwall, 1972) and 1.5-9.5 l/h (Andreasen et al, 1978).

The excretion rates did not differ significantly in the steady-state portions of each part of the study, as this is a dose related parameter this might have been attributed to variations in individual pH, but no association was found. This finding may reflect the need for a larger number of subjects to be studied.

4.8. CONCLUSION

The comparability of the kinetics of the two preparations indicate that there is equivalent bio-availability, the urinary concentrations achieved are adequate for use in urinary tract infection, however the 'steady-state' serum concentrations in the 300 mg o.d. regimen were consistently below 2 mg/l, the MIC for sensitive organisms. Where serum concentrations are a guide to efficacy the kinetics suggest that a 300 mg o.d. regimen would be inappropriate for the treatment of infections where penetration to the site of infection is not subject to favourable pH gradients, indeed this could select for resistance, (Platt, Guthrie and Langan 1983) although this is probably rare (Lacey, 1982). Due to the higher dose and hence higher serum levels the 200 mg b.d. preparation would be preferable.

Se no è vero ma è ben trovato.

(It may not be true but it is well contrived).

Giordano Bruno 1548-1600

CHAPTER 5

THE RELATIONSHIP BETWEEN SALIVA AND SERUM TMP CONCENTRATIONS

INTRODUCTION

5.1. SALIVA AND DRUG MONITORING

Since saliva can be obtained non-invasively it has been advanced as an acceptable alternative to serum for investigating compliance, obtaining pharmacokinetic information or for therapeutic drug monitoring.

A strong case has been made for the use of saliva in the monitoring of anticonvulsants in paediatric patients (Knott and Reynolds, 1984) however in two recent reviews (Danhof and Breimer, 1978; Mucklow, 1982) it was agreed that the anticonvulsants phenytoin and carbamazepine could be monitored using saliva, but opinion differed for phenobarbitone and ethosuximide; it was agreed that for digoxin, theophylline, procainamide and a variety of other drugs a poor correlation was obtained between saliva and serum levels.

5.1.1. Sources of saliva

Saliva is secreted by the three major paired salivary glands, the parotid, submandibular and sublingual. In addition there are a number of smaller glands (labial, buccal and palatal); the composition of saliva from these glands varies considerably and the combined product is termed 'mixed' saliva. The composition and flow rate of saliva changes rapidly following stimulation; mixed saliva contains the electrolytes potassium, sodium, chloride and bicarbonate each at concentrations of approximately 20 mmol/l plus calcium and magnesium at 10% and 1% respectively of these levels, the other main components

are enzymes, the chief of which is amylase, and mucus, of which the main constituents are the sialomucins and the fucomucins which are sialic acid rich mucoproteins and fucose-rich mucopolysaccharides respectively; the mucins are present at a concentration of 2.7 g/l (Documenta Geigy, 1975). Although the nature of the stimulus to salivation does not affect the composition of the saliva produced, the proportions of parotid and sub-mandibular saliva can vary according to stimulus and as these glands give saliva of different composition, the mixed saliva produced will reflect these differences (Dawes and Jenkins, 1964). So far as the effect on drug concentrations is concerned the most important factor is the hydrogen ion concentration of the saliva since this will determine the extent of partition of ionised drugs. Resting saliva, both parotid and submandibular, has a pH of 6.8-7.2 (Danhof and Breimer, 1978) whereas saliva secreted following stimulation may show a rise in pH of as much as two pH units. The extent of the pH change has been reported as being related to flow rate (Dawes and Jenkins, 1964).

5.1.2. Stimulation of Salivary Flow

Encouragement of saliva flow can be achieved either by mastication or by a gustative stimulus. A number of materials have been used for mastication-stimulated salivary flow including paraffin wax (Hoeprich and Warshauer , 1974), parafilm (Koup, Jusko and Goldfarb, 1975), teflon (Boxenbaum et al, 1975) or a washed rubber

band (Hallstrom, Lader and Curry, 1980). Parafilm suffers from the disadvantage that it is likely to 'extract' lipophilic drugs (Chang and Chiou, 1976; Taylor, Kaspi and Turner, 1978) and such a criticism is likely to apply also to paraffin wax. A relatively inert material such as teflon or a rubber band is to preferred provided that substances which may interfere in the assay are not leached from them.

A number of sapid stimuli were used by Dawes and Jenkins (1964) including citric acid, oxo and salt; citric acid is the most commonly used of these (Reynolds et al, 1976; Stephen and Speirs, 1976).

5.1.3. Collection of Saliva

Following stimulation an adequate pool of mixed saliva is allowed to collect in the mouth and is smoothly expelled into a preweighed collecting tube, avoiding expectoration (Mucklow, 1982). The use of a preweighed vial and known collection time allows calculation of the salivary flow rate (Hallstrom et al, 1980). The salivary hydrogen ion concentration needs to be determined when measuring ionized drugs (Dvorchik and Vesell, 1976). Loss of bicarbonate will result in falsely elevated estimates of salivary hydrogen ion concentration at the point of secretion (Borzelleca and Putney, 1970; Mucklow, 1982), although once saliva has been collected the pH has been found to be stable for 24 hours (Hallstrom et al, 1980).

A number of devices have been described for the

sampling of individual glands (Lashley, 1916; Shannon and Chauncey, 1967; Stephen and Speirs, 1976) although these do not lend themselves readily to routine use.

5.1.4. Passage of Drugs into Saliva

It is generally accepted that the passage of drugs from the plasma, through the salivary gland cells to the salivary ducts is effected by passive diffusion; therefore the difference in concentration across the gradient will determine the rate of penetration from plasma to saliva. In addition the physical properties of the drug will determine its ability to cross the membranes of the salivary gland cells, the more lipophilic a compound the better will be its diffusion across a lipid membrane; the degree of ionisation at physiological plasma pH will determine the extent of penetration and its degree of ionisation at physiological saliva pH will affect its rate of back-diffusion. Prediction of plasma drug concentrations from saliva is best for drugs that are mainly un-ionised at physiological plasma pH (Mucklow, et al, 1978).

There appears to be little evidence of drug binding to the mucins, drug in saliva is usually considered to be unbound (Mucklow, 1982).

A number of antimicrobial agents have been investigated to determine whether there is a relationship between saliva and serum concentrations. Day and Houston (1980) were able to establish acetylator phenotypes using salivary sulphapyrimidine and acetylsulphapyrimidine

levels, although results using salivary dapsone gave conflicting results (Lammintausta, Kangas and Lammintausta, 1979; Peters et al, 1981). Pyrimethamine appears to be a suitable candidate for salivary monitoring (Ahmad and Rogers, 1981) whereas there was no relationship between saliva and serum chloramphenicol levels (Koup et al, 1979).

It has been proposed that the ratio between saliva (S) and plasma (P) (or S/P ratio) can be predicted for ionised drugs as was successfully shown for tolbutamide by Matin, Wan and Karam, (1974).

Since TMP is a basic drug with a pKa of 7.3 and is substantially ionised at physiological plasma pH it is a useful probe for assessing the predictability of the equation of Matin et al (1974). The saliva and serum concentrations of TMP have previously been investigated by several groups of workers in dogs (Granato, Gross and Stamey, 1973) and in humans: (Quayle and Hailey, 1973; Hansen, et al 1973a; Koup, et al 1975; Eatman et al 1977; Bernard et al, 1978; Sardi, et al, 1981 and Kamme, Melander and Nilsson, 1983).

Of these reports those of Koup et al (1975), Eatman et al (1977) and Sardi et al (1981) have considered the predictability of the TMP S/P ratio using the equation of Matin et al (1974).

Significant correlation between serum and saliva TMP concentrations has been reported by Eatman et al (1977) and Sardi et al (1981). Good agreement between observed and predicted S/P ratios is claimed by Koup et al

(1975), Eatman et al (1977) and Sardi et al (1981).

Saliva and serum concentrations obtained during the study reported in Chapter 4 were used to assess the predictability of the equation of Matin et al (1974) for TMP. In addition the relationship between saliva and serum TMP concentrations and the influence of saliva hydrogen ion concentration and saliva flow rate on the observed TMP S/P ratio was examined. Two dosage forms (200 mg b.d. and 300 mg o.d.) were also studied since Sardi et al (1981) have suggested that saliva might be a useful medium for examining the relative disposition of different dosage forms of TMP.

METHODS

5.2. CLINICAL

5.2.1. Study Design

Saliva samples were collected as described in section 2.8.1 from the participants of the study described in section 4.2.2. The samples were collected at the same time as blood samples i.e. 2 and 10 hours after the initial dose and immediately prior to and 2 hours after the dose administered at 72 hours. These samples were analysed for TMP as described in Chapter 2 as were serum ultrafiltrates using the modified TMP assay and the second and latterly the third instrument configuration (section 2.6.2.(2) and 2.6.2.(3)).

5.3. MATHEMATICS

5.3.1. Prediction of Saliva/Serum Ratio

The Henderson-Hasselbalch equation (5.1) describes the relationship between degree of ionisation and pH. The partition of TMP between plasma (or serum) and saliva is pH dependent.

The Henderson-Hasselbalch equation is given below:

$$pH = pKa + \log \frac{|A^-|}{|HA|} \qquad \text{Equation 5.1.}$$

This may be rewritten as:

$$\frac{|A^-|}{|HA|} = 10^{(pH-pKa)} \qquad \text{Equation 5.2.}$$

But,

$$[A^-] + [HA] = [A] \qquad \text{Equation 5.3.}$$

Combining equations 5.2 and 5.3 gives the following result:

$$[A] = 1+10^{(pH-pKa)} \qquad \text{Equation 5.4.}$$

Clearly the ratio of saliva and plasma (or serum) concentrations is a ratio of equation 5.4 i.e.

$$\frac{[A] \text{ saliva}}{[A] \text{ plasma}} = \frac{1+10^{(pH_s-pKa)}}{1+10^{(pH_p-pKa)}} \qquad \text{Equation 5.5.}$$

Equation 5.5. applies to acids, equation 5.6. describes the predicted ratio for bases i.e.

$$\frac{[B] \text{ saliva}}{[B] \text{ plasma}} = \frac{1+10^{(pKa-pH_s)}}{1+10^{(pKa-pH_p)}} \qquad \text{Equation 5.6.}$$

A factor which allows for the proportion of the drug which is free (i.e. unbound) also needs to be included.

$$\frac{S}{P} = \frac{[B] \text{ saliva}}{[B] \text{ plasma}} = \frac{1+10^{(pKa-pH_s)}}{1+10^{(pKa-pH_p)}} \cdot \frac{f_p}{f_s} \qquad \text{Equation 5.7.}$$

In these equations pKa is the pKa of TMP (7.3), pH_s is the saliva pH (as measured on collection), f_p is the fraction of unbound drug in plasma (0.6) (Schwartz and Zeigler, 1969) and f_s is the fraction of unbound drug in saliva (assumed to 1.0) (Mucklow, 1982).

Equation 5.7 corresponds to the equation given by Matin et al (1974) for bases and used by other workers for TMP.

Actual individual S/P ratios were determined from the measured serum and saliva TMP concentrations; these were compared with the calculated ratios.

5.4. STATISTICS

5.4.1. Calculation of 95th Percentile

The 95th percentile line on Figs. 5.1 and Figs. 5.3 was calculated from the following formula:

$$S\hat{y} = Sm\sqrt{\sigma_x^2 + (x_i - \bar{x})^2} \qquad \text{Equation 5.8.}$$

where $S\hat{y}$ is the standard error of y

σ_x^2 is the variance of x

Sm is the standard error of the gradient of x to y

x_i is the ith observation

and $\bar{x}$ is the mean of x

5.4.2. Estimates of Precision and Bias

In addition to the association comparison of observed TMP plasma level and calculated TMP plasma level, predictive evaluation was performed according to the recommendations of Sheiner and Beal (1981a) on

calculated TMP levels from equation 5.7 and TMP levels calculated from the regression line of saliva TMP versus serum TMP (Fig 5.1)

A measure of the precision of the prediction is given by the mean squared prediction error (mse)

$$mse = \frac{1}{n} \sum_{i=1}^{n} pe_i^2 \qquad \text{Equation 5.9.}$$

where pe (prediction error) is the difference between the predicted and observed value; root mse (rmse) is

$$rmse = \sqrt{mse} \qquad \text{Equation 5.10.}$$

and is a measure of precision in the original units of the original quantities.

Bias is obtained by calculating the mean prediction error (me) which is defined as

$$me = \frac{1}{n} \sum_{i=1}^{n} pe_i \qquad \text{Equation 5.11.}$$

Relative precision is calculated as the difference between two predictions i.e.

$$\Delta mse = mse_1 - mse_2 \qquad \text{Equation 5.12.}$$

similarly relative bias is calculated by difference

i.e. $$\Delta me = me_1 - me_2 \qquad \text{Equation 5.13.}$$

A naive standard is an absolute predictive comparator, only predictions better than this standard are preferred for forecasting. The naive standard assumes that all predicted levels are equivalent to the mean of the observed data.

The confidence estimates aid comparison, essentially if the confidence interval does not include zero, the smaller mse is the most precise, if it does include zero the difference is not significant at the 0.05 level (using a 2 tailed test) although the smaller mse is usually favoured (Sheiner and Beal, 1981a).

Δmse and Δme are measures of relative precision and bias, essentially the smaller the value the better they are, indeed a negative value favours that predictor as a better estimate than the predictor to which it has been compared.

Confidence limits were calculated using the following equations:

$$\bar{x} - t_{0.975}^{(n-1)} se_{\bar{x}} < x_t < \bar{x} + t_{0.975}^{(n-1)} se_{\bar{x}}$$

Equation 5.14.

where:

$$se_{\bar{x}} = \left[\frac{1}{n(n-1)} \sum_{i=1}^{n} (x_i - \bar{x})^2 \right]^{\frac{1}{2}}$$

Equation 5.15.

The $se_{\Delta mse}$ was calculated using:

$$se_{\Delta mse} = \left[\frac{1}{n(n-1)} \sum_{i=1}^{n} \left[(pe_{1i}^2 - pe_{2i}^2) - mse \right]^2 \right]^{\frac{1}{2}}$$

Equation 5.16.

and for $se_{\Delta me}$:

$$se_{\Delta me} = \left[\frac{1}{n(n-1)} \sum_{i=1}^{n} \left[(pe_{1i} - pe_{2i}) - me \right]^2 \right]^{\frac{1}{2}}$$

Equation 5.17

RESULTS

Only mean data are presented in this section, data from individuals are given in appendix C.

5.5. TMP PARTITION IN SALIVA

5.5.1. Comparison of Saliva and Serum Concentrations

There was a statistically significant linear correlation between observed saliva TMP and serum TMP, this relationship held for both dosage regimes and for all time points ($p<0.01$) (Fig 5.1.); 8.2% of points are outside the 95% confidence limits. Saliva and serum TMP concentrations closely paralleled each other for both dosage regimes (Fig 5.2.). Occasional saliva levels were notably greater than the serum level.

5.5.2. Observed S/P Ratios

The mean S/P ratio for all time points was 0.879 ± 0.22 for the 300 mg o.d. regimen, 0.835 ± 0.20 for the 200 mg b.d. regimen and 0.874 ± 0.21 for both regimens. The ratios for each time point are given in table 5.1.

There was no statistically significant difference between times, treatments or individuals (t.test).

5.5.3. Effect of Saliva Flow Rate and Hydrogen Ion Concentration on Salivary TMP Concentration

The mean saliva flow rate was 1.386 ± 0.426 ml/min and the mean hydrogen ion concentration was 35.92 ± 24.2 nmol/l. Using non-parametric statistics a significant (non-linear) correlation was found between salivary flow rate and hydrogen ion concentration ($p<0.01$,

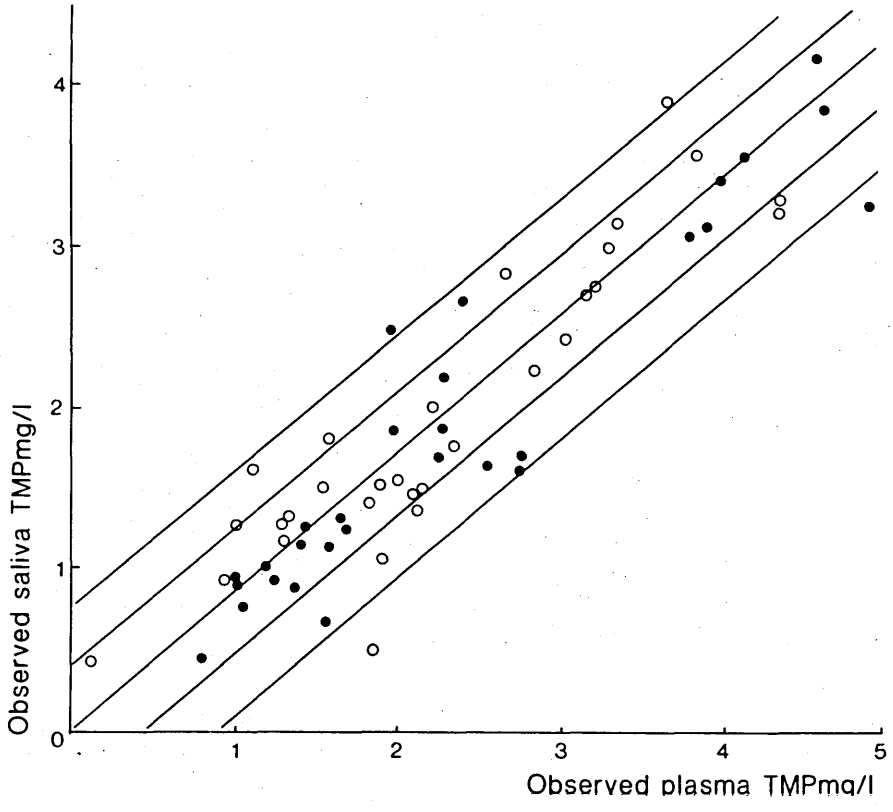


FIGURE 5.1. Association of observed plasma TMP versus observed saliva TMP. The regression line is bounded by 68 and 95% confidence limits.

● = 200 mg TMP b.d.
o = 300 mg TMP o.d.

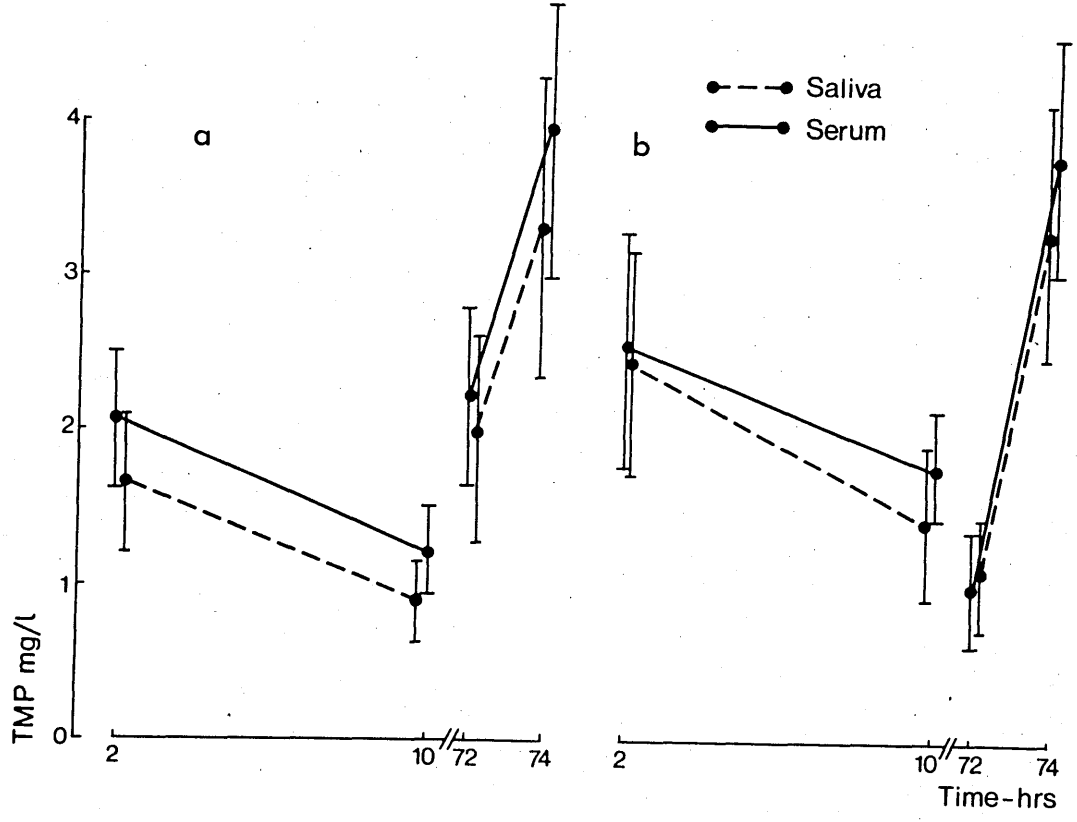


FIGURE 5.2. Relationship between serum and saliva TMP during dosing
a) 200 mg b.d. b) 300 mg o.d.

TABLE 5.I

TMP S/P ratios at different time points for 200 mg and 300 mg dosage regimens.

Time (hrs)		2	10	72	74
200 mg	mean	0.90	0.75	0.94	0.84
regimen	sd	0.30	0.17	0.24	0.11
300 mg	mean	0.86	0.79	1.07	0.87
regimen	sd	0.13	0.24	0.23	0.12

Kendall rank correlation coefficient). Salivary flow rate appeared reasonably reproducible within an individual although there were insufficient data points to test this hypothesis statistically. Hydrogen ion concentration was less consistent within individuals between study legs.

There was no significant correlation between salivary TMP concentration and salivary hydrogen ion concentration, but there was a weak negative correlation with salivary flow rate ($r=-0.288$, $p<0.05$).

No correlation was found between observed S/P ratio and either salivary hydrogen ion concentration or salivary flow rate.

5.5.4. Predicted S/P Ratios

The predicted range of S/P ratios using equation 5.7 and the assumptions previously noted, was 0.4-0.9. This is similar to the observed range of 0.4-1.59 over an observed pH range of 6.5-8.2. However, there was no correlation between individual observed and predicted S/P data points using either parametric or non-parametric tests of association.

A comparison between predicted and observed TMP S/P ratios from this study and those reported by other workers is given in table 5.2.

5.5.5. Prediction and Measurement of fp

The free plasma concentration can be calculated using equation 5.7 by substituting observed S/P and pHs; the result of this manipulation are presented in

TABLE 5.2

Reported observed and predicted TMP S/P ratios

AUTHORS	Observed TMP S/P ratio	Calculated TMP S/P ratio
Koup et al (1975) using data of Hansen et al 1973a	1.0-8.3	0.7-7.6
Eatman et al (1977)	1.26±0.345	1.27 (mean)
Sardi et al (1981)	0.839±0.171	0.64-0.73
This study	0.874±0.21 (range 0.4-1.59)	0.4-0.9

Fig 5.3, clearly prediction is poor with wide intra-individual scatter.

TMP concentrations in serum ultrafiltrate were estimated at 2 and 72 hours during the 200 mg b.d. regimen, the mean value was 0.2 (± 0.08) and 0.54 (± 0.12) mg/l respectively, representing 9.8% and 13.0% of the serum concentrations respectively.

5.5.6. Relative Predictive Performance

From Fig 5.3. it is clear that fp cannot be predicted, there was however an association between the observed serum TMP concentration and that predicted from equation 5.7, (Fig 5.4) although 14/60 (23%) of points are outwith the 95% confidence limits.

As the regression obtained for observed saliva TMP concentration versus observed serum TMP concentration was better (Fig 5.1) than the performance of prediction using equation 5.7, a comparison of the predictive performance of these two approaches was required. Sheiner and Beal (1981) have criticised the use of regression lines for predictive purposes and had developed an approach for the estimation of precision and bias of predictions using equations 5.9-5.17; these were applied to assess the predictability of equation 5.7 and linear regression (Fig 5.1) for predicted values as compared to observed values for the 300 mg o.d. data (appendix C, table C.2), the results of this exercise are summarised in table 5.3. Linear regression performed best, equation 5.7 performed poorly, worse than the

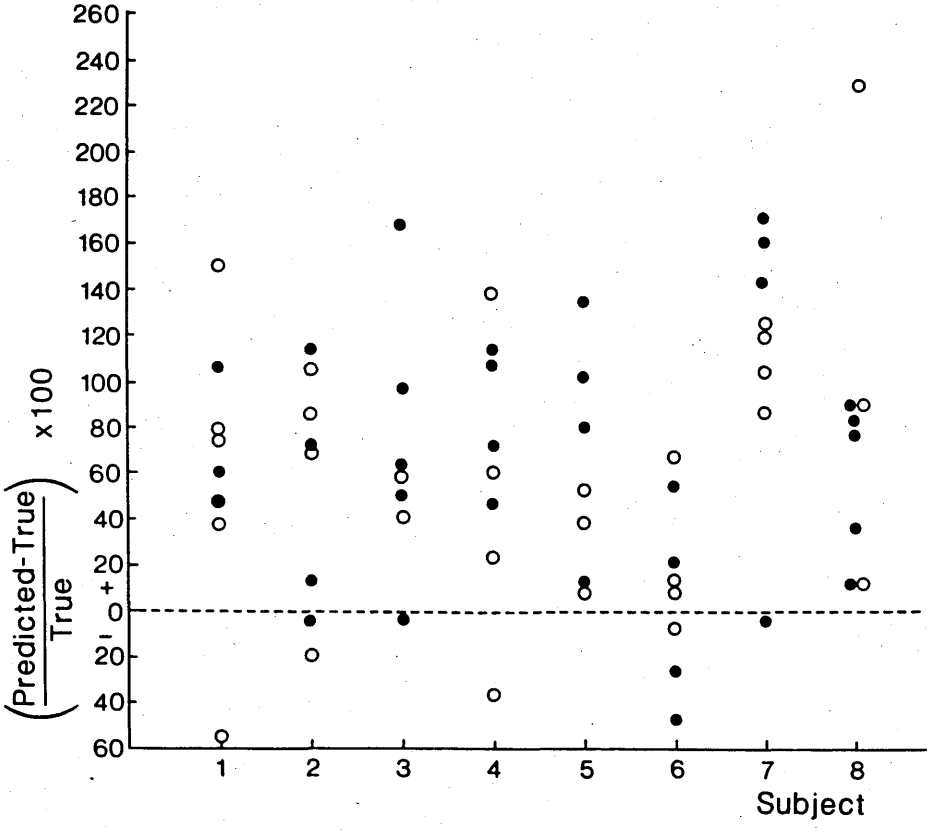


FIGURE 5.3. Prediction of fp from equation 5.7 for each subject at each observed time point.

● = 200 mg TMP b.d.
o = 300 mg TMP o.d.

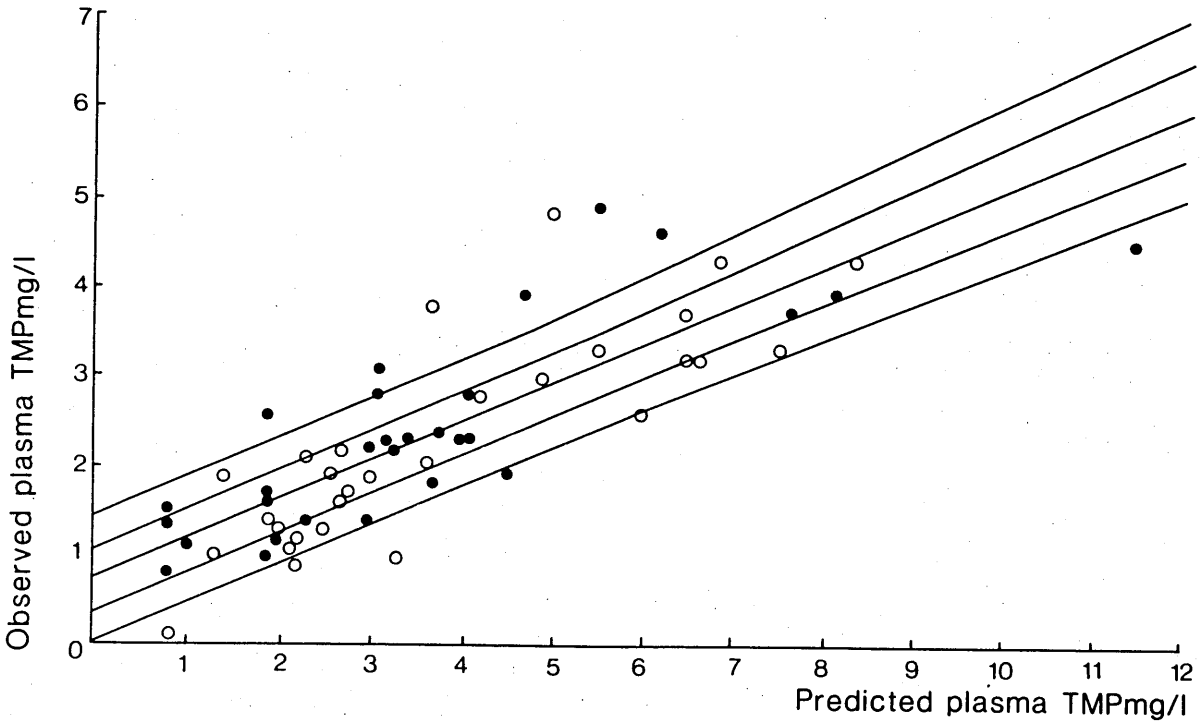


FIGURE 5.4. Association between predicted plasma TMP concentration from equation 5.7 and observed plasma TMP concentration. The regression line is bounded by 68 and 95% confidence limits. ● = 200 mg TMP b.d. o = 300 mg TMP o.d.

TABLE 5.3

Relative predictive performance of equation 5.7 and linear regression of saliva TMP concentration versus serum TMP concentration. Values in parentheses are the 95% confidence limits. See text for an explanation of the terms.

Precision	A(Equation 5.7)	B(Linear regression)	Naive Standard
mse	3.63(1.74, 5.52)	0.18(0.10, 0.26)	1.44
rmse	1.91(1.32, 2.35)	0.42(0.32, 0.51)	1.20
Δmse (to naive standard)	2.19(0.19, 4.19)	-1.26(-1.82, -0.7)	
Δmse (BvA)		-3.45(-6.73, -0.17)	
Bias			
me	1.41(0.92, 1.91)	0.04(-0.11, 0.19)	0(by definition)
Δme (to naive standard)	1.41(0.54, 2.28)	0.04(-0.41, 0.49)	
Δme (BvA)		-1.49(-2.68, -0.3)	

absolute (naive) standard and was a poor predictor.

5.6. BIOEQUIVALENCE: SERUM VERSUS SALIVA

5.6.1. TMP Concentrations in Saliva and Serum

Salivary and serum TMP concentrations were significantly different between treatments 2, 10 and 72 hours ($p<0.01$, t.test). There was no significant difference between dose regimens at 74 hours for either serum or saliva concentrations.

Calculation of $C_{ss}MAX$ (Equation 3.5) predicts levels of 4.5 mg/l and 4.7 mg/l for the 200 mg b.d. and 300 mg o.d. regimens (table 4.1) thus no difference would be expected.

5.6.2. Concentration Ratios between Regimens

Using the equations previously described (Equations 3.4 and 3.7) the anticipated serum level ratio (200mg/300mg) for the two regimens can be calculated at 10 and 72 hours; the results of this along with the mean serum ratios and mean saliva ratio are given in table 5.4. There was greater variation associated with the saliva results than with serum results, this was significant at 10 hours ($p< 0.05$, F test) but not at 72 hours.

TABLE 5.4.

Predicted mean serum and mean saliva concentration ratios (200mg/300mg) at 10 and 72 hours post initial dose.

Time (hrs)		10	72
Predicted ratio		0.67	2.16
Serum ratio	$\bar{x}$	0.68	2.13
	sd	0.11	0.45
Saliva ratio	$\bar{x}$	0.76	2.0
	sd	0.51	0.51

DISCUSSION

5.7. TMP PARTITION IN SALIVA

5.7.1. Comparison of Saliva and Serum Concentrations

The highly significant correlation between saliva and serum concentrations is in agreement with previous reports (Eatman et al, 1977; Bernard et al, 1978; Sardi et al, 1981); calculation of the results reported by Hansen et al, (1973a) also shows good correlation.

5.7.2. Observed S/P Ratios

The mean observed TMP S/P ratio compares well with that noted by Sardi et al,(1981) but is 30% lower than that quoted by Eatman et al (1977), however these authors measured TMP in blood rather than serum and on the basis of a blood/plasma concentration ratio determined by *in vitro* experimentation, adjusted all their observed blood levels by the mean of this result. The ratios found by Hansen et al (1973a) are much higher (up to 8.5) which would suggest some form of active secretion with the 'ion-trapping' effect of a saliva pH which was lower than serum pH. The same authors noted reproducible S/P ratios at different times within one individual.

5.7.3. Effect of Saliva Flow Rate and Hydrogen Ion Concentration on Salivary TMP Concentration

There is a non-linear relationship between salivary flow rate and hydrogen ion concentration (Dawes and Jenkins, 1964), this work confirms this finding. Since the scatter observed was large the only statement which

can be made is the general one that increasing salivary flow rate results in a decrease in hydrogen ion concentration.

TMP partition will be profoundly affected by minor fluctuations in salivary pH (and hence flow rate), a view supported by Mucklow et al (1978) and noted for lidocaine (Barchowsky et al, 1982) and for chloramphenicol (Koup et al, 1979).

The relationship between saliva TMP concentration and saliva pH is not discernable, and although there is a weak correlation between saliva TMP and flow rate, this only accounts for 8.3% of the variability and is of marginal significance.

5.7.4. Predicted S/P Ratios

Various authors have endeavoured to use the equation of Matin et al (1974), the observed range of S/P ratios of 0.4-1.59 (mean 0.87) compares with the range calculated using equation 5.7 of 0.4-0.9 (saliva pH 7.1-8.2), superficially there seems to be agreement. Koup et al (1975) calculated TMP S/P ratios from the data of Hansen et al (1973a) and predicted a range of 0.7-7.6 which compared with an observed range of 1.0-8.3, this was despite the fact that Hansen et al (1973a) did not give pH values for their saliva samples. Koup et al (1975) used a saliva pH range of 6.3-8.0, using the equation they quoted (equivalent to equation 5.6) the calculated range is actually 0.7-6.1, presumably an error in calculation was made, in addition these authors neglected

the fp component of equation 5.7 (Matin et al, 1974) incorporating this the calculated range of S/P ratios becomes 0.4-3.7.

Eatman et al (1977) claimed close agreement between an observed S/P ratio of 1.26 and a calculated ratio of 1.27, however they assumed that the carbon dioxide loss from collected saliva would result in a pH reduction of one unit and calculated their results at a pH of 6.5, despite the fact that in their study salivary flow was stimulated by mastication of a piece of teflon tape, this will result in a more alkaline saliva (Dawes and Jenkins, 1964; Sardi et al, 1981). Their assumption as to the extent of carbon dioxide loss after collection is probably an overestimate, (Hallstrom et al, 1980; Mucklow, 1982). In the present study the pH of the collected saliva was determined immediately, and although there may be carbon dioxide loss between the instant of secretion and the moment of collection it is unlikely to be such a large change although evidence for collection loss has been reported (Borzelleca and Putney, 1970; Levy et al 1980). Eatman et al (1977) considerably overestimated the TMP S/P ratio as 2.44 wheras without pH "correction" a value of about 0.7 would have been obtained. This would also have avoided the dubious manoeuvre of correcting their calculated TMP S/P ratio for "buccal mucosa partitioning" by 48% giving an S/P ratio of 1.27, this hypothesis was discounted by Sardi et al (1981).

Sardi et al (1981) assumed a decrease in saliva

pH between secretion and collection of 0.2-0.3 pH units based on the findings of Dawes and Jenkins, (1964) and Mucklow et al (1978), although the degree of loss is speculative. Sardi and co-workers claim comparability between the calculated and observed parameters although the former are higher than the observed by 15-30%; the comparability was not tested statistically.

Calculation of S/P ratio using equation 5.7 requires assumptions as to plasma pH, f_s and f_p. Most authors assume plasma pH to be 7.4. The fraction unbound in serum (f_p) has been measured by Schwartz and Zeigler (1969) and Sardi et al (1981) and found to be 0.58 and 0.51 ± 0.02 for TMP; other authors have assumed a value of 0.5 or 0.6 for this variable; the latter assumption was made in this work. Eatman et al (1977) quote Devine et al (1969) as the source of this figure.

The fraction unbound in saliva is taken to be 1.0 unless there is evidence to the contrary (Mucklow, 1982), this has also been the practice of other workers.

5.7.5. Prediction and Measurement of fp

Calculation of the free serum fraction (fp) was wildly inaccurate and did not reflect previously reported values. As calculated fp is generally greater than 0.6 (the observed fp) this suggests that fs was not overestimated as this would result in an underestimate of fp, however the extent of CO_2 loss will affect predicted fp; it will be overestimated if there is a decrease in hydrogen ion concentration, no adjustments were made to

the measured pHs in this study. In view of the over-estimate found some adjustment would appear appropriate, however there was no consistency in the estimates for calculated S/P ratio or calculated fp. Although the plasma ultrafiltrate levels suggested binding of the order of 90% this does not agree with any literature reports and is probably an artefact caused by binding of TMP to the ultrafiltration membrane.

The lack of consistency in any predictions attempted, there being no reproducible over or underestimate, indicate the inapplicability of the equation of Matin et al to drugs that are largely ionised at normal serum and saliva pH; this expands the caution expressed by Mucklow et al (1978).

5.7.6. Relative Predictive Performance

Using the criteria suggested by Sheiner and Beal (1981a) it is seen that predictions made from the linear regression line (Fig 5.1) of saliva TMP concentration versus serum TMP concentration gives estimates which are closer to those observed than values obtained using equation 5.7. The former estimates show better precision and less bias as measured by mse and me respectively and compare well with the absolute (naive) standard (Δmse and Δme), the finding of a negative estimate between the two predictors confirms linear regression as being superior.

Since the relative precision of equation 5.7 is worse than the naive standard (positive Δmse for A minus

naive) the relevance of this equation as an adequate mathematical description of partitioning between saliva and plasma (serum) is open to considerable doubt.

The theoretical advantages of using saliva rather than blood samples as a means of predicting the plasma concentration of TMP, which is significantly ionised at physiological pH, are not borne out by this work. The correlation observed between saliva and serum TMP concentrations allows an approximate estimate of the serum concentration to be made from analysis of saliva.

5.8. BIOEQUIVALENCE:SERUM VERSUS SALIVA

As would be expected,observed saliva TMP data on both treatment regimens compared well with the data obtained from serum, however there was a larger variance, thus there would be an increased error in any parameters derived from saliva values; for bioequvalence studies saliva values would probably be adequate.

An aspect of salivary monitoring that is mentioned infrequently is the aesthetics of this approach to sampling; most volunteers found the mastication technique mildly unpleasant and the sample collection more so and expressed their views that a blood sample was easier and quicker to obtain and, for them at least, this was desirable.

5.9. CONCLUSION

In conclusion the use of saliva concentrations for studies on TMP are unsatisfactory due to the unpredictability of the relationship between serum and

saliva TMP. If these findings are characteristic of all well ionised drugs saliva monitoring should probably be restricted to a few drugs (Danhof and Breimer, 1978) and then only in clearly defined circumstances (Mucklow, 1982).

Errors using inadequate data are much less than those using no data at all.

Charles Babbage 1792-1871

CHAPTER 6

THERAPY OF ACUTE EXACERBATIONS OF CHRONIC BRONCHITIS IN GENERAL PRACTICE WITH TRIMETHOPRIM ALONE: CURE RATES AND TRIMETHOPRIM CONCENTRATIONS IN SPUTUM

INTRODUCTION

6.1. ACUTE EXACERBATIONS OF CHRONIC BRONCHITIS

Acute exacerbations of chronic bronchitis are significant because of their contribution to morbidity, leading to more severe respiratory problems, possibly culminating in eventual respiratory failure.

The role of antimicrobials has been established in exacerbations with a bacterial aetiology, however in a number of exacerbations either no bacteriological cause can be found or the suspected pathogen is not eradicated and yet there is an apparent clinical cure. Although the situation is very complex the former has been ascribed to H. Influenzae (Hughes et al, 1969), and the latter to a further infection or incomplete eradication following initial therapy (Chodosh et al, 1982).

6.1.1. Trimethoprim in Therapy

Co-trimoxazole has been used in a number of comparative studies in the treatment of exacerbations of chronic bronchitis in which it was as effective or better than the following antibiotics: ampicillin (Hughes, 1969; Chodosh et al, 1982), tetracycline (Lal and Bhalla, 1969) dimethylchlortetracycline (Beumer, 1972), doxycycline (General Practitioner Research Group, 1972; Pandy, 1979) ampiclox (Kaplan and Stegman, 1972), amoxycillin (Carroll et al, 1977; Pines et al, 1977) and cephalexin (Cooper and McGillion, 1978). Efficacy equivalent to that found for co-trimoxazole has been demonstrated for co-trifamole (Knothe et al, 1978) and co-trimazine (sulphadiazine and trimethoprim),(Leone et al, 1984).

Trimethoprim alone has been compared with co-trimoxazole in respiratory infections (including chronic bronchitis) and been shown to be as effective as co-trimoxazole (Lacey et al, 1980; Ashford and Downey, 1982) with fewer side-effects (Lacey et al, 1980); therefore TMP alone would seem preferable in the treatment of chronic bronchitis rather than co-trimoxazole.

6.1.2. Trimethoprim in Sputum

Trimethoprim has been shown to accumulate in lung tissue (Hansen et al, 1973a, b; Wieser, Haiderer and Takacs, 1981), and to a greater extent when the tissue is inflamed (Hansen et al, 1973b), TMP levels in sputum have been shown to be of the same order as lung tissue levels (Hansen et al 1973a). It is probable therefore that levels of TMP in the sputum of chronic bronchitics in exacerbation will be sufficiently high to ensure eradication of the common causative organisms.

TMP sputum levels were measured as part of a study performed in domiciliary practice to determine the comparative efficacy of TMP and ampicillin in the treatment of acute exacerbations of chronic bronchitis. It was the intention to determine whether either bacteriological or 'clinical' cure were related to achievement of concentrations of TMP in sputum which were greater than the MIC for the pathogen isolated.

METHODS

6.2. CLINICAL

This was a two phase study with clinical and bacteriological assessment of patients and was performed in conjunction with Dr. C.E. Langan (Baillieston Health Centre, Glasgow) who co-ordinated the clinical aspects of the trial and Dr. D.J.Platt (Dept. Bacteriology, Glasgow Royal Infirmary) who performed the associated bacteriology.

6.2.1. Patient Selection

i) Admissions

Patients that satisfied all of the following criteria were admitted:

a) A history of recurrent lower respiratory tract infection (LRTI) associated with a productive cough (for 2 years or longer). Clinical and bacteriological assessment of the patients were performed between exacerbations i.e. baseline assessment.

b) An acute exacerbation associated with a macroscopically purulent sputum having previously undergone baseline assessment.

c) The respiratory tract pathogens present in sputum were sensitive to both TMP and ampicillin: these drugs were administered under double-blind conditions in doses of 200 mg b.d. and 500 q.i.d. respectively for 10 days.

The patients were allowed to receive concomitant bronchodilator therapy and other treatment e.g. steroids

for respiratory tract disease, other antimicrobial agents were not permitted. Presence of organic disease was not a criterion for exclusion.

ii) Exclusions

Patients with the following histories were excluded:

a) Hypersensitivity to TMP or βlactams.

b) suspected, recent or unhealed tuberculosis.

c) Diagnosis pf pneumonia, carcinoma of the bronchus or empyema.

d) Severe renal, hepatic or cardiac impairment.

e) Malabsorption.

f) Blood dyscrasias, severe dermatitis or infectious mononucleosis.

g) Current use of oral contraceptive steroids.

h) Pregnant or lactating.

i) Requiring parenteral chemotherapy.

j) Previous recent (14 days) exposure to preparations containing TMP or a penicillin.

6.2.2. Patient Assessments

Assessments were performed pre-treatment (baseline), on presentation with an exacerbation (day 0) and days 3, 11 (cessation of treatment) and 38 (4 weeks post treatment).

Clinical and bacteriological assessment of the volume and appearance of sputum were performed according to the MRC criteria (Medical Research Council, 1965). A full medical history, with particular reference to cough, breathlessness and sleeplessness, was obtained. The significance and

sensitivity of the organisms isolated was determined.

6.3. ANALYTICAL

6.3.1. Sputum TMP Estimations

Sputum was analysed for TMP once steady state had been achieved. Samples were usually obtained at day 3 or occasionally at other times.

Sputum TMP assay was performed as described in section 2.8.2. using the instrument configuration described in section 2.6.2.(3). Specimens ofsputum were obtained by expectoration and clotted blood by venepuncture on visiting the surgery. Where two samples of sputum were provided a clotted blood sample was taken as close as possible to the timing of the second sputum sample.

RESULTS

6.4. BACTERIOLOGICAL AND CLINICAL CURE

The main aspects of the trial were clinical and bacteriological assessment of cure, the findings are summarised below. (The permission of Drs Langan and Platt is acknowledged).

6.4.1. Clinical

Highly significant improvements were found between day 0 and day 11 for many of the clinical variables. The improvements in performance were statistically significant ($p<0.005$, paired t test) for both treatment groups; there was no difference in the extent of improvement between groups. There were 118 initial entrants to the trial.

There was a difference between the GP's assessment of macroscopical sputum purulence and that of the bacteriologist, but the degree of change found with treatment corresponded well.

6.4.2. Bacteriological

Haemophilus influenzae and Streptococcus pneumoniae were considered to be primary pathogens and were isolated in 20 patients in the ampicillin group and 16 in the TMP group on day 0, but were eradicated in 15/20 ampicillin and 13/16 TMP cases by day 11. Although there was significant clinical improvement in 78/83 assessable patients only 17/83 had a complete cure i.e. at or better than baseline response, the remainder had not improved to this extent but were better by day 11 than they had been at day 0.

On comparison of all variables such as relapse rate, ventilatory capacity change, sputum appearance etc. there was no significant difference between groups at any stage. The only differences were: a higher proportion of patients withdrawn due to lack of efficacy in the ampicillin group (TMP 0/41, ampicillin 5/42) and a higher side-effect rate in the TMP group (TMP 15/41, ampicillin 9/42).

6.5. TRIMETHOPRIM IN SPUTUM

Paired serum and sputum samples were required and some losses were experienced as is evident from table 6.1., there were no serum samples for several individuals, subjects for whom no sputum sample was received have been omitted from the table.

6.5.1. Clinical and Bacteriological Aspects

The breakdown of available data is as follows. Of the 118 entrants to the trial 35 were withdrawn for clinical reasons, 41 were allocated to TMP therapy (i.e. 41 TMP, 42 on ampicillin) bacteria were isolated in 33 of these; there were samples for sputum TMP analysis for 35/41, of these 29 had matching sputum and serum samples; there were pathogens detected at baseline in 14/33, none detected in 11/33, and information not available in the remainder i.e. 8/33 (5/33 clinical withdrawals and 3/33 lost to follow-up).

Of the group on whom sputum TMP levels were available, there were 19/25 clinical cures at day 11 although 4 subsequently relapsed 4 were failures and

TABLE 6.1

Serum and sputum concentrations of TMP at steady-state

SUBJECT	SERUM TMP mg/l	SPUTUM mg/l	$\frac{\text{SPUTUM}}{\text{SERUM}}$ RATIO
1	4.7	8.7	1.9
2	8.2	9.4	1.2
6	0.2	0.2	1.0
8	0.2	0.1	0.5
12	0.5	3.0	6.0
14	4.4	13.0	3.0
18	6.5	2.5	0.4
24	4.2	7.2	1.7
25		5.3	
30	1.7	7.7	4.5
32	3.6	1.5	0.4
35	4.0	<0.1	-
36	4.5	<0.1	-
37	2.0	5.3	2.7
38	5.4	5.1	0.9
40	2.8	3.2	1.1
41		6.5	
42	2.8	4.7	1.7
45	5.8	17.5	3.0
56	0.1	0.4	-
66)		(2.5	
66)		(2.3	

TABLE 6.1 (cont.)

SUBJECT	SERUM TMP mg/l	SPUTUM mg/l	$\frac{\text{SPUTUM}}{\text{SERUM}}$ RATIO
71		13.3	
72	6.6	4.9	0.7
76		4.3	
78)	5.0	(18.7	1.2
78)		(6.2	
80		15.3	
81)	4.0	(5.0	1.8
81)		(7.1	
82	5.6	12.3	2.2
84)	4.3	(4.2	1.1
84)		(4.8	
88	1.1	7.8	7.1
90	3.6	6.2	1.7
93	1.6	2.3	1.4
95	1.6	4.2	2.6
104	3.5	6.5	1.9
114	2.6	3.6	1.4
$\bar{x}$	3.48	5.97	2.04
sd	2.11	4.68	1.63
n	29	39	26

2 were withdrawn for other reasons. Of the 14 with isolates on day 0, by day 11, 9 had been eradicated, in 2 the same organism was present, in 2 a different organism was found and one was lost to follow-up. Of the 9 bacteriological successes, all had sputum TMP concentrations of >2 mg/l, the mean was 6.2 mg/l with a range of 2.5-12.3 mg/l.

6.5.2. Sputum Kinetics of Trimethoprim

In general sputum levels were greater than serum levels although there was considerable variation between individuals, the relationship of sampling to dose was not clear in many individuals.

Fig 6.1. shows consecutive sputum TMP levels in relation to dosing for those patients for whom this information is available.

There was no consistent change in sputum TMP concentration with time. In subject 95 a number of sputum samples were obtained, C_{MAX} is 2.75 hours post dose which is within the limits found for serum TMP C_{MAX}. Approximately 4 hours post dose the sputum concentration is fairly steady; however the magnitude of concentration varies widely as did the direction of change, with subjects 45, 81 and 84 all showing increases during the 3 hours following dosing, subject 66 showing little change and subject 78 having a high TMP concentration shortly after dosing with a rapid fall some 2 hours later.

Subject 95 was the only patient willing to give

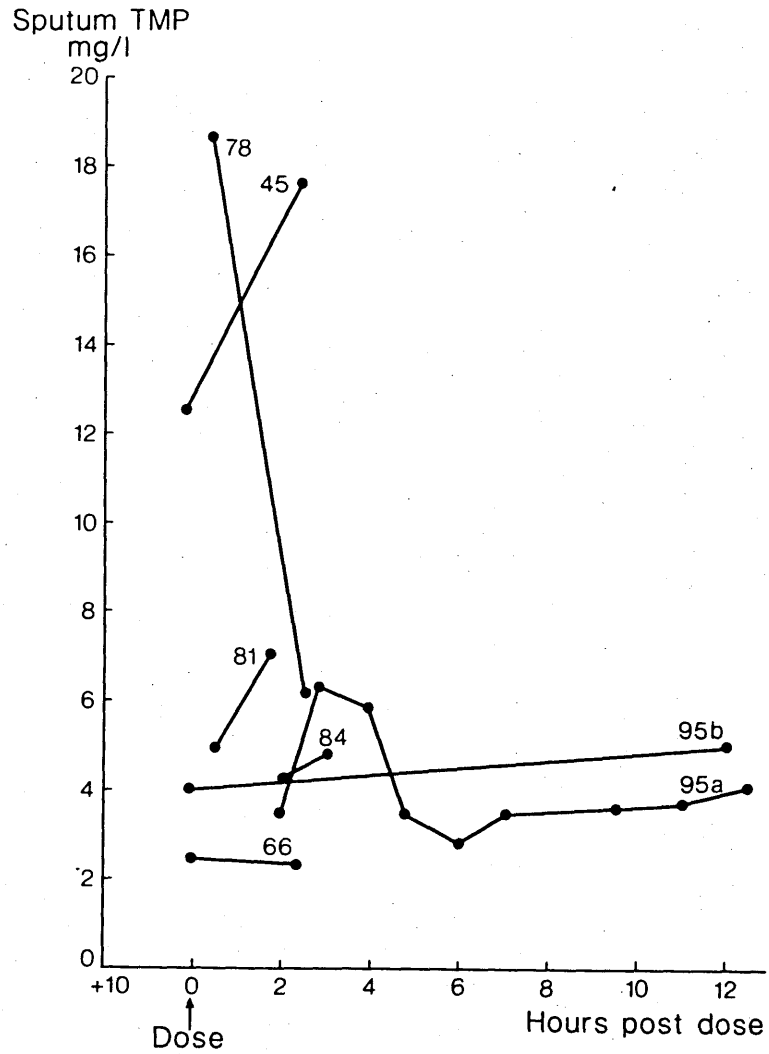


FIGURE 6.1. Sputum TMP concentration versus time curves. The numbers on each line relate to patient identification.

samples on two consecutive days, following the initial dose (95_A) there is some evidence of accumulation following the second dose (95_B), unfortunately this patient had to withdraw due to side-effects (vomiting) at day 3.

There was an association between serum TMP concentration and sputum TMP concentration: slope = 1.32, intercept 2.26, r = 0.459, (n = 23), this association although not strong is statistically at the level of significance ($0.01<p<0.05$)

6.5.3. Bacteriological Cure and Sputum Trimethoprim Concentration

Data on serum and sputum TMP levels were available in 25 subjects for whom there was also information on bacteriological outcome. The following terminology was used: "cure" was the eradication of the pathogen which had been isolated on day 0, "no cure" was the failure to eradicate the pathogen and "negative" was the failure to identify the pathogen at day 0. The outcome is plotted in Fig 6.2 in relation to sputum and serum TMP. Cures were associated with sputum levels ranging from 0.1-13 mg/l, however those with no cure spanned the range 0.1-18.7 mg/l.

The clinical criteria used were more variable, but no association between sputum TMP levels and clinical improvement could be demonstrated. There were two individuals who developed a thymidine-requiring mutant of the same biotype as their original pathogen, these patients had sputum and serum TMP levels of <0.2 mg/l.

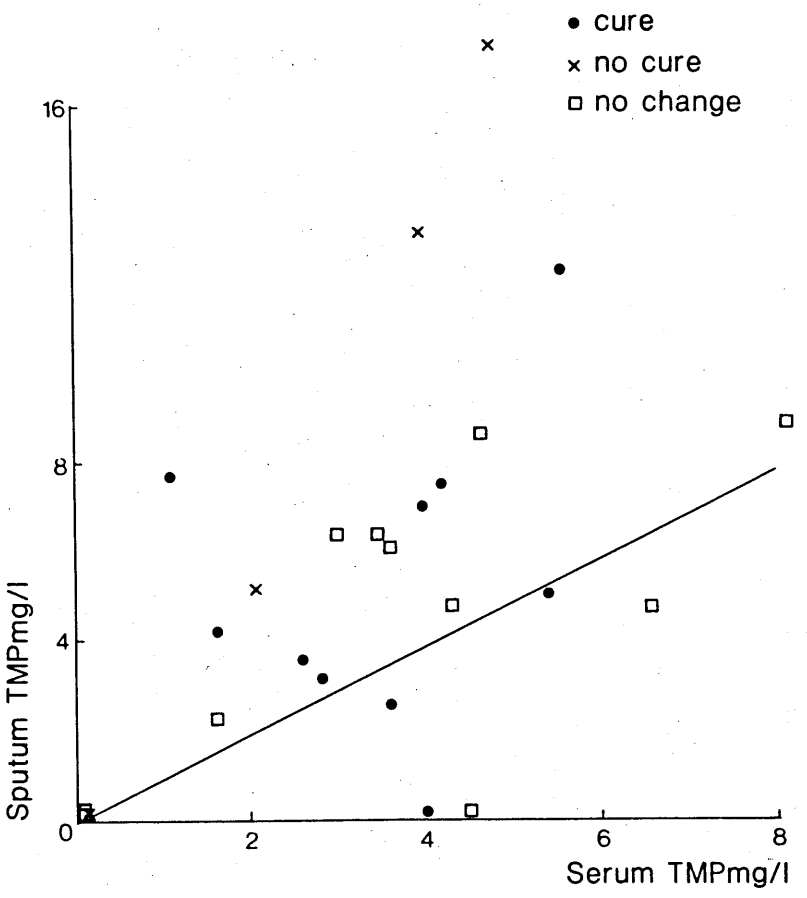


FIGURE 6.2. Association between serum TMP and sputum TMP concentrations with an indication of outcome for each case.

(Platt, Guthrie and Langan, 1983).

6.6. TRIMETHOPRIM AND RENAL FUNCTION

There was a slight but statistically significant increase ($p<0.05$, t test) in creatinine level following treatment with TMP between day 0 and day 11.

DISCUSSION

6.7. BACTERIOLOGICAL AND CLINICAL CURE

6.7.1. Clinical

This study confirms previous findings that TMP alone is effective in the treatment of LRTI (eg Lacey et al, 1980, Hughes and Russell, 1982). The equivalence of efficacy of TMP alone with ampicillin has not previously been reported although there is equivalence between co-trimoxazole and ampicillin (Hughes, 1969; Chodosh et al, 1982). As in the report of Chodosh et al (1982) there is a notable improvement in symptomatic rating score and sputum appearance and volume from initiation of therapy to its conclusion. Treatment with either antimicrobial has therefore been consistent with clinical cure.

6.7.2. Bacteriological

Although sputum presentation and symptomology are objective measures of change, another appropriate indicator is the eradication of the pathogen involved, the isolation rate in this study was 67%, this compares with 54% isolated by Hughes et al (1969). The same author reported a clinical failure rate for co-trimoxazole of 6% and a bacteriological failure rate of 16%, in this study the corresponding rates were 0% and 19% respectively.

Interpretation of results can be complicated by patient compliance, non-compliance with therapy will present as a clinical failure but with a sensitive

pathogen, if one were detected initially. During this study two patients (subjects 6 and 8) were both found to have thymidine requiring Haemophilus influenzae strains in their sputum after TMP therapy; on biotyping these strains were found to be identical with those isolated at exacerbation. Reference to table 6.1 shows that on day 3 these subjects (6 and 8) both had low TMP levels in their serum and sputum, indicating non-compliance.

These levels, which were at or below the organisms' MIC for TMP, selected for resistance, but the mutants were present only transiently suggesting they were poorly adapted to their environment (Platt et al, 1983).

6.8. TRIMETHOPRIM IN SPUTUM

6.8.1. Sputum Sampling

There are some limitations related to the sputum sampling procedure used here. Sputum was expectorated therefore there is likely to be contamination with saliva and possibly nasopharyngeal secretion, with or without pus. Saliva has a slightly lower TMP concentration than serum (Section 5.5.2.) and nasopharyngeal secretion should have similar concentrations (Hoeprich, 1971), thus expectorated sputum would be expected to show TMP levels lower than the 'true' value. It is well known in salivary drug monitoring that care is required to ensure no tablet or capsule residue is in the mouth leading to spuriously high levels (Mucklow, 1982). Expectorated sputum may therefore have impaired sample integrity. The only satisfactory procedure for sampling

sputum is transtracheal aspiration, especially for pharmacokinetic studies, however such procedures are impractical in general practice.

6.8.2. Kinetic Aspects

The data shown in table 6.1 indicates that in 33 of 39 samples the sputum TMP concentrations exceed the TMP MIC of 2 mg/l. It has been shown by Hansen et al (1973a, b) that TMP accumulates in the lung and that the concentration in lung tissue may be 1.5-3.5 times greater than the corresponding serum level with sputum attaining ratios of 0.5-4 times. This is a narrower range than found in this study.

The time to C_{MAX} in sputum for subject 95 was slightly later than that usually found in serum studies, suggesting delay in penetration, however as lung tissue is highly perfused the rate of delivery to lung cells will be rapid; although uptake and passage may well be impaired in lungs damaged by bronchitis.

6.8.3. Trimethoprim Penetration and Pathological Changes

Acute on chronic bronchitis is a complex situation and there will be a number of factors that will affect the rate of TMP penetration into lung tissue, including:

i) Decreased hepatic blood flow secondary to increased hypoxia (Kirby, Cooke and Finley, 1976), resulting in decreased TMP metabolic clearance and higher plasma concentration which would be reflected in tissue levels.

ii) Decreased pH due to CO_2 retention may have two effects:

a) There will be an increase in ionisation of TMP from that found at normal (or fully compensated) blood pH from 44% at pH 7.4 to 56% at pH 7.2. Presuming penetration to be passive then there will be a decrease in the proportion of TMP available for diffusion.

b) There could be an increase in TMP fp as acidosis has been associated with decreased plasma protein binding, (Tillement, Lhoste and Giudicelli, 1978)

iii) As in many other diseases chronic bronchitis is associated with hypoalbuminaemia, resulting in an increase in TMP fp.

iv) TMP penetration into lung cells will be enhanced in the presence of inflammation (Hansen et al, 1973b).

6.8.4. Factors Affecting Trimethoprim Concentrations in Sputum

Once TMP has penetrated into the lung cell there is a tendency for it to accumulate (Hansen et al, 1973a, b) subsequent secretion reflects these levels. Once sputum is *in situ* there are a number of factors which will affect the apparent TMP concentration, these include evaporation of the sputum resulting in concentration, expectoration in which part of the sputum (at least) is lost and a new sputum drug concentration would be established and, possible mixing with other sputum with different concentrations of TMP. The levels achieved

will also depend on the rate of sputum formation and residence time, the general trend perceived in fig 6.1 was for an increase in concentration following a dose of TMP. Patient 78 may have contaminated his initial sample with residual drug in the oral cavity causing a spuriously high level and an apparent subsequent fall.

There was a weak association between serum and sputum TMP concentrations, this is in contrast to a preliminary publication in which no association was reported (McIntosh et al, 1983). This was due to the larger numbers that have since been examined giving a more statistically meaningful sample.

6.8.5. Bacteriological Cure and Sputum Trimethoprim Concentration

Association of cure rate, either clinical or bacteriological, with TMP sputum level is not proven, in Fig 6.2 although there were no cures at levels below the MIC, there was also failure at very high sputum levels. As described earlier measurement of TMP concentrations in serum and urine could prove non-compliance and provide a rationale for the emergence of a thymidine requiring mutant.

Since the rate of resistance to ampicillin in British hospitals is 6.2% in H. influenzae isolates (Philpott-Howard and Williams, 1982) TMP has been looked to as a useful alternative, however the same authors have noted an increase in the rate of resistance to TMP from 0.2% to 1.4% from 1970-1977, the current rate is

unknown.

6.9. TRIMETHOPRIM AND RENAL FUNCTION

The rise which was found in serum creatinine is marginal, but confirms the findings of other workers who have noted this in patients treated with co-trimoxazole, but have identified TMP as the causative agent (Kainer and Rosenberg, 1981; Dijkmans et al, 1981 ; Roy et al, 1982). The rise is reversible and may be associated with the free TMP concentration (Dijkmans et al, 1981). It has been suggested that the cause is interference with creatinine secretion (Kainer and Rosenberg, 1981; Roy et al, 1982), since total renal function as determined by ^{51}Cr-EDTA clearance is not impaired (Kainer and Rosenberg, 1981).

6.10. CONCLUSION

In conclusion, this study has shown TMP levels well in excess of the MIC (2mg/l) for sensitive pathogens can be achieved in sputum. There was bacteriological success in a significant proportion of individuals and clinical improvement in numbers equivalent to those improved by the use of the 'reference' antibiotic. TMP used alone can 'cure' bacteriologically induced exacerbations of chronic bronchitis.

I have yet to see any problem, however complicated, which, when you looked at it the right way, did not become still more complicated.

Poul Anderson

New Scientist 25th Sept. 1969.

CHAPTER 7

INDIVIDUAL PLASMA CONCENTRATIONS AND PHARMACOKINETIC PARAMETERS: COMPARISON OF OBSERVED VALUES AND ESTIMATES OBTAINED FROM OPT, A PROGRAMME USING BAYESIAN STATISTICS AND ESTIMATES OF MAXIMUM LIKELIHOOD

INTRODUCTION

7.1. STANDARD APPROACH TO OBTAINING 'POPULATION' KINETICS

Following administration of a standard dose to a number of matched individuals, carefully timed samples are withdrawn and analysed to determine the drug concentration. The number of exponential terms required to describe the serum drug concentration time curve indicate whether a one, two or three compartment model is appropriate for fitting the data. A monoexponential term requires a one compartment model, a biexponential a two-compartment model and a triexponential a three-compartment model (Fig 7.1), the choice of appropriate model can however be complicated by considerations as to the compartment in which elimination etc is occurring (Wagner, 1975). In many cases the simplest model, the one-compartment model, provides an adequate description allowing prediction of future Cp and dosage to be performed relatively easily.

If a one-compartment model is chosen then the equations and procedures described in section 3.6.3. can be used to obtain estimates of the parameters: Vd, Cl, ka etc. Taking the mean of the individual parameters will yield population parameters, however there may be errors in such estimates due to the variance of each Cp contributing to mispecification of the curve, this is particularly true if models of greater complexity than one-compartment are used. An approach which avoids

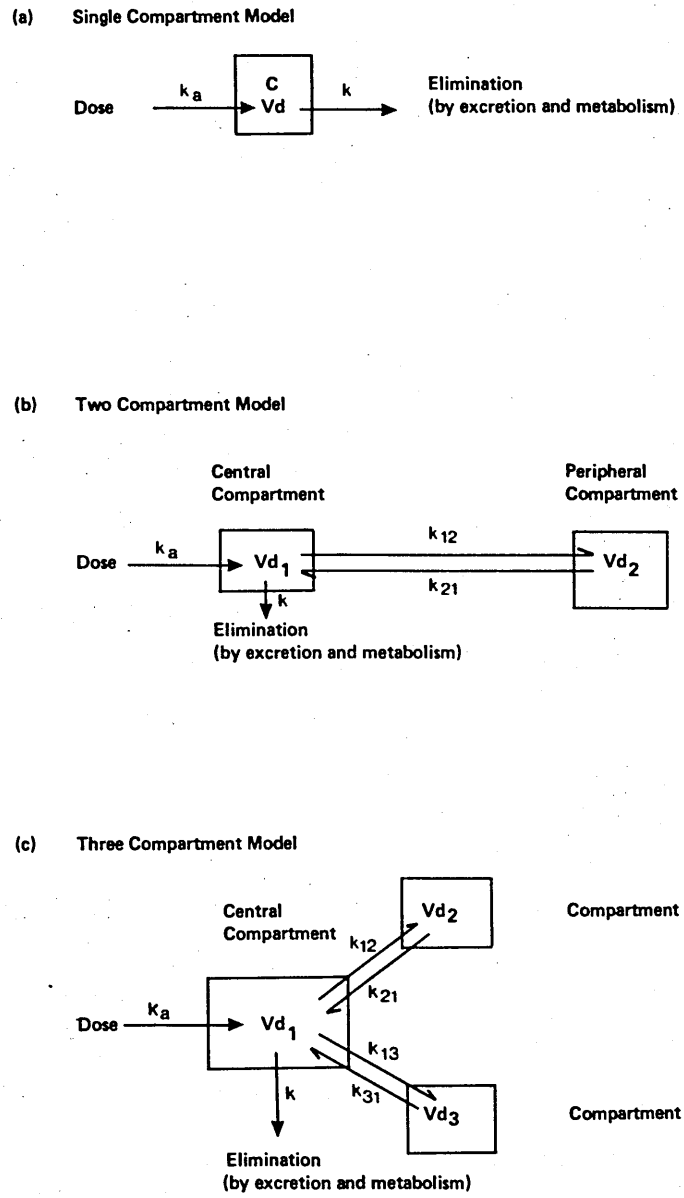


FIGURE 7.1. One, two and three compartment models. It is assumed that absorption and elimination occur through the central compartment.

this difficulty is to use time averaged concentrations i.e. at a particular time all the concentrations are averaged and the parameters determined, from the meaned data; this provides a good estimate of the population kinetic parameters but provides no information on the variance of the parameters.

Parameters are usually calculated following a single dose but estimates may be obtained in steady-state (Benet and Massoud, 1984).

7.2. BAYESIAN STATISTICS

Statistical methods are usually based on the frequency view of probability, however Bayes's Theorem, (named after an English clergyman, Thomas Bayes, 1702-1761) is appropriate in situations in which foreknowledge of the possibility of an event i.e. prior probability is used to judge the likelihood of the event; once an observation(s) has been made then the probability of the event will be retrospectively modified i.e. posterior probability. By taking the most likely parameter it may be inferred that this is the best estimate of that parameter (Fig. 7.2.); this is the method of maximum likelihood. This may be expressed as: posterior probability is proportional to likelihood multiplied by prior probability.

With the combination of Bayesian statistics and estimates of maximum likelihood it is possible by entering minimal information to estimate parameters for

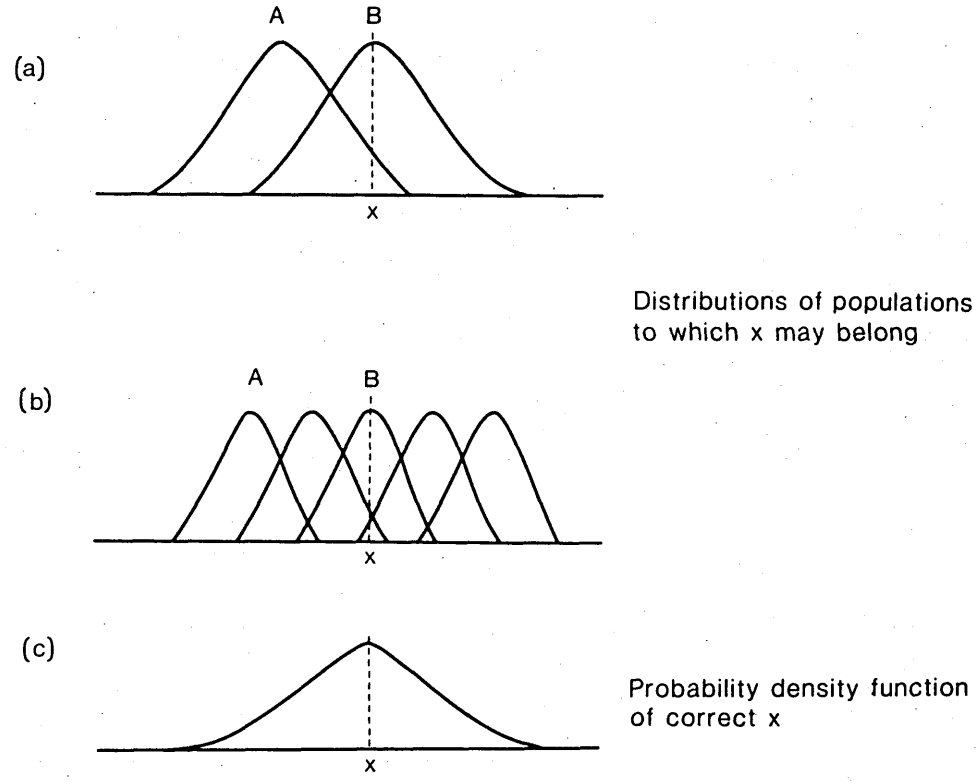


FIGURE 7.2. Diagramatic representation of maximum likelihood
a) Observation x and its place in population A and B
b) Probability of distribution of populations to which x may belong
c) Probability density function of correct assignment of x

which there is a previously obtained reference.

7.3. BAYESIAN ESTIMATION OF INDIVIDUAL KINETICS

The plasma drug concentration (Cp) after a known dose and time is a function of an individual's pharmco-kinetics; there will be variance associated with the Cp eg.analytical and pharmacokinetic, and there will be a distribution of likely estimates. In relation to this individual the population distribution of parameters may be taken as a prior probability, Cp is the observation which will enable an estimate to be made to be used for modified posterior probability and therefore the pharmaco-kinetic parameters estimated via this procedure will be 'individualised' due to the influence of Cp. (Fig 7.2)

Sheiner et al (1979a) used a Bayesian technique to forecast individual pharmacokinetics in a group of patients taking digoxin; the parameters F, Vd and Cl were estimated and used to predict future Cp as dosage was changed.

In using population kinetics for a particular drug the use of co-variates eg. sex, weight, disease state can be used to modify the prior probability of eg. Cl., however the prediction will be open to considerable error because of the variation possible in the population. Full specification of the measurable features pertinent to an individual's kinetics (eg. GFR) was found to be of less significance than the input of one plasma drug level (Sheiner et al, 1979a) which allowed

'feedback' and improvement in the forecast kinetics. Sheiner et al (1979a) validated their approach using digoxin and found that with two 'feedback' Cp's the accuracy and precision of forecast Cp's was very good and that estimates of Vd and Cl also improved.

7.4. THE OPT(IMISATION) PROGRAMME

This approach has been adopted and adapted as the microcomputer programme OPT (Kelman, Whiting and Bryson, 1982a; Kelman, Whiting and Bryson, 1982b) which has been shown to be of significant predictive value in a clinical setting (Joel et al, 1983; Whiting et al, 1984; Howie et al, 1985). OPT is able to model the irregular dosing schedules seen in clinical practice, also it may not require that samples for Cp be taken at any particular time in relation to dosing (Howie et al, 1985) but is governed by compatability with the model. The only requirements for effective use of the programme are : the individual's measurable features, the dosage, time of dosage and the time at which the sample was taken in relation to the dose. Although Sheiner et al (1979a) included the population variances for their individualisation of digoxin, the variances used in OPT are equal for all drugs and fixed (Kelman et al 1982a, b) although the authors state these may be reviewed if more accurate studies are performed.

The programme as configured by these authors is applicable to : digoxin, disopyramide, lignocaine,

procainamide, theophylline, carbamazepine, phenobarbitone and the aminoglycoside antibiotics, (Whiting et al, 1984) phenytoin is also available but only in steady-state as saturation kinetics require a different approach (Kelman et al, 1982a). The drugs included have been chosen on the basis of their relevance to good therapeutic drug monitoring practice i.e. they are all well established drugs usually with a low therapeutic index where individualisation of dosage is important.

It was of interest to ascertain how generally applicable OPT could be, and to determine if Cp can be reliably predicted.

The intention was to assess the ability of OPT to predict serum levels when different numbers of Cp were available and to determine how acceptable a forecast could be achieved for C_{ss}MIN and a point approximating to C_{ss}MAX for two dosage regimens.

7.5. ESTIMATION OF POPULATION KINETICS

Analyses using the standard pharmacokinetic approach on reasonably well subjects is an expensive exercise in terms of assay time, bed occupancy and personnel; for severely ill patients the rigours required for assessing the change in pharmacokinetics in the diseased state may be unacceptable and thus reliable information could prove difficult to obtain. For these reasons Sheiner and others proposed statistically sophisticated analysis of fragmentary sets of well

documented data from individuals. To do this requires a knowledge of population kinetics with an estimate of the parameter variances, this is obtained using NONMEM and has been succesfully used in the estimation of the kinetics of several drugs (Sheiner, Rosenberg and Melmon, 1972; Sheiner, Rosenberg and Marathe, 1977; Beal and Sheiner, 1980; Sheiner and Beal, 1981b; Sheiner and Beal, 1984). Such population data can then be used as the basis for individualised Bayesian predictions.

METHODS

7.6. COMPUTING

7.6.1. Software

OPT was kindly made available by Drs. A. Kelman and B Whiting (Dept. Materia Medica, Stobhill Hospital, Glasgow) originally for the meeting Clinical Drug Analysis, Techniques and Interpretation held in March 1982 in Glasgow; Dr. B. Clark (Dept. Biochemistry, Western Infirmary, Glasgow) made the software modifications required to enable OPT to be used on Apple IIe plus computers. The procedure for use, and the files available were as described by Kelman et al (1982a, b).

Dr. B. Clark subsequently modified the input to enable any drug with first order, one compartment open model kinetics to be considered.

7.6.2. Hardware

The OPT programme as used was transferred to a Winchester disc. The following hardware was used: an Apple IIe plus microcomputer with a language card, a floating point board, a buffered Grappler™ serial interface card, an additional memory card, a soft switch, one floppy disc drive and a Corvus Winchester disc drive (10Mb), a monitor and an Epson MX-100 dot matrix printer. The basic microcomputer and floppy disc drive were obtained from Scotlab Instrument Sales Ltd. (Bellshill, Scotland), all other hardware was obtained through Strathand Ltd. (Glasgow, Scotland).

Full details of the software and hardware requirements may be obtained from Dr. B. Clark or Dr. A. Kelman.

7.6.3. Data

The ability of OPT to predict Cp for the individuals described in the TMP study in Chapter 4 were examined. Using data from the 300 mg o.d. regimen, increasing numbers of Cp from 0 to 4 at 2, 12, 48 and 72 hours were incorporated cumulatively in chronological order into the model; the bias and precision of the prospective Bayesian prediction were compared with the observed Cp at 96 (C_{ss}MIN) and 98 (C_{ss}MAX) hours post commencement of the 300 mg o.d. regimen, in addition the accuracy of forecast Cp for the 200 mg b.d. regimen at 96 and 98 hours post initiation of dosing were also examined. The population kinetics used were obtained during the studies described in Chapter 4, the time averaged parameters compared well with the averaged parameter values. The parameter values used were ka 2.5 h^{-1}, Vd 75 l and Cl 5.4 l/h.

It is recognised that the use of data to test a prediction that also forms the reference base should result in a better than anticipated performance. Predictive performance was evaluated for precision and bias using the statistical approach suggested by Sheiner and Beal (1981a). (Section 5.4.2.).

RESULTS

7.7. OPT OUTPUT

Following 'optimisation' of an individual with population data a result table is printed, which gives details of dosage regimens, predicted and observed serum drug concentrations and derived parameter estimates. A typical result table is shown in Fig 7.3. A graphical printout of serum concentration versus time is also available, a + indicates a measured point (Fig 7.4).

7.8. PREDICTIVE PERFORMANCE OF OPT FOR Cp

7.8.1. Prospective Predictions of Cp for TMP in Steady State Versus Observed Cp

Table 7.1 summarises the mean and range of the prediction error for each Bayesian estimate with increasing Cp. The systematic component of pe is considered below under bias.

Mean pe decreased with increasing Cp input; the ranges were wide, although they tended to decrease with increasing Cp input. Subject 5 was excluded from pe and bias calculations in 300 mg o.d. 96 hours due to a very large pe suggestive of an observational Cp error.

Comparison of precision and bias for each forecast Cp i.e. 300 o.d. 96 and 98 hours post commencement of the 300 o.d. regimen and 96 and 98 hours post commencement of the 200 b.d. TMP regimen using 0, 1, 2, 3 or 4 Cp or the naive standard (Section 5.4.2.) were often

```
                    DRUG OPTIMISATION PROGRAM  - 1982
                    ---------------------------------

TMP TEST 4                                          UNIT NO:
                                                    **************************
  DOSE                      TIME FROM       ROUTE OF        * DRUG= TRIMETHOPRIM     *
INTERVAL       DOSE        LAST DOSE     ADMINISTRATION     * NCONC= 4               *
    1         300.00          0.           ORAL
    2         300.00         24.           ORAL
    3         300.00         24.           ORAL
    4         300.00         24.           ORAL
                        CONCENTRATION IN UG/ML
  DOSE
INTERVAL        TIME      PRED.CONC.    MEAS.CONC.     SD      DIFF
    1           7.00        2.47          2.10         .76     -.37
    2           2.00        4.01          3.40        1.02     -.61
    3          24.00        1.16          1.00         .19     -.16
    4          24.00        1.17          1.40         .21      .23
-------------------------------------------------------------------------------
          PREDICTED PARAMETER ESTIMATES
          -----------------------------
                                BAYESIAN ESTM.   SD       EXPECTED
CONC.AT START                   --------------   --       --------
 OF MEAS. PERIOD          =        .10            .09          .10   UG/ML
CLEARANCE                 =       4.97            .39         5.40   L/HR
VOL. OF DISTR.            =      84.78           9.84        74.98   L
KA(ORAL), KM(IM)          =     2.5064         1.4471       2.5000   /HR
ELIM. RATE CONST.         =      .0586 /HR
ELIM. HALF LIFE           =      11.83  HR
-------------------------------------------------------------------------------

( ACCEPTED THERAPEUTIC RANGE IS   2.0- 10.0 UG/ML)
RECOMMENDED DOSE REGIME FOR STEADY STATE ORAL ADMINISTRATION
DOSE =  300.000 MG     DOSING INTERVAL =  24. HOURS & ON THIS' DOSE
CMIN =    1.177      CMAX =    4.314      CPSS =    2.517
TIME TO PEAK =  1.4 HOURS.
CONCENTRATIONS IN UG/ML
-------------------------------------------------------------------------------
```

FIGURE 7.3. Table format output from OPT

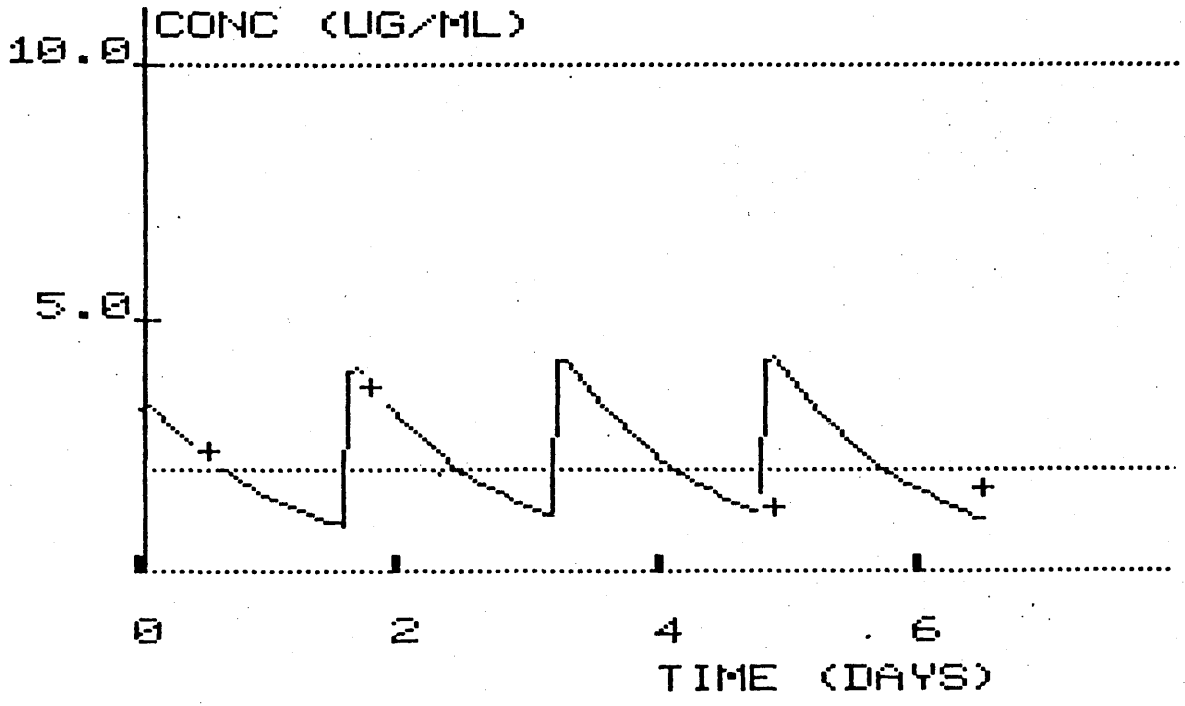


FIGURE 7.4. Serum concentration versus time graphics output from OPT. + indicates an observation.

TABLE 7.1.

Percent prediction error for Bayesian forecasts of Cp compared with observed mean and range for TMP

No Cp		300 mg o.d. 96 hrs	300 mg* o.d. 98 hrs	200 mg b.d. 96 hrs	200 mg b.d. 98 hrs
0	$\bar{x}$	34	-17	6	-3
	range	-55 to 92	-30 to -4	-60 to 53	-27 to 18
1	$\bar{x}$	42	1	19	18
	range	-50 to 109	-19 to 13	-52 to 77	-19 to 48
2	$\bar{x}$	49	4	22	21
	range	8 to 147	-10 to 26	-28 to 51	-1 to 42
3	$\bar{x}$	19	-6	4	6
	range	-42 to 117	-20 to 20	-45 to 25	-9 to 33
4	$\bar{x}$	18	-7	3	7
	range	-12 to 109	-13 to 16	-50 to 28	-13 to 31

* excluding subject 5

not statistically significantly different. Statistical evaluations of the precision and bias of the forecasts are contained in appendix D. The number of input Cp resulting in the best and worst absolute precision and bias are contained in table 7.2.

i) Precision

a) absolute

The number of input Cp reported in table 7.2 as yielding significant predictive imprecision are consequently poor predictors, although those noted for 3 and 4 Cp (300 mg o.d., 98 hours) had unusually tight confidence limits. The best mse for each group predicting steady-state Cp are also summarised in table 7.2. Precision of the naive standard was comparable to that obtained for 3 or 4 Cp.

b) relative

In general the best precision was noted with 3 or 4 Cp and the worst with 1 or 2 Cp.

ii) Bias

a) absolute

Two occurrences of statistically significant absolute bias are noted in table 7.2, in the other groups the worst bias was associated with 2 Cp (300 mg o.d. 96 hours) and 2 Cp (200 mg b.d., 96 hours). Best absolute bias is also noted in table 7.2.

b) relative

Least bias was noted most often for 3 or

TABLE 7.2.

Best and worst absolute precision and bias in forecasting Cp at 96 and 98 hour after 300 mg TMP o.d. and 200 mg TMP b.d.

		300 mg o.d. 96 hrs	300 mg o.d. 98 hrs	200 mg b.d. 96 hrs	200 mg b.d. 98 hrs
mse (precision)	Best	4 Cp	1 or 2 Cp	4 Cp	0, 3 or 4 Cp
	Worst ($p<0.05$)	0 Cp	0, 3 or 4 Cp	0, 1, 2 or 3 Cp	1 or 2 Cp
me (bias)	Best	3 or 4 Cp	1 Cp	3 or 4 Cp	3 or 4 Cp
	Worst ($p<0.05$)	-	0 Cp (-ve bias)	-	2 Cp (+ve bias)

TABLE 7.3.

Precision (mse) and bias (me) estimates for the parameters Vd, Cl and $t_{\frac{1}{2}}$: comparison of Bayesian estimates utilising increasing numbers of observed Cp and the estimates obtained using standard pharmacokinetic techniques
mse (or me) ±95% confidence interval

No Cp	Vd me	Vd mse	Vd rmse	Cl me	Cl mse	Cl rmse	$t_{\frac{1}{2}}$ me	$t_{\frac{1}{2}}$ mse	$t_{\frac{1}{2}}$ rmse
0	2.24 (±11.4)	119 (±247)	10.9	-0.6 (±1.7)	4.9 (±5.4)	2.2	-	-	-
1	17.4* (±11.1)	409 (±522)	20.2	1.3 (±2.8)	9.1 (±5.2)*	3.0	-0.6 (±5.1)	25.8 (±25.4)*	5.1
2	15.6 (± 8.5)*	307 (±348)	17.5	2.1 (±0.8)*	4.3 (±3.1)	2.1	-1.8 (±4.6)	7.8 (±12.5)	2.8
3	20.7 (±13.6)*	590 (±748)	24.3	0.3 (±1.3)	2.2 (±2.1)*	1.5	1.6 (±3.9)	13.1 (±12.5)*	3.6
4	14.3 (±14.8)	374 (±699)	19.3	1.1 (±2.4)	6.8 (±4.4)*	2.6	0.2 (±5.3)	12.2 (±13.4)	3.5

* SIGNIFICANT ($p<0.05$)

4 Cp and most bias with 0, 1 or 2 Cp.

7.9. PREDICTIVE PERFORMANCE OF OPT FOR PHARMACOKINETIC PARAMETERS

7.9.1. Comparison of Observed and Predicted Parameters

The precision and bias of predictions for Vd, Cl and $t_{\frac{1}{2}}$ when compared to those obtained using standard methods are presented in table 7.3. There was significant positive bias in estimates for Vd and significant imprecision in estimates for Cl. The performance of the prediction for each parameter in each individual are considered in the remainder of this section.

i) Volume of distribution

The values for the observed Vd i.e. standard multiple sample method and that obtained by Bayesian estimate are compared in table 7.4. Bayesian estimates were higher than observed values, three estimates were within one s.d., three within two s.d. and two outwith two s.d. from the estimate.

ii) Clearance

A comparison of clearances obtained as above are presented in table 7.5. Four subjects had estimates within 2 s.d. of the observed, however there were some marked differences between estimated and observed Cl for the remaining subjects.

iii) Elimination half-life

Elimination half-life was considered

TABLE 7.4

Comparison of Vd (l) obtained by standard pharmacokinetic method and Bayesian estimate (using 4 observed Cp)

Subject	Standard technique	Bayesian estimate	(±sd)
1	82.6	100.5	(±13.6)
2	60.3	68.6	(± 7.3)
3	110.0	110.8	(± 6.4)
4	81.4	127.2	(±17.3)
5	72.9	90.2	(±10.5)
6	67.2	74.8	(± 8.0)
7	64.1	70.6	(± 7.5)
8	60.9	71.2	(± 7.6)

TABLE 7.5

Comparison of Cl(l/h) obtained by standard pharmacokinetic method and Bayesian estimate (using 4 observed Cp)

Subject	Standard technique	Bayesian estimate	(±sd)
1	5.2	4.9	(±0.5)
2	3.6	4.8	(±0.4)
3	8.4	14.4	(±0.8)
4	9.3	7.1	(±0.6)
5	3.3	5.5	(±0.4)
6	4.5	5.2	(±0.4)
7	4.4	4.8	(±0.4)
8	4.8	4.8	(±0.4)

as it is related to k_{el} (table 7.6), five estimates were different from the observed value by more than 15%.

7.9.2. Comparison of Final and Sequential Forecasting of Parameters

i) Volume of distribution

The final forecast Vd with maximum (i.e.4) Cp input was not statistically significantly different for either absolute bias or precision from the estimate obtained with 0 Cp (table 7.7); least imprecision and bias were noted for 3 Cp and most for 0 Cp. The order of error in the units of measurement (rmse) is large, of the order of 12-18% of the observed mean value (table 7.4).

ii) Clearance

There was no statistical difference for estimates of absolute bias or precision for forecast Cl (table 8.8), bias tended to be positive, the least imprecision was associated with 2 and 3 Cp. The rmse indicate errors of between 41-70% of the observed mean values (table 7.5).

iii) Elimination half-life

Precision was best after 2 or more Cp, there was most bias with 1 Cp (table 7.7), in terms of the observed mean values (table 7.6) the error of prediction was between 29-45%.

iv) Absorption rate constant

Generally adjustments in forecast k_a showed minimal change with no significant bias or

TABLE 7.6.

Comparison of $t_{\frac{1}{2}}\beta$ (h) obtained by standard pharmacokinetic method and Bayesian estimate (using 4 observed Cp)

Subject	Standard technique	Bayesian estimate
1	11.0	14.2
2	11.6	10.0
3	9.1	5.4
4	6.1	12.4
5	15.4	11.4
6	10.4	9.9
7	10.0	10.1
8	8.7	10.4

TABLE 7.7

Precision and bias estimates for the parameters Vd, Cl, $t_{\frac{1}{2}}$ and ka: comparison of Bayesian estimates utilising increasing numbers of observed Cp and the 'final' Bayesian estimate (Cp=4). mse (or me) ± 95% confidence interval

No Cp	Vd me	Vd mse	Vd rmse	Cl me	Cl mse	Cl rmse	$t_{\frac{1}{2}}$ me	$t_{\frac{1}{2}}$ mse	$t_{\frac{1}{2}}$ rmse	ka me	ka mse	ka rmse
0	10.3 (±11.1)	193 (±294)	13.9	1.0 (±3.3)	10.7 (±28.6)	3.3	-	-	-	0.28 (±0.76)	0.5843* (±0.570)	0.764
1	-4.7 (± 9.6)	104 (±267)	10.2	0.1 (±4.1)	14.7 (±30.8)	3.8	0.5 (±4.9)	21.4 (±38.8)	4.6	0.053 (±0.057)	0.0055 (±0.0057)	0.074
2	0.75(± 9.8)	81 (±150)	9.0	0.8 (±2.8)	4.7 (± 5.8)	2.2	1.7 (±5.3)	9.4 (±10.9)	3.1	0.041 (±0.042)	0.0029 (±0.0030)	0.054
3	5.3 (±15.5)	128 (±338)	11.3	0.7 (±2.5)	5.8 (±15.9)	2.4	-1.2 (±4.0)	8.7 (±18.5)	3.0	-0.01 (±0.038)	0.0013 (±0.0016)	0.036

* SIGNIFICANT ($p<0.05$)

imprecision (table 7.7), least bias and best precision was achieved with 3 Cp. There was a statistically significant imprecision associated with 0 Cp, as subject five was subsequently predicted to have a k_a of 0.23-0.34 (h^{-1}). If the typical k_a of TMP is 2.5 h^{-1} then the rmse errors are between 1.4-30.6%; the high rate includes subject 5, excluding this individual an rmse of 0.033 was found, the range of error then becomes 1.3-3.0%.

DISCUSSION

7.10. PREDICTIVE PERFORMANCE OF OPT FOR Cp

The intention of OPT is to predict appropriate dosage from a minimum of pharmacokinetic information, it follows from this that appropriate prediction of Cp is an integral part of such strategy when effect and Cp are related to a therapeutic range. The prediction of Cp requires the optimisation of the parameters of Vd, Cl and k_a; given the approach used these are unlikely to have a unique solution but should tend towards to the correct values (Sheiner et al 1979a).

This evaluation was not intended to consider the mathematical and statistical modelling in OPT but to examine the predictive efficiency of OPT for a drug and to consider what utility such an approach might offer.

There is the underlying assumption in the assessment of Cp that the reference value i.e. observed Cp, is correct and that prediction must attempt to achieve comparability with such a value. Inevitably there are a number of contributions to the variance of the observed Cp including pharmacokinetic and analytical factors, the extent of such variance around a point observation for this data is not known; if the data were to be submitted to NONMEM (Sheiner and Beal, 1981b,) then a population value could however be estimated. With this caveat the prediction of Cp in a practical

setting would be expected to cope with such difficulties and thus it is valid to assess predictive performance in relation to observed data.

7.10.1. Prospective Predictions of Cp versus Observed Cp for TMP at Steady-State for Two Dosage Regimens

The mean prediction error decreased with increasing entry of Cp, the ranges however were wide and reflect the individuals with extreme errors, this may relate to observational Cp error although only one subject was excluded for that reason. (It is worth noting that a 15% error is assumed for each Cp entered into OPT) (Kelman et al, 1982b).

The errors in forecast Cp were worse for predicted Cp at 96 hour concentrations (i.e. trough) than for that predicted at 98 hours. This is surprising in view of the fact that the observed Cp estimates of Cl as the 12 hour and two trough level Cp's (48 and 72 hours) are most influenced by Cl, whereas the 2 hour sample point is the only observed estimate of k_a and any error would result in poor estimates of Cp at 98 hours. Error in forecasting 96 hour Cp was not influenced by drug induced changes in clearance as the steady state values were achieved and maintained (see Table 4.1). The significance of these errors require consideration in terms of clinical relevance. Sheiner et al (1979a) suggested a "clinical acceptable error SD" based on the

therapeutic range, unfortunately while most would agree that the lower limit of the effective concentration of TMP is 2 mg/l there is no indication of any desirable upper limit for TMP concentration. The highest observed TMP Cp was 5.3 mg/l, for the purposes of computation an upper limit of 6 mg/l is arbitrarily assumed to be acceptable. Using the assumptions and approach of Sheiner the clinically acceptable error SD is 1 mg/l i.e. 95% of true Cp's will be within 2 mg/l. By this criterion the acceptable percentage errors are: 75% (300 mg o.d. 96 hours) 26% (300 mg o.d. 98 hours), 48% (200 mg b.d. 96 hours) and 25% (200 mg b.d. 98 hours). On these criteria predictions using 3 or more Cp were acceptable with the exception of one individual at 200 mg b.d. 98 hours and two individuals at 300 mg o.d. 96 hours. Where TMP is concerned the most important range is that about the lower reference level, where an error of 50% is unacceptable, since it would not allow differentiation between effective and ineffective therapy.

In assessing the performance of prediction it is necessary to consider the precision and bias of the forecast at the different levels of stipulated Cp. Statistically significant bias was found on two occasions, one positive and the other negative, there was thus no consistent systematic component to any error. Consideration of relative bias, particularly

as it relates to the naive standard, which by definition has no bias, indicated that bias decreased with increasing Cp input; least bias was most consistently achieved with 3 or 4 Cp. Imprecision was most commonly associated with 0 Cp with statistically significant imprecision on 3 of 4 occasions. As the number of stipulated Cp increased so precision improved although the naive standard has as good precision as Bayesian estimates up to 3 Cp or 4 Cp. When statistically significant imprecision was noted, this was associated with unusually narrow confidence limits (table D.1).

Performance could have been adversely affected by the choice of observed Cp giving a poor mix of points and not enabling a good balance of influences on Cp to be assessed and not resulting in adequate posterior modification of the prior probabilities. This could be further compounded if the population data used as a reference base to establish the prior probabilities were inadequate; the values used were close to those obtained from time averaged parameters obtained from each of the studies described in Cpaters 3 and 4. It is unlikely that significant error was entered through the population reference base although a larger, more independent (i.e. less correlated) population sample would be desirable.

On the basis of these results 3 or 4 Cp must be input for each individual in order to obtain the most

precise and least biased forecasts of Cp, allowing dosing prediction, based on these Cp, to be performed with confidence. This finding is in accord with the findings of a recent review comparing predictive drug dosing methods which showed that Bayesian statistical predictions perform best (Burton, Vasko and Brater, 1985). The authors implied that less than 3 Cp are required, although this does not coincide with the findings reported here it is most dependent on what the clinically acceptable level of error in dosing is.

7.10.2. Retrospective Predictions of Cp for TMP

As OPT has a time weighted factor which decreases the influence of earlier Cp on prediction (Kelman et al, 1982b) it is hardly surprising that the quality of prediction of Cp retrospectively declines. This is of no consequence unless an attempt is made to interpret findings retrospectively; awareness of this limitation should avoid misinterpretation.

7.11 PREDICTIVE PERFORMANCE OF OPT FOR PHARMACOKINETIC PARAMETERS

The prediction of Cp requires that a balance between the three main pharmacokinetic variables be achieved. As described by Kelman et al (1982b) there are variances associated with each parameter. As Cl is more likely to vary than Vd it is set at 50%, k_a has the same weighting as Cl; reflecting potential variables in absorption. In addition an uncertainty principle

is included to account for previous possibly non-compliant dosing and a variance of 15% is allocated to each Cp.

The balance of parameters and variances result in a 'model' which fits the observed Cp, however the parameters produced need not reflect actual observed measurement as the solution for Cp using these parameters can have a number of solutions.

7.11.1. Comparison of Observed and Predicted Parameters

Sheiner et al (1979a) contend that estimates of observed Cl and Vd can be obtained from an individualised Bayesian pharmacokinetic approach, the degree of error reported in section 7.9.1. do not confirm this claim for any of the parameters (Cl, Vd, $t_{\frac{1}{2}\beta}$) considered; all predictions have large s.d., this will result in the parameter being poorly determined (Boxenbaum, Riegelman and Elashoff, 1974).

7.11.2. Comparison of Final and Sequential Forecasting of Parameters

Sheiner et al (1979a) found decreased pe for Cl and Vd with increasing stipulated Cp. The intention therefore was to consider how close to the final point estimate each forecast was for the parameters Cl, Vd, $t_{\frac{1}{2}\beta}$ and k_a, as more Cp were input.

There was a trend for decreasing positive bias for estimates of k_a, there was no systematic bias for the other parameters, as a Bayesian system has an

inherent bias towards the prior probability this is perhaps surprising; such bias may not be statistically significant.

With the exception of Vd, in which no trend was discernable there was improved precision of estimates of Cl, $t_{\frac{1}{2}\beta}$ and k_a with increasing Cp. A poor predictive performance for k_a (table 7.7) was due to the observed value for Cp not being consistent with the expected value, the error is therefore observational.

Although the performance improved with increasing stipulated Cp the degree of error was large as reflected in the rmse, these values reflect 20-30% of the final point estimate, therefore the pharmacokinetic parameters produced by OPT (Fig 7.1) are of little relevance and should not be reported, although they are useful for simulation of alternative doses.

7.12. PERSPECTIVES OF THE MODEL

Despite the use of fixed variances which may not reflect the actual population variances reasonable estimates of Cp could be obtained prospectively, and the protagonists of Bayesian forecasting attest to its efficacy in dosage prediction (Sheiner et al, 1979a); Kelman et al 1982a,b; Joel et al, 1985). Bayesian forecasting has limitations; as with any pharmacokinetic predictive model it is entirely reliant on good compliance and without this prediction is meaningless. Inclusion of an uncertainty factor in the model presupposes that

subsequent measurements are performed in an environment in which compliance can be assumed, this is a reasonable assumption for some groups of hospital in-patients but not for out-patients or those attending their G.P. Even in an 'educated' group of bronchitics in general practice taking theophylline only 52-58% were fully compliant. When results were fed back to the patients non-compliance apparently dropped from 28 to 8% although this was subsequently found to be 38% on an unannounced domiciliary visit (Langan and Watson, unpublished work).

A valuable aspect of OPT in the above study was the education of the general practitioner about pharmacokinetics and rational prescribing however after three months his clinical judgement had become sufficiently attuned to enable him to prescribe doses comparable to those predicted by OPT, provided both had Cp feedback.

7.13. CONCLUSION

OPT is useful in forecasting future Cp and dosage. The most fundamental limitation to the technique is compliance. OPT will perform satisfactorily provided adequate Cp data are available and care is excercised in interpreting its output. An appreciation of kinetic principles would be a desirable pre-requisite for a potential user.

'Hard' replied the Dodger.

'As nails', added Charley Bates.

Charles Dickens

Oliver Twist Ch.9.

CHAPTER 8

LIQUID CHROMATOGRAPHIC ASSAYS FOR CLAVULANIC ACID AND THE ISOMERS OF TICARCILLIN IN SERUM AND URINE

INTRODUCTION

8.1. PENICILLINS

8.1.1. Microbial Assay

Typically penicillins, including ticarcillin (TIC) have been assayed by agar-diffusion microbiological assay which may be highly sensitive (Sutherland and Rolinson, 1978), such an assay has been described for TIC (Bannatyne and Cheung, 1981). Several workers have used Sarcina lutea as the test organism as clavulanic acid (CLAV) does not cause inhibition of this organism (Witkowski et al, 1982; Adam, De Visser and Koeppe, 1982; Schaad, Casey and Cooper, 1983).

8.1.2. Spectrophotometric Assay

Acid labile penicillins may be measured spectrophotometrically following isomerization to the corresponding penicillenic acids (De Weck, 1962), which although labile may be rendered more stable by the addition of copper (Saccani and Pitrolo, 1969) or mercuric salts (Brandriss et al, 1963). However the penicillins undergo side reactions and the penicillenic acids are unstable (Bundgaard, 1971).

Formation of the mercuric mercaptides of imidazole-penicillenic acids results in a stable product that has been used as the basis for a procedure for the assay of penicillins (Bundgaard and Ilver, 1972), but was not applied to the estimation of penicillins in biological fluids.

Fluorimetric assays for ampicillin (Jusko, 1971) and amoxycillin (Davidson, 1976) suffer from the disadvantage

that they also measure the penicillinoic acid metabolites.

8.1.3. Liquid Chromatographic Assay

There are a number of reports of HPLC assays for penicillins including one for amoxicillin in biological fluids using post-column reaction with imidazole (Carlqvist and Westerlund, 1979), however only a few reports on liquid chromatographic assays relate to TIC. Gupta and Stewart (1980) examined a number of βlactams including TIC in a study on the stability of intravenous mixtures; they noted two peaks for TIC but did not investigate this finding further. Kwan et al (1982) claimed to obtain a single peak for TIC in their assays for serum and urine concentrations, however they had noted two peaks which they suggested might be isomers. As the acetylating agent used in the preparation of semi-synthetic penicillins from 6-aminopenicillanic acid is isomeric it would be expected that the final product might contain isomeric penicillins (Salto, 1978).

The assay developed by Kwan et al (1982) had absolute recovery of TIC of 76% and used a technically demanding extraction procedure and did not assay the isomers of TIC. These isomers are of particular interest in renal failure as one or both may accumulate, therefore an assay for the isomers applicable to both serum and urine was required.

8.2 CLAVULANIC ACID

8.2.1. Microbial Assays

Clavulanic acid (CLAV) is a relatively new compound.

Its structure, established by X-ray crystallography (Howarth, et al 1976), has only been known for eight years. There have not been many methods developed for its determination in biological fluids, microbial assays are commonly used. The principle of these assays is the inhibition of the βlactamase of the test organism resulting in it being susceptible to penicillin seeded in the medium (Adam et al, 1982).

8.2.2. Liquid Chromatographic Assays

CLAV is unstable at alkaline pH and in strong acids and therefore is difficult to extract from biological fluids for subsequent liquid chromatographic assay. The first assay developed was by Haginaka et al (1981) and used UV absorbance for detection; usually CLAV does not absorb significantly above 210 nm, however a bathochromic shift is induced in methanol which permits measurement at higher wavelengths. This assay was further developed and refined; the use of methanolic sodium hydroxide degraded clavulanate to produce methyl-4-(2 hydroxyethyl)-pyrrole-3-carboxylate which absorbs at 270 nm, by using a methanolic HPLC eluant and a sodium hydroxide post-column reaction a sensitive assay for CLAV in serum and urine was developed (Haginaka, et al, 1983). Although sample preparation was simple, minimal performance data were presented and only spiked samples were used. The chromatogram contained a number of peaks, but apparently none interfered with the measurement of CLAV.

CLAV can also be measured following derivatisation with imidazole, a procedure applicable to serum and urine has been described (Foulstone and Reading, 1982), derivatisation was achieved prior to injection. Assay of serum was carried out either directly or on ultrafiltrates.

The imidazole-CLAV product, 1-(8 hydroxy-6-oxo-4-azaoct-2 enoyl)-imidazole absorbs at 311 nm and thus potential interference is minimised. Surprisingly this assay was not validated for precision, sensitivity etc. An assay using elements of this work has been published with detailed validation (Watson, 1985) and is based on the work described in this thesis.

MATERIALS AND METHODS

8.3. MATERIALS

8.3.1. Chemicals

i) Ticarcillin

Ticarcillin sodium (Beecham Pharmaceuticals, Brockham, Surrey) (pure free acid equivalent 80.8%) was used to prepare calibrators, and thienylbutyric acid (Sigma Chemical Co., London, England) was used as the internal standard. All other reagents used were BDH Analar grade unless specified. β Lactamase (E.C. 3.5.2.6.) was obtained from Whatman Biochemicals Ltd. (Maidstone, England) and reconstituted according to the manufacturers directions.

ii) Clavulanic Acid

Clavulanate potassium (Beechams Pharmaceuticals) (pure free acid equivalent 82.5%) was used to prepare calibrators. Imidazole reagent was used with and without mercuric chloride as described by Foulstone and Reading (1982) and detailed below. Imidazole was obtained from BDH (for penicillin analysis grade) and Aldrich Chemical Co. (Gillingham, England).

8.3.2. Imidazole Reagents

i) Imidazole reagent with mercuric chloride

Imidazole (16.5 g) was dissolved in 24 ml distilled water plus 4 ml of 5M hydrochloric acid and 4 ml of 1.35% mercuric chloride, the pH was adjusted to 6.8 and the volume then made up to 40 ml with distilled water.

ii) Imidazole reagent without mercuric chloride

Imidazole (8.25 g) was dissolved in 24 ml distilled water and 10 ml of 5M hydrochloric acid added, the pH was adjusted to 6.8 and the solution stored in an amber bottle at room temperature.

8.4. EQUIPMENT

8.4.1. U.V. Spectrophotometry

U.V. spectrophotometry was performed on a Beckman DU-7HS spectrophotometer (Beckman RIIC Ltd, High Wycombe, England).

8.4.2. HPLC Equipment

A Gilson 302 B pump fitted with a series 5 head, latterly a series 5C head and a Gilson 802 manometric module (Gilson, Paris, France) was used with an Altex 160 fixed wavelength filter detector (Beckman). Injections were made with a Waters 710B WISP autosampler (Waters Associates, Northwick, England). Results were integrated using a Shimadzu C-R2AX integrator and latterly a Shimadzu C-R3A integrator (Dyson Instruments, Houghton-le-Spring, England). Columns were slurry packed using a Shandon column packer (Shandon Southern Products, Runcorn.)

i) Ticarcillin

A 10 cm x 5 mm i.d. column packed with Hypersil 3µ ODS was used for the assay of TIC.

ii) Clavulanic acid

A 25 cm x 4.6 mm i.d. Spherisorb 5µ ODS was used in the determination of the CLAV-imidazole product. Prior to this Hypersil 5µ ODS in a 10 cm x 5 mm i.d. column had been used.

8.5. DEVELOPMENT OF LIQUID CHROMATOGRAPHIC ASSAYS

8.5.1. Imidazole Reactions

i) Imidazole reagent with mercuric chloride

To 4 ml of aqueous TIC or CLAV solution (50 mg/l) was added 1 ml of reagent and the mixture incubated at 60°C for 25 minutes. The solution was cooled and scanned versus a blank of water and reagent (4:1) over the range 200-400 nm.

ii) Imidazole reagent without mercuric chloride

To 1 ml of aqueous CLAV solution (50 mg/l) was added 1 ml of reagent and after incubation at room temperature for 10 mins was scanned over the range 200-400 nm.

8.5.2. Eluants

Eluant flow rates were 1 ml/min unless specified.

i) Ticarcillin

Initially a methanol/1% acetic acid (30:70) v/v eluant was used followed by methanol/1%acetic acid/ tetraethylammonium bromide (Aldrich) (30:70:0.05M) v/v/w with detection at 233 nm. Finally a methanol/0.05M phosphoric acid eluant v/v was used, the proportion of methanol used was 25 or 30% depending on required elution speed with a flow rate of 2 ml/min.

ii) Clavulanic Acid

The eluant used by Foulstone and Reading (1982)(methanol/0.1M phosphate buffer pH 3.2 (6:100)), was subsequently modified to methanol/0.1M NaH_2PO_4 containing 0.05M pentane sulphonic acid (Fisons, Loughborough,

England) (10:90). These eluants were used on the 5μ Hypersil ODS material. On the change to Spherisorb ODS packing the eluant was changed to its final form of methanol/ 0.1M NaH_2PO_4 containing 0.05M pentane sulphonic acid and 0.1M ethanolamine, this eluant was pumped at 1.5 ml/ min.

8.5.3. Sample Preparation

i) Ticarcillin

Initially ultrafiltration of serum was attempted using the Syva 'Free' drug assembly (Syva, Maidstone, England). Subsequently an acid extract into diethyl ether was employed with evaporation of the organic phase to dryness, the residue being dissolved in the HPLC eluant prior to analysis.

The finalised extraction procedure is as noted below:

To 1 ml of sample in a Z10 plain tube (Brunswick, Ballymoney, Northern Ireland) was added 50 ul of 1 g/ l methanolic thienylbutyric acid and 6 ml of diethyl ether. To each tube was then added 100 ul of 3M hydrochloric acid, the tube was immediately capped and inverted 2 or 3 times to ensure complete mixing. It was essential to perform this step immediately as TIC was very labile in acid conditions. The tubes were shaken for 5 mins on a lateral shaker (Griffin and George, London, England). The ether layer was transferred to a second Z10 tube containing 500 ul of 0.1M Phosphate buffer pH 7.0 and the ether removed under a stream of air at 40°C; of the

remaining aqueous layer 200 ul was transferred to a PRO tube (Sarstedt Ltd., Leicester , England) from which the upper 5 mm had been removed, these were placed in the centre of a spring insert in the WISP autosampler vial and the septum screw caps applied, the carousel was then loaded and the samples injected. The procedures for serum,diluted urine and peritoneal fluid were identical.

ii) Clavulanic acid

Apart from the substitution of the Syva 'Free' Drug Assembly for the Amicon MPS-1 system the sample preparation was similar to that described by Foulstone and Reading (1982).

The procedure used for serum was as follows: To 500 ul of serum in a Z10 tube was added 500 ul of 0.1M phosphate buffer, pH 7.0, after mixing this was transferred to the Syva 'Free' Drug Assembly and these were centrifuged for 20 mins at room temperature at 1500 g. To 100 ul of ultrafiltrate in a PRO tube was added 100 ul of imidazole reagent without mercuric chloride, the tube was capped, mixed by flicking and inversion, the top 5mm removed and inserted into the WISP autosampler as described above. The technique for diluted urine and peritoneal dialysate are as described for ultrafiltrate.

8.5.4. Sample Storage

Clavulanic acid is labile, as is ticarcillin to a lesser extent. CLAV is labile in alkali, strong acid and is also thermolabile, TIC is acid labile but

relatively stable at room temperature.

In view of these problems the following precautions were taken.

i) Serum

To allow good clot retraction the blood samples were stored at room temperature for one hour then centrifuged and the serum stored at -80°C prior to analysis.

ii) Urine

24 hour urine collections could not be used therefore urine was volumed following micturition and an aliquot taken and diluted 1 in 10 with 0.1M citrate buffer pH 6.5, the diluted samples were stored at -80°C prior to analysis.

8.5.5. Linearity and Calibration

From a freshly prepared stock of 1 g/l ticarcillin sodium or clavulanate potassium were prepared serum calibrators covering the range 0-500 mg/l for TIC and 0-50 mg/l for CLAV using horse serum No 3 (Wellcome Reagents, England). Urine calibrators were prepared in fresh human urine diluted 1 in 10 with 0.1M citrate buffer pH 6.5 covering the range 0-2000 mg/l for TIC and 0-500 mg/l for CLAV.

8.5.6. Recovery

Recovery of TIC relative to the internal standard was assessed from the ratio of peak areas of directly injected TIC/internal standard solution to comparable assayed serum standards at 80 mg/l (n=20). For CLAV a

comparison with directly injected aqueous standards at a concentration of 5 mg/l was performed. The recovery of aqueous ultrafiltrate (n=5) and serum ultrafiltrate (n=5) was compared to assess losses.

8.5.7. Precision

Precision was performed within and between batch, at 5 mg/l and 80 mg/l for TIC (n=21 and n=60 respectively within batch and n=58 and n=62 respectively between batch) and at 1 mg/l and 8 mg/l for CLAV (n=40 and n=40 respectively within batch and n=44 and n=48 respectively between batch).

8.5.8. Accuracy

Samples from individuals on a number of drugs were studied and the relative error determined (Equation 2.2) for TIC at 5 mg/l and CLAV at 1 mg/l.

8.5.9. Quantitation

The concentration of TIC was calculated using equation 2.3 using total peak area as TIC eluted as two distinct peaks. In any calculation of TIC isomer concentration, peak height was used. Results obtained using summed peak height for isomers 1 and 2 were comparable to those obtained using total peak area. The relative contributions of the two isomers was calculated by proportion of peak height to the total ticarcillin concentration assuming equal detector response on a weight for weight basis.

As an internal standard was not used in the CLAV assay external standardisation was used in a manner

analogous to that described for TMP in section 2.7.5.

The use of a target calibrator (section 2.7.5.) also served to correct for within-run degradative losses and was applied to both TIC and CLAV assays. The target calibrator is prepared in 0.1M phosphate buffer pH 7.0 at concentrations of 250 mg/l and 20 mg/l for TIC and CLAV respectively.

8.5.10. Ticarcillin Isomer Equilibration

The equilibrium of the TIC isomer was investigated by collecting the leading or trailing edge of the first or second TIC peak respectively and re-analysing it, this was repeated on the original fractions until the ratio of the two peaks, as determined by peak height, had reverted to that originally found.

RESULTS

8.6. DEVELOPMENT OF LIQUID CHROMATOGRAPHIC ASSAYS

8.6.1. Imidazole Reaction Products

Both TIC and CLAV react with imidazole reagent, and products absorbing in the UV would be useful in enhancing specificity. TIC only reacts with imidazole containing mercuric chloride, CLAV reacts with either of the reagents described in section 8.3.2. to produce a product with an identical UV spectrum and chromatographic characteristics.

Derivatives of CLAV were routinely made using the procedure outlined in section 8.5.1. (ii) and for TIC by the procedure in 8.5.1. (i), the UV spectra for the products are similar (Fig 8.1) and are an improvement on their UV spectra as there is no UV absorbance above 220 nm for either compound prior to derivitisation.

The procedure outlined in section 8.5.1. (ii) was routinely used in all subsequent investigations of CLAV and the product measured by HPLC.

Adequate sensitivity was available using UV detection of TIC at 214 nm and the derivitisation technique was not further developed.

8.6.2. Eluants

i) Ticarcillin

Chromatography of TIC with methanol/1% acetic acid (30:70) revealed two peaks (Fig 8.2a); confirmation that both of these peaks were penicillins was achieved by the addition of penicillinase to the TIC solution

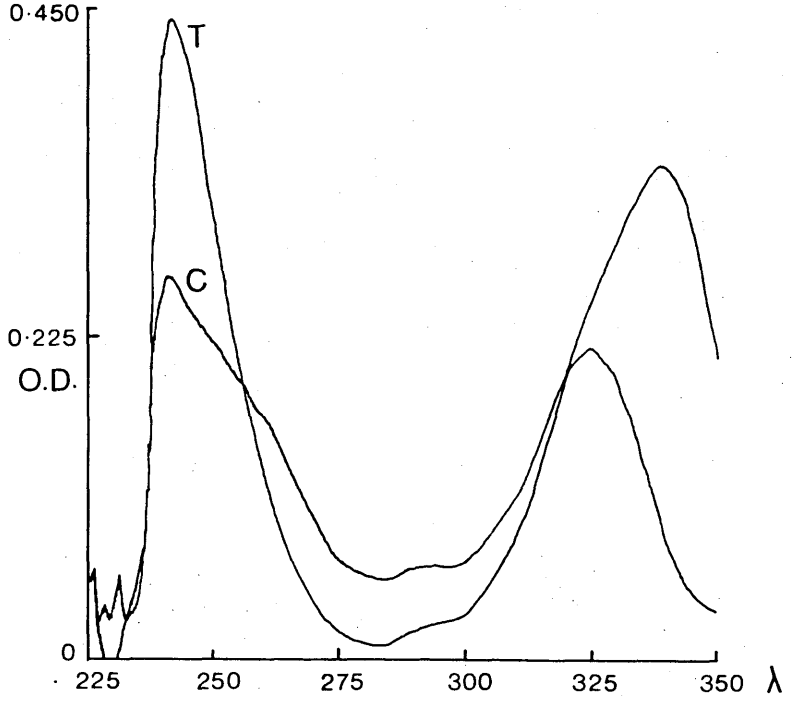


FIGURE 8.1. Ultraviolet scans of the imidazole reaction products of ticarcillin and clavulanic acid.

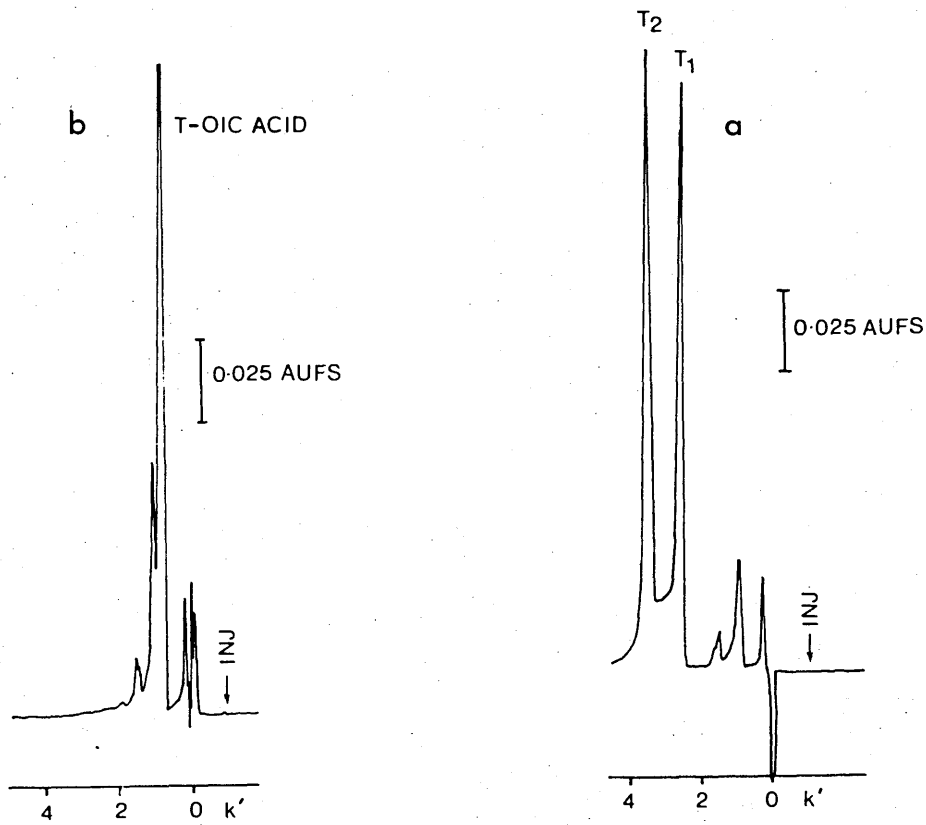


FIGURE 8.2. LC of ticarcillin : eluant Methanol/1% acetic acid (30:70).

a) ticarcillin enantiomers,
b) ticarcillinoic acid (s).

which would result in the disappearance of any peak(s) relating to TIC and produce ticarcillinoic acid; both peaks were absent and a large, earlier eluting peak was detected which was ascribed the identity of ticarcillinoic acid (Fig 8.2b). The k' for ticarcillin isomer 1, isomer 2 and 'ticarcillinoic acid' were 2.6, 3.6 and 0.85 respectively. This was a chromatographically efficient system (TIC isomer 2, h=0.6), the addition of tetre-ethylammonium bromide to the eluant resulted in a reduction in efficiency for this peak to h=1.9.

Serum ultrafiltrates were studied using spiked horse serum with the second eluant. Fig 8.3 shows a serum containing TIC and Fig 8.3b shows a drug free serum, there was a peak that co-eluted with TIC isomer 1 (α=1.0) and also late eluting endogenous material. Due to these problems the methanol/acetic acid eluant was preferred, however sensitivity at the wavelength used (233 nm) was inadequate for adequate pharmacokinetic profiling and a fixed wavelength detector with a zinc lamp and a 214 filter was commissioned. Acetic acid absorbs at this wavelength and to improve the signal/noise ratio, 0.05M phosphoric acid was used instead.

ii) Clavulanic acid

Using methanol/0.1M phosphate buffer pH 3.2 (6:100) the CLAV imidazole product had a k' of 9, but there were some peaks that co-eluted. Addition of 0.05M pentanesulphonic acid resulted in an increase in k' to 29. The eluant was amended to methanol/0.1M sodium

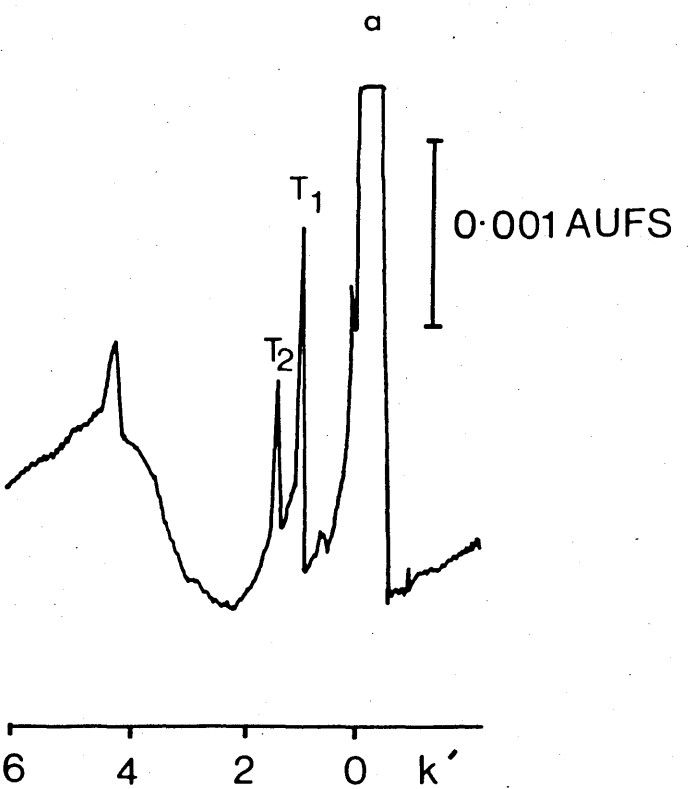


FIGURE 8.3a LC of TIC : serum ultrafiltrate , 20 mg/l TIC in serum

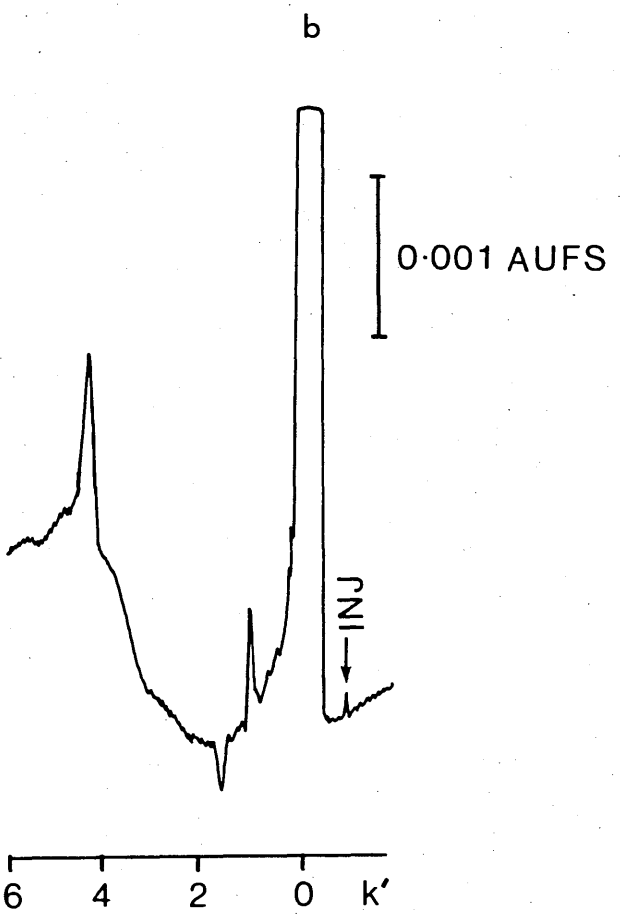


FIGURE 8.3b. LC of TIC : serum ultrafiltrate , TIC free serum

dihydrogen phosphate containing 0.05M pentanesulphonic acid (10:90), a typical chromatogram of an aqueous standard is shown in Fig 8.4. The imidazole reagent gave rise to a number of peaks; one of these co-eluted with the CLAV peak. This problem only occurred with imidazole supplied by Aldrich, use of imidazole (for penicillin analysis) (BDH) avoided this problem. As shown in Fig 8.5 there was significantly more UV absorbing material in the former than the latter.

Following an experiment involving the direct injection of serum the column performance was degraded ($As_{10\%} = 2.0$). Previously the $As_{10\%}$ had been 1.5, (the CLAV peak was symmetrical but with tailing) however on replacement of the column with 5u ODS spherisorb there was very significant asymmetry (Fig 8.6) using the solvent of Foulstone and Reading (1982). The use of pentane sulphonic acid as ion-pair resulted in the chromatogram shown in Fig 8.7, however addition of a counterion, ethanolamine, resulted in a marked improvement in peak symmetry (Fig 8.8) and reduction in k'. The eluant was slightly modified thereafter for routine use.

8.6.3. Sample Preparation

i) Ticarcillin

a) ultrafiltration

There was a 2% loss of TIC on the filters, but recovery in the ultrafiltrate was 54% (the remaining 44% being protein bound). Preliminary investigation of the precision indicated that the CV was greater than 10%

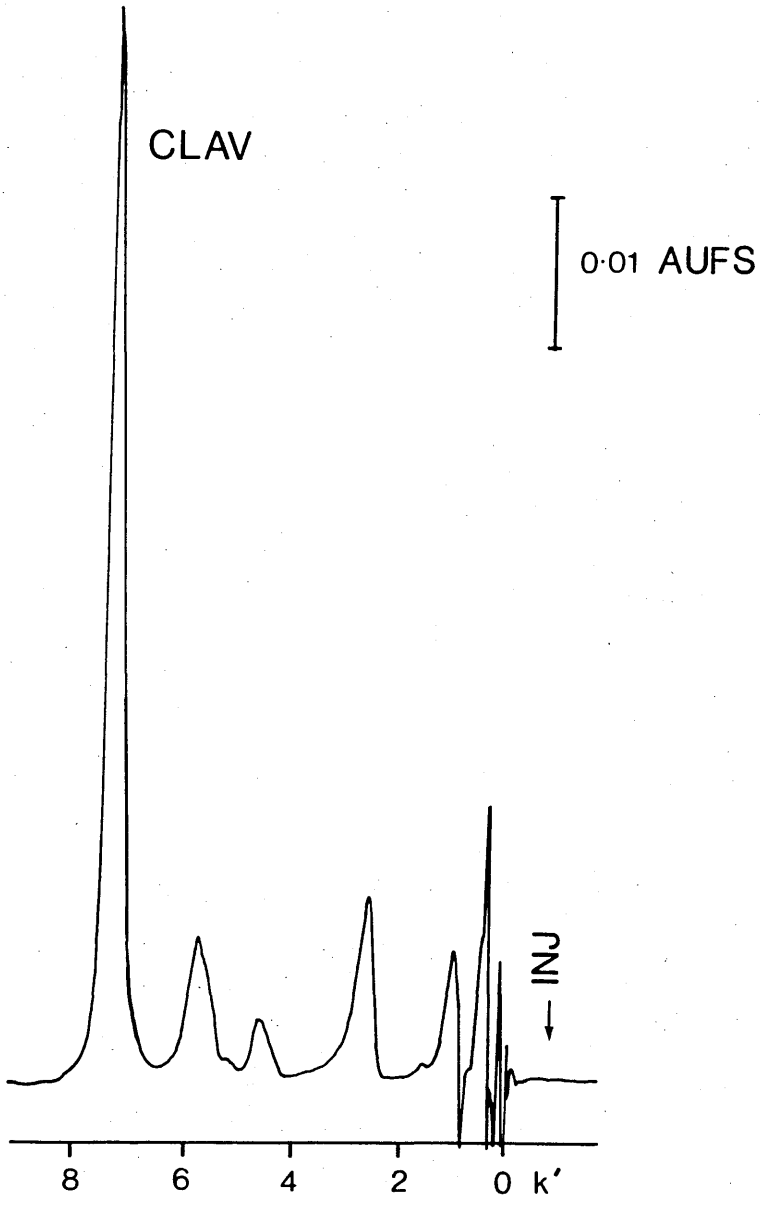


FIGURE 8.4. LC of CLAV : aqueous CLAV imidazole product, other peaks from Aldrich imidazole. Eluant methanol/0.1M phosphoric acid (6:94); column 5µHypersil ODS.

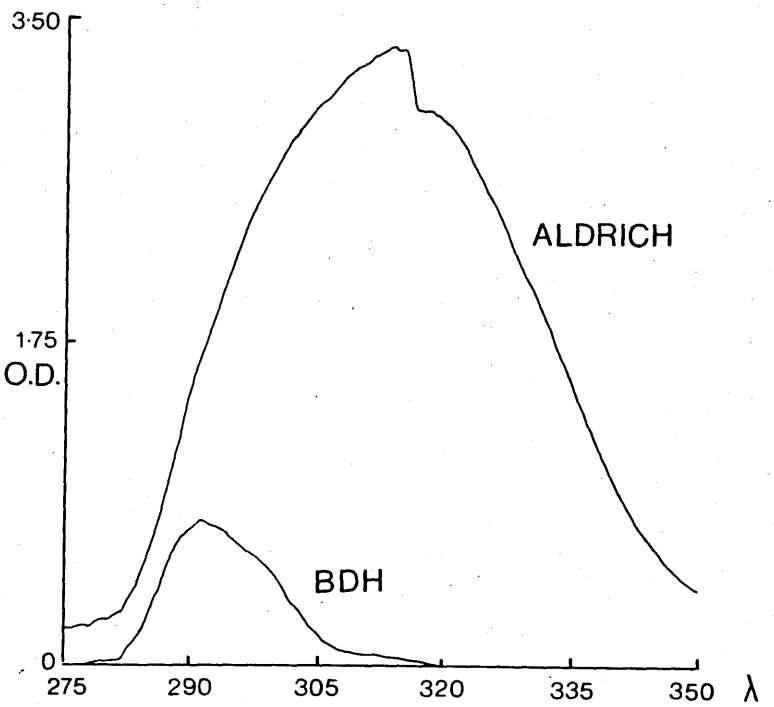


FIGURE 8.5. Ultraviolet spectrophotometry of 20% aqueous solutions of imidazole supplied by Aldrich and BDH.

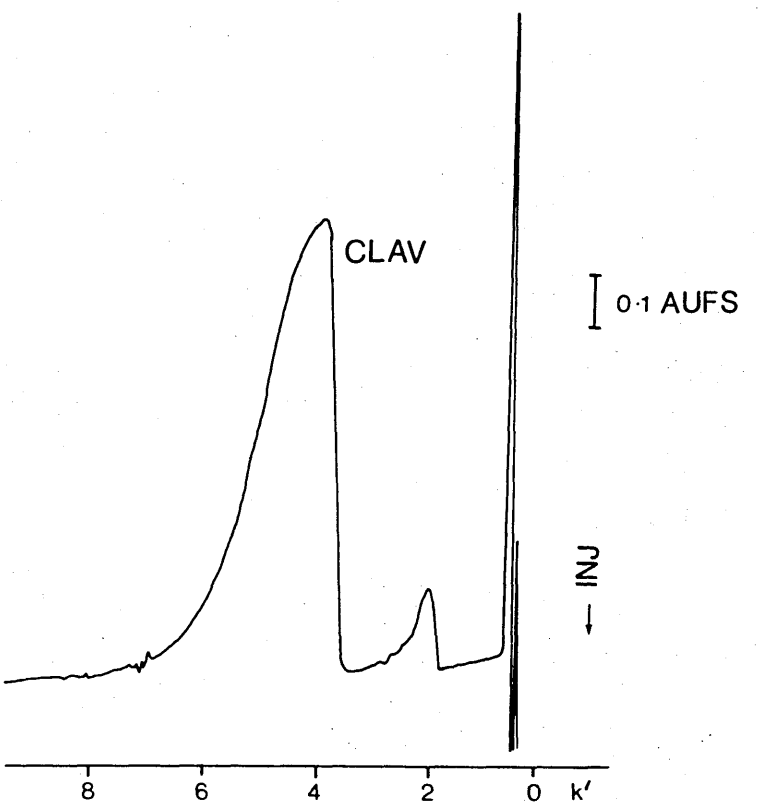


FIGURE 8.6. LC of CLAV : aqueous CLAV-imidazole product, eluant methanol:0.1M phosphoric acid (6:94); column 5μ Spherisorb ODS.

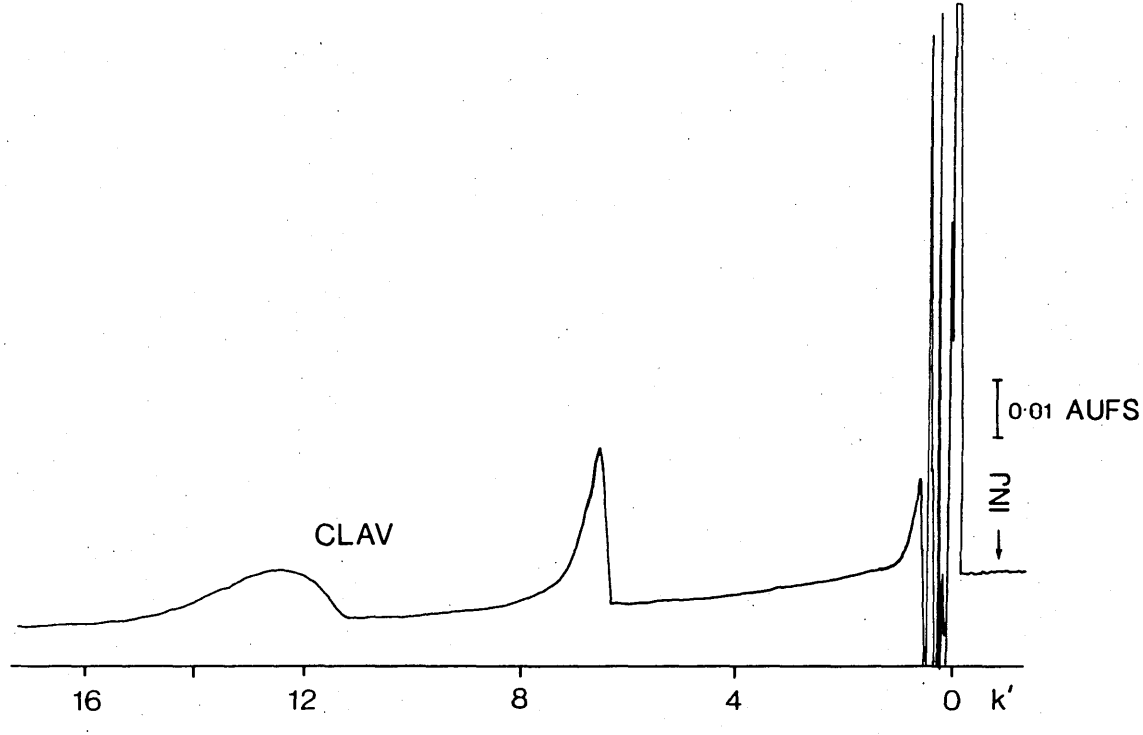


FIGURE 8.7. LC of CLAV : aqueous CLAV-imidazole product, eluant methanol:0.1M sodium dihydrogen phosphate: pentanesulphonic acid (7:93:0.05M) (v/v/w); column 5µ Spherisorb ODS.

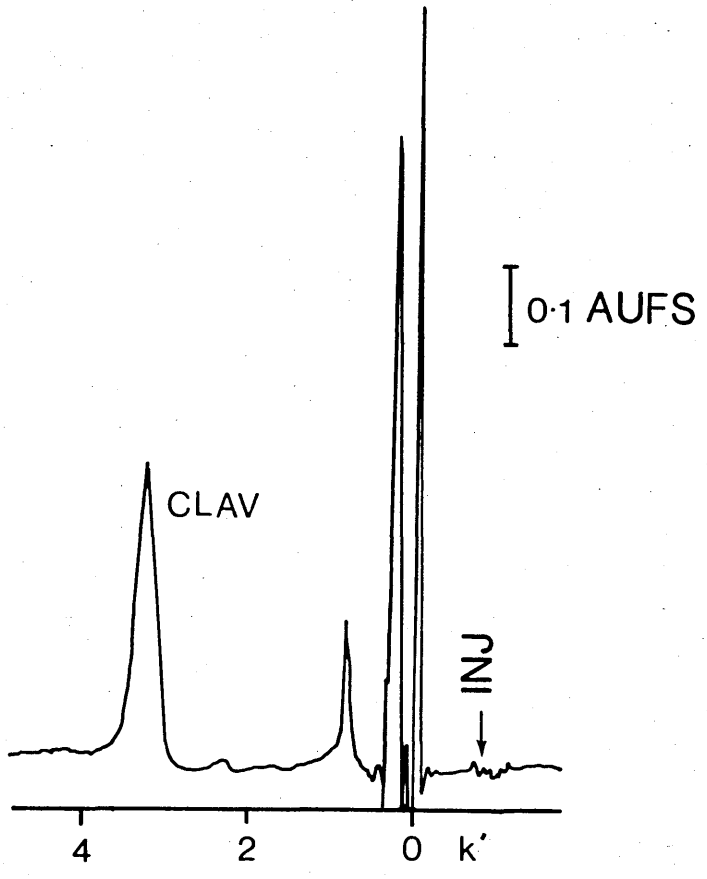


FIGURE 8.8. LC of CLAV : aqueous CLAV-imidazole product, eluant methanol:0.1M sodium dihydrogen phosphate:pentanesulphonic acid:ethanolamine (7:93:0.05M:0.2M) (v/v/w/w); column 5µ Spherisorb ODS.

therefore this approach was abandoned.

b) liquid/liquid extraction

As penicillins are acids, an acidic extraction into diethyl ether was appropriate, however TIC, in common with other penicillins is acid labile. Initially recoveries were very variable, but addition of ether to the TIC sample prior to the addition of the acid followed by IMMEDIATE inversion improved the consistency of recovery, however losses were found at the evaporation stage, caused by residual acidity from the evaporated ether phase. Removal of the organic phase over 0.1M phosphate buffer, pH7, circumvented this difficulty. Further imprecision was associated with variable recovery of the internal standard; mechanisation of extraction on a lateral shaker ensured reproducible recovery of this component. Absolute recovery was then dependent on the amount of organic phase transferred.

ii) Clavulanic acid

Only serum samples required ultrafiltration prior to CLAV analysis. It was possible to analyse serum for CLAV by injecting serum on column following reaction with imidazole, (Fig 8.9), as noted earlier (section 8.6.2. ii) this rapidly led to a loss of column performance. Buffering of serum prior to ultrafiltration counteracted the alkaline pH of the ultrafiltrate (pH 8.0) which resulted in degradation of CLAV.

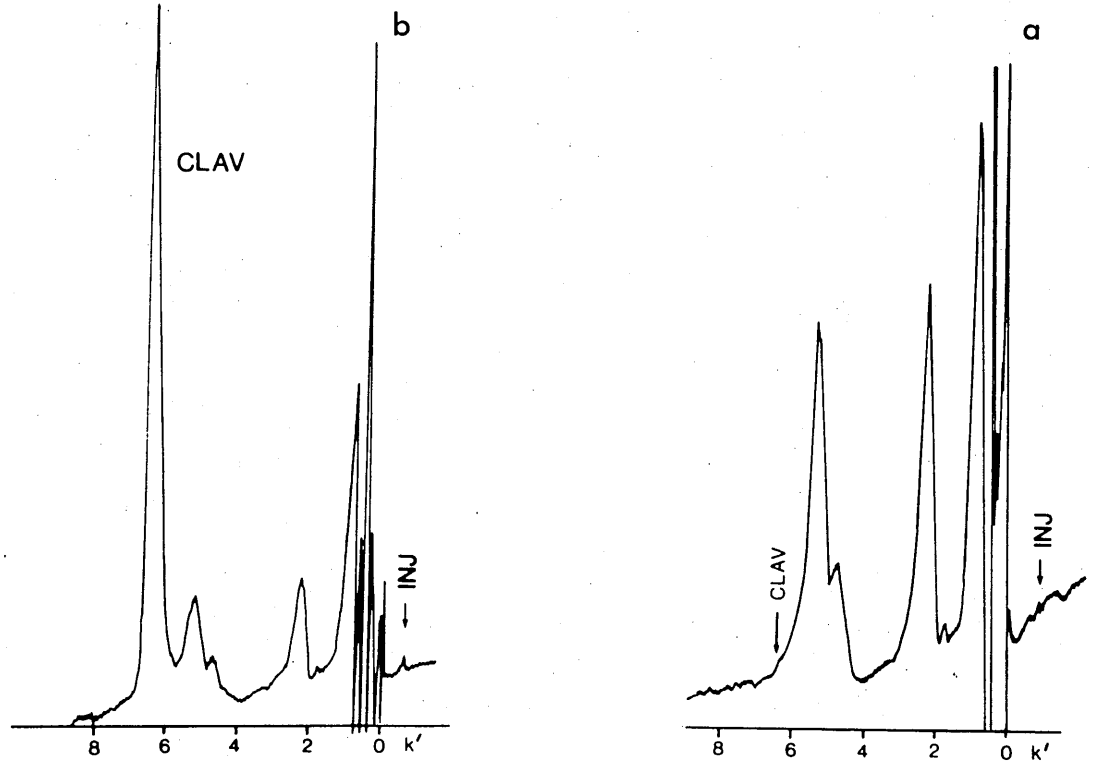


FIGURE 8.9. LC of direct injection of serum a) no CLAV present, b) CLAV present. Conditions as in FIG 8.4.

8.6.4. Sensitivity

Sensitivity, defined as twice the signal/noise ratio was 100ng on-column weight for each TIC isomer, i.e. total TIC sensitivity is 200 ng on-column weight and 4 ng on-column weight for CLAV. These levels are equivalent to 4 mg/l and 40 mg/l TIC and 0.1 mg/l and 0.5 mg/l CLAV for serum and urine respectively.

8.6.5. Linearity

i) Ticarcillin

The summed area of the ticarcillin peaks was found to be linear over the range 0-30 ug on-column weight, this is equivalent to 0-600 mg/l for serum samples and 0-6000 mg/l for urine samples.

ii) Clavulanic acid

Linearity was established for CLAV peak area over the range 0-20 ug on-column weight, this is equivalent to 0-500 mg/l for serum and 0-2500 mg/l for the urine assay.

8.6.6. Recovery

Recovery relative to the internal standard for 80 mg/l TIC was 98.1±8.3% (n=20). The absolute recovery of CLAV from serum was 79.2±4.2% (n=5). It was determined that the average losses were 6.2% due to ultrafiltration and 12.6% due to protein binding.

8.6.7. Precision

i) Ticarcillin

The within batch precision at 5 mg/l and 80 mg/l was 7.8% and 6.0% respectively; between batch

precision was 12.4% and 8.4% respectively.

ii) Clavulanic acid

The within batch precision at 1 mg/l and 8 mg/l was 4.5% and 3.0% respectively; between batch precision at these levels was 6.6 and 4.3% respectively.

8.6.8. Accuracy

i) Ticarcillin

The relative error was not statistically significant (t=1.0). The co-eluting endogenous peak had an area equivalent to 3.5 mg/l TIC. No medication has yet been encountered which has given rise to spurious results.

ii) Clavulanic acid

The relative error was not statistically significant (t=0.02). Samples from patients on a great variety of co-medication have been assayed with no apparent interference.

8.6.9. Assay in Biological Fluids

i) Serum

Chromatograms of CLAV obtained from serum ultrafiltrates prior to dosing and following dosing are shown in Fig 8.10.

Using a 25% methanol eluant the endogenous peak was separated from the TIC isomers, and eluted between them; chromatograms obtained pre and post dose are shown in Fig 8.11.

ii) Urine

Slower chart speeds were used to obtain the

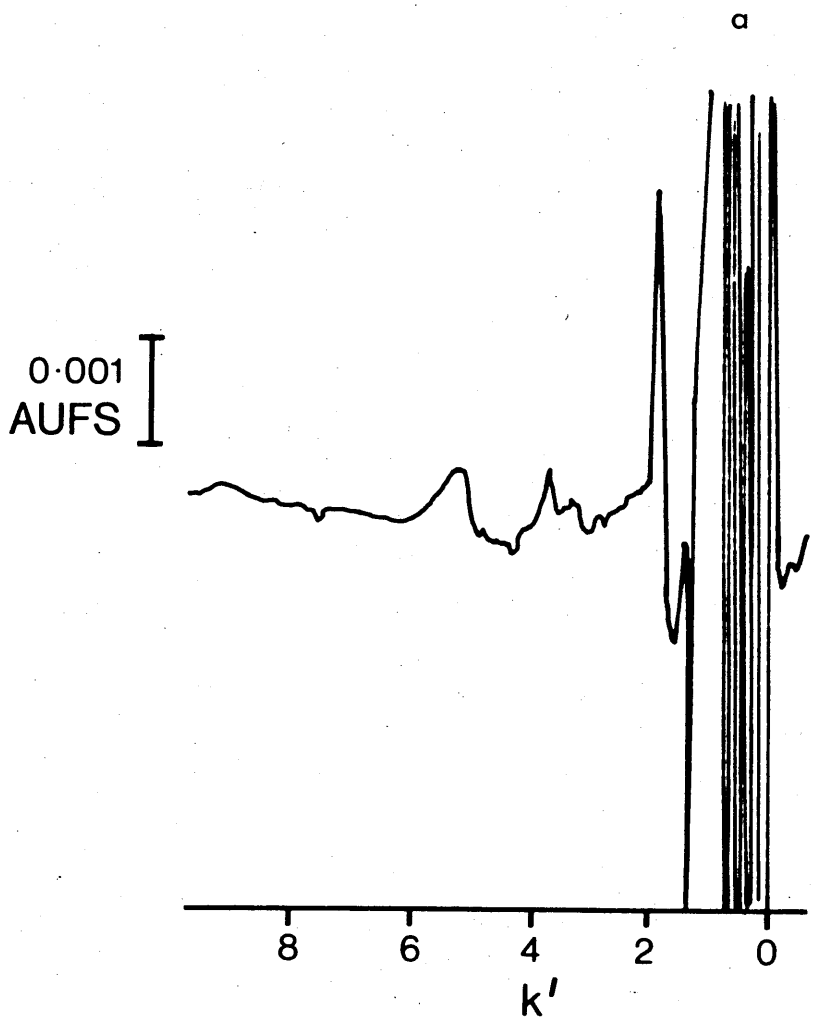


FIGURE 8.10a LC of CLAV : serum ultrafiltrate prior to dosing eluant, methanol:0.1M sodium dihydrogen phosphate: pentanesulphonic acid:ethanolamine (10:90:0.05M:0.1M), flow rate 1.5 ml/min, column Spherisorb 5µ ODS.

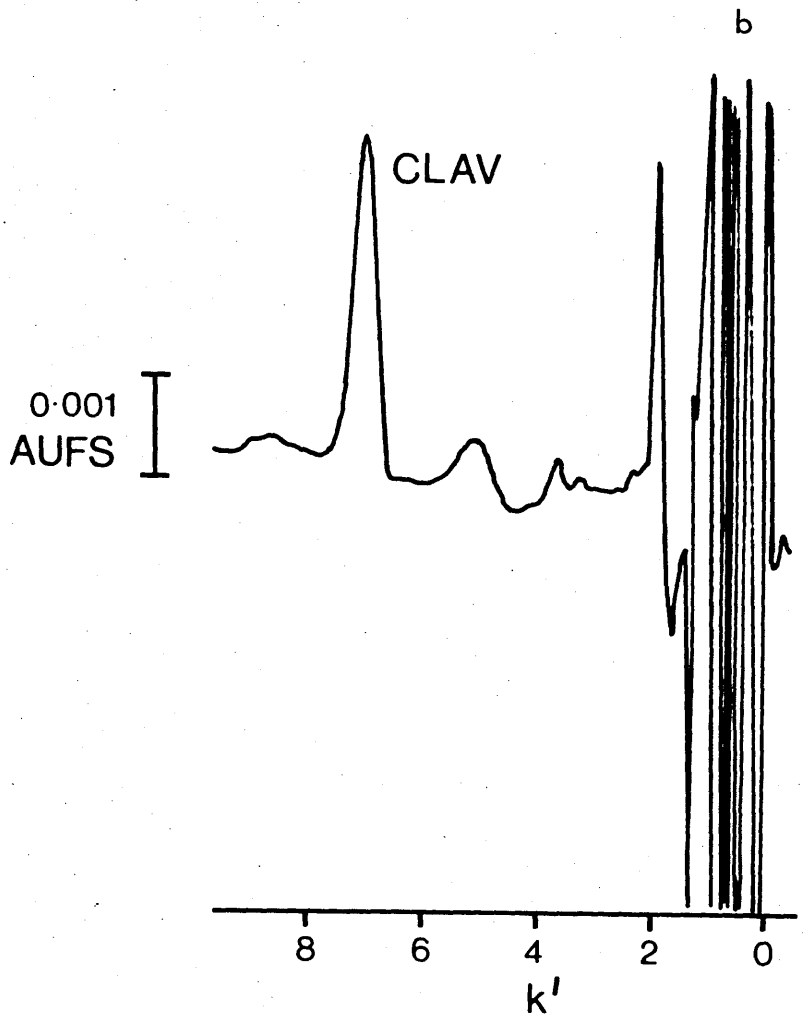


FIGURE 8.10b LC of CLAV : serum ultrafiltrate 8 hours post dose. Conditions as in FIG. 8.10a.

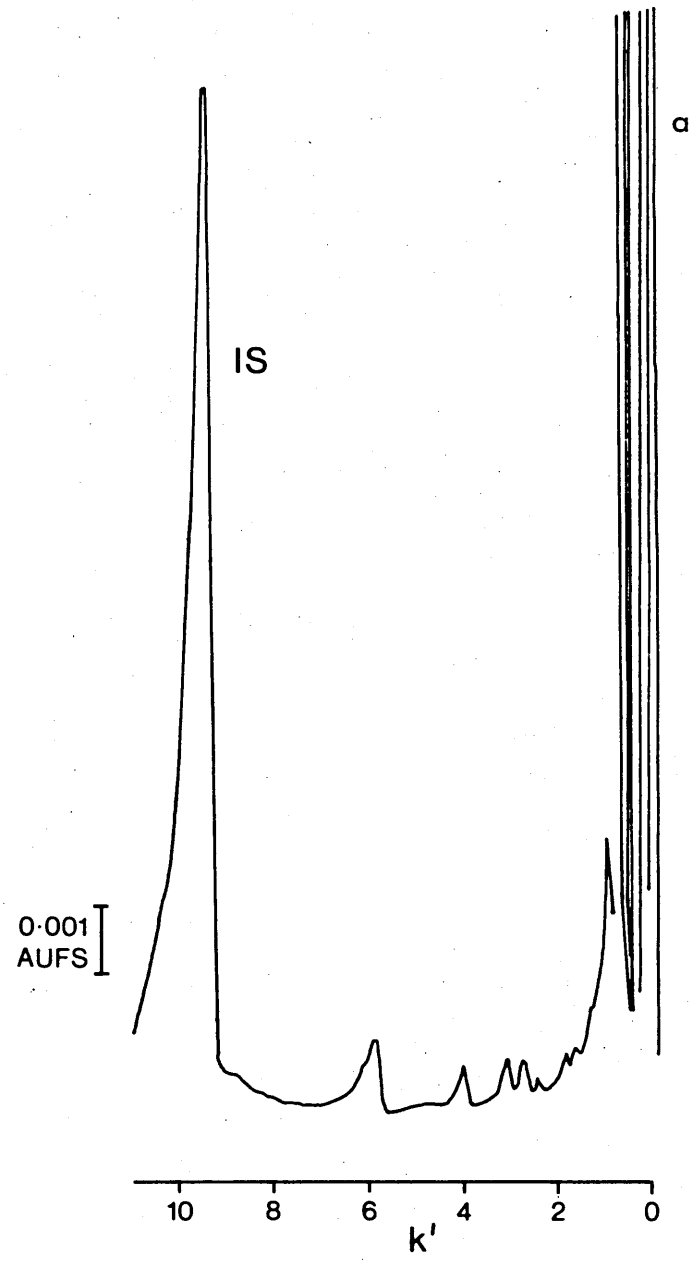


FIGURE 8.11a LC of TIC : serum extract prior to dosing. Eluant methanol:0.05M phosphoric acid (25:75), flow rate 2 ml/min, column Hypersil 3µ ODS. Internal standard (IS) thienylbutyric acid.

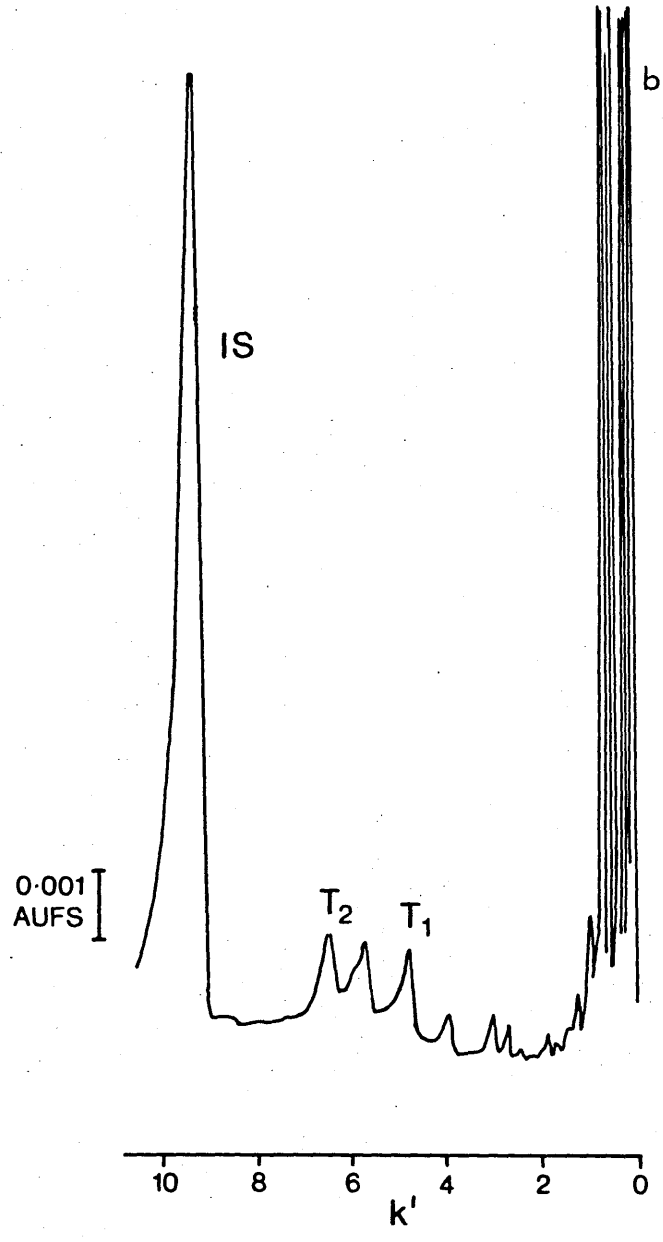


FIGURE 8.11b. LC of TIC : serum extract 8 hours post dose, conditions as in FIG 8.11a.

chromatograms of CLAV and TIC in urine (Figs 8.12 and 8.13). The 30% methanol eluant was used for the TIC assay.

8.6.10 Ticarcillin Isomer Equilibration

Equilibria between the isomers of TIC are rapidly re-established following perturbation by collection. TIC_1 re-established equilibrium with a half-life of 6.0 minutes, and TIC_2 with a half-life of 1.4 minutes, a plot of log peak height deviation versus time was linear and had apparent first-order re-equilibration kinetics.

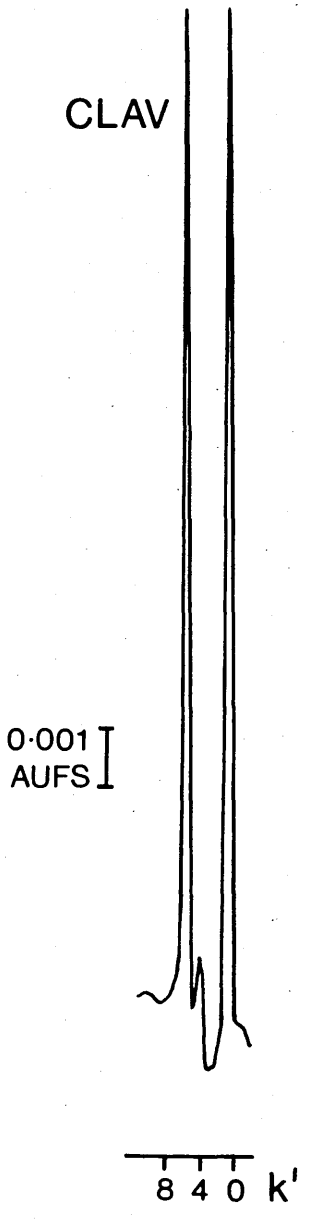


FIGURE 8.12. LC of CLAV : Assay of CLAV in urine 8 hour post dose. Conditions as in FIG. 8.10a.

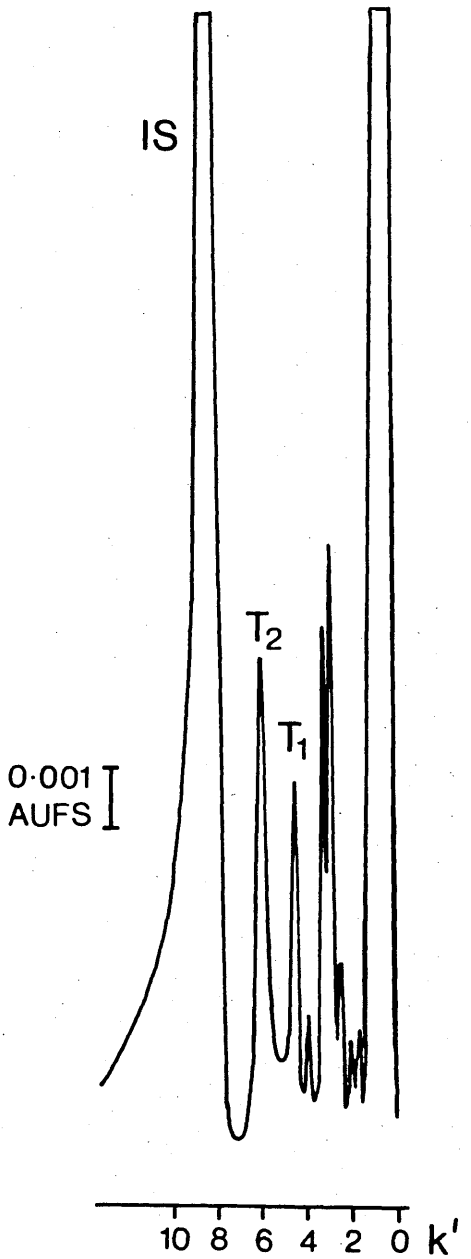


FIGURE 8.13. LC of TIC : Assay of TIC in urine 8 hours post dose. Eluant : methanol:0.05M phosphoric acid (30:70) other conditions as in FIG 8.11a.

DISCUSSION

8.7. ASSAY FOR TICARCILLIN

Of interest in the assay described for TIC was the ability to separate and quantitate the two ticarcillin isomers. Kwan et al (1982) eluted the TIC isomers as a fused peak having noted that the isomers had been the cause of earlier asymmetric peaks, although they found that by close control of the eluant pH it was possible to obtain TIC as two fused peaks. Gupta and Stewart (1980) separated the isomers of TIC, but did not apply the assay to biological fluids. The assay of Kwan et al (1982) had similar sensitivity to that reported here and was applicable to both serum and urine although the sample procedure was somewhat involved; the performance characteristics are very similar to the TIC assay reported here and elsewhere (Watson, 1985), however the major difference is the ability to quantitate the isomers, in addition this assay is simpler.

It has not proved possible to independently isolate the enantiomers to enable identification as they rapidly re-equilibrate in solution; the R-isomer is slightly more active than the S although this is pH dependent, it is most probable that the R isomer elutes first (B. Slocumbe, personal communication).

However if immediate analysis of urine by direct injection were to be perfomed it would be possible to investigate any differential excretion of TIC isomers although this is unlikely to be important therapeutically.

The interference by the small serum peak could be avoided by increasing the retention to allow resolution, by subtraction of the peak area found in a pre-dose sample or by running a penicillinase blank; the second option was most rapid and was routinely adopted.

As would be anticipated manual measurement and summation of TIC peak height for total TIC was less precise than integrated summation of area with correction of background, especially if faster elution times are used. However, measurement of peak height for each isomer and summation compared well with blank corrected integrated area; using peak height in this way a negligible contribution from the endogenous peak was noted.

Despite investigating a number of penicillins and their penicillinoic acids none had a k' appropriate for a useful internal standard, Kwan et al (1982) used carbenicillin which is also an isomeric mixture (Gupta and Stewart, 1980) and although the eluant used by Kwan et al (1982) is substantially different from that used here it might prove to be a more appropriate internal standard, particularly as there was internal standard induced imprecision introduced into the assay reported here. Thienylbutyric acid was suitable in terms of its chromatographic behaviour but it was not until a lateral shaker was used for extraction that acceptably reproducible recoveries of internal standard were obtained.

In addition to this problem there were other areas of potential error: in the extraction step it was found

to be essential to add the acid after the diethyl ether and mix immediately as delay resulted in significant losses of the acid-labile TIC. Losses could also occur due to residual acidity when the organic phase was evaporated to dryness, extracts were therefore concentrated by evaporation in phosphate buffer.

The sensitivity and precision of the LC assay for TIC is superior to that reported for a TIC bioassay (Bannatyne and Cheung, 1981) although the latter only required 40 ul of serum.

8.8 ASSAY FOR CLAVULANIC ACID

The sample preparation procedure used here was closely related to that of Foulstone and Reading (1982) but had not been validated by them for a routine procedure. The Syva assemblies used are very similar to the Amicon MPS-1 system and acceptable results should be obtainable using this. The buffer used prior to ultrafiltration was required to stabilise CLAV, derivitization prior to injection was preferred although it should be possible to develop a satisfactory post-column reaction procedure as has succesfully been done for amoxicillin using a mercuric-imidazole reagent (Carlqvist and Westerlund, 1979). A novel post-column reaction procedure for CLAV has been reported by Haginaka et al (1983) in which post- column alkaline degradation was used; these authors exhaustively examined a number of CLAV degradation product and procedures. An ion-pair separation of CLAV followed by alkaline degradation was found to provide

sufficient sensitivity to allow pharmacokinetic studies to be performed. Interesting though this assay is, it has limitations, direct injection of serum was used and this has been found to degrade column performance (Watson, 1985). Two different eluants were required for analysis of serum and urine and there were problems of noise associated with the post-column reactor. After each run, to protect the post-column reaction equipment and plumbing, it was necessary to flush out the alkali with dilute nitric acid. Thus although sensitivity and precision appear comparable to those reported here this would appear to be a more demanding system to operate.

An ion/counter-ion eluant was developed for the assay described in this work. The reasons for its composition are as follws:- The phosphate salt was used to maintain an acid pH at which the clavulanate derivative was relatively stable, methanol was an organic modifier, pentane sulphonic acid was required to ion-pair the reaction product (1-(8-hydroxy-6-oxo-4azaoct-2-enoyl)-imidazole) to improve its retention and ethanolamine acted as a counter-ion. No products were found for TIC or its penicillinoic acid when incubated with imidazole reagent.

When a well capped reverse phase material was used some tailing of the peak shape resulted, however if 'end-capping ' was incomplete it was found that peak tailing was excessive; in either case excellent peak shape could be obtained by the addition of a cation

which covered the residual silanol groups and also acted as a counter-ion, ethanolamine was found to be satisfactory and adjustment of its concentration could be used to control the retention of the clavulanate-imidazole reaction product; an increase causing a decrease in k' and a decrease on increased k'. The use of longer chain alkyl cationic surfactants resulted in excessive desorption with a decrease in solute retention to a k' <1.

An internal standard was unnecessary as there was little sample preparation for urine samples and a simple preparation for serum with a reproducible recovery. The product stability was such that adjustment for degradation loss within run was necessary although reproducible within the run; this was well identified with the repeat sampling of the 'target' standard.

The precision of the assay was better than that previously noted with a microbiological assay (Bennet et al, 1983).

8.9. CONCLUSION

Since both TIC and CLAV are labile, correct storage and handling of samples are essential for accurate results; the use of liquid chromatography provides an ideal approach for the fast, reliable, precise and accurate assay of both analytes in serum and urine.

The assays developed are suitable for estimation of both CLAV and TIC (and its isomers) in clinical studies.

The whole is more than the sum of the parts.

Aristotle 384-322BC

Metaphysica 1045a .10

CHAPTER 9

CLAVULANATE POTENTIATED TICARCILLIN : PHARMACOKINETICS IN PATIENTS WITH RENAL FAILURE AND IN PATIENTS UNDERGOING DIALYSIS

INTRODUCTION

9.1. CLINICAL USE OF CLAVULANIC POTENTIATION OF PENICILLINS

Clavulanic acid is a β-lactam first isolated from Streptomyces clavuligerus. It is an antimicrobial agent with a very low specific antimicrobial activity (Cole, 1980), however it has a very high affinity for β-lactamases especially the plasmid mediated TEM enzymes (Reading and Cole, 1977); its action may be reversible or irreversible (Fisher, Charnas and Knowles, 1978: Cole, 1980). Thus CLAV can be used in combination with β-lactamase-sensitive agents to potentiate their action (Cole, 1980).

9.1.1. Amoxycillin/CLAV

A number of β-lactams have been shown to be potentiated by CLAV in vitro (Wise, Andrews and Bedford, 1978; Reeves, Bywater and Holt, 1978; Cole, 1980), one that was particularly effective was CLAV and amoxycillin, this was shown to have significant activity against amoxycillin-resistant organisms in vitro (Miller, Baker and Thornsberry, 1978) and in vivo (Ninane at al, 1978).

The combination of amoxycillin/CLAV has since been used to treat a wide variety of infections including respiratory tract infection (Ninane et al, 1978; Stevens and Abbas, 1980) and simple, complicated or recurrent urinary tract infection (Reeves et al, 1980; Gurwith, Stein and Gurwith, 1983; Brumfitt and Hamilton-Miller, 1984).

9.1.2. Ticarcillin/CLAV

In complicated urinary tract infection significant re-infection may occur, in one study using amoxycillin/CLAV,17% of 'cures' suffered re- infection by Pseudomonas aeruginosa (Leigh et al, 1980) which is not susceptible to amoxycillin. More recently CLAV in combination with ticarcillin (TIC) has become available; TIC is a broad-spectrum penicillin with activity against Pseudomonas aeruginosa, unlike amoxycillin it is acid labile and can therefore only be administered intravenously.

Carbenicillin and TIC were the earliest penicillins to be shown to be effective against P. aeruginosa, in vitro testing indicated that TIC was 2-3 times as potent as carbenicillin against this organism (Klastersky and Daneau, 1972; Fuchs et al, 1977; King et al, 1980). Both drugs are effective against gram-negative organisms with TIC the more active. Although not primarily intended for such use,TIC and carbenicillin may be used against gram-positive organisms, carbenicillin is more active (Fuchs et al, 1977).

Resistance to TIC, by a variety of species eg. E. Coli (Rodriguez et al, 1973) is due to its susceptability to their β lactamases (for E.Coli, TEM β lactamase) CLAV irreversibly inhibits this enzyme and therefore combining it with TIC would be expected to broaden its spectrum. The combination of TIC and CLAV is more active than TIC alone in vitro (Paisley and Washington II,

1978; Chattopadhyay and Hall, 1984; Clarke and Zemcov, 1984; Fuchs et al, 1984) and has succesfully been used in vivo (Cox, 1983; Williams,Horobin and Price, 1984; Kosmidis et al, 1984)

9.2. PHARMACOKINETICS OF CLAVULANATE/PENICILLIN COMBINATIONS IN NORMAL SUBJECTS

9.2.1. Amoxycillin/CLAV

The kinetics of amoxycillin and CLAV are comparable whether administered alone or in combination (Jackson et al, 1980; Ball et al, 1980; Adam, et al, 1982; Witkowski et al, 1982). Elimination half-life for both amoxycillin and CLAV is about 1.1 hours (Adam et al, 1982; Witkowski et al, 1982), there is approximately a 20% reduction in CLAV recovered in urine if the dose is administered orally rather than intravenously (Jackson et al, 1980) which may be due to lability in the gut or to a first-pass effect.

9.2.2. TIC/CLAV

The combination of TIC/CLAV is newer than the amoxycillin/CLAV combination and consequently there is a lack of data on the TIC/CLAV combination. It has been reported that the kinetics of TIC and CLAV in combination are comparable in combination to those obtained when the drugs are given independently (Bennett et al, 1983) and this has recently been confirmed (Tetzel et al, 1984)

i) Volume of distribution

Bennett et al (1983) found a Vd of 10.8 l for TIC, this compares with other reports (Libke, et al, 1975; Findlay et al, 1981; Davies et al, 1982), these values are in contrast to the Vd reported by Mayers et al (1980) of 41.8 l/100 kg. All the values quoted in these studies were obtained using microbiological assays.

The Vd reported for CLAV (20 l) by Bennett et al (1983) compares with those computed from earlier reports (Adam et al, 1982; Witkowski et al, 1982)

ii) Elimination half-life

The elimination half-life for TIC and CLAV in normal subjects was reported as 1.1 hr and 1.5 hr respectively Bennett et al (1983); their TIC half-life is comparable to that reported by Libke et al (1975), however the CLAV half-life is double that reported by other workers (Adam et al, 1982; Witkowski, et al, 1982), Ball et al (1980) found a CLAV half-life of around one hour and Tetzel et al (1984) a half-life of 1.3 hours.

iii) Clearance

According to Bennett et al (1983) in normal subjects serum clearance of TIC is 7.0 l/h of which 5.2 l/h is renal and for CLAV the serum clearance was 14.5 l/h of which 6.4 l/h was renal. Libke et al (1975) found a total serum clearance for

TIC of 9.2 l/h of which 7.9 l/h was renal, serum clearances for TIC of 6.5 l/h and 11.8 l/h have been reported by Findlay et al (1981) and Meyers et al (1980) respectively, the data of Tetzel et al (1984) agree with the former figure.

The serum clearance for CLAV was calculated from the data of Adams et al (1982) and Witkowski et al (1982) and found to be approximately 14 l/h and 16 l/h respectively. The serum clearance calculated from the data of Staniforth et al (1983) was 12.4 l/h with a renal clearance of 5.3 l/h, Tetzel et al (1984) reported a serum clearance of 9.5 l/h.

9.3. RENAL FAILURE AND TICARCILLIN/CLAVULANIC ACID THERAPY

9.3.1. Clinical

There are a wide variety of chemotherapeutic agents available for the treatment of infections of the urinary tract. These include TMP either alone or in combination with a sulphonamide, a penicillin (often amoxycillin), a cephalosporin, and possibly nitrofurantoin or nalidixic acid. The quinolone derivatives eg. norfloxacin, ofloxacin are derived from nalidixic acid, are new broad spectrum antibiotics with significant activity against P. aeuroginosa. These drugs have been used succesfully for the treatment of urinary tract infection in pre-launch clinical trials (Bint, Pedler and Marshall, 1984; Leigh and Emmanuel 1984) and may

be taken orally. How long it will take for significant resistance to develop is a matter for conjecture.

In renal failure, due to the impairment of excretion, care is required with dosing, particularly for drugs with a narrow therapeutic index. Patients with renal failure tend to be infected with organisms with multiple resistance, in which circumstance treatment with an aminoglycoside is often used; monitoring of the serum levels may avoid toxicity but the use of a nephrotoxic agent on patients with renal failure is less than desirable. Due to problems of resistance, cephalosporins and penicillins are not favoured, however with the claims that CLAV lowers the MIC of penicillins in amoxycillin resistant organisms, (Miller et al, 1978; Fuchs et al, 1984), then clavulanate potentiated β-lactams could prove useful in these situations. To date there have been no reports of either combination being extensively used in patients with renal failure.

9.3.2. Pharmacokinetics

i) Renal Function

As noted in section 9.2.2.iii., nearly 75% of TIC and 45% of CLAV are cleared by renal excretion and consequently accumulation would be expected in renal failure. Probenecid, which blocks secretion of organic acids, has been shown to cause accumulation of TIC (Libke et al, 1975), but not CLAV, although there was

a statistically significant decrease in renal clearance (Staniforth et al, 1983).

TIC has been available for a number of years and its disposition investigated (Davies, Morgan and Anand 1974; Parry and Neu, 1976; Hoffler, Dalhoff and Koeppe, 1978). There was a relationship between the degree of renal failure, as assessed by creatinine clearance and TIC elimination half-life (Parry and Neu, 1976, Hoffler et al, 1978; Boelaert et al 1984; Dalet et al, 1984a; Watson and Boulton-Jones, 1984) dosage regimes to avoid TIC accumulation have been proposed (Parry and Neu, 1976; Hoffler et al, 1978). Pharmacokinetic parameters other than elimination half-life were not noted.

There are limited reports on the effect of renal failure on CLAV kinetics. There is an increase in CLAV half-life with increasing renal failure (Dalet et al, 1984b) and although insufficient patient numbers were examined no difference between patients for Vd was noted, this has been confirmed in studies on TIC/CLAV (Boelaert et al, 1984; Dalet et al, 1984a; Watson and Boulton-Jones, 1984).

ii) Haemodialysis and peritoneal dialysis

Removal of TIC during haemodialysis is relatively efficient with half-lives of around 4 hours (Davies et al, 1974; Wise, Reeves and Parker, 1974; Parry and Neu, 1976) although clearance via peritoneal

dialysis is poor (Wise et al, 1974; Parry and Neu, 1976). There is a considerable difference of opinion as to the required dosing frequency for patients undergoing dialysis: Davies et al (1974) recommend dosing half-way through dialysis, Wise et al (1974) recommend dosing every fifteen minutes and Parry and Neu (1976) recommend dosing at the end of dialysis.

The reports on the haemodialysis of CLAV suffer from lack of data; Dalet et al (1984b) studying amoxycillin/CLAV, reported a half-life of 1.1 hours in 5 patients and recommended dosing half-way through dialysis with a further dose. The efficiency of CLAV removal has been confirmed in a report on a single patient. (Slaughter, Kohli and Brass, 1984) and Dalet et al (1984a) confirmed their earlier findings. There is currently no information available on CLAV in peritoneal dialysis although it is known to rapidly penetrate into peritoneal fluid (Wise et al, 1983a).

The aim of this work was to determine the pharmacokinetics of the TIC/CLAV combination in patients with varying degrees of renal impairment and patients undergoing either haemodialysis (HD) or continuous ambulatory peritoneal dialysis (CAPD) and to devise an appropriate dosing schedule for use in these patient groups.

MATERIALS AND METHODS

9.4. CLINICAL

9.4.1. Subjects

All subjects received a single IV bolus injection consisting of 3 g TIC and 200 mg CLAV, blood samples were obtained from the cephalic vein of the arm not used for drug administration.

All subjects were volunteers and informed consent and ethical permission were obtained.

Patients with either known hypersensitivity to cephalosporins, or concurrent infection were excluded.

9.4.2. Study Design

i) Renal failure

The degree of renal impairment was categorised by creatinine clearance (CrCl) it was proposed that 6 subjects in each of the following categories be investigated: with a CrCl >60 ml/min, CrCl 30-60 ml/min, CrCl 10-30 ml/min, CrCl <10 ml/min and no urine flow (anephric).

ii) Haemodialysis

Six patients undergoing routine haemodialysis treatment would be used with haemodialysis starting two hours after dosing and lasting for four hours.

iii) Continuous ambulatory peritoneal dialysis

It was proposed to examine the return dialysate levels of TIC/CLAV following an I.V. bolus

administration and also to examine the penetration of TIC/CLAV to the systemic circulation when administered in the dialysate. Patients with peritoneal scarring from previous episodes of infection were excluded from the study. Six patients in each group were to be studied with three bag changes each with a four hour dwell time, CAPD to commence at time of dosing. Bags may be 1 or 2 l depending on body size.

9.4.3. Sampling Protocols

i) Renal failure

a) serum

Blood samples (10 ml) were drawn from an indwelling catheter situated in the cephalic vein, patency was maintained with heparin. Samples were obtained pre-dose and at 0.083, 0.33, 0.67, 1, 1.5, 2, 4, 8, 12 and 24 hours post dose. The samples were separated soon after collection and stored at -80°C prior to analysis (see 8.5.4.i.).

b) urine

The bladder was emptied prior to dosing and then aliquoted, diluted and stored as described in section 8.5.4.ii.

ii) Haemodialysis

a) serum

A sample was obtained pre-dose, then at 1.5 hours post dose at which point dialysis was commenced and lasted for 4 hours, samples were obtained

0.25, 0.5, 1, 2 and 4 hours post-commencement of haemodialysis, following cessation of dialysis samples were collected at 1 and 4 hours post completion of dialysis.

b) urine

Although most patients were anephric, any urine passed was collected and stored for analysis as described in section 8.5.4.ii.

iii) Continuous ambulatory peritoneal dialysis

a) serum

A pre-dose sample was obtained, dosing and commencement of dialysis coincided and samples (10 ml) were obtained at 0.083, 0.5, 1 and 4 hours post commencement of dialysis , following the first bag change (i.e. at 4 hours) a sample was obtained and a further sample just prior to completion of dialysis with the second bag. Following instillation of the third bag a sample was obtained, finally a sample at 24 hours post dose was obtained (i.e. 16 hours post 3rd bag instillation).

b) dialysate

Return dialysate was collected (10 ml) at 0.25, 0.5, 1. 2. 3 and 4 hours post commencement of dialysis. Aliquots were taken of dialysate upon completion of the first, second and third bag changes, these were immediately frozen at - 80°C and stored until analysis.

c) urine

Any urine passed was stored as described in section 8.5.4.ii.

9.5. ANALYTICAL

9.5.1. Assays

The assays for TIC and CLAV in serum, urine and dialysate were used as described in section 8.5.3.

9.5.2. Pharmacokinetics

The studies used single i.v. bolus doses only. The design of the sampling protocols used in the HD and CAPD studies did not allow estimates other than elimination half-life to calculated.

i) Clavulanic acid

The ratio of the α and β slopes for CLAV did not differ by more than 2 and thus use of a single compartment model was appropriate (Curry 1980). Estimates of $t_{\frac{1}{2}\beta}$, Vd, Cl_s and Cl_r were performed using equations 3.1, 3.2, 3.3 and 4.1 respectively.

ii) Ticarcillin

The α/β ratio for TIC was greater than 2 and a two compartment model was applied.

The equation which applies to a two compartment model is:

$$Cpt = Ae^{-\alpha t} + Be^{-\beta t} \qquad \text{Equation 9.1}$$

where A and B are the intercepts on y axis (i.e. concentration at time t = 0) from the plot of log Cp

versus t, α and β are the slopes (rate constants) of the exponential components of the curve. The parameters Vd, Cl_s and $t_{\frac{1}{2}\beta}$ were obtained from a computer programme using least squares regression to obtain the best curve described in general terms by equation 9.1.

The programme was VASP (Nonlinear regression) with calculation of Vd, Cl and $t_{\frac{1}{2}\beta}$ by the MICRO 2 programme, access to these programmes at Dept. Materia Medica, Stobhill Hospital, Glasgow was through Dr. P. Meredith.

Renal clearance was calculated from the equation below:

$$Cl_r = k_{el} \cdot fe \cdot Vd \qquad \text{Equation 9.2.}$$

When Vd is noted for TIC without a sub-script this is the sum of the Vds of compartments 1 and 2 ($Vd_1 + Vd_2 = Vd$).

Elimination was assumed to occur only from the central compartment (Vd_1).

RESULTS

9.6. PHARMACOKINETICS IN RENAL FAILURE

Unless otherwise noted all results reported in tables etc are mean values, the individual data are contained in appendix E.

9.6.1. Serum Concentration Ratios

The serum concentration ratio of TIC/CLAV was not statistically significantly different between the different categories of renal impairment at the different times (t-test). As can be seen in Fig 9.1 there is nearly an order of magnitude difference at 20 minutes post dose, the range being between 10 and 70, the ratio deteriorated with time with the spread becoming wider and the ratio tending to increase. Clearance during HD was rapid, but in CAPD was less so, similar trends as to concentration ratios could however be detected.

9.6.2. Serum Kinetics

i) Clavulanic acid

The pharmacokinetics of CLAV are given in table 9.1. The indices of elimination decrease with increasing renal impairment, however in the anephric group there is a statistically significant improvement in clearance ($p < 0.05$, t-test) when compared with the CrCl <10 ml/min group.

Omitting the anephric group, there was significant linear association between CrCl and Cl_s or $t_{\frac{1}{2}\beta}$ ($r=0.747$ and $r=-0.728$, $df = 22$) ($p < 0.001$, linear regression). Vd was independant of renal function, - no relationship was found.

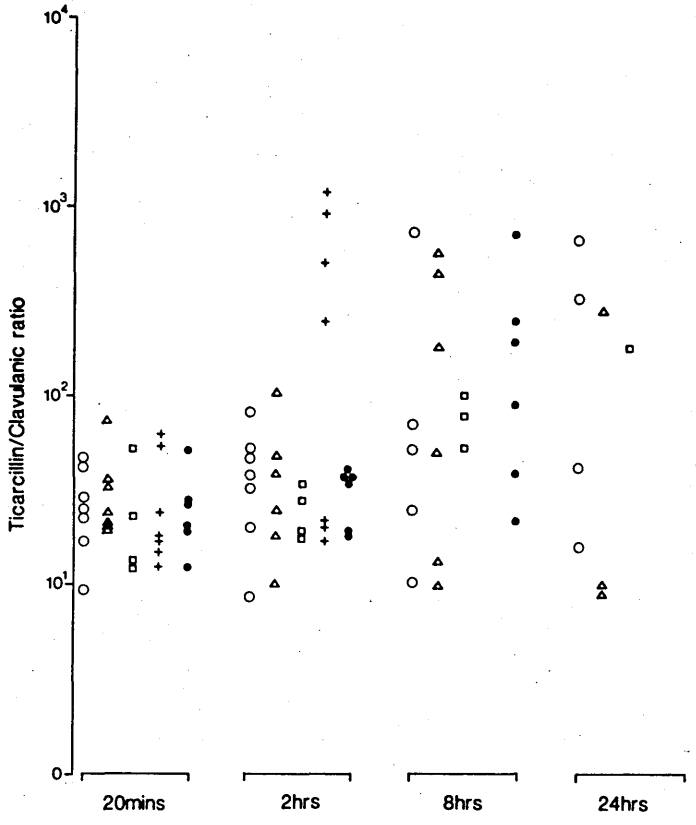


FIGURE 9.1. Range of TIC/CLAV ratio with time for different categories of renal function.
o <10 ml/min, Δ 10-30 ml/min, ¤ 30-60 ml/min, + >60 ml/min
● Anephric. No plot if CLAV not detectable.

TABLE 9.1

Pharmacokinetics of CLAV following a single i.v. dose of 200 mg CLAV and 3 g TIC, one compartment model

Creatinine Clearance (ml/min)			ANEPHRIC	<10	10-30	30-60	>60
PARAMETER							
(l)	Vd	$\bar{x}$	16.33	13.34	15.79	15.65	11.50
		sd	6.51	4.18	6.61	3.06	4.84
(l/h)	Cl_s	$\bar{x}$	7.12	3.46	6.89	7.23	19.71
		sd	2.97	1.10	4.35	3.57	6.36
(h)	$t_{\frac{1}{2}\beta}$	$\bar{x}$	1.65	2.74	2.0	1.83	0.41
		sd	0.65	0.60	0.79	0.93	0.27
(h^{-1})	k_{el}	$\bar{x}$	0.42	0.25	0.35	0.38	1.69
		sd	0.17	0.05	0.14	0.19	1.13
	n		6	7	7	4	7

ii) Ticarcillin

The pharmacokinetics of TIC in the different renal impairment groups is given in table 9.2.

There was an association between a decrease in estimates of elimination (CrCl v Cl_s : r = 0.842; CrCl v $t_{\frac{1}{2}}\beta$: r = -0.761, df = 21) and decreasing renal function, the differences between the following groups were not statistically significant (α = 0.05) : CrCl <10 ml/min and 10-30 ml/min, anephric and <10 ml/min 10-30 ml/min and 30-60 ml/min. There was no change in total Vd with changing renal function, nor with the Vd of either compartment.

9.6.3. Urine Kinetics

i) Clavulanic acid

The renal clearance, fe, and cumulative amount of CLAV excreted are summarised in table 9.3. There was a statistically significant association (r = 0.805; 22 df) between decreasing renal clearance and decreasing renal function (p <0.001, linear regression) there was correlation between the fraction excreted unchanged (fe) and renal function (r = 0.691, 31 df) (p <0.01),renal excretion predominated only in the >60 ml/min group.

ii) Ticarcillin

The urine kinetics of TIC are given in table 9.4, there was a significant linear correlation between Cl_r and CrCl (r=0.831, df 21) (p<0.001),

TABLE 9.2

Pharmacokinetics of TIC following a single i.v. dose of 200 mg CLAV and 3 g TIC, two compartment model

Creatinine Clearance (ml/min)		ANEPHRIC	<10	10-30	30-60	>60
PARAMETER						
(l) Vd (CENTRAL COMPT)	$\bar{x}$	7.30	5.20	5.46	5.35	5.69
	sd	3.05	1.67	1.76	3.27	2.27
Vd (PERIPHERAL COMPT)	$\bar{x}$	8.73	4.14	6.19	6.75	6.33
	sd	3.10	1.89	3.14	1.32	1.44
Vd (TOTAL)	$\bar{x}$	16.37	9.33	11.64	12.10	11.70
	sd	5.05	3.07	3.25	2.59	3.45
(l/h) Cl_s	$\bar{x}$	1.23	0.91	2.26	2.50	9.04
	sd	0.46	0.46	1.16	1.33	2.37
(h) $t_{\frac{1}{2}\beta}$	$\bar{x}$	9.48	6.87	5.44	3.38	0.94
	sd	3.81	2.52	1.85	1.82	0.21
(h^{-1}) k_{el}	$\bar{x}$	0.073	0.100	0.127	0.205	0.737
	sd	0.029	0.037	0.043	0.110	0.165
n		6	7	7	4	7

TABLE 9.3

Urine kinetics of Clavulanic acid

Creatinine Clearance (ml/min)		ANEPHRIC	<10	10-30	30-60	>60
Cl_r	$\bar{x}$ ± sd	0	0.82 ± 0.65	1.60 ± 1.29	2.86 ± 1.61	14.82 ± 6.10
fe	$\bar{x}$ ± sd	0	0.21 ± 0.11	0.27 ± 0.12	0.37 ± 0.13	0.58 ± 0.17
cumulative amount (mg) excreted	$\bar{x}$ ± sd	0	41.70 ± 21.80	55.00 ± 23.30	73.30 ± 26.20	114.90 ± 34.90
n		6	7	6	4	7

TABLE 9.4

Urine kinetics of Ticarcillin

Creatinine Clearance (ml/min)		ANEPHRIC	<10	10-30	30-60	>60
Cl_r	$\bar{x}$	0	0.22	0.39	0.81	5.88
	± sd		0.13	0.13	0.88	2.08
fe	$\bar{x}$	0	0.23	0.25	0.24	0.49
	± sd		0.07	0.12	0.19	0.26
cumulative amount (mg) excreted	$\bar{x}$	0	684	760	724	1657
	± sd		203	353	573	627
n		6	7	6	4	7

but the relationship between fe and CrCl is less clearcut ($r = 0.468$, df 21) ($0.01<p<0.05$). As with CLAV renal excretion only predominated in the >60 ml/min group.

9.7. DISPOSITION DURING DIALYSIS

9.7.1. Disposition During Haemodialysis

Three patients were studied during HD; disposition prior to dialysis was assumed to be as previously found in the anephric group.

i) Clavulanic acid

The half-life of removal following the commencement of HD was 0.6, 0.55 and 0.5 hours respectively (Fig 9.2). Only one patient (patient 3) had 'rebound' serum levels following cessation of therapy (0.3 mg/l, 0.9 hours after dialysis).

ii) Ticarcillin

The half-lives of removal for TIC were slower than for CLAV and were 2.9, 1.7 and 2.1 hours respectively, all patients had rebound serum levels (Fig 9.3) although the extent of the rebound varied.

9.7.2. Disposition During CAPD

There was great difficulty in recruiting CAPD patients consequently only two patients were studied following i.v. administration of the TIC/CLAV combination. The concentrations for TIC and CLAV in serum and dialysate are given in appendix F.

i) Clavulanic acid

There was steady accumulation of CLAV

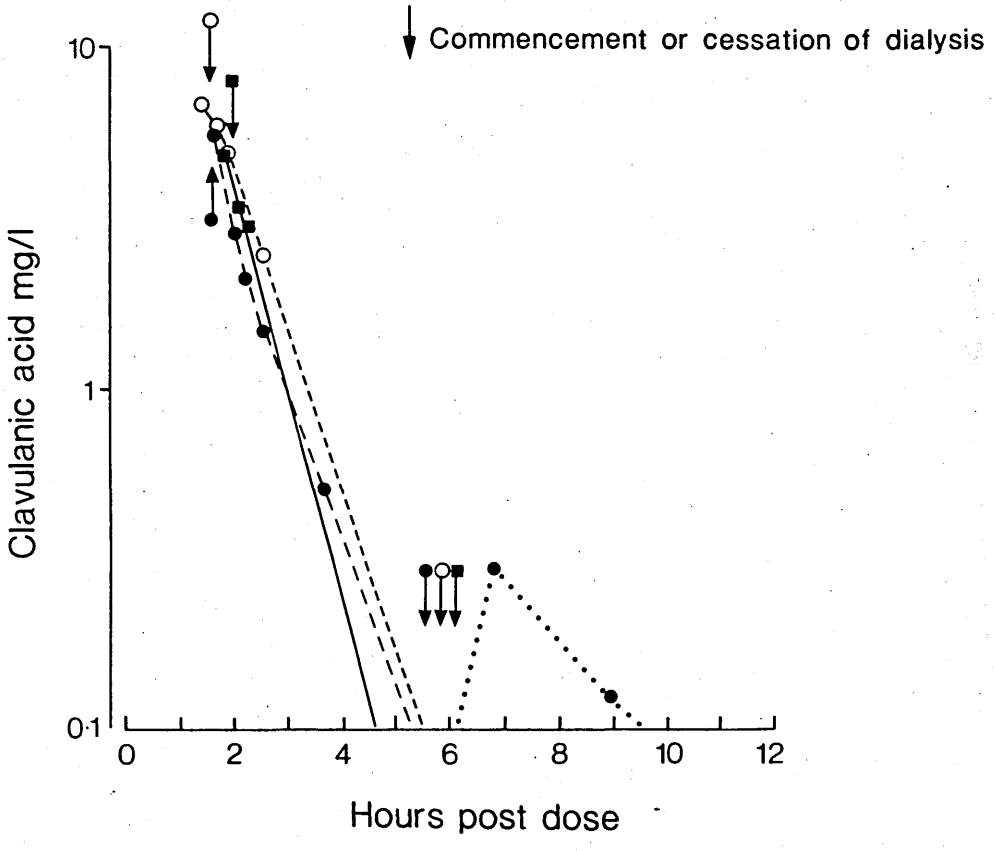


FIGURE 9.2. Disposition of CLAV during haemodialysis. Dotted line is the approximate time course of the 'rebound' levels.

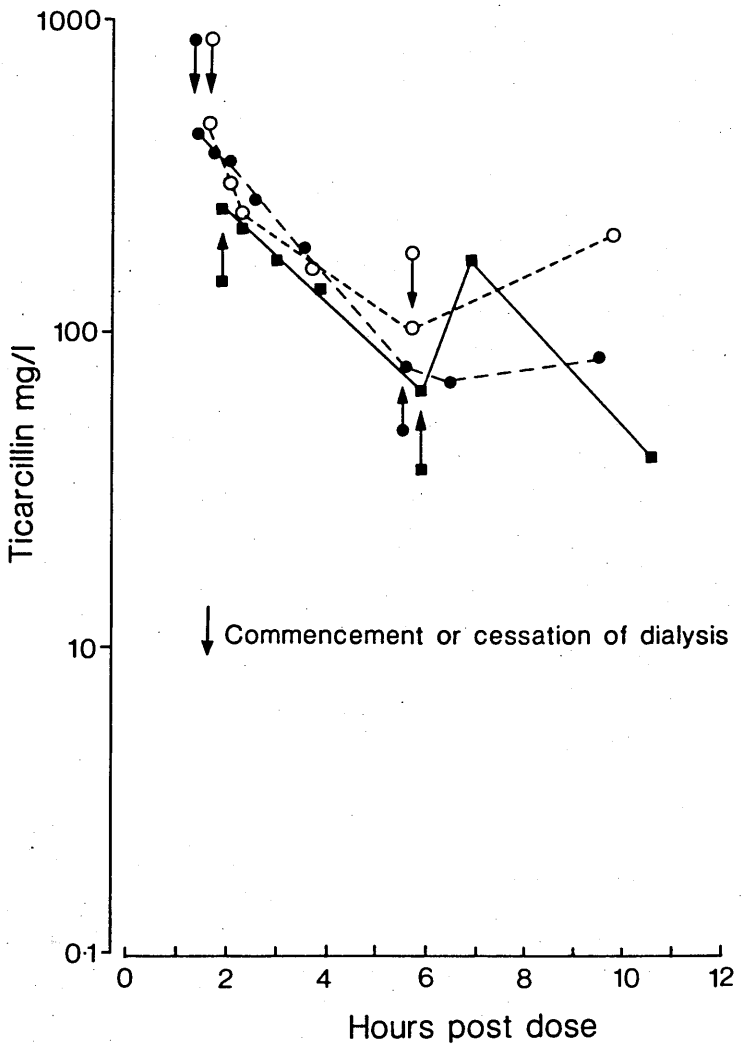


FIGURE 9.3. Disposition of TIC during and following haemodialysis.

in the dialysate over the first 4 hour period (Fig 9.4), following the first bag change there was a drop in dialysate CLAV levels for patient 2 at changeover however no sample was taken immediately following the second bag for patient 1; CLAV apparently did not accumulate during the second period of dialysis as the dialysate CLAV concentration was less after the second period of dialysis.

The serum CLAV concentrations indicate a rapid fall during the first dialysis period, a sample was not drawn following changeover to the second bag nor during the residence time of the second bag, but serum CLAV concentrations had fallen below the detectable limits of the assay by 8 hours post dose. Although the half-life of removal of CLAV in patient 2 is approximately 0.6 hr, the elimination in patient 1 appears biphasic with half-lives of 0.3 and 7 hours respectively, although too few measurements were made to substantiate this, however it is worthy of note that the rate of accumulation in the dialysate of patient 1 is slower and more pronouncedly biphasic than for patient 2, however the extent of recovery was similar at 115.2 mg (57.6%) and 100.3 mg (50.2%) from patients 1 and 2 respectively.

ii) Ticarcillin

Ticarcillin accumulated continuously in the dialysate during the first period of dialysis (Fig 9.5) for both patients, patient 2 had a more

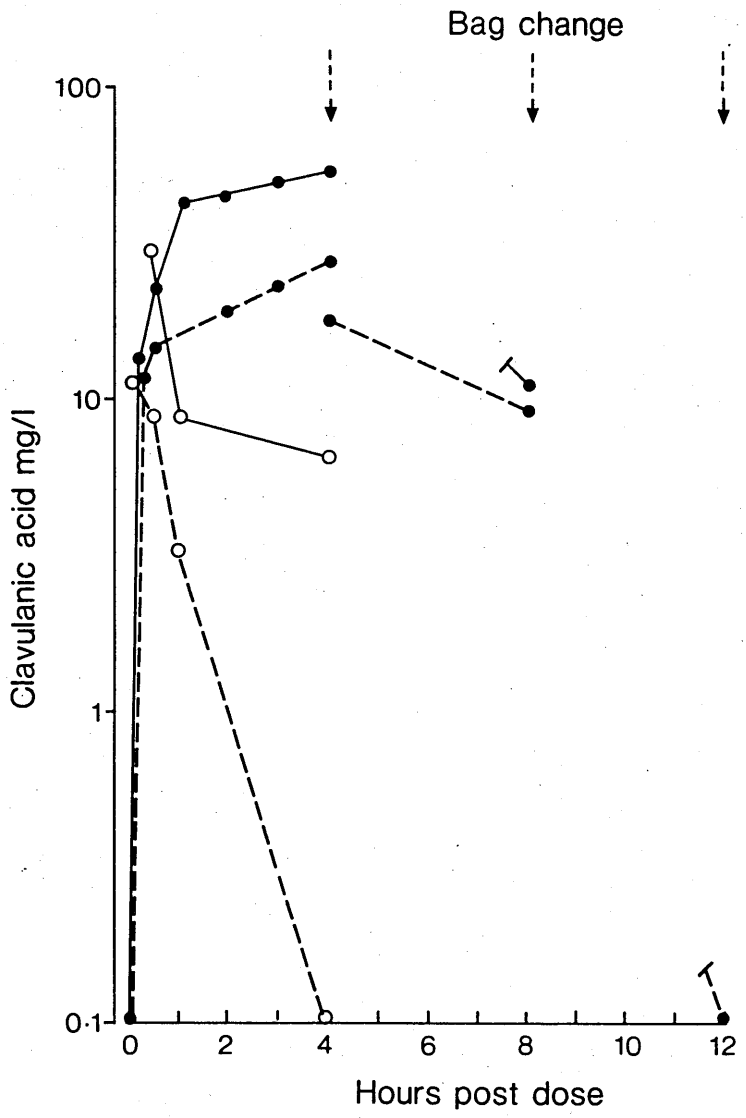


FIGURE 9.4. Disposition of CLAV during CAPD.

o = Serum concentrations
● = Dialysate concentrations

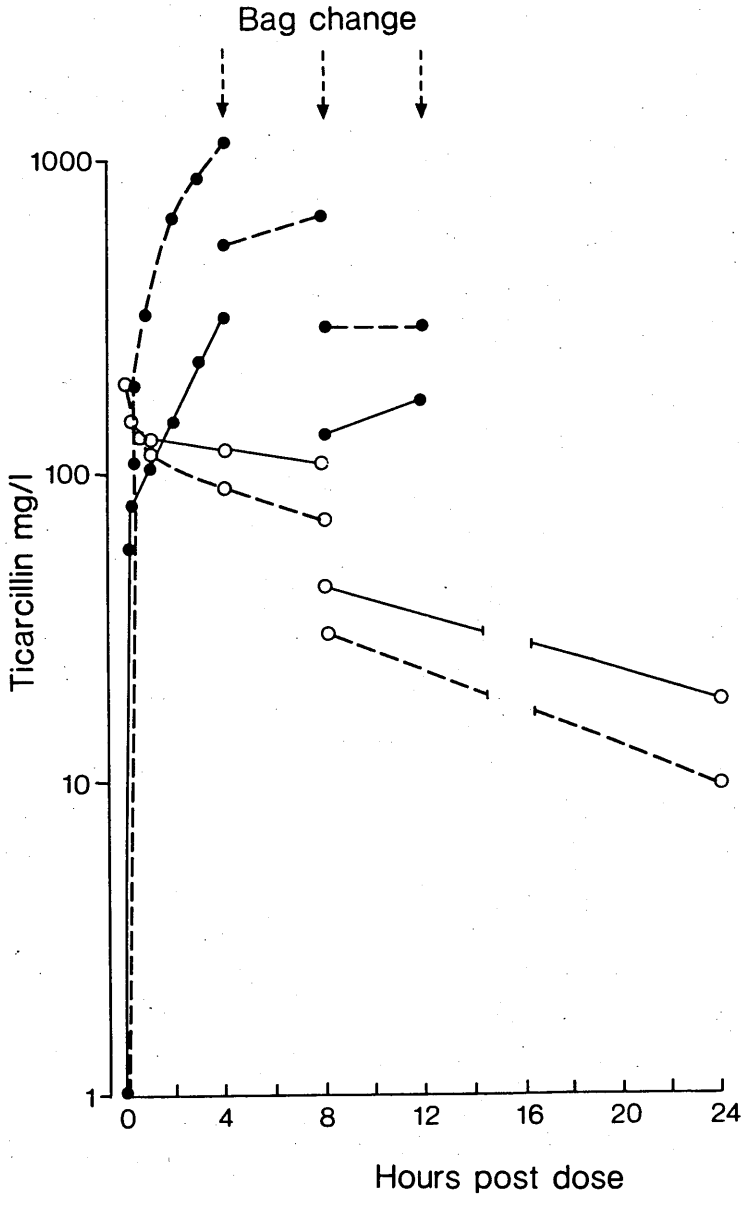


FIGURE 9.5. Disposition of TIC during CAPD

o = Serum concentrations
● = Dialysate concentrations

rapid and greater accumulation of TIC than patient 1. In the second four hours the dialysate TIC level dropped on dialysate changeover but there was some accumulation by four hours (patient 2), there was little change in this individual in the third period although some accumulation was noted for patient 1.

There was good recovery of TIC, 1637 mg (54.6%) and 2377 mg (77.9%) respectively from patients 1 and 2.

The elimination was however not reflected in rapid elimination half-lives, estimated at between 13-24 hours for patient 1 and approximately 10 hours for patient 2, i.e. similar to the anephric group elimination half-lives.

9.8. DOSING REGIMENS IN RENAL FAILURE AND DIALYSIS

Having cognisance of the kinetics of TIC and CLAV in the dosage combination used in this study, a recommended dosage schedule has been designed (table 9.5).

The CAPD group have been excluded as there is insufficient consistent information to allow prediction of serum levels following dosage.

TABLE 9.5

Dosing schedule for 3.2 g TIC/CLAV in renal failure and haemodialysis

Creatinine Clearance (ml/min)	Maintenance Dose and Frequency	Predicted serum concentrations			
		TIC (mg/l)		CLAV (mg/l)	
		C_{ss}AVE	C_{ss}MIN	C_{ss}AVE	C_{ss}MIN
>60	3.2gTIC/CLAV/4h (3gTIC+0.2gCLAV)	72	12	3.2	0.1
30-60	3.2gTIC/CLAV/8h	161	64	4.2	0.9
10-30	1.6gTIC/CLAV/8h	126	73	2.3	0.4
<10	1.6gTIC/CLAV/12h	137	71	2.5	0.4
ANEPHRIC	1.6gTIC/CLAV/12h	104	66	1.5	0.09
Haemodialysis	3.2gTIC/CLAV post-dialysis and then 1.6gTIC/CLAV 12 hourly. Assuming 4 hour haemodialysis on a 48 hour cycle serum concentration immediately prior to next dialysis will be TIC 90 mg/l and CLAV 0.2 mg/l.				

DISCUSSION

9.9. PHARMACOKINETICS IN RENAL FAILURE

9.9.1. Serum Concentration Ratios

In the discussion of TMP/sulphonamide serum concentration ratios (section 3.9.4.) the significance of the ratios achieved centred round whether these were within the range of optimal synergy and the implications of this for therapeutic effect. The combination of TIC/CLAV is administered in the ratio of 15:1, in plasma it was found to be between 10:1 and 70:1 twenty minutes after administration, the latter is over double that noted by Bennet et al (1983) in normal males. The ratio increased with time to up to 1000:1 in some instances, the highest ratios occurred in the anephric group followed by the groups with the least renal function. The cause of these wide ratios is the notable difference in elimination between TIC and CLAV in renal failure, CLAV is more rapidly eliminated than TIC, the differential increasing with increasing renal failure.

If the TIC/CLAV ratio were of the same significance as the TMP/sulphonamide ratio then supplementation of CLAV during dosing might be necessary, however the mechanisms of action of these two formulations are in the general case fundamentally different. In TMP/sulphonamide there is reversible competitive inhibition at the enzyme(s) active site whereas, in susceptible organisms, the mode of action of CLAV is by irreversible competitive inhibition, thus it is not the ratio of TIC/CLAV that is

significant but the level of CLAV that is achieved.

The length of duration above the required threshold may be significant, but when CLAV action is due to irreversible inhibition the inevitable drop in CLAV concentration due to distribution/elimination will not be so significant. The relationship between pharmacokinetics and bacterial growth kinetics requires much closer examination especially for the novel combination of clavulanate potentiated penicillins.

In vitro studies have indicated that concentrations of at least 5 ug/ml must be achieved for potentiation of TIC against TIC resistant strains (Paisley and Washington II, 1978) and *in vitro* potentiation of TIC at this concentration of CLAV has been confirmed (Clarke and Zemcov, 1984); reflecting expected *in vivo* levels Fuchs et al (1984) recommended *in vitro* testing of CLAV at 2 ug/ml. Bennet et al (1983) suggest however that CLAV need only be present for a short period of time *in vivo* to exert its effect basing this on pharmacokinetic and clinical experience, it is probable therefore that the lower levels achieved in *vivo* are effective and thus the TIC/CLAV ratio is not important and supplementation of dosing is not required.

9.9.2. Kinetics

i) Clavulanic acid

a) volume of distribution

Bennett et al (1983) found a Vd for normal volunteers 20.3 ± 8.0 l and calculation from the data

of Witkowski et al (1982) gives a Vd of approximately 17.5 ± 5 l and from the data of Adam et al (1982) gives a Vd of 17 l, these compare with the Vd for CLAV reported here although the range of values is wide but is comparable to values reported by Bennett et al(1983).

In one study of CLAV in renal failure, the Vd in 5 patients was found to have a mean Vd of 11.1 ± 1.4 l (Dalet et al 1984b), data from a study on the effect of probenecid on CLAV kinetics (Staniforth et al 1983) was calculated to give a Vd double that found by Dalet et al (1984b), subsequently Dalet et al (1984a) found a Vd of 22.9 l in patients with renal failure on a TIC/CLAV combination, in a similar study an equivalent Vd was found (Boelaert et al 1984), these Vd's are slightly higher than reported here. Although this work reports the kinetics of 31 subjects with renal failure and is the most wide ranging study to date on these subjects these differences may not be significant. Comparability between other reports on normal subjects and the lack of effect of probenecid all suggest that the Vd of CLAV is not affected by renal failure.

b) elimination

The elimination half-life of CLAV in the CrCl >60 ml/min group, when compared with that reported for individuals with renal function, is lower than that reported by other workers for normal subjects, most estimates giving a half-life of about 0.7 hours (Adam

et al, 1982; Witkowski et al, 1982; Schaad, et al, 1983) and indeed some half-lives have been reported as being approximately one hour (Ball et al, 1980). The main difference between this study and the values reported in the literature is the use of a specific liquid chromatography procedure instead of the microbiological assays used by other workers. If a microbiologically active metabolite were present then this would also be measured with an apparent increase in half-life, currently there is little information on CLAV metabolism.

All of the above studies used a one-compartment model for CLAV kinetics, Bennett et al (1983) used a two-compartment model and obtained $t_{\frac{1}{2}}\alpha$ = 0.42 hours and $t_{\frac{1}{2}}\beta$ = 1.5 hours, the observed difference between the slopes α and β did not approach 2 and the use of two-compartment model was not deemed to be justified (section 9.5.2.i.).

Probenecid increased the half-life of CLAV from 1.23 hours to 1.32 hours but this was not statistically significant (Staniforth et al 1983). An increase in $t_{\frac{1}{2}}\beta$ was noted with increasing renal failure by Dalet et al, (1984b) and also by Boelaert et al, (1984) but although an association was noted it was not examined statistically; a report arising from this work (Watson and Boulton-Jones, 1984) summarised the findings in sections 9.6.2.i. and 9.6.3.i., there is statistically significant association between indices

of elimination ($t_{\frac{1}{2}\beta}$, k_{el}, Cl_s) and decreasing renal function for CrCl from <10 ml/min to >60 ml/min and this association holds for indexes of renal elimination (Cl_r and fe), i.e. increasing renal failure leads to decreased elimination of CLAV. These results for clearance etc are similar to those reported by Boelaert et al (1984).

In the anephric group a faster rate of elimination was noted than for the CrCl <10 ml/min group, this has not previously been noted, the probable explanation is that hepatic metabolism is induced, this view was supported by other workers when originally proposed (Watson and Boulton-Jones, 1984) at the 4th Mediterranean Conference on Chemotherapy.

ii) Ticarcillin

a) volume of distribution

As for CLAV it was found that the Vd for TIC was unaffected by the degree of renal dysfunction. As α and β differed by more than two, a two-compartment model was applied, the volumes of distribution of the peripheral and central compartments were not influenced by renal failure, although such Vd's do not relate directly to physiologically valid compartments. In normal volunteers, using two-compartment open models the following Vd's have been reported (Libke et al, 1975), 9.9 ± 2 l (Findlay et al, 1981) and 10.8 ± 4.3 l (Bennett, 1983), the last study being performed on a

TIC/CLAV combination, these values compare with total Vd as reported in this work; there is one report of a Vd of 41.8 l/100Kg (≈30 l) for TIC in normal volunteers (Meyers et al, 1980), this is almost certainly erroneous the AUC reported for this study seem low and it is probable that under estimation of serum concentrations was the cause; a microbiological assay was used.

One report of TIC Vd in renal failure gave a mean Vd of 14.8 l in a study of 61 patients, a one-compartment model was used, there was no apparent relationship between Vd and dose but any association with degree of renal function was not discussed (Hoffler et al, 1978). Mean Vd's for TIC in renal failure have been reported of the same order as those found in normals (Boelaert et al, 1984; Dalet et al, 1984a).

b) elimination

The half-life of elimination of TIC for the group CrCl >60 ml/min of 0.94 ± 0.21 hours compares with half-lives obtained in subjects with normal renal function of 1.1 ± 0.1 hours, 1.2 hours, 1.3 ± 0.16 hours and 0.96 hours (Bennett et al, 1983; Libke et al, 1975; Findlay et al 1981 and Meyers et al, 1980) respectively.

Ticarcillin, as a penicillin, would be expected to accumulate in renal failure due to decreased renal clearance and the increase in elimination half-life with increasing renal failure has been well established

(Davies et al 1974; Parry and Neu, 1976; Hoffler et al, 1978) and probenecid decreases TIC clearance(Libke et al 1975; Davies et al, 1982). In keeping with these findings decreasing clearance was found with increasing renal impairment; the association was statistically significant between CrCl and $t_{\frac{1}{2}\beta}$, k_{el} and Cl_s, the association between CrCl and Cl_r was also highly significant although the relationship between CrCl and fe was equivocal although this is probably an artefact as the TIC kinetics reported by Boelaert et al (1984) are very similar to those reported here and they found a decrease in fe with a decrease in renal function.

Unlike CLAV the anticipated increase in half-life in the anephric group was observed for TIC, the elimination rate being an order of magnitude lower than found in normals.

As noted in Section 8.6.10.equilibration between the TIC epimers is rapid, thus any difference in elimination could not be detected using the storage and assay procedures described here; difference in the clearance of penicillin epimers in renal failure has been noted for temocillin which is structurally similar to TIC, half-lives on normals were R epimer 3.5 hours, S epimer 5.5 hours, the rates reflecting the degree of protein binding, activity varies to some extent with pH (Slocumbe, personal communication).

9.10 ELIMINATION DURING DIALYSIS

Study of TIC/CLAV disposition during dialysis was

hampered by lack of patients and limitations placed on sampling.

The haemodialysis study suffered limitations as to the number of blood samples the clinical staff were willing to allow, thus limiting the amount of pharmacokinetic information attainable. In addition recruitment was slow and the numbers available were further diminished by the loss of two complete sets of samples due to a freezer failure.

Recruitment to the CAPD study was minimal, only two patients agreeing. Exclusion due to peritoneal scarring from previous episodes of infection, and problems of access to facilities undoubtedly reduced numbers ; the principle factor was the reluctance of patients and staff to interrupt sterile dialysis to obtain dialysate samples during dwell time, as this could lead to a significant risk in infection and possible loss of perfusable peritoneal surface for dialysis, in the worst case this could lead to CAPD being ineffective and most patients were unwilling to take the risk. Dropping the requirement for dialysate samples and using an aliquot of exchanged dialysate did not, however, lead to further recruitment. In addition there was reluctance to obtain too many blood samples again limiting the amount of obtainable pharmacokinetic information.

9.10.1. Haemodialysis

The elimination of CLAV was far more rapid than TIC once haemodialysis commenced, respective mean half-lives of elimination 0.55 and 2.23 hours. Elimination during dialysis depends on rate of blood flow and membrane surface area and thus figures for elimination in the literature may not be strictly comparable. TIC elimination half-life has been reported as being 4.7 ± 0.8 hours (Davies et al, 1974), 4.9 hours (Wise et al, 1974), 3.4 ± 0.8 hours (Parry and Neu, 1976) and 2.2 hours (Dalet et al, 1984a). Clinically haemodialysis is expected to be equivalent to a creatinine clearance of around 40 ml/min, this is consistent with the half-life found.

The effect of haemodialysis on CLAV elimination is less well documented. Dalet et al (1984b)in a paper in Spanish described the kinetics of an amoxycillin/CLAV preparation during haemodialysis, Vd was comparable to that found in a group with renal failure and half-life of elimination was 1.15 hours, the same group later reported a half-life of 1.0 hour(Dalet et al 1984a). In a report of a single case Slaughter et al (1984) were unable to determine CLAV half-life during dialysis due to "erratic absorption" of the oral dose, although this was 3 hours post dose.

None of the above papers comments on the redistribution of TIC from the peripheral to central compartments

following cessation of haemodialysis, although this was consistently observed in this study, this feature of TIC disposition has been noted by others (Dalet, personal communication). Slaughter et al (1984) did not note redistribution of CLAV at the end of dialysis, this was noted in one patient in this study, but the rate of elimination of CLAV during haemodialysis and the fact that these subjects are probably anephric and therefore liable to have a greater metabolic clearance will make detection of redistribution less likely.

9.10.2. CAPD

Peritoneal dialysis has been reported as having a moderate effect on TIC elimination (Wise et al, 1974), the extent of removal was not studied although Parry and Neu (1976) claims only a small fraction of the dose (amount unspecified) was recovered with dialysate levels 10-30% of simultaneous serum levels; however only 0.17-0.25 hour dwell times were used and the rate of penetration may have been too slow to allow equilibration in this short time. Certainly ampicillin has been shown to penetrate peritoneal fluid effectively (Wise et al, 1983b) and TIC has been shown to be stable in peritoneal dialysate (Sewell and Golper, 1982).

There is no comparable data on CLAV penetration into peritoneal dialysate although CLAV levels in patients with normal renal function undergoing elective abdominal surgery had peritoneal fluid levels 66% of

serum levels indicating good penetration through the normal peritoneum (Wise et al 1983a).

The recovery of TIC is remarkable as is that of CLAV and interpretation should be cautioned on two counts : the accuracy of the volume of dialysate instilled and the adequacy of the flushing of the line prior to sampling; the stability of CLAV in dialysate fluid is a further variable.

If TIC/CLAV is a useful antibiotic for use in CAPD patients, further studies are required, not only comparing peritoneal penetration following i.v. or dialysate administration, but also examining their penetration through inflamed or scarred peritoneum.

9.11. DOSING REGIMENS IN RENAL FAILURE AND DIALYSIS

The dosing regimens described in section 9.8. are based on the following assumptions : that CLAV will be $\geq$5 ug/ml and that TIC will not accumulate excessively; although patients will be subjected to higher TIC levels than would be the case using the standard regimen in individuals with normal renal function.

A common method of dosage adjustment in renal failure is to halve the frequency of dosing or to halve the dose while maintaining the frequency, The former has been proposed (Dalet et al, 1984b) but will lead to unacceptably long periods without antibiotic cover, the approach recommended in table 9.5. was compared with that of Dalet al (1984a) at the 4th Mediterranean Congress

on Chemotherapy and the schedules proposed in table 9.5 and elsewhere (Watson and Boulton-Jones, 1984) were agreed.

Approaches as to the optimisation of drug dosage has recently been reviewed (Chennavasin and Brater, 1981) who noted that procedures based on nomograms could be useful but that they could under-or over-predict quite markedly but that a combination approach to lowered dose, decreased frequency and 'targeting' of Cp_{ss}MAX, MIN and/or AVE) was preferrable, however the best solution was to measure Cp.

The design of this dosing schedule was complicated by the need to balance the requirements of two drugs with diverging rates of clearance with increasing renal impairment yet to yield a dosing schedule with logical progression. Thus two decrements in frequency of 4 hours each and one halving of the dose led to the recommended maintenance dose for the anephric and CrCl <10 ml/min group; although the anephric group have a higher Cls for CLAV than the CrCl <10 ml/min group the C_{MAX} CLAV is still >6 ug/ml and thus no change is necessary if the assumption of Bennett et al (1983) is correct that CLAV levels >5 ug/ml are only required for short periods of time; CLAV C_{ss}AVE was noticeably lower than for the CrCl <10 ml/min group but should not carry a penalty; the TIC C_{ss}AVE is also lower but is a function of using the mean data

for the anephric group and should not be considered as being statistically different from the CrCl <10ml/min group.

The regimen recommended for haemodialysis is designed on the assumption that clearance following haemodialysis of TIC and CLAV is effectively complete, therefore a loading dose must be given, as the maintenance dose for anephrics is 1.6 g TIC/CLAV per 12 hours then the maintenance dose is double this; low levels of TIC will be found in the serum post-dialysis from dosing prior to dialysis, this factor has been taken into account when calculating the projected levels quoted in the table. It is probable that a similar approach can be adopted for CAPD but further detailed kinetic studies are required.

9.12. CONCLUSION

TIC and CLAV have decreasing clearance with increasing renal impairment but CLAV clearance is increased in anephrics possibly due to induction of metabolism. Haemodialysis and CAPD both clear TIC and CLAV effectively, the former more efficiently than the latter. Allowing for the different kinetics in the groups with renal failure and those undergoing dialysis a pharmacokinetically valid, 'logical' dosing schedule for a TIC and CLAV combination consisting of 3 g of the former and 200 mg of the latter is proposed.

In science, read, by preference, the newest works, in literature the oldest.

Edward Bulmer-Lytton 1803-1873

Caxtoniana Essay X

CHAPTER 10

GENERAL DISCUSSION

10.1. SPECIFIC CHEMICAL METHODS

This thesis has been concerned with the measurement of drugs having antimicrobial activity. In the past these drugs have been measured using biological assays, which have been criticised for many years as being both imprecise and inaccurate (Boxer et al 1948; Reeves and Wise, 1978). The errors in such assays may be further compounded in the case of agents with active metabolites and when drugs are used in combination. Until recently most alternative assays were photometric (eg. Rieder, 1972) and could be liable to inaccuracy and imprecision.

To date most pharmacokinetic studies on antimicrobials have been performed using such assay procedures. Departments of Microbiology are infrequently equipped with gas or liquid chromatographs, which, with suitable sample preparation can yield accurate and precise quantitative results, allowing the determination of definitive pharmacokinetics and possibly leading to a more critical interpretation of the relationship between plasma concentration and clinical effect.

Due to the inadequacies of analytical technique and, in some cases, poor study design, it was felt that a re-examination of the pharmacokinetics of antimicrobial agents using newly developed accurate, precise and sensitive liquid chromatographic assays would be worthwhile. As a start the performance of

the colorimetric procedure of Rieder (1972) was evaluated and found to be adequate, not all antimicrobial agents lend themselves to photometric assay, liquid chromatography is a good alternative to microbiological assay.

Comparison of the kinetics obtained for TMP and the sulphonamides showed equivalence with some studies and marked differences with the results from others; it is probable, but not absolutely certain, that these differences are due to the use of inadequate methodology. The methods used for studying trimethoprim and the sulphonamides were usually fluorimetric and colorimetric respectively.

To date all values for the kinetics of ticarcillin and clavulanic acid in the literature have been produced using microbiological assays. In one publication the Vd for clavulanic acid is underestimated and in one the Vd for ticarcillin was overestimated. It may be that these 'outliers' reflect some systematic error in the assays used. The Vd found in this study for clavulanic acid is slightly lower than reported by others in the literature and it may be that this reflects greater specificity in the determination of clavulanic acid by LC, however the values of Vd obtained for ticarcillin are comparable with other reports. The addition of an extraction stage can improve the accuracy of chemical assays such as that of Rieder without a notable loss in precision.

There are several major advantages in the use chromatographic assays. Of prime importance is the

separation of parent drug from metabolites and other sources of interference. The disadvantage however is the sequential nature of LC assay and the time taken to complete the large number of analyses required for pharmacokinetic studies when compared to the batch analysis approach usually used in bacteriological assays which have a higher throughput. The rate of analysis could be improved for LC by using 3μ packing and high flow rates, which could notably reduce chromatography time. Sample preparation procedures would then become rate-limiting; automation of this step is possible and use of the Du Pont PREP system was attempted for CLAV and TIC but it was not flexible enough to deal with the CLAV sample preparation.

If the sensitivity and selectivity of chromatography is required to be retained with the advantages of batch analysis an alternative approach to LC is high performance thin-layer chromatography with scanning densitometry. This approach has not yet been developed widely. A useful source of reference on the subject is available (Zlatkis and Kaiser, 1977).

This present study has demonstrated the applicability of LC to the analysis of several different chemical species of antimicrobial agent. During the course of this work an increasing number of publications on assays of antimicrobials in particular and other

drugs in general using LC indicate that this is a trend which will continue.

10.2. PHARMACOKINETICS

10.2.1. General

Along with increasing appreciation of the importance of analytical factors in determining the disposition of antimicrobials there has been an increased awareness of the necessity for the application of appropriate pharmacokinetic analysis. Whilst the use of one-compartment open models with first-order kinetics is sufficient for most purposes, the need for more accurate models has become clear. As effective chemotherapy of either of infection or neoplasia is dependent on drug penetration to the site of action, it may be that consideration of the kinetics of such agents needs to go beyond simple descriptive kinetics. The rate of penetration into various compartments is characterised by rate constants and the compartments have nominal volumes which do not relate to any physiologically measurable compartments. Some physiological variables may be considered as appropriate to certain compartments, an approach which has been used in identifying the kinetic factors which predispose to toxicity during chemotherapy with methotrexate (Reich et al, 1977).

If the rate of penetration of an agent to a desired site of action can be described then, from a

model, it should be possible to design an optimal dosing regimen that would enable the desired effect to be achieved while minimising toxicity. It has been proposed that pharmacodynamics be modelled in conjunction with pharmacokinetics (Sheiner et al, 1979b; Whiting and Kelman, 1980). The models considered hitherto have been for drugs which have a directly measurable pharmacological effect eg. the force of thumb adduction following d-tubocurarine (Sheiner et al, 1979b); QT prolongation following disopyramide and left ventricular ejection time following digoxin (Kelman and Whiting, 1980).

10.2.2. Fixed Dose Combinations of Antimicrobial Agents

From the results of the kinetics of TMP/sulphonamide combinations it is clear that the optimum synergistic concentrations determined from *in vitro* studies were not attained *in vivo*, but this fact was masked by the overall efficacy of the preparation, whereas the TIC/CLAV kinetics suggest that the required levels can be attained *in vivo*. The significance of the findings awaits clarification.

What is quite clear is that although SMZ was chosen to match TMP on the basis of kinetics, the kinetic data used in making the decision were inadequate. As the comparison of *in vivo* Cp ratio with the desired *in vitro* concentration ratios shows, no account of

penetration from the central compartment was considered. Allowing for such a limitation, then TMP and SMZ did show roughly parallel elimination kinetics, however TIC and CLAV did not, especially in patients with renal failure. The underlying principle of action is however different and it is probable that TIC/CLAV ratio is of little importance, even in vitro. There may be lessons here for antineoplastic chemotherapy when, as seems inevitable, new combinations of drugs are designed to be pharmacologically and kinetically compatible. It is possible that the drug delivery systems that are now being investigated for anti-neoplastic agents might be able to deliver such agents in combination and achieve the the optimum target concentration ratios. Such delivery systems might also be used for delivery of combinations of antimicrobial agents.

Even allowing the necessity for fixed combinations the continuation of potentiation may be difficult to sustain for agents that rely for their action on reversible competitive inhibition, in such circumstances, unless there is parallel kinetic behaviour, particularly penetration and elimination, then supplementation of one or other of the components may be necessary prior to the next combined dose.

10.2.3. Prediction of Cp and Dosing

There is an underlying assumption in kinetics that Cp proportionally reflects the concentration at

the site of action allowing for temporal effects, thus using indirect measurement of Cp eg. saliva,or by measurement of a few Cp, it should be possible to predict either Cp at the time of observation or future Cp respectively.

There is a temporal relationship between saliva and serum concentrations; however predictions using the equation of Matin et al (1974) do not describe the relationship adequately for a drug, such as TMP, that is significantly ionised at physiological serum and saliva pH. The limitations of the equation are its reliance on time-independent factors to describe the partition relationship, no time-related factor such as salivary flow-rate or penetration lag time being included, although there is parallelism with serum and saliva drug concentrations. Thus while saliva may be used to indirectly measure total TMP plasma concentration the same information cannot be obtained using the Matin equation despite the extra data required, nor can it be relied upon to give reasonable estimates of the free fraction.

Bayesian prediction has proved satisfactory provided that sufficient observations (three or more) are obtained. If the intent is to forecast Cp and hence dosage the technique is useful with good precision and bias and predicts well for individuals. It must be understood that the parameters produced, although

'individualised' are not true parameters and must not be construed as such,however they are useful for the simulation of alternative dosage regimens and calculations for predicting Cp from such regimens. As noted in Chapter 7 use of OPT resulted in the 'education' of a G.P.in therapeutic monitoring but had limitations on its forecasting due to non-compliance.

Consideration of compliance and informed clinical judgement with Cp feedback would suggest that the need for OPT becomes less apparent from a service aspect and thus commitment to the cost or the upgrading of the required hardware, if not already available, may be difficult to justify,but experience over three years with OPT routinely available has shown it to be invaluable on occasions. Therefore if Cp forecasting is required then Bayesian forecasting is undoubtedly the procedure of choice, as has already been noted (Burton et al 1985).

Prediction of Cp allows prediction of dosage an alternative aspect to this was the prediction of dosing for patients with different degrees of renal failure who had received the TIC/CLAV combination. The recommended dosing regimens were dependent on the degree of renal failure and on the use of dialysis. The prime requisite for any such dosing regimen is that it progress logically and be relatively uncomplicated; this was achieved. Within each sub-set

individualised prediction would have suggested an alternative dose, but it is doubtful whether this would have differed significantly from that which is practicable due to dosage formulation.

If adequate effect and minimal toxicity can be attained with a 'standard' dose then this should be used, only if this is not the case is 'individualisation' required.

10.2.4. Antimicrobial and Antineoplastic Chemotherapy

In vivo cell death is a measure of chemotherapeutic efficacy, however the modes of action employed to achieve this are many and varied. There are similarities between the intent and action of antimicrobial and antineoplastic chemotherapeutic agents but there are also fundamental differences; cell wall disruption, such as occurs with penicillins is not appropriate for antineoplastic drugs, their chief line of attack is aimed at factors governing cell division eg. mitotic microtubule inhibition by vinca alkaloids. The differences between malignant eukaryotic cells and host cells are much less than between host and bacterial cells, thus antineoplastics will tend to have a low therapeutic index, while this may be true for some antimicrobials eg. the aminoglycosides, this is more an exception.

The target cells and therapeutic strategies

are more easily manipulated and comprehended in antimicrobial chemotherapy, since for antineoplastic chemotherapy there is a comparative lack of flexibility imposed by the nature of the disease process, however it is a reasonable concept that consideration of antimicrobial chemotherapy may allow insight into the potential for developments in antineoplastic chemotherapy.

In both types of therapy failure may occur due to the emergence of a resistant cell line, however it is at this point that the differences in the maturity of the respective therapeutic strategies are revealed, in vitro microbiological testing determines to what agents the resistant cell line is susceptible and therapy is then instituted. There is no parallel in oncology; further microbiological regimens may be so designed as to 'cover' the emergence of resistance eg. fixed combination therapy, again there is no parallel in oncology. The reason for these differences is that therapy in infection is, if required, staged i.e. an initial best guess therapy which if not succesful is examined in vitro, therapy is amended accordingly and the system re-examined and amended again as necessary, in other words the chemotherapy of infection is individualised. In contrast due to the lack of adequate effective in vitro methods such an approach has not been adopted in antineoplastic therapy. The agents are always used in combination to a set regimen of cycles

and certain specified regimens are applied to specified neoplasms.

If a resistant line emerges as is often the case, or the degree of cellular differentiation is such as to be associated with poor response any flexibility in the chemotherapeutic regimen is often empirically imposed although other clinical and histopathological evidence may be considered. Anti cancer drugs are used in combinations and times that are intended to attack the malignant cells at different stages of the cell cycle so histological and biochemical evidence is therefore of limited value. There is therefore a need for individualisation of antineoplastic chemotherapy, if only to attack the problem of resistance.

Relapse following antineoplastic chemotherapy due to tumour regrowth requires the identification of the causative factors, and may be related to the degree of cell differentiation, the ability of the cell to bypass the site of drug action and the achievement and maintenance of adequate levels at the neoplastic site. The degree of penetration will depend on the rate of drug delivery and thus exceptional routes of administration eg. intrathecal may be utilised; if the tumour is poorly vascularised or necrotic drug penetration will be slow and possibly inadequate and such difficulties require individualisation of therapy not only kinetically but in terms of the drugs employed.

If individualised *in vitro* testing could indicate the patient's requirements and knowledge of the required drug concentrations at the site of action were known, then measurement of drug levels at the site and also in plasma could be of value in assessing the adequacy of therapy.

Current investigations on the penetration of chemotherapeutic agents tend towards measuring achieved drug concentrations in various sites eg. sputum, CSF, peritoneal fluid, lung tissue etc. such point estimates are virtually meaningless and require systematic study of their kinetics of penetration, this information is gradually becoming available but the ethical limitations inherent in such studies inhibits investigation. The modelling and investigation of penetration of chemotherapeutic agents to their site of action requires more work.

If this knowledge could be obtained then consideration of fixed dose combination antineoplastic therapy might become a viable proposition, eg. the use of nitroimidazole to 'sensitise' hypoxic cells to a second agent eg. methotrexate. If this is a synergistic effect then there is a need for parallel kinetic behaviour c/f the arguments of TMP/sulphonamide ratios. If the potentiation is due to independent effects then the situation with clavulanic acid and the penicillins is the closer analogy.

10.3. FUTURE PERSPECTIVES

All of the above discussion has centred on the attainment and maintenance of Cp or tissue concentrations which are adequate for effective action. For routine purposes this may be appropriate, however in chemotherapy the intention is to kill cells, to do this requires that the agent penetrate the cells and disrupt some vital metabolic function thereby rendering them non-viable, susceptible to the host's immune defence system or at least unable to generate resistance to chemotherapy. Such resistance can arise in a number of ways,but lack of cell-wall penetration and intra-cellular metabolic adjustment are significant problems, these problems can be identified in vitro, through tissue culture but rely more on growth kinetics than direct examination. In bacteria resistance may be acquired by plasmid transfer,this mechanism does not apply to cancer cells, but a sub-population of cells may be inherently resistant to a chemotherapeutic agent. It may prove possible by examination of a cell population to determine how cell resistance is most likely to be made manifest, thus enabling therapy to be designed to avoid resurgence. As antimicrobial chemotherapy is usually more succesful than antineoplastic chemotherapy it may prove to be that the latter would benefit most from a knowledge of the intra-cellular milieu, i.e. direct examination.

With current technology direct examination of

cellular contents is not feasible. However developments in LC technology suggest this may not be impossible; it will be feasible in the foreseeable future to design detectors that can detect 10^6 molecules (Poppe, 1984), such a number of molecules is equivalent to femtogram amounts, these are detectable with todays technology using open tubular LC with laser fluorescence detection (Novotny, 1984). Although examination of a single cell is not yet possible it has been suggested as a realistic prospect for the future (Jorgensen et al, 1984), this may not be achievable, however monoclonal techniques make it possible to consider cloning a single cell line to examine its products.

Combining capillary LC and human cell culture methods for antineoplastic agents or bacteriological cell culture methods for antimicrobial agents could prove to be a highly informative progression.

It is possible to envisage that research into chemotherapy involving a combination of more physiologically descriptive pharmacokinetic models, (using accurate, precise assays), limited point estimates of drug concentrations in tissue and other sites, Bayesian predictive forecasting and highly efficient capillary LC investigations into intra-cellular chemotherapeutic action, will result in significant advances in the design of chemotherapeutic regimens.

APPENDIX A

TABLE A.1

Sex and weight of subjects

	Subject	Sex	Weight (kg)
1	A.J.	M	63
2	A.B.	F	62
3	R.McD.	M	58
4	M.B.	F	66
5	J.McK.	M	73
6	C.R.	F	65
7	R.C.	F	60
8	G.T.	M	80
9	S.B.	F	64
10	G.B.	M	71

TABLE A.2

Time to steady state (hrs) for SDMO, SMZ and TMP following co-trifamole or co-trimoxazole therapy

SUBJECT	SMZ	SDMO	TMP	
			co-trimoxazole	co-trifamole
1	72	24	72	24
2	72	24	24	48
3	48	48	48	48
4	72	24	48	24
5	72	24	72	24
6	48	24	24	24
7	48	24	24	48
8	48	24	48	24
9	24	24	24	24
10	48	24	48	48
median	48	24	48	24
range	24-72	24-48	24-72	24-48

TABLE A.3

Individual areas under the serum concentration time curve for TMP, SDMO and SMZ following co-trifamole or co-trimoxazole therapy (n=10) (AUC in mg h l^{-1})

SUBJECT	SMZ	SDMO	TMP	
			co-trimoxazole	co-trifamole
1	1377	1204	64.2	35.8
2	907	997	29.8	34.4
3	877	1146	57.3	31.3
4	804	1453	30.9	33.6
5	811	477	24.5	25.1
6	905	997	32.1	32.6
7	917	1193	57.7	35.4
8	649	526	21.8	24.1
9	590	634	23.8	37.9
10	902	1677	28.6	28.0

TABLE A.4

Observed peak serum concentration (mg/l) for SDMO, SMZ and TMP following co-trifamole or co-trimoxazole initial dose

SUBJECT	SMZ	SDMO	TMP	
			co-trimoxazole	co-trifamole
1	69	74	1.7	1.7
2	74	105	2.1	2.2
3	76	82	1.8	1.7
4	56	96	2.0	2.0
5	61	58	1.4	1.3
6	69	132	2.0	1.6
7	70	96	1.9	1.9
8	58	65	2.3	1.4
9	91	89	1.8	2.3
10	57	80	1.5	1.3

TABLE A.5

Time to peak serum concentration (hours) for SDMO, SMZ and TMP following co-trifamole or co-trimoxazole

SUBJECT	SMZ	SDMO	TMP	
			co-trimoxazole	co-trifamole
1	3	2	2	3
2	2	1	2	½
3	2	4	2	3
4	4	2	4	1
5	2	1	1	1
6	3	2	2	2
7	2	2	2	3
8	2	3	1	2
9	2	1	½	1
10	2	2	2	2

TABLE A.6

Apparent volume of distribution for SDMO, SMZ and TMP following co-trifamole or co-trimoxazole (Vd (l))

SUBJECT	SMZ	SDMO	TMP	
			co-trimoxazole	co-trifamole
1	11.6	10.4	65.4	72.6
2	10.4	7.4	59.8	59.7
3	9.9	9.0	64.4	78.5
4	13.5	7.9	56.4	64.6
5	13.8	14.2	109.0	106.5
6	10.5	7.7	63.3	80.1
7	11.1	8.2	65.6	63.5
8	14.0	10.8	71.9	92.1
9	9.2	9.0	71.1	61.8
10	16.2	9.2	86.3	98.8

TABLE A.8

Urinary SDMO concentrations (mg/l)

TIME (HRS) / SUBJECT	0-12	12-24	24-48	48-72	72-96	96-108
1	146	169	144	93	122	-
2	205	186	124	194	255	106
3	103	280	276	211	255	206
4	153	179	219	266	135	211
5	100	61	53	62	57	60
6	203	171	194	229	189	178
7	118	140	115	188	152	124
8	231	86	48	85	50	51
9	177	135	91	53	69	93
10	115	98	77	65	77	78

TABLE A.9

Urinary SMZ levels (mg/l) following co-trimoxazole therapy

TIME (HRS) / SUBJECT	0-12	12-24	24-48	48-72	72-96	96-108
1	147	251	169	244	330	168
2	129	219	176	177	118	119
3	252	357	361	292	396	349
4	268	135	265	215	96	-
5	51	78	109	90	85	90
6	390	245	115	179	247	211
7	-	103	180	95	207	153
8	201	92	136	89	148	133
9	108	144	187	290	264	326
10	62	228	178	80	166	258

TABLE A.10

Urinary TMP levels (mg/l) following co-trifamole therapy

TIME (HRS) / SUBJECT	0-12	12-24	24-48	48-72	72-96	96-108
1	78	113	84	58	75	-
2	67	110	72	64	65	34
3	41	118	89	72	85	84
4	54	78	114	71	52	52
5	36	81	72	85	75	74
6	72	84	91	93	95	108
7	82	99	63	86	46	42
8	104	165	73	94	54	57
9	70	107	73	51	89	79
10	59	67	65	65	60	63

TABLE A.11

Urinary TMP levels (mg/l) following co-trimoxazole therapy

TIME (HRS) / SUBJECT	0-12	12-24	24-48	48-72	72-96	96-108
1	80	101	128	125	175	129
2	83	165	99	118	121	103
3	90	173	122	148	105	100
4	101	225	215	222	167	-
5	30	200	140	134	119	88
6	114	143	181	227	186	174
7	44	174	118	143	107	112
8	62	231	113	136	98	98
9	80	105	99	197	127	107
10	41	103	126	98	148	100

TABLE A.7

Elimination half-life, elimination rate constant and serum clearance for SDMO, SMZ and TMP following co-trifamole or co-trimoxazole ($t_{\frac{1}{2}}$(h), k_{el}(h^{-1}), Cl(lh^{-1}))

	SMZ			SDMO			TMP					
							co-trimoxazole			co-trifamole		
SUBJECT	$t_{\frac{1}{2}}$	k_{el}	Cl	$t_{\frac{1}{2}}$	k_{el}	Cl	$t_{\frac{1}{2}}$	k_{el}	Cl	$t_{\frac{1}{2}}$	k_{el}	Cl
1	13.9	0.050	0.6	11.0	0.063	0.7	18.2	0.038	2.5	11.3	0.062	4.5
2	8.2	0.084	0.9	6.4	0.108	0.8	7.7	0.090	5.4	8.9	0.078	4.7
3	7.6	0.092	0.9	8.9	0.078	0.7	16.0	0.043	2.8	10.6	0.065	5.1
4	9.4	0.074	1.0	10.0	0.069	0.6	7.5	0.092	5.2	9.4	0.074	4.8
5	9.7	0.072	1.0	5.9	0.118	1.7	11.6	0.060	6.5	11.6	0.060	6.4
6	8.2	0.084	0.9	6.6	0.104	0.8	8.8	0.079	5.0	11.3	0.061	4.9
7	8.9	0.078	0.9	8.4	0.082	0.7	15.7	0.042	2.8	9.8	0.071	4.5
8	7.9	0.088	1.2	4.9	0.141	1.5	6.8	0.102	7.3	9.6	0.072	6.7
9	4.7	0.148	1.4	5.0	0.140	1.3	7.3	0.095	6.7	10.2	0.068	4.2
10	12.6	0.055	0.9	13.2	0.052	0.5	10.7	0.065	5.6	12.0	0.058	5.7

APPENDIX B

TABLE B.1.

Demographic data for subjects

Subject	Sex	Age	Weight (kg)
1	M	33	79.5
2	M	29	55.5
3	M	29	110.0
4	M	30	101.0
5	M	22	56.5
6	F	22	63.5
7	F	53	56.0
8	F	26	55.0

TABLE B.2.

Maximum concentration (C_{MAX}) and time to maximum concentration (T_{MAX}) for the 200 mg b.d. and 300 mg o.d. preparations following the initial dose.

PARAMETER / SUBJECT	C_{MAX}		T_{MAX}	
	200 b.d.	300 o.d.	200 b.d.	300 o.d.
1	2.0	2.9	1.0	0.5
2	2.4	3.4	1.0	1.0
3	1.7	2.6	4.0	1.0
4	1.7	2.3	2.0	4.0
5	2.5	2.8	1.0	4.0
6	2.3	3.4	1.0	1.0
7	2.5	3.5	1.0	1.0
8	2.8	4.6	2.0	1.0

TABLE B.3

Individual area under the serum concentration-time curve for each preparation following the initial dose ($mg.h.l^{-1}$)

REGIMEN / SUBJECT	200 mg	300 mg
1	39.8	57.9
2	37.8	83.2
3	28.9	35.9
4	32.3	32.4
5	65.1	91.3
6	51.6	67.0
7	34.8	67.9
8	30.1	62.1
PREDICTED	56.5	86.5

TABLE B.4.

Volumes of distribution for each subject for 200 mg and 300 mg doses following the initial dose (l)

SUBJECT \ DOSAGE	200 mg	300 mg
1	86.2	82.6
2	69.2	60.3
3	91.1	110.0
4	99.7	81.4
5	64.0	72.9
6	67.8	67.2
7	62.8	64.1
8	62.5	60.9
mean	75.4	74.9
sd	14.7	16.6

Vd calculated from: co-trifamole 77.8±16.6 l

co-trimoxazole 71.3±15.6 l

TABLE B.5

Pharmacokinetic elimination parameters for each subject following the initial dose of either 200 mg or 300 mg

PARAMETER / SUBJECT	$t_{\frac{1}{2}\beta}$(h)		k_{el}(h^{-1})		Cl_s(lh^{-1})	
	200	300	200	300	200	300
1	11.9	11.0	0.058	0.063	5.0	5.2
2	9.1	11.6	0.077	0.060	5.3	3.6
3	9.1	9.1	0.076	0.076	6.9	8.4
4	11.2	6.1	0.062	0.114	6.2	9.3
5	14.4	15.4	0.048	0.045	3.1	3.3
6	12.1	10.4	0.057	0.067	3.9	4.5
7	7.6	10.0	0.092	0.069	5.8	4.4
8	6.5	8.7	0.107	0.079	6.7	4.8
mean	10.2	10.3	0.072	0.072	5.4	5.4
sd	2.6	2.7	0.020	0.020	1.3	2.2

values for:

co-trifamole	10.5±1.0	0.067±0.007	5.1±0.8
co-trimoxazole	11.0±4.2	0.071±0.024	5.0±1.7

TABLE B.6

Normalised values for apparent volume of distribution ($l.kg^{-1}$) for each subject following both dose regimens

SUBJECT \ REGIMEN	200 mg	300 mg
1	1.08	1.04
2	1.25	1.09
3	0.83	1.00
4	0.99	0.81
5	1.13	1.29
6	1.07	1.06
7	1.12	1.14
8	1.14	1.11
mean	1.08	1.07
sd	0.12	0.14

TABLE B.7

Time to steady state (T_{ss}), and mean C_{ss} peak and C_{ss} trough for each individual on 200 mg b.d. and 300 mg o.d.

PARAMETER / SUBJECT	T_{ss} (h)		C_{ss} peak (mg/l)		C_{ss} trough (mg/l)	
	200 b.d.	300 o.d.	200 b.d.	300 o.d.	200 b.d.	300 o.d.
1	48	48	4.3	3.7	2.5	1.4
2	48	48	5.1	4.7	3.1	1.3
3	24	24	2.5	2.5	1.1	0.5
4	24	48	2.9	3.2	1.7	1.3
5	48	48	3.9	3.9	1.9	1.3
6	72	48	3.8	3.8	2.4	0.9
7	48	48	4.5	4.8	2.6	1.1
8	48	25	4.3	4.2	2.2	1.3
mean	-	-	3.9	3.8	2.2	1.1
sd	-	-	0.9	0.8	0.6	0.3
median	48	48				
range	24-72	24-48				

APPENDIX C

TABLE C.1

Saliva and serum TMP concentrations during the 200 mg b.d. regimen.

Time (hrs)	2		10		72		74	
Subject	serum	saliva	serum	saliva	serum	saliva	serum	saliva
1	1.8	1.5	1.2	0.9	2.4	2.7	4.6	4.2
2	2.3	2.0	1.4	1.3	3.1	2.6	4.9	3.3
3	1.4	0.9	0.9	0.5	1.4	1.2	2.3	1.7
4	1.7	1.3	1.0	0.9	1.6	1.2	2.8	2.3
5	2.3	2.2	1.7	1.3	1.9	2.5	4.4	3.9
6	2.2	1.8	1.5	0.7	2.6	1.7	3.9	3.2
7	2.1	3.4	1.1	0.8	2.6	3.2	4.4	4.6
8	2.8	1.7	1.0	0.9	2.3	1.9	4.2	3.6

TABLE C.2

Saliva and serum TMP concentration during the 300 mg 6.d. regimen

Time (hrs)	2		10		72		74	
Subject	serum	saliva	serum	saliva	serum	saliva	serum	saliva
1	2.6	2.9	1.6	1.8	1.3	1.3	3.7	3.9
2	3.2	2.7	1.9	1.1	1.2	1.0	4.3	3.2
3	1.9	1.4	1.3	1.2	0.1	0.4	2.3	1.8
4	2.2	1.5	1.3	0.5	0.9	0.9	3.2	2.8
5	1.0	3.7	2.2	2.0	1.3	1.3	3.8	3.6
6	3.3	3.0	2.1	1.4	1.0	0.9	3.8	3.1
7	3.3	3.1	2.1	1.7	1.1	1.6	4.8	4.6
8	3.0	2.5	1.9	1.5	1.0	1.3	4.3	3.3

TABLE C.3

Observed S/P ratios for both regimens

Time (hrs)	2		10		72		74	
Subject	200	300	200	300	200	300	200	300
1	0.87	1.08	0.75	1.17	1.12	1.01	0.91	1.07
2	0.86	0.86	0.89	0.6	0.84	0.87	0.67	0.75
3	0.65	0.74	0.58	0.92	0.82	3.58	0.75	0.76
4	0.81	0.69	0.94	0.38	0.73	1.0	0.8	0.87
5	0.98	3.69	0.75	0.92	1.28	1.02	0.89	0.94
6	0.85	0.92	0.44	0.65	0.65	0.85	0.81	0.81
7	1.59	0.94	0.73	0.82	1.22	1.47	1.04	0.96
8	0.62	0.81	0.89	0.82	0.83	1.28	0.86	0.76

TABLE C.4

Mean individual salivary flow rate (ml/min) over both legs of study

	200		300	
Subject	$\bar{x}$	sd	$\bar{x}$	sd
1	1.31	0.21	1.28	0.16
2	0.93	0.19	1.07	0.28
3	1.84	0.41	2.21	0.19
4	1.20	0.26	1.04	0.28
5	1.72	0.21	1.38	0.21
6	1.66	0.31	1.74	0.25
7	1.11	0.15	0.97	0.28
8	1.48	0.17	1.22	0.08

TABLE C.5

Mean individual salivary hydrogen ion concentration (nmol/l) over both legs of the study

	200		300	
Subject	$\bar{x}$	sd	$\bar{x}$	sd
1	30.4	24.8	31.5	14.0
2	34.8	27.4	24.4	21.9
3	26.0	8.2	22.0	10.8
4	24.8	10.7	22.8	12.4
5	29.2	14.1	45.2	26.6
6	58.4	21.0	48.0	22.9
7	37.6	20.8	26.2	15.1
8	19.6	9.2	19.2	13.4

APPENDIX D

TABLE D.1

Mean squared prediction error (precision) of predicted Cp at 96 and 98 hours post commemcement of therapy for both 300 mg TMP o.d. and 200 mg TMP b.d.
mse ± 95% confidence interval

No Cp	300 mg o.d. 96 hours	300 mg o.d. 98 hours	200 mg b.d. 96 hours	200 mg b.d. 98 hours
0	0.543 ± 0.534*	0.470 ± 0.369*	0.588 ± 0.580*	0.321 ± 0.392
1	0.675 ± 0.690	0.146 ± 0.182	0.972 ± 0.872*	1.385 ± 1.367*
2	0.742 ± 1.279	0.171 ± 0.318	0.490 ± 0.440*	0.929 ± 0.929*
3	0.485 ± 0.841	0.253 ± 0.205*	0.370 ± 0.367*	0.376 ± 0.585
4	0.144 ± 0.242	0.236 ± 0.085*	0.248 ± 0.367	0.398 ± 0.582
N.S.	0.278 ± 0.278	0.391 ± 0.630	0.358 ± 0.341	0.580 ± 0.634
Best mse	4 Cp	1, 2 Cp	4 Cp	0, 3, 4 Cp
Worst mse	0, 1, 2 Cp	0 Cp	1 Cp	1 Cp

* significant ($\alpha = 0.05$)

NS Naive Standard

TABLE D.2.

mean prediction error (bias) of predicted Cp at 96 and 98 hours post commencement of therapy for both 300 mg TMP o.d. and 200 mg TMP b.d.
me ± 95% confidence interval

No. Cp	300 mg o.d. 96 hours	300 mg o.d. 98 hours	200 mg b.d. 96 hours	200 mg b.d. 98 hours
0	0.456 ± 0.622	-0.628 ± 0.284*	0.120 ± 0.875	-0.104 ± 0.599
1	0.599 ± 0.647	0.049 ± 0.380	0.408 ± 0.963	0.733 ± 0.997
2	0.653 ± 0.605	0.144 ± 0.389	0.470 ± 0.557	0.833 ± 0.523*
3	0.251 ± 0.699	-0.243 ± 0.443	0.090 ± 0.671	0.250 ± 0.599
4	0.238 ± 0.304	-0.249 ± 0.420	0.060 ± 0.531	0.290 ± 0.602
N.S.	0 ± 0.567	0 ± 0.640	0 ± 0.642	0 ± 0.818
best mse	3, 4 Cp	1, 2 Cp	3, 4 Cp	0 Cp
worst mse	1, 2 Cp	0 Cp Excluding subject 5	1, 2 Cp	2 Cp

* significant ($\alpha = 0.05$)

NS Naive Standard

TABLE D.3.

Relative mean squared prediction error of predicted Cp of 96 hours post commencement of therapy for 300 mg TMP o.d. (x v y)
Δmse ± 95% confidence interval

No Cp y \ x	0	1	2	3	4
1	-0.132 ± 0.261	-	-	-	-
2	-0.199 ± 1.046	-0.067 ± 1.071	-	-	-
3	0.058 ± 0.551	0.190 ± 0.625	0.257 ± 0.531	-	-
4	0.399 ± 0.338*	0.531 ± 0.480*	0.598 ± 0.645	0.341 ± 0.332*	-
N.S.	0.265 ± 0.440	0.397 ± 0.625	0.464 ± 1.054	0.207 ± 0.659	-0.134 ± 0.341

* significant ($\alpha = 0.05$)

NS naive standard

TABLE D.4.

Relative mean squared prediction error of predicted Cp at 98 hours post commencement of therapy for 300 mg TMP o.d. (x v y)
Δmse ± 95% confidence interval

No Cp	x	0	1	2	3	4
y						
1		0.324 ± 0.250*	-	-	-	-
2		0.299 ± 0.599	-0.025 ± 0.412	-	-	-
3		0.217 ± 0.403	-0.107 ± 0.318	-0.082 ± 0.267	-	-
4		0.234 ± 0.472	-0.090 ± 0.315	-0.065 ± 0.151	-0.017 ± 0.176	-
N.S.		0.079 ± -.379	-0.245 ± 0.474	-0.220 ± 0.776	-0.138 ± 0.733	-0.155 ± 0.651

* significant ($\alpha = 0.05$)

Subject 5 excluded

NS naive standard

TABLE D.5.

Relative mean square prediction error of predicted Cp at 96 hours post commencement of therapy for 200 mg TMP b.d. (x v y)
Δmse ± 95% confidence interval

No Cp	x	0	1	2	3	4
y						
1		-0.382 ± 1.026	-	-	-	-
2		0.098 ± 0.784	0.480 ± 0.898	-	-	-
3		0.218 ± 0.676	0.600 ± 0.821	0.120 ± 0.636	-	-
4		0.340 ± 0.659	0.722 ± 0.972	0.242 ± 0.636	0.122 ± 0.361	
N.S.		0.230 ± 0.324	0.612 ± 0.867	0.132 ± 0.625	0.012 ± 0.492	-0.110 ± 0.355

* significant ($\alpha = 0.05$)

NS naive standard

TABLE D.6.

Relative mean square prediction error of predicted Cp at 98 hours post commencement of therapy for 200 mg TMP b.d. (x v y)
Δmse ± 95% confidence interval

No Cp Y	X	0	1	2	3	4
1		-1.064 ± 1.514	-	-	-	-
2		-0.608 ± 1.165	0.456 ± 1.071	-	-	-
3		-0.055 ± 0.279	1.009 ± 0.935*	0.553 ± 0.548*		
4		-0.077 ± 0.257	0.987 ± 1.460	0.531 ± 0.864	-0.022 ± 0.617	-
N.S.		-0.259 ± 0.287	0.805 ± 1.506	0.349 ± 1.341	-0.204 ± 0.980	-0.182 ± 0.857

* significant ($\alpha = 0.05$)

NS naive standard

TABLE D.7.

Relative mean prediction error of predicted Cp at 96 hours post commencement of therapy for 300 mg TMP o.d. (x v y)
Δme ± 95% confidence interval

No Cp / Y	X	0	1	2	3	4
1	-0.103 ± 0.131	-	-	-	-	
2	-0.197 ± 0.494	-0.094 ± 0.509	-	-	-	
3	0.205 ± 0.506	0.308 ± 0.554	0.402 ± 0.361*	-	-	
4	0.218 ± 0.591	0.321 ± 0.617	0.415 ± 0.330*	0.013 ± 0.349	-	
N.S.	0.456 ± 0.642	0.559 ± 0.318*	0.653 ± 0.233*	0.251 ± 0.332	0.238 ± 0.395	

* significant ($\alpha = 0.05$)

NS naive standard

TABLE D:8.

Relative mean prediction error of predicted Cp at 98 hours post commencement of therapy for 300 mg TMP o.d. (x v y)
Δm e ± 95% confidence interval

No Cp	x 0	1	2	3	4
y					
1	-0.677 ± 0.324*	-	-	-	-
2	-0.722 ± 0.267*	-0.095 ± 0.440	-	-	-
3	-0.385 ± 0.341*	0.292 ± 0.597	0.387 ± 0.244*	-	-
4	-0.379 ± 0.412*	0.298 ± 0.639	0.393 ± 0.222*	-0.060 ± 0.202	-
N.S.	-0.628 ± 0.571*	0.049 ± 0.332	-0.247 ± 0.923	-0.243 ± 0.850	0.249 ± 0.923

* significant ($\alpha = 0.05$)

NS naive standard

TABLE D.9.

Relative mean prediction error of predicted Cp at 96 hours post commencement of therapy for 200 mg TMP b.d. (x v y)
Δme ± 95% confidence interval

No Cp x / y	0	1	2	3	4
1	-0.288 ± 0.199*	-	-	-	-
2	-0.350 ± 0.599	-0.062 ± 0.655	-	-	-
3	0.030 ± 0.850	0.318 ± 0.801	0.380 ± 0.548	-	-
4	0.060 ± 0.864	0.348 ± 0.915	0.410 ± 0.460	0.030 ± 0.531	-
N.S.	0.120 ± 0.250	0.408 ± 0.406*	0.470 ± 0.367*	0.090 ± 0.582	0.060 ± 0.671

* significant ($\alpha = 0.05$)

NS naive standard

TABLE D.10

Relative mean prediction error of predicted Cp at 98 hours post commencement of therapy for 200 mg TMP b.d. (x v y)
Δme ± 95% confidence interval

No Cp	x 0	1	2	3	4
y					
1	-0.837 ± 0.591*	-	-	-	-
2	-0.937 ± 0.634*	-0.100 ± 0.793	-	-	-
3	-0.354 ± 0.767	0.483 ± 0.895	0.583 ± 0.420*	-	-
4	-0.394 ± 0.798	0.443 ± 0.989	0.543 ± 0.412*	-0.040 ± 0.446	-
N.S.	-0.104 ± 0.273	0.733 ± 0.449*	0.833 ± 0.818*	0.250 ± 0.929	0.290 ± 0.998

* significant ($\alpha = 0.05$)

NS naive standard

APPENDIX E

TABLE E.1

Volume of distribution of CLAV

Subject	No	Vd (l)	Weight (Kg)	Vd (l/Kg)
MK	1	10.9	55.2	0.20
VD	2	9.5	54.8	0.17
AS	3	18.1	81.8	0.22
EB	4	8.7	48.6	0.18
JP	5	17.1	84.0	0.20
SJ	6	10.5	57.0	0.18
HL	7	12.0	56.0	0.21
AA	8	11.1	65.0	0.17
AL	9	14.2	77.3	0.18
DS	10	15.2	66.4	0.23
RMcL	11	10.4	85.8	0.12
SK	12	-	85.0	INAPPROPRIATE
MC	13	-	64.0	SAMPLING
MR	14	18.5	47.0	0.39
DH	15	11.3	61.0	0.19
DM	16	11.2	60.0	0.19
MS	17	21.6	50.0	0.43
JH	18	12.6	81.9	0.15
JMI	19	22.9	82.0	0.28
NW	20	17.1	76.6	0.22
PMcN	21	14.4	66.1	0.22
EM	22	17.0	66.1	0.26
JMA	23	27.0	65.0	0.42
AH	24	7.7	48.6	0.16
PS	25	HAEMODIALYSIS		
FMcL	26	"		
NMcD	27	22.0	88.9	0.25
UK	28	9.4	50.7	0.19
MS	29	8.1	69.1	0.12
PM	30	9.3	49.7	0.19
MP	31	HAEMODIALYSIS		
JMcL	32	9.7	64.5	0.15
TMcK	33	13.0	70.0	0.19
-	34	STORAGE FAILURE		
JT	35	CAPD		
KS	36	4.4	75.0	0.06
AG	37	CAPD		
LMcD	38	17.8	55.2	0.32
MA	39	4.1	77.8	0.05

TABLE E.2

Volumes of Distribution of TIC

Vd_1=Vd CENTRAL compartment, Vd_2=Vd PERIPHERAL compartment

Subject	No	Weight (Kg)	Vd_1 (l)	Vd_2 (l)	Vd_{TOT} (l)	Vd_1 (l/Kg)	Vd_2 (l/Kg)	Vd_{TOT} (l/Kg)
MK	1	55.2	7.9	6.0	13.9	0.14	0.11	0.25
VD	2	54.8	5.4	11.1	16.5	0.10	0.20	0.30
AS	3	81.8	1.6	6.5	8.1	0.02	0.08	0.10
EB	4	48.6	2.0	2.2	4.2	0.04	0.05	0.09
JP	5	84.0	9.9	4.9	14.8	0.12	0.06	0.18
SJ	6	57.0	5.2	2.0	7.2	0.09	0.04	0.13
HL	7	56.0	6.2	2.9	9.1	0.11	0.01	0.12
AA	8	65.0	5.5	2.3	7.8	0.08	0.04	0.12
AL	9	77.3	6.6	6.3	12.8	0.09	0.08	0.17
DS	10	66.4	4.4	4.4	8.8	0.07	0.07	0.13
RMcL	11	85.8	3.0	8.6	11.6	0.03	0.10	0.14
SK	12	INAPPROPRIATE						
MC	13	SAMPLING						
MR	14	47.0	4.4	3.4	7.8	0.09	0.07	0.16
DH	15	61.0	4.9	4.6	9.4	0.08	0.08	0.16
DM	16	60.0	9.7	10.8	20.5	0.16	0.18	0.34
MS	17	50.0	12.3	11.2	23.5	0.25	0.22	0.47
JH	18	81.9	6.2	6.8	13.0	0.08	0.08	0.16
JMI	19	82.0	8.8	7.0	15.8	0.11	0.09	0.20
NW	20	76.6	6.7	3.1	9.8	0.09	0.04	0.13
PMcN	21	66.1	7.9	9.4	17.3	0.12	0.14	0.26
EM	22	66.1	6.9	7.0	13.9	0.10	0.11	0.21
JMA	23	65.0	5.0	5.8	10.8	0.08	0.09	0.17
AH	24	48.6	5.5	7.6	13.1	0.11	0.16	0.27
PS	25	HAEMODIALYSIS						
FMcL	26							
NMcP	27	88.9	8.3	7.3	15.6	0.09	0.08	0.17
UK	28	50.7	4.3	11.5	15.7	0.08	0.23	0.31
MS	29	69.1	2.7	9.9	12.6	0.04	0.14	0.18
PM	30	49.7	5.1	2.6	7.8	0.10	0.05	0.15
MP	31	HAEMODIALYSIS						
JMcL	32	64.5	4.1	9.0	13.1	0.06	0.14	0.20
TMcK	33	70.0	6.2	6.1	12.2	0.09	0.09	0.18
-	34	STORAGE FAULT						
JT	35	CAPD						
KS	36	75.0	8.3	4.6	12.9	0.11	0.06	0.17
AG	37	CAPD						
LMcD	38	55.2	2.4	6.1	6.5	0.04	0.11	0.12
MA	39	77.8	2.2	5.0	7.2	0.03	0.06	0.09

TABLE E.3

Clearance and elimination half-lives of TIC and CLAV

Subject	No	CrCl (ml/min)	CLAV $t_{\frac{1}{2}\beta}$ (h)	CLAV k_{el} (h^{-1})	CLAV Cl_s (l/h)	TIC $t_{\frac{1}{2}\beta}$ (h)	TIC k_{el} (h^{-1})	TIC Cls (l/h)
MK	1	6.8	3.3	0.210	2.3	2.4	0.289	1.9
VD	2	14.0	3.1	0.224	2.1	5.3	0.131	1.4
AS	3	34.0	1.0	0.693	12.8	3.0	0.231	1.3
EB	4	4.8	2.8	0.248	2.1	7.9	0.088	0.3
JP	5	49.0	1.5	0.462	7.7	2.5	0.277	2.7
SJ	6	13.0	1.7	0.408	4.4	6.1	0.144	0.9
HL	7	9.4	2.3	0.301	3.7	6.2	0.112	0.8
AA	8	6.8	2.8	0.248	2.7	5.5	0.126	1.0
AL	9	78.0	0.6	1.155	15.5	0.8	0.866	8.7
DS	10	19.0	2.0	0.347	5.3	1.4	0.495	3.9
RMcL	11	55.0	1.4	0.495	5.0	1.6	0.433	4.6
SK	12	INAPPROPRIATE						
ML	13	SAMPLING						
MR	14	6.0	2.3	0.301	5.5	9.9	0.070	0.6
DH	15	9.5	1.9	0.364	4.1	6.0	0.116	0.9
DM	16	AN	1.3	0.533	5.9	12.2	0.057	0.8
MS	17	AN	3.1	0.224	4.9	16.7	0.041	1.1
JH	18	97.0	0.9	0.770	9.7	1.1	0.630	8.2
JMI	19	22.0	3.2	0.217	4.9	6.9	0.100	2.1
NW	20	26.0	1.2	0.578	10.0	4.6	0.151	4.1
PMcN	21	70.0	0.6	1.155	16.1	1.1	0.630	13.4
EM	22	40.0	3.4	0.203	3.4	6.4	0.108	1.4
JMA	23	20.0	1.8	0.385	16.1	7.0	0.099	1.5
AH	24	6.0	3.8	0.182	3.8	10.2	0.068	0.9
PS	25	HD	0.3	-	-	1.2	-	-
FMcL	26	HD	0.5	-	-	1.7	-	-
NMcP	27	AN	1.4	0.495	12.4	7.3	0.095	1.8
UK	28	AN	1.3	0.533	4.8	5.6	0.124	1.9
MS	29	14.0	1.0	0.693	5.4	6.8	0.102	1.9
PM	30	AN	1.3	0.533	4.8	7.9	0.088	0.7
MP	31	HD	0.3	-	-	2.0	-	-
JMcL	32	AN	1.5	0.462	9.9	7.2	0.096	1.1
TMcK	33	108.0	0.3	2.310	30.5	1.1	0.630	6.6
-	34	STORAGE FAULT						
JT	35	CAPD	-	-	-	-	-	-
KS	36	110.0	0.14	4.950	20.9	0.85	1.155	8.7
AG	37	CAPD	-	-	-	-	-	-
LMcD	38	118.0	0.24	2.890	25.6	1.0	0.693	5.7
MA	39	125.0	0.10	6.930	19.7	0.35	1.980	6.9

TABLE E.4

Urine Kinetics of TIC and CLAV

Subject	No	CrCl (ml/min)	CLAV Cl_r (l/h)	CLAV AMOUNT EXCRETED Mg	CLAV fe	TIC Cl_r (l/h)	TIC AMOUNT EXCRETED mg	TIC fe
MK	1	6.8	0.21	18.4	0.09	0.47	470	0.16
VD	2	14.0	0.74	70.0	0.35	0.27	370	0.12
AS	3	34.0	5.00	78.2	0.39	0.34	570	0.19
EB	4	4.8	0.31	28.4	0.14	0.10	840	0.28
JP	5	49.0	3.30	83.6	0.42	2.32	1698	0.57
SJ	6	13.0	0.25	11.6	0.06	0.16	570	0.19
HL	7	9.4	0.79	43.8	0.22	0.20	576	0.19
AA	8	6.8	0.42	30.8	0.15	0.12	537	0.18
AL	9	78.0	9.41	122.4	0.61	1.67	453	0.15
DS	10	19.0	1.38	52.4	0.26	0.51	360	0.12
RMcL	11	55.0	2.60	101.0	0.51	0.40	270	0.09
SK	12	INAPPROPRIATE						
ML	13	SAMPLING						
MR	14	6.0	2.03	72.8	0.36	0.11	600	0.20
DH	15	9.5	1.56	75.6	0.38	0.24	660	0.22
DM	16	AN	0.00	00.0	0.00	0.00	0	0.00
MS	17	AN	0.00	00.0	0.00	0.00	0	0.00
JH	18	97.0	5.60	115.0	0.58	5.90	2160	0.72
JMI	19	22.0	1.76	70.8	0.35	0.46	861	0.29
NW	20	NO URINE COLLECTED						
PMcN	21	70.0	9.30	115.4	0.58	5.85	1611	0.54
EM	22	40.0	0.52	30.2	0.15	0.18	357	0.12
JMA	23	20.0	4.28	82.4	0.41	0.43	1224	0.41
AH	24	9.2	0.43	22.4	0.11	0.33	1104	0.37
PS	25	HD	-	-	-	-	-	-
FMcL	26	HD	-	-	-	-	-	-
NMcP	27	AN	0.00	00.0	0.00	0.00	0	0.00
UK	28	AN	0.00	00.0	0.00	0.00	0	0.00
MS	29	14.0	1.20	42.8	0.21	0.50	1176	0.39
PM	30	AN	0.00	00.0	0.00	0.00	0	0.00
MP	31	HD	-	-	-	-	-	-
JMcL	32	AN	0.00	00.0	0.00	0.00	0	0.00
TMcK	33	108.0	22.30	148.4	0.74	5.62	2232	0.74
	34	STORAGE FAULT						
JT	35	CAPD	-	-	-	-	-	-
KS	36	110.0	17.2	157.6	0.79	8.3	2370	0.79
AG	37	CAPD	-	-	-	-	-	-
LMcD	38	118.0	19.2	104.5	0.52	5.4	1216	0.41
MA	39	125.0	20.7	41.3	0.21	8.4	1560	0.52

APPENDIX F

TABLE F.1.

Serum and peritoneal dialysate concentrations of ticarcillin and clavulanic acid during CAPD (mg/l).

TIME (h)	SUBJECT 1				SUBJECT 2			
	SERUM		DIALYSATE		SERUM		DIALYSATE	
	TIC	CLAV	TIC	CLAV	TIC	CLAV	TIC	CLAV
0	ND	ND	-	-	ND	ND	-	-
0.083	730*	160.9*	-	-	194	11.3	-	-
0.025	-	-	57	14.4	-	-	104	11.7
0.5	142	29.3	75	23.6	130	8.8	187	15.2
1	125	9.2	102	42.4	117	3.3	319	8.3†
2	-	-	141	46.6	-	-	640	19.5
3	-	-	225	50.2	-	-	846	22.6
4 (pre exchange)	113	6.6	303	52.6	90	0.1	2230	28.2
4 (post exchange)	-	-	-	-	87	<0.1	536	17.9
8 (pre exchange)	104	ND	-	-	70	ND	662	9.6
8 (post exchange)	42	ND	130	11.6	30	ND	288	ND
12 (post exchange)	-	-	165	ND	-	-	272	ND
24	18	ND	-	-	10	ND	-	-

* Sample rejected - contamination from line?

† " " - ? storage inadequate

ND - not detected

\- - no sample

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