The pharmacokinetic interaction between cyclosporine and methoxsalen

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Abstract

Cyclosporine forms the cornerstone of therapy to prevent rejection after organ transplantation. However, the clinical use of the drug is compromised by a narrow therapeutic window and a wide inter- and intra-individual variation in metabolism. Cyclosporine is metabolised by the CYP3A4 isoenzymes in both the liver and intestine, while it has been reported that the metabolism of the drug can be inhibited by certain furocoumarin derivatives in grapefruit juice. Methoxsalen (8-methoxypsoralen) is a furocoumarin and a potent inhibitor of the cytochrome P450 system in both the liver and intestine. The study was conducted to investigate the possibility whether methoxsalen may inhibit the metabolism of cyclosporine and thereby increase the bioavailability of the drug. The interaction is of clinical relevance since both drugs are used in the treatment of psoriases.

The study, conducted in 12 healthy male volunteers, was a three-way comparative bioavailability study with a wash out period of one week between treatments. The patients received 40 mg methoxsalen, 200 mg cyclosporine or a combination of the two on three separate occasions. Blood samples of 10 ml were collected by venupuncture at the following times: 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 12 and 24 hours after drug administration. Methoxsalen was analysed by a high pressure liquid chromatograph method (HPLC) with UV detection (LOQ = 10 ηg/ml), while cyclosporine was analysed using a fluorescence polarisation immunoassay (FPIA) technique.

The results were as follows:

Cyclosporine and Cyclosporine/Methoxsalen combination.

	Cyclosporine in presence of Methoxsalen	**Cyclosporine**	**Ratio**	**90% Confidence interval**	***P -value**	****η^2 value**
AUC_{0-t} ηg.h/ml	915.75 ± 474.63	762.64 ± 482.99	120.50	103 – 138	0.68	0.133
$AUC_{0-\infty}$ ηg.h/ml	932.97 ± 473.48	774.06 ± 487.02	122.00	103 – 141	**0.04 *** **	**0.191*** **
C_{max} ηg/ml	285.70 ± 148.16	266.04 ± 136.26	108.00	102 - 114	**0.004*** **	**0.458*** **
$t_{½}$ (h)	3.03 ± 1.78	2.85 ± 1.54	95.00	68 – 122	0.57	0.091
t_{max} (h)	1.91 ± 0.71	1.72 ± 0.34	107.50	100 - 115	0.86	0.048

*P-value = Paired t-test. **η^2–value = Clinical Significant test. *** = Statistical significant.

Methoxsalen and the Methoxsalen/Cyclosporine combination.

	Methoxsalen in presence of cyclosporine	**Methoxsalen**	**Ratio**	**90% Confidence interval**	***P -value**	****η^2 value**
AUC_{0-t} ηg.h/ml	246.91 ± 236.58	362.24 ± 278.04	77	41 – 113	0.15	0.001
$AUC_{0-\infty}$ ηg.h/ml	247.00 ± 236.56	362.54 ± 279.37	77	41 – 113	0.15	0.000
C_{max} ηg/ml	96.48 ±65.97	150.12 ± 104.88	70	41 – 99	0.06	0.093
$t_{½}$ (h)	1.02 ± 0.54	1.04 ± 0.89	114.5	88 – 141	0.68	-0.091
t_{max}(h)	2.13 ± 1.13	1.87 ± 0.62	118	73 – 163	0.26	-0.011

*P-value = Paired t-test. **η^2-value = Clinical Significant test. *** = Statistical significant.

There was a statistical significant difference in $AUC_{0-\infty}$ and C_{max} for cyclosporine when methoxsalen was added to the drug regimen. When the methoxsalen levels were compared with those in the presence of cyclosporine, the levels were lower, although the difference was not statistical significant. We conclude that methoxsalen increase the levels of cyclosporine by inhibiting the P450 system enzymes in the liver and intestine. However, the absorption of methoxsalen is highly variable in the same individual which needs to be considered before this interaction can be regarded as being of any clinical relevance.

Key words: *Cyclosporine, Methoxsalen, Furocoumarin, Grapefruit juice, Cytochrome P450, Cytochrome P450 3A4 (CYP3A4), Intestinal metabolism, HPLC.*

Opsomming

Siklosporien is die keusemiddel om verwerping na orgaan oorplanting te voorkom. Die kliniese gebruik van siklosporien word ingeperk deur die nou terapeutiese reikwydte van die middel asook die groot inter- en intra-individuele verskille in metabolisme. Die geneesmiddel word deur die CYP3A4 iso-ensieme in die lewer en dunderm gemetaboliseer. Daar is in die literatuur gerapporteer dat die metabolisme van siklosporien geïnhibeer kan word deur sekere furokumarien derivate wat in pomelosap voorkom. Metoksalen (8-metoksipsoraleen) is ook 'n furokumarien derivaat en 'n sterk inhibeerder van die sitochroom P450 sisteem in die dunderm en lewer. Die doel van hierdie studie was om ondersoekin te stel of metoksalen die metabolisme van siklosporien kan onderdruk en dus die biobeskikbaarheid van die middel kan verhoog. Die twee middels word beide vir die behandeling van psoriase gebruik en die interaksie kan dus kliniese voordele inhou.

Die studie is op 12 gesonde manlike vrywilligers uitgevoer. Dit was 'n drie fase vergelykende biobeskikbaarheid studie. Die uitwasperiode was een week tussen die drie behandelings. Die pasiënte het op die drie geleenthede een van die volgende doserings ontvang: metoksalen 40 mg, siklosporien 200 mg, of metoksalen 40 mg en siklosporien 200 mg. Tien milliliter bloed is op die volgende tye versamel: 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 12 en 24 uur. Die metoksalen was bepaal met 'n hoëdrukvloeistof chromatografie metode met ultraviolet deteksie (laagste meetbare konsentrasie = 10 ηg/ml). Siklosporien was bepaal met 'n immunobepaling wat gebruik maak van 'n gepolariseerde fluoresensie tegniek.

Die resultate was as volg:

Siklosporien en die Siklosporien/Metoksalen kombinasie.

	Siklosporien in die teenwoordigheid van metoksalen	**Siklosporien**	**Verhou-ding**	**90% Vertrouensinterval**	***P - waarde**	****η^2 waarde**
AOK_{0-t} ηg.h/ml	915.75 ± 474.63	762.64 ± 482.99	120.50	103 – 138	0.68	0.133
$AOK_{0-\infty}$ ηg.h/ml	932.97 ± 473.48	774.06 ± 487.02	122.00	103 – 141	**0.04 *****	**0.191*****
C_{maks} ηg/ml	285.70 ± 148.16	266.04 ± 136.26	108.00	102 - 114	**0.004*****	**0.458*****
$t_{½}$ (h)	3.03 ± 1.78	2.85 ± 1.54	95.00	68 – 122	0.57	0.091
t_{maks} (h)	1.91 ± 0.71	1.72 ± 0.34	107.50	100 - 115	0.86	0.048

*P-waarde = Gepaarde t-toets. **η^2–waarde = Klinies betekenisvol. *** = Statisties betekenisvol.

Metoksalen en die Metoksalen/Siklosporien kombinasie.

	Metoksalen in die teenwoordigheid van siklosporien	**Metoksalen**	**Verhouding**	**90% Vertrouensinterval**	***P - waarde**	****η^2 waarde**
AOK_{0-t} ηg.h/ml	246.91 ± 236.58	362.24 ± 278.04	77	41 – 113	0.15	0.001
$AOK_{0-\infty}$ ηg.h/ml	247.00 ± 236.56	362.54 ± 279.37	77	41 – 113	0.15	0.000
C_{maks} ηg/ml	96.48 ±65.97	150.12 ± 104.88	70	41 – 99	0.06	0.093
$t_{½}$ (h)	1.02 ± 0.54	1.04 ± 0.89	114.5	88 – 141	0.68	-0.091
t_{max}(h)	2.13 ± 1.13	1.87 ± 0.62	118	73 - 163	0.26	-0.011

*P-waarde = Gepaarde t-toets. **η^2–waarde = Klinies betekenisvol. *** = Statisties betekenisvol.

Daar was 'n statisties beduidende verskil in siklosporien $AUC_{0-\infty}$ en C_{maks} wanneer metoksalen saam met siklosporien toegedien word. Die metoksalen vlakke het verlaag wanneeer siklosporien bygevoeg is maar hierdie verlaging was nie statisties beduidend nie. Daar kan dus afgelei word dat metoksalen wel die P450 ensiem sisteem in die lewer en dunderm inhibeer en sodoende die biobeskikbaarheid van siklosporien verhoog. Die absorpsie van metoksalen is baie wisselvallig in dieselfde persoon en dit sal aangespreek moet word voordat hierdie kombinasie van siklosporien en metoksalen klinies lewensvatbaar kan wees.

Sleutelwoorde: *Siklosporien, Metoksalen, Furokumarien, Pomelosap, Sitochroom P450, Sitochroom P450 3A4 (CYP3A4), Ingewande metabolisme, Hoëdrukvloeistofchromatografie.*

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"En wat besit jy wat jy nie ontvang het nie?"
1 Kor. 4:7

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Glossary

The definition or meanings of the various symbols, abbreviations and terminology used in this dissertation are as follows:

Cyclosporine: : According to the American dictionary

%CV : percentage coefficient of variation
8-MOP : 8-methoxypsoralen, methoxsalen
5-MOP : 5-methoxypsoralen, bergapten

ANOVA : analysis of variance
ATP : adenosine triphosphate
ADP : adenosine diphosphate
AHH : aryl hydrocarbon hydroxylase
AUC : area under the curve
$AUC_{8\text{-}MOP}$: area under the curve for methoxsalen
AUC_{TMP} : area under the curve for trioxsalen
$AUC_{0\text{-}t}$: area under the curve from time 0 to the time of last blood sample
$AUC_{0\text{-}\infty}$: area under the curve from time 0 to infinity

BMI : body mass index
BNF : β-naphtoflavone

CA : caffeine
C_{max} : maximum concentration of drug in the plasma
CHE : Christian higher education
Cl : clearance
C_t : plasma concentration at corresponding time
CV : coefficient of variation
CYP : cytochrome P450
CYP3A4 : cytochrome P450 3A4

DAD : diode and multiple wavelength detector
DNA : deoxyribonucleic acid

DPH	: 5,5-dipheylhydantoin
E	: enzyme
ES	: enzyme-substrate complex
ECG	: electrocardiogram
FAD	: flavin adenine dinucleotide (oxidised form)
FMN	: flavin mononucleotide (oxidised form)
FPIA	: fluorescence polarisation immunoassay
GIT	: gastrointestinal tract
HB	: hexobarbital
HDL	: high density lipoprotein
HIV	: human immuno deficiency virus
HPLC	: high performance liquid chromatography
HPPH	: 5-(4'-hydroxyphenyl)-5-phenylhydantoin
k_e	: terminal elimination rate constant
LDL	: low density lipoprotein
LOD	: limit of detection
LOQ	: limit of quantification
MEDUNSA	: Medical University of South Africa
NAD	: nicotinamide adenine dinucleotide (oxidised form)
NADH	: nicotinamide adenine dinucleotide (reduced form)
NADP	: nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH	: nicotinamide adenine dinucleotide phosphate (reduced form)
PB	: phenobarbital
PEG400	: polyethylene glycol
PU for CHE	: Potchefstroom University for Christian Higher Education
PUVA	: psoralen ultra violet therapy

S	: substrate
SD	: standard deviation
SEM	: standard error of the mean
$t_{½}$	: elimination half life
$t_{½\alpha}$	: distribution half-life
$t_{½\beta}$	: elimination half-life
t_{max}	: time to reach maximum plasma concentration
TMP	: 4,5',8-trimethylpsoralen, trioxsalen
UV	: ultra violet
Vd	: volume of distribution
Vd_{ss}	: volume of distribution at steady state

1. Introduction

1.1. Background

Cyclosporine, is a cyclic undecapeptide, highly lipid soluble compound, isolated from Norwegian soil fungi in 1970. Cyclosporine suppresses cytokine production (especially interleukin-2) by T cells. Less pronounced immunosuppression is the result of this selective action, while efficacy over older agents is improved. For these reasons, it forms the cornerstone of therapy for maintenance in immunosuppression following transplantation, while its role in a variety of diseases which involve the immune mechanisms is increasing (Jones, 1997).

However, its clinical use is complicated by its narrow therapeutic index, the daily dose of cyclosporine required to suppress organ rejection in some patients can cause renal and neurologic toxicity in others (Lown *et al*, 1997). To maintain blood levels within a relatively narrow range (approximately 150-400 ng/ml), can be a difficult task due, in large part, to the wide inter-patient variability in oral bioavailability ranging from less than 5% to as much as 90% (Ducharme *et al*, 1993).

Absorption of cyclosporine occurs only in the upper gastrointestinal tract (Jones, 1997). When administered intravenously, the drug distributes rapidly with a bi-phasic distribution pattern. The first phase of intravenous distribution occurs rapidly and represents distribution into erythrocytes, where cyclosporine is bound to hemoglobin. A second distribution phenomenon occurs as the drug distributes into peripheral tissues. Cyclosporine's elimination half-life ranges from 3 to 40 hours. The average half-life is approximately 10 hours. When given orally, the peak blood concentration occurs within 2-3 hours. The blood concentration then remains statically elevated at approximately the same concentration for up to six hours, representing a prolonged period of absorption (Moyer *et al*, 1986). The poor and variable bioavailability of cyclosporine may relate to pre-systemic metabolism of cyclosporine in the gut wall (Ducharme *et al*, 1993).

The cytochrome P450 (CYP3A4) isoenzyme, found in both the liver and enterocytes, is the major enzyme responsible for cyclosporine metabolism. Interindividual variability in intestinal CYP3A4 activity has been demonstrated and that is why the metabolism of cyclosporine might explain the huge interpatient variability in absorption. It was thought that metabolism occurred primarily in the liver, but the importance of intestinal metabolism was established by Kolars &

co-workers (1991) and it may even exceed hepatic metabolism (Jones, 1997). If the extent of intestinal first-pass metabolism is proportional to the intestinal content of CYP3A4, it would seem to be likely that variation in intestinal CYP3A4 activity could account for a portion of the unexplained variation in the oral pharmacokinetics of cyclosporine (Lown *et al*, 1997).

Another potential source of variation in oral cyclosporine pharmacokinetics may be related to the expression of P-glycoprotein, the multi drug resistance gene (*mdr1*) product, a transmembrane protein in the intestine (Edwards *et al*, 1999). P-glycoprotein is a versatile transporter that is able to pump a wide variety of xenobiotics including cyclosporine. P-glycoprotein is located almost exclusively within the brush border on the apical (luminal) surface of the enterocyte where it pumps xenobiotics from the cytoplasm to the exterior of the cell (i.e., from the enterocyte back into the intestinal lumen) (Lown *et al*, 1997). This protective function is shared with cytochrome P450 enzymes such as CYP3A4, and many compounds have been identified as substrates or inhibitors of both CYP3A4 and P-glycoprotein (Edwards *et al*, 1999). As with CYP3A4, there is significant interindividual variation in the intestinal expression of P-glycoprotein. High intestinal levels of P-glycoprotein may interfere with drug absorption and contribute to the variation in cyclosporine oral pharmacokinetics (Lown *et al*, 1997).

Blood levels of cyclosporine are increased if taken with grapefruit juice. The mechanism by which grapefruit juice improves cyclosporine bioavailability is of interest. Because systemically available cyclosporine is almost entirely eliminated by hepatic metabolism and grapefruit juice had no effect on intravenous cyclosporine, the increase in plasma concentrations and bioavailability with oral dosing is the result of an effect on the gut rather than the liver. Ducharme & co-workers (1995) indicated that grapefruit juice affects cyclosporine absorption and/or gut wall metabolism.

There is some increase of interest in the co-administration of cyclosporine with inhibitors of CYP3A4 in order to increase concentrations, in patients having difficulty attaining therapeutic levels and to decrease the cost of therapy with this expensive drug (Ducharme *et al*, 1993).

Methoxsalen (8-methoxypsoralen) is a naturally occurring tricyclic furocoumarin (Bickers *et al*, 1982) present in several plant species (Fouin-Fortunet *et al*, 1985). Methoxsalen is used clinically in the treatment of selected dermatological diseases including vitiligo, psoriasis, and mycosis fungoides (Bickers *et al*, 1982). Methoxsalen is orally administered at 0.6 mg/kg for psoralen (PUVA) therapy (Mays *et al*, 1987).

Tinel and co-workers (1987) reported that methoxsalen is activated by rat liver cytochrome P450 into reactive metabolites which covalently bind to microsomal proteins and inactivate cytochrome P450. As a consequence of both competitive inhibition by methoxsalen itself, and suicide inactivation by methoxsalen metabolites, the drug is a potent inhibitor of rat liver cytochrome P450. They concluded that methoxsalen destroys CO-binding to cytochrome P450, and markedly decreases monooxygenase activities in human liver microsomes (Tinel *et al*, 1987). Thus effect can be extended to the inhibition of gut wall P450 enzymes. It seems, from the literature, that the methoxsalen effect on the metabolism can be compared to the effect of grapefruit juice on the metabolism of cyclosporin. The clearance of caffeine decreased from 110 ml/min to 34 ml/min in patients who received methoxsalen an hour before administration (Mays *et al*, 1987).

1.2. Study objectives

The primary aims of this study are to:

- Investigate the effects of methoxsalen (a psoralen) on the pharmacokinetics and bioavailability of cyclosporine.
- Determine the plasma levels of methoxsalen to investigate the inter-individual variation of the drug between individuals.
- To develop a HPLC method for the determination of methoxsalen concentrations in human plasma after oral adminstration.

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2. The cytochrome P450 (CYP) enzyme system

2.1. Introduction

There are two types of metabolism reactions. Phase I reactions which includes such reactions as oxidation, hydroxylation, reduction and hydrolysis and phase II reactions, which involve the addition of a new functional group, therefore called conjugation (Watkins, 1992). The cytochrome P450 (CYP) mixed function oxidases are a family of enzymes (Dresser *et al,* 2000), present in the endoplasmic reticulum of many cells in the body with the highest concentration in hepatocytes (Watkins, 1992). CYP is a host of enzymes that use iron to oxidise potentially harmful substances by making them more water-soluble (Van Schalkwyk & Van Schalkwyk, 2003) and accounts for the majority of oxidative biotransformations of xenobiotics and endogenous biochemicals (Dresser *et al,* 2000). CYP catalyses a variety of reactions including epoxidation, *N*-dealkylation, *O*-dealkylation, *S*-oxidation, hydroxylation (Van Schalkwyk & Van Schalkwyk, 2003), aliphatic oxidation, aromatic hydroxylation, oxidative deamination, and sulfoxide formation. It can also activate carcinogenic substances such as the aflatoxins and polycyclic aromatic hydrocarbons (Watkins, 1992). The overall reaction can be summarises as:

$$DH + NAD(P)H + H^+ + O_2 = DOH + NAD(P)^+ + H_2O$$

DH is the drug, NADH or NADPH is a reduced nicotinamide adeninedinucleotide co-factor, and NAD or NADP is an oxidised co-factor. In this reaction molecular oxygen serves as the final electron acceptor. During the catalytic reaction, the heme iron in the enzyme undergoes a cycle that begins in the ferric oxidation state, when the drug binds to CYP. The heme iron undergoes reduction to the ferrous state, binds oxygen, and the molecular oxygen bound to the active site is reduced to a reactive form that inserts one oxygen atom into the drug substrate with the other oxygen being reduced to water, with the eventual regeneration of the ferric state of the heme iron. Free radical or iron-radical groups are formed at one or more parts of the cycle. The reaction cycle is summarised in figure 2.1. (Hollenberg & Brody, 1998).

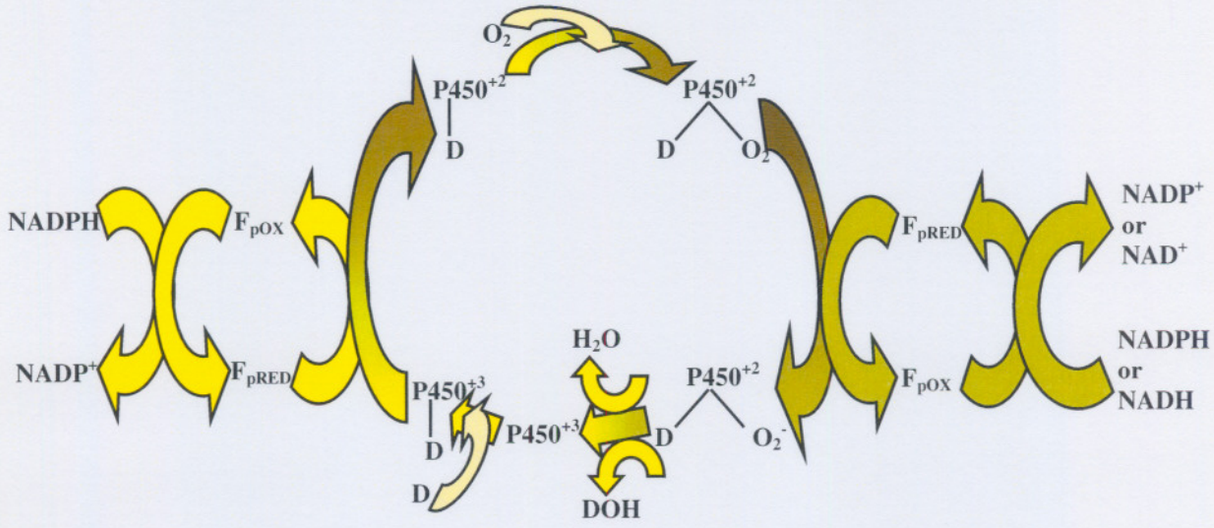


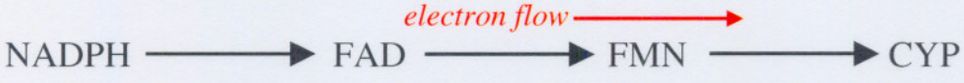
Figure 2.1: Simplified model of CYP mixed-function oxidase reaction sequence. D is the drug undergoing oxidation to product DOH. Molecular oxygen serves as the final electron acceptor. Flavin protein co-factor (F_p) systems are involved at several sites. The iron of the CYP is involved in binding oxygen and electron transfer with changes in valence state (Hollenberg & Brody, 1998)

It is known that the gene for CYP has existed for more then 3.5 billion years (Hayes, 2003). The massive heterogeneity of these oxidases is thought to reflect the complex interdependence between plants and animals (Van Schalkwyk & Van Schalkwyk, 2003). CYP was only capable of reductive metabolic reactions. Gradually oxygen started to accumulate in the atmosphere and the function of the enzyme changed. Oxidative capabilities developed which are of interest to us in understanding drug metabolism in humans (Ilett, 1997). Plants develop new alkaloids to limit their consumption by animals, and animals develop new enzymes to metabolise the plant toxins. It appears that the number of CYP genes exploded at about the time when organisms moved from the oceans to dry land – around 400 million years ago (Van Schalkwyk & Van Schalkwyk, 2003).

To identify the CYP component, the microsomal portion of the cell has to be extracted, resulting in a rather opaque suspension. The microsome-containing solution is divided into two, after adding an agent that reduces any heme that might be present. One part is exposed to carbon monoxide, if this solution strongly absorbs light at a wavelength of 450 nm compared with the original solution, it must contain CYP. This is called "difference spectroscopy" and investigators are finding the "reduced CO difference spectrum". The P in P450 stands for pigment. The reason why absorption occurs at this wavelength is related to one of the six ligands associated with the iron atom contained in the heme (Van Schalkwyk & Van Schalkwyk, 2003).

2.2. CYP chemistry

The bond between the two atoms in an oxygen molecule is rather strong. This implies that a substantial amount of energy is required to break the bond - energy that is supplied by addition of electrons to the iron atom of heme. These electrons in turn come from the last protein in an "electron transfer chain". There are two such chains in cells that end up at CYP. The first is in the endoplasmic reticulum (ER), and the protein involved is called NADPH CYP reductase. The electron flow is demonstrated below:



NADPH = nicotinamide adenine dinucleotide phosphate (reduced form); FAD = flavin adenine dinucleotide (oxidised form); FMN = flavin mononucleotide (oxidised form); CYP = cytochrome P450.

The second chain lurks within mitochondria. A complex bucket brigade of proteins hands the electrons down to heme. NADPH passes electrons to ferredoxin reductase, thence to ferredoxin (which itself has an iron-sulphur cluster), and from there to CYP (Van Schalkwyk & Van Schalkwyk, 2003).

2.3. Families, subfamilies and nomenclature of the P450 cytochrome system

The CYP's have been referred to as *Supergene family* for their significant homology among each other in few regions of the proteins, suggesting a common ancestry. They comprise a superfamily of hemeproteins that contain a single-iron protoporphyrin IX prosthetic group (Lin *et al,* 1999). Nonetheless, there is heterogeneity in CYP structures with the genes distributed in different chromosomes. 74 Gene families were known in 1996, 14 families exist in all mammals with 26 subfamilies from which 20 of these subfamilies have been mapped to the human genome. Families have > 40% homology in their amino acid sequence while subfamilies have > 55%. The nomenclature are as follows:

Root:	**CYP**
Family:	CYP**2**
Subfamily:	CYP2**D**
Gene:	CYP2D**6**

(Flockhardt, 1999).

There are three gene families, CYP1, CYP2 and CYP3, thought to be responsible for drug metabolism. CYP1A2, 2A6, 2B6, 2C, 2D6, 2E1 and 3A isoforms account for approximately

70% of human liver CYP. CYP3A (CYP3A4 and CYP3A5) and CYP2C (CYP2C8, 2C9, 2C18 and 2C19) are the most abundant subfamilies, accounting for 30% and 20% of total CYP, respectively (Lin *et al,* 1999).

Although one form of CYP may be capable of metabolising a number of drugs, a given drug may rely largely or exclusively on a single form of CYP for its normal metabolism (Watkins, 1992).

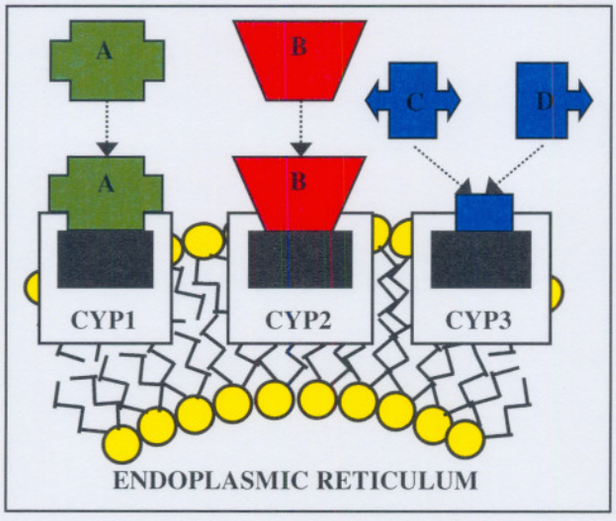


Figure 2.2: Drug metabolism by the CYP's. CYP are heme-containing enzymes (indicated by the dark squares) that are attached to the endoplasmic reticulum. Most CYP's involves in drug metabolism are members of three families: CYP1, CYP2 and CYP3. Individual CYP's have distinct catalytic properties. For example, drug A is metabolised by CYP1 but not by CYP2 or CYP3, because drug A only fits into the substrate-binding site of CYP1. Likewise, drug B is metabolised only by CYP2. Nevertheless, any one CYP appears to be capable of binding and metabolising multiple drugs, as shown with drugs C and D. These drugs differ in structure, but both can bind to the substrate-binding site of CYP3 (Watkins, 1992).

2.4. Distribution

Tissue like liver, kidneys, lungs, small intestine, brain, skin and lymphocytes contain the CYP enzyme system (Fouin-Fortunet *et al*, 1986). It is not surprising that much of the CYP in man is found in the liver, the main organ involved in drug and toxin removal, but a remarkable amount is also found in the small intestine. Metabolic clearance of drugs is not the only function of CYP – recently, it has been found that CYP is intimately involved in vascular autoregulation, particularly in the brain. CYP is vital to the formation of cholesterol, steroids and arachidonic acid metabolites (Van Schalkwyk & Van Schalkwyk, 2003).

Some isoforms are found throughout the body, for example CYP51, while other are limited to one specific tissue (CYP11B2, found mainly if not exclusively in the glomerulosa zone of the adrenal gland) (Van Schalkwyk & Van Schalkwyk, 2003). CYP3A5 is predominant in the lung and stomach and is present in the small bowel and renal tissue. CYP3A7 is absent in adults but is found in fetal livers (Watkins, 1992). CYP1A2 is not expressed in neonates, making them particularly susceptible to toxicity from drugs such as caffeine (Van Schalkwyk & Van Schalkwyk, 2003). CYP3A4 is the predominant isoform of CYP3A in adult humans (Watkins, 1992).

Differential expression of some CYP's in different organs may also have clinical consequences, especially where the degradation of a drug results in a toxic metabolite. The degradation of paracetamol by CYP2E1 results in a highly active intermediate product, N-acetyl-benzoquinoneimine, which in sufficient quantities can result in fulminant liver failure by depleting hepatic glutathione. Anti-oxidants protect against this catastrophe; in contrast, chronic ethanol consumption induces CYP2E1 and may increase the likelihood of toxicity (Van Schalkwyk & Van Schalkwyk, 2003).

2.4.1. CYP in the liver

The liver is the most important site of drug metabolism (Watkins, 1992). CYP3A4 was found to be the most abundant CYP in both human liver and small intestine, although, the liver exhibited a 2- to 5-fold higher level of the enzyme than the intestine (Lin *et al*, 1999). Table 2.1. list the major CYP substrates and inducers in the human liver.

CYP4A5 is only present in one out of five adults. Watkins (1992) also found that women have more hepatic CYP3A activity than men (Watkins, 1992).

Table 2.1: Characteristics of some human liver CYP's (Watkins, 1992).

Gene	Enzyme	Substrates	Probable inducers
CYP1A2	P450IA2	Acetaminophen, caffeine, theophylline	Cigarette smoke, charcoal-broiled foods, omeprazole
CYP2C	P450IIC	Mephenytoin, hexobarbital diazepam, tolbutamide, sulfinpyrazone, phenylbutazone	None identified
CYP2D6	P450IID6	Debrisoquine, dextromethorphan, metoprolol, other β-blockers, perhexiline, amitriptyline, other neuroleptics, encainide, flecainide, codeine	None identified
CYP2E1	P450IIE1	Acetaminophen, ethanol	Ethanol, isoniazid
CYP3A4	P450IIIA4	Erythromycin, cyclosporine, steroids, estrogens, midazolam/triazolam, nifedipine, diltiazem, lidocaine, triacetyloleandomycin (TAO), ketoconazole, miconazole, quinidine, lovastatin, FK506	Glucocorticoids, rifampicin, phenytoin, carbamazepine, sulfinpyrazone, phenylburazone

2.4.2. CYP in the intestine

In rats, some CYP's within the three families are also present in the apex of enterocytes at the tip of the villus, lying just below the microvillus border. In both rats and humans the major enterocyte CYP appears to be a member of the CYP3A family, in humans it is CYP3A4. Kolars & co-workers (1991) strongly suggest that CYP3A present in enterocytes catalyses significant first-pass metabolism of orally administered cyclosporine. They have done studies on microsomes prepared from rat jejunal enterocytes and in patients during the anhepatic phase of liver transplant operation. Because there is significant inter-patient heterogeneity in the expression of CYP3A4 in intestinal mucosa, just as there is in the liver, the extent to which cyclosporine is metabolised in the gut probably varies significantly among patients.

When erythromycin is administered intravenously to patients on cyclosporine therapy, little change in the cyclosporine pharmacokinetics is observed. When erythromycin is administered orally to patients on cyclosporine therapy, there is a large increase in the maximal blood concentration and the area-under the blood-concentration of cyclosporine-versus-time curve but

little effect on the rate of systemic clearance of cyclosporine. Erythromycin increases the oral bioavailability of cyclosporine by inhibiting CYP3A within the enterocyte. Induction of enterocyte CYP3A4 may also explain many drug interactions with cyclosporine (Watkins, 1992).

2.5. Inter-individual variability of CYP and drug metabolism

There is a wide interindividual variability and it is likely, in part, to be caused by ethnic or cultural differences, perhaps related to an interaction between race and diet. Other factors known to play a role in activity are age and the presence of small bowel or liver disease (Dresser *et al,* 2000). Both environmental and genetic factors can control the rate of drug disposition in the individual patient (Van Schalkwyk & Van Schalkwyk, 2003). Genetic variability is known for drug absorption, drug metabolism and for drug interactions with receptors. This forms the basis for slow and rapid drug absorption, poor, efficient or ultrarapid drug metabolism and poor or efficient receptor interactions. Environmental influence includes induction and inhibition of drug transport and metabolism (Ingelman-Sundberg, 2001). Genetic variation in a population is termed *polymorphism* when both gene variants exist with a frequency of at least one percent. Such differences in activity may have profound clinical consequences, especially when multiple drugs are given to a patient (Van Schalkwyk & Van Schalkwyk, 2003). Mutations of these genes can cause the formation of an enzyme that is (i) defect or partially defect, (ii) has an altered substrate specificity or (iii) is expressed at a lower or higher level (Ingelman-Sundberg, 1998).

The clinical importance of the different polymorphic CYP enzymes, with respect to drug metabolism, could be summarised as follows:

Poor metabolisers:

- diminished first pass metabolism, increased bioavailability, exaggerated response;
- diminished drug elimination, side effects or toxic effects;
- the active metabolite is not formed; loss of therapeutic efficacy.

Ultrarapid metabolisers:

- lack of therapeutic effect at standard doses;
- too much of the active metabolite;
- explanation for suspected bad compliance.

(Ingelman-Sundberg, 1998).

In table 2.2., an overview of the polymorphic phase I enzymes of functional importance for the metabolism of xenobiotics is given (Ingelman-Sundberg, 2001).

Table 2.2: Functional importance of polymorphism in phase I CYP enzymes (Ingelman-Sundberg, 2001).

Enzymes	Importance for metabolism	Polymorphism, significance
CYP1A1	Carcinogens	Unproven
CYP1A2	Drugs and carcinogens	Induction
CYP1B1	Carcinogens, oestrogen (?)	Rare null alleles, many variants of uncertain significance
CYP2A6	Nicotine, drugs, carcinogens	Important functional polymorphism
CYP2B6	Drugs	Polymorphically expressed
CYP2C8	Some drugs	
CYP2C9	Drugs	Very significant
CYP2C19	Drugs	Very significant
CYP2D6	Drugs	Very significant
CYP2E1	Carcinogens, some drugs	Not shown hitherto
CYP3A4	Drugs, carcinogens	Rare functional variants
CYP3A5	Drugs	Polymorphically expressed

Interindividual distribution of many of the CYP forms varies strikingly and their extensive polymorphism is likely, to a great extent, the result of dietary adaptation of different populations in the world. No important endogenous substrates have been described for any of the polymorphic CYP's and their primary function is in the metabolism of dietary components (Ingelman-Sundberg, 2001).

The high expression of CYP2D6 in many persons of Ethiopian and Audi Arabian origin is very interesting. 2D6 is not inducible, so these people have developed a different strategy to cope with the (presumed) high load of toxic alkaloids in their diet – multiple copies of the gene. These CYP's therefore metabolise a variety of drugs, making them ineffective – many antidepressants and neuroleptics are an important example. Conversely, prodrugs will be extensively activated – codeine will be turned in vast amount into morphine. In contrast, many individuals lack functional 2D6. These subjects will be predisposed to drug toxicity caused by antidepressants or neuroleptics, but will find codeine (and indeed tramadol) to be inefficacious due to lack of activation. Other drugs that have caused problems in those lacking 2D6 include dexfenfluramine, propafenone, mexiletine, and perhexiline. Perhexiline was in fact withdrawn from the market due to neuropathy caused in 2D6 inactive patients. Another potentially disastrous polymorphism is deficient activity of CYP2C9. This is because patients possessing

this enzyme variant are ineffective in clearing (S)-warfarin – so much so that they may be fully anticoagulated on just 0.5 mg of warfarin a day. The same CYP is important in removal of phenytoin and tolbutamide, both potentially very toxic drugs in excess. The flip-side is that the prodrug losartan will be poorly activated and inefficacious with 2C9 deficiency. Azole antifungals, sulphinpyrazone and even amiodarone may cause a similar effect by inhibiting the enzyme. Cure rates for peptic ulcer treated with omeprazole are substantially greater in individuals with defective CYP2C19, owing to the sustained, high plasma levels achieved (Van Schalkwyk & Van Schalkwyk, 2003).

Each patient have a unique liver "P450 fingerprint", which may change over the time. CYP3A activity varies at least 10-fold among patients. Liver activity of CYP3A largely predicts blood level of cyclosporine as shown by Watkins & co-workers (1992). They found that patients with higher CYP3A activity have lower blood levels of cyclosporine at any given daily dose of the drug (Watkins, 1992).

2.6. CYP in drug interactions

CYP dependent monooxygenases are of biological importance, since the metabolism of several drugs, intensity and duration of their pharmacological action can be determined. Reactive metabolites can form which are often mutagens, carcinogens or cytotoxic agents (Tsamboas *et al*, 1978).

The intestinal and/or hepatic CYP3A4 enzymes can be induced or inhibited which can result in the elevating or lowering of blood cyclosporine concentrations (Jones, 1997). Induction of the CYP enzyme system leads to increased biotransformation of cyclosporine, and therefore decreased effect of the drug (Wideman, 1983). Inhibition of CYP3A4 increase the plasma concentration of cyclosporine and this interaction can be of great clinical value. Cost savings and enhanced efficacy are beneficial clinical consequences of CYP3A4 interactions (Dresser, *et al*, 2000). In Chapter 4, section 4.3.5, drug interactions with cyclosporine manifesting in alterations of blood concentrations are described completely.

2.6.1. Intestinal enzyme induction

Induction is defined as the increase in the amount and activity of drug-metabolising enzymes, mainly CYP of the families 1 to 4 (Van Schalkwyk & Van Schalkwyk, 2003). Some mammalian

drug-metabolising enzymes like CYP3A4 are inducible. Induction may lead to an increased first-pass effect and, in turn, to a decreased oral bioavailability (Lin *et al,* 1999). When taking with a CYP3A inducer, the substrate metabolism will increase (Watkins, 1992). The most important inducers of CYP3A are antimicrobials such as rifampicin, and anticonvulsants like carbamazepine and phenytoin, but potent steroids such as dexamethasone may also induce CYP3A. The long list of agents metabolised by the enzyme include opioids, benzodiazepines and local anaesthetics, as well as erythromycin, cyclosporine, haloperidol, calcium channel blockers, cisapride and pimozide (Van Schalkwyk & Van Schalkwyk, 2003). Induction of CYP3A by rifampicin may explain the failure of oral contraceptives, smokers require more theophylline and are more tolerant to caffeine, presumably because CYP1A2 in their livers is induced. Warfarin's metabolism is complex and is metabolised by multiple members of each of the three families of CYP. Drugs that alter activities of individual CYP's cause an interaction with warfarin only in those patients relying on those enzymes for warfarin metabolism (Watkins, 1992).

Since cyclosporine is a CYP3A substrate, the metabolism of the drug can increase with the inducers mentioned above.

2.6.2. Enzyme inhibition

Inhibition of CYP means a decrease in drug metabolising enzymes. Inhibition of the CYP's can cause clinical important, interactions between drugs on different mechanisms:

2.6.2.1. Presystemic metabolism

If substrates with high presystemic elimination (low oral bioavailability) and which is dependent upon CYP3A4 for elimination, is administered with an inhibitor of its metabolism, it can be expected to produce substantial change in the substrate's pharmacokinetics under single dose conditions. Higher drug peak plasma drug concentration (C_{max}) will result from reduced presystemic metabolism and a greater area under the drug concentration-time curve (AUC) will be found possible from both lower presystemic and systemic elimination (Dresser *et al,* 2000).

Table 2.3: Drugs metabolised by cytochrome CYP3A4 and the extent of presystemic metabolism (Dresser *et al*, 2000).

Presystemic metabolism	Oral bioavailability (%)	Drugs
VERY HIGH	< 10	Astemizole, buspirone, ergotamine, lovastatin, nimodipine, nisoldipine, saquinavir, simvastatin, terfenadine
HIGH	10-30	Estradiol*, atorvastatin, felodipine, indinavir, isradipine, nicardipine, nitrendipine, propafenone*, tacrolimus
INTERMEDIATE	30-70	Amiodarone*, amprenavir, carbamazepine, carvedilol*, cisapride, **cyclosporine**, diltiazem*, ethinylestradiol, etoposide, losartan*, midazolam, nifedipine, nelfinavcir, ondensetron, pimozide, sildenafil*, triazolam, verapamil*
LOW	> 70	Alprazolam, amlodipine, dapsone, dexamethasone, disopyramide, donepezil, quinidine*, ritonavir, temazepam
*Metabolic pathways in addition to CYP3A4 may play a significant role.		

2.6.2.2. Competitive inhibition.

As seen in figure 2.2., page 9, drug C and D compete for binding to and metabolism by CYP3. The grade of the competitive inhibition relies on the drugs' respective relative affinities for binding to CYP3, concentration in the endoplasmic reticulum, dependence on the CYP for elimination and the potential to produce toxicity. Some drugs may even bind to a certain CYP and act as competitive inhibitor but are not metabolised by that CYP (Watkins, 1992). Potent inhibitors of CYP3A4 that has been associated with clinically relevant interactions can be seen in table 2.4., and include azole antifungals, macrolide antibiotics, nefazodone, HIV protease inhibitors and grapefruit juice.

Table 2.4: Inhibitors of CYP3A4 (Dresser *et al*, 2000).

Mechanism of action	Inhibitors
REVERSIBLE	Amprenavir, clarithromycin, **cyclosporine**, diltiazem, erythromycin, itraconazole, indinavir, ketoconazole, mibefradil, nefazodone, nelfinavir, ritonavir
MECHANISM-BASED (SUICIDE)	Bergamottin (grapefruit juice) dihydroxybergamottin (grapefruit juice)

Azole antifungals and first generation HIV protease inhibitors appear to act via competitive inhibition by rapid, reversible binding of the inhibitor or its metabolite to CYP3A4. Macrolide antibacterials produce slowly reversible, non-competitive inhibition. The furanocoumarins in grapefruit juice cause irreversible, mechanism-based (suicide) inhibition. 8-MOP is also an

inhibitor of several CYP's in the liver and grapefruit juice is also an example of an inhibitor that appears to be clinically active against only enteric CYP3A4 (Dresser *et al,* 2000).

Recent studies of Piver and co-workers (2001) showed that resveratrol (RESV), present at concentrations of about 10 μM in red wine, inhibits events associated with tumor initiation, promotion and progression. They suspected that the mechanism involved could be the inhibition of activities catalysed by CYP's, which activate procarcinogens. They investigated the inhibitory effect of RESV on CYP1A, CYP2E1 and CYP3A enzymatic activities and compared it to that of non-volatile compounds present in red wine. Their study showed that red wine solids (RWS) are particularly potent inhibitors of CYP1A1/1A2, CYP2E1 and CYP3A4 activities in human liver microsomes. A probably mechanism-based inhibitory effect for CYP3A4 were displayed (Piver *et al,* 2001)

The activity of CYP3A appears to be rate limiting in the metabolism of cyclosporine. Any drug that binds to CYP3A has the potential to inhibit cyclosporine metabolism competitively, resulting higher blood levels of cyclosporine and possibly toxicity. Pichard & co-workers (1990) exposed drugs, reported to elevate cyclosporine blood levels, such as erythromycin, to cultured human hepatocytes. The rate of cyclosporine metabolism by the hepatocytes decreased. They found that drugs that inhibited cyclosporine metabolism in the hepatocyte usually also inhibited cyclosporine metabolism in the microsomes in a competitive way, by binding to CYP3A.

Any drug shown to be metabolised by CYP3A should be metabolised at a reduced rate when a patient is also taking a CYP3A inhibitor (Watkins, 1992). Non-sedating antihistamines have resulted in fatal arrhythmias, as has occurred with cisapride administration in combination with an inhibitor. Erythromycin in combination with theophylline may cause theophylline toxicity (Van Schalkwyk & Van Schalkwyk, 2003).

2.7. P-glycoprotein

Cross-resistance of some primary and many recurrent human tumours to multiple chemotherapeutic drugs is a major problem in the chemotherapy of cancer. Multidrug-resistant tumour cells have been shown to have a highly active efflux mechanism for chemotherapeutic drugs, which prevents accumulation of these drugs in the cytoplasm of multidrug-resistant cells. Multidrug-resistance has been shown to be due to the product of a gene (*MDR1*) that confers the multidrug-resistant phenotype. A transmembrane glycoprotein has been identified and named

P170 or P-glycoprotein (Thiebaut *et al,* 1987). There are two types of p-glycoproteins in mammals: the drug-transporting p-glycoprotein and phospholipid-transporting p-glycoproteins (Lin *et al,* 1999). P-glycoprotein has been shown to be present and function as a transporter in the plasma membranes of many normal tissues (Terao *et al,* 1996). P-glycoprotein is localised on the bile canalicular surface of hepatocytes, apical surface of proximal tubules in kidneys and columnar epithelial cells of intestine and capillary endothelial cells of brain and testis. It can therefore protect the body against toxic xenobiotics by excreting these compounds into bile, urine, and the intestinal lumen, and by preventing their accumulation in brain and testis (Lin *et al,* 1999). P-glycoprotein is located almost exclusively within the brush border on the apical (luminal) surface of the enterocyte where it pumps xenobiotics from the cytoplasm to the exterior of the cell (i.e., from the enterocyte back into the intestinal lumen.) (Lown *et al,* 1997).
Table 2.5. represents a summary of tissues expressing p-glycoprotein.

Table 2.5: Localisation of p-glycoprotein in normal human tissues (Cordon-Cardo *et al,* 1990; Fojo *et al,* 1987; Sugawara et al, 1997; Thiebaut *et al,* 1987; Tsuji *et al,* 1992).

Tissues expressing p-glycoprotein	
Kidney: Proximal tubules and medulla	Digestive tract: esophagus, stomach, jejunum, colon, rectum
Pancreas: ductules	Placenta: trophoblas
Adrenal cortex and medulla	Testis: seminiferous tubules
Brain: endothelial cells	Sweat glands
Heart: muscle	Smooth muscle
Lung: bronchi and trachea	Liver: Bile canaliculi and biliary ducts

2.7.1. Structure of p-glycoprotein

P-glycoprotein has 1280 amino acids with a calculated molecular weight of 141.48 daltons. It consists of two approximately equal parts sharing considerable amino acid sequence homology with each other. Each part can be subdivided into a short hydrophilic region at the N-terminus, a long hydrophobic region and a relatively long hydrophilic region near the C-terminus. Each hydrophobic region includes six 21 amino acid long transmembrane domains (Figure 2.3) (Chen *et al,* 1986).

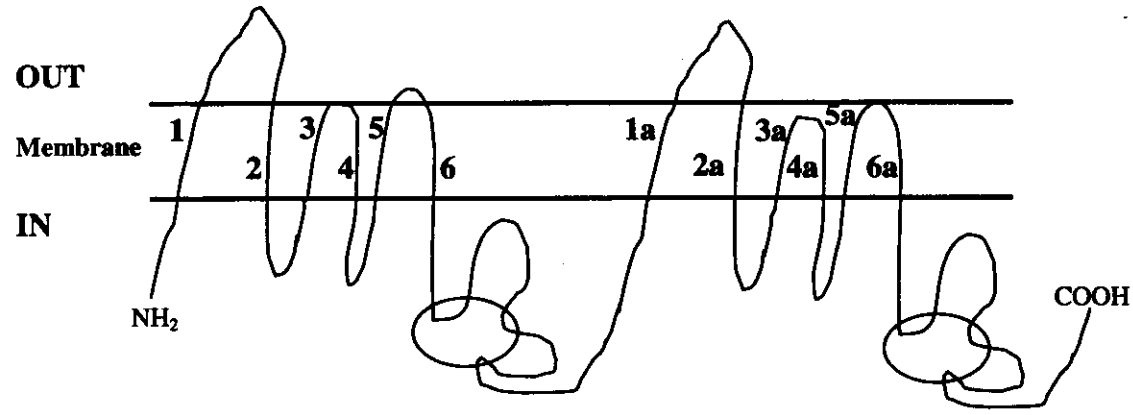


Figure 2.3: Model of the transmembrane orientation of p-glycoprotein (Chen *et al*, 1986).

2.7.2. Drug binding sites of p-glycoprotein

Previously, only two regions of photoaffinity labelling were identified, one within each half of p-glycoprotein (Morris *et al*, 1994). These regions were not part of a single drug-binding site, but represented two separate drug-interaction domains (Dey *et al*, 1997). In another study, it was demonstrated that p-glycoprotein contained two drug binding sites that exhibited positive cooperativity for dug transport and distinct substrate specifications (Shapiro & Ling, 1997). Recent studies indicated the existence of a third drug binding site on p-glycoprotein with a positive allosteric effect on drug transport, however, this site is not capable of transporting drugs (Shapiro *et al*, 1999). In another study, at least four distinct drug interaction sites on p-glycoprotein were detected, three of these sites were classified as transport sites and the fourth site as a regulatory site (Martin *et al*, 2000).

2.7.3. The ATP dependence of p-glycoprotein

By using colchicine, it was shown that drug transport by p-glycoprotein was ATP-dependent and required ATP-hydrolysis (Sharom *et al*, 1993). P-glycoprotein-ATPase generates an ATP-hydrolytic activity (Rao & Scarborough, 1994). P-glycoprotein seemed different from many other membrane-bound ATPase in that it hydrolysed ATP at a very high rate in the absence of drug substrates (Sharom *et al*, 1993).

Rosenberg & co-workers (2001) proposed a mechanism for the ATPase cycle of p-glycoprotein. The transport cycle is initiated by drug binding to a high-affinity site on the transmembrane domains for the inner leaflet of the lipid bilayer (Figure 2.4., step 1). ATP is then bound by the nucleotide binding domains, inducing a conformational change that results in a reduction in the

affinity of drug binding and re-orientation of the site such that it is exposed to the extracellular environment (Figure 2.4., step 2). Thus, ATP binding, rather than hydrolysis, appears to provide the energy for drug translocation. Following ATP hydrolysis, but prior to release of ADP/P_i, there is a further conformational change, although the drug-binding site retains its low affinity state (Figure 2.4., step 3). Following release of ADP and/or P_i, the protein returns to its starting configuration and regains its high affinity drug binding (Figure 2.4., step 4).

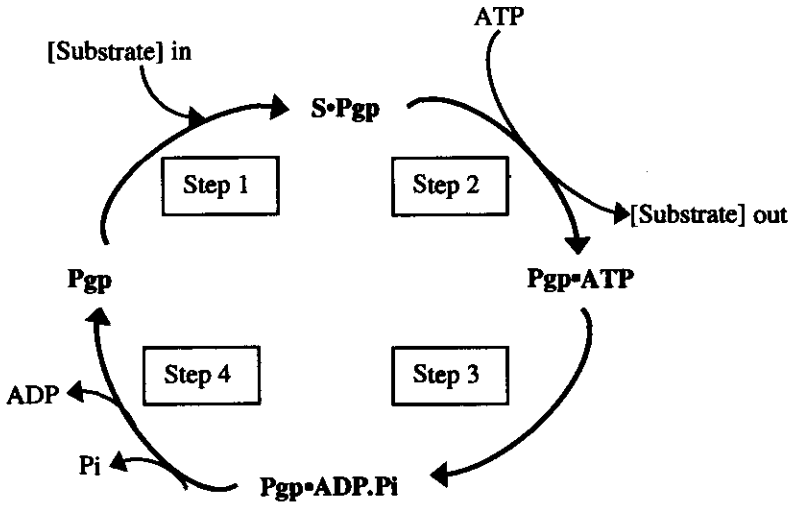


Figure 2.4: The ATPase cycle of p-glycoprotein binding. Pgp = p-glycoprotein, S = substrate (Rosenberg *et al*, 2001).

2.8. Cytochromes P450 and P-Glycoprotein

There is a striking overlap between substrates for CYP3A4 and p-glycoprotein, including cyclosporine, FK506, diltiazem verapamil, etoposide and pactaxol (Lin *et al,* 1999). Studies with well-known inhibitors of CYP3A4, such as verapamil, ketoconazole and erythromycin, have documented that these compounds also inhibit the activity of p-glycoprotein (Edwards *et al,* 1999). Intestinal CYP3A-dependent drug metabolism and p-glycoprotein-mediated counter-transport may act in a co-ordinated manner to determine oral drug bioavailability and pharmacokinetic variability, and play an important role in drug interactions (Soldner *et al,* 1999). See a summary of inhibitors and inducers of both CYP3A4 and p-glycoprotein in table 2.6.

Table 2.6: Inhibitors and inducers of both CYP3A4 and p-glycoprotein (Lin *et al,* 1999).

Inhibitor	Inducer
Ketoconazole, itraconazole, erythromycin	Phenobarbital, isosafrole, rifampin, clotrimazole, reserpine, dexamethasone

There is however some evidence from the studies of Lown & co-workers (1997) and Schuetz & co-workers (1995), that suggests that the expression of CYP3A4 and p-glycoprotein is independently and non-co-ordinately regulated. The combination of CYP and p-glycoprotein reflects the perfection of Mother Nature to protect the body against toxic xenobiotics (Lin *et al,* 1999).

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3. Methoxsalen

3.1. Introduction

Methoxsalen is a naturally occurring furocoumarin (Mays *et al,* 1987) present in several plants, including some edible ones such as figs, celery, parsley or parsnip (Tinel *et al,* 1987). Several psoralen derivatives including psoralen, 8-methoxsalen (methoxsalen = 8-MOP), 5-methoxsalen (bergapten = 5-MOP) and trioxsalen (trimethylpsoralen = TMP), have photosensitising properties (Letteron *et al,* 1986). Psoralens are inactive after oral or topical administration unless combine with long-wave ultraviolet light (UVA) (de Wolff & Thomas, 1986). The dramatic efficacy of the combination, oral psoralens (8-MOP) and UVA defined as "photochemotherapy", was first demonstrated by Parrish and co-workers (1974) and confirmed by a large number of subsequent clinical trials (Tsamboas *et al,* 1978). Psoralen UVA-therapy (PUVA) is used in the treatment of psoriasis, vitiligo and cutaneous T-cell lymphoma (Donath *et al,* 1999). Vitiligo is a amelanotic skin disease (Bickers *et al,* 1982) and psoriases is a chronic recurrent inflammatory skin disease of unknown etiology. The underlying problem is one of uncontrolled epidermal cell growth coupled with a rapid cellular turnover, which leads to the formation of thick scales or plaques of epidermal tissue (Stolk & Siddiqui, 1988).

3.2. Structures and clinical formulation

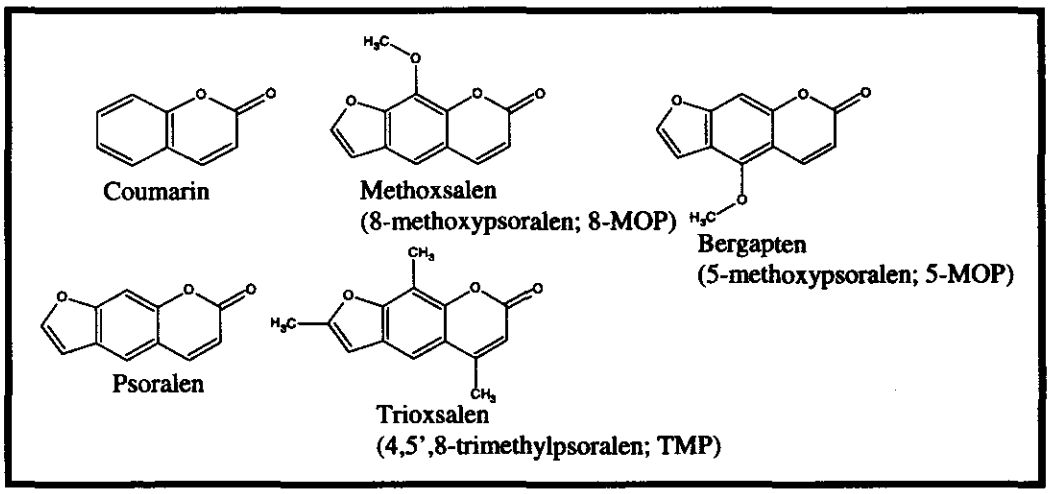

Figure 3.1: Chemical structures of the psoralens (Koeings *et al,* 1997, Letteron *et al,* 1986, de Wolff & Thomas, 1986).

8-MOP is poorly soluble in water, the dissolution of 8-MOP in water is therefore slow and this may cause irregular absorption (Stolk & Siddiqui, 1988). Stolk & co-workers (1987) were the first to demonstrate that the area under the serum concentration-time curve (AUC_{0-4h}) could be significantly increased by administering 8-MOP as an aqueous solution instead of powder in a gelatin capsule (de Wolff & Thomas, 1986). They also found that the maximum concentrations were higher, earlier and more predictable. Favourable results were also observed with soft gelatin capsules containing 8-MOP dissolved in polyethylene glycol (PEG 400). With liquid oral preparations nausea is experienced more frequently (Stolk & Siddiqui, 1988). After topical application of a 8-MOP emulsion Neild & Scott (1982) found that the plasma concentrations obtained are of the same order as those found following oral administration. Disadvantages of topical administration may be local burns due to over dosage and cosmetically unacceptable patchy pigmentation (de Wolff & Thomas, 1986). Rapid and predictable absorption and subsequent quick elimination of 8-MOP, together with the absence of nausea, rectal administration might have certain advantages over the oral route (Stolk & Siddiqui, 1988), although the latter mode of administration is likely to be preferred by most patients (de Wolff & Thomas, 1986).

3.3. Pharmacokinetics

In table 3.1. is a summary of the pharmacokinetic parameters of 8-MOP.

Table 3.1: Pharmacokinetic parameters of 8-MOP 0.5 to 0.6 mg/kg orally[a] in 18 psoriatic patients (de Wolff & Thomas, 1986).

	Mean (± SD)	**Range**
t_{max} (h)	1.6 (0.47)	0.8–2.5
C_{max} (µg/L)	168 (80.9)	53-277
AUC_{0-t} (µg/L x h^{-1})	479 (327.0)	93–1066
$t_{½}$ (min)	67 (27.1)	36–144
CL (L/h)[b]	180 (178.5)	43–658
Vd (L/kg)[b]	3.2 (2.55)	0.9–8.9

[a] Meladinine® (Basotherm) tablets

[b] Assuming complete absorption.

3.3.1. Absorption

The chemical stability of psoralen compounds is quite good, and absorption from the GIT of the dissolved chemical is rapid. Bioavailability problems, however, may stem from poor dissolution, changes in gastric emptying and first pass effects (Brickl *et al,* 1984).

Table 3.2: Factors influencing bioavailability of drugs (Brickl *et al,* 1984).

Parameter	Mechanism of bioavailability loss
Dissolution	Part of drug not dissolved
Drug stability	Chemical or enzymatic decomposition
Gastric emptying	Emptying delayed or decreased
Intestinal absorption	Incomplete uptake from GIT
First pass effect	Drug conversion to inactive metabolites

Early pharmacokinetic work with 8-MOP pointed towards a high interpatient variability of commonly measured pharmacokinetic parameters. As mentioned earlier, the absorption characteristics of the drug are affected by concomitant food ingestion and by differences in pharmaceutical formulation (Ehrsson *et al,* 1979). A high protein, light meal, seems preferable as it suggests a decrease in intra-individual variation and increased bioavailability (Schafer-Korting & Korting, 1982). These results were confirmed with other studies in the literature (Ehrsson *et al,* 1979). It was also reported that levels decrease after a meal or if the drug was taken with fatty food (Brickl *et al,* 1984).

3.3.2. Distribution

Rate and extent of distribution of a drug to the different tissues is determined by several factors, such as affinity to tissue in question, affinity to blood constituents (protein binding, penetration into red blood cells), perfusion of tissues with blood, and ability of drug to penetrate membranes. The lipophilicity of 8-MOP favours diffusion through biological membranes, and thus absorption and penetration into tissues and cells. 8-MOP is non-ionised under physiological conditions and therefore its absorption is pH-independent. Psoralens spread rapidly to most organs, but binding seems to be short-lived and reversible (Brickl *et al,* 1984).

3.3.2.1. Protein binding

8-MOP was demonstrated to bind strongly to human serum albumin. The protein may work as a carrier of the drug, it may alter the elimination and metabolism of the drug, and its affects distribution of the drug, as usually only free drug passes biological membranes (Brickl *et al,* 1984). About 75–80% of the drug will be reversibly bound to serum proteins and only 20–25% of the total concentration represents the effective (or bioavailable) drug concentration, which is free to diffuse and equilibrate with the various skin layers (Artuc *et al,* 1979). At therapeutic concentrations only tolbutamide may displace 8-MOP from its binding site to a clinically relevant extent, the concomitant use of tolbutamide and 8-MOP may therefore lead to enhanced photosensitivity (De Wolff & Thomas, 1986).

3.3.3. Metabolism

8-MOP is activated by CYP into chemically reactive metabolites, which bind covalently to microsomal proteins and inactivate CYP (Letteron *et al,* 1986). 8-MOP is therefore a potent suicide inhibitor of hepatic and renal CYP. Inactivation of CYP is associated with a small loss in CO-binding capacity (Labbe *et al,* 1989) in the presence of an NADPH regenerating system (Tinel *et al,* 1987) and a greater decrease in monooxygenase activities in rats (Labbe *et al,* 1989). Aromatic oxidations are usually mediated by the CYP via an epoxide pathway (Brickl *et al,* 1984). 8-MOP is extensively oxidised on its furan ring (Tinel *et al,* 1987) to 6-(7-hydroxy-8-methoxycoumaryl)-acetic acid (Mays *et al,* 1987) with the presumed formation of a reactive epoxide (Tinel *et al,* 1987). Epoxides, which bind covalently to macromolecules and cause tissue damage, have been proposed as reactive intermediates of other furans, including furosemide and 4-ipomeanol. Furthermore, 8-MOP is also metabolised in the rat to a quinone-hydroquinone redox pair. Redox recycling of similar compounds with concomitant production of reactive oxygen species has been suggested as one of the mechanisms for the cytotoxic effects of benzo(a)pyrene. An analogous quinone imine, which binds to sulphur-containing nucleophiles, apparently causes the hepatoxicity of acetaminophen (Mays *et al,* 1987). Even at low substrate concentrations, aromatic oxidations are often saturated. This is probably the reason for the on-dose linearity of the 8-MOP plasma levels (Brickl *et al,* 1984). Many monooxygenase activities are decreased markedly, as well as the metabolic activation of acetaminophen, carbon tetrachloride and chloroform, suggesting that the inactivation process affects, to some extent, several CYP isoenzymes (Labbe *et al,* 1989). 8-MOP has been shown to undergo very little O-demethylation in rats, dogs and humans (Letteron *et al,* 1986).

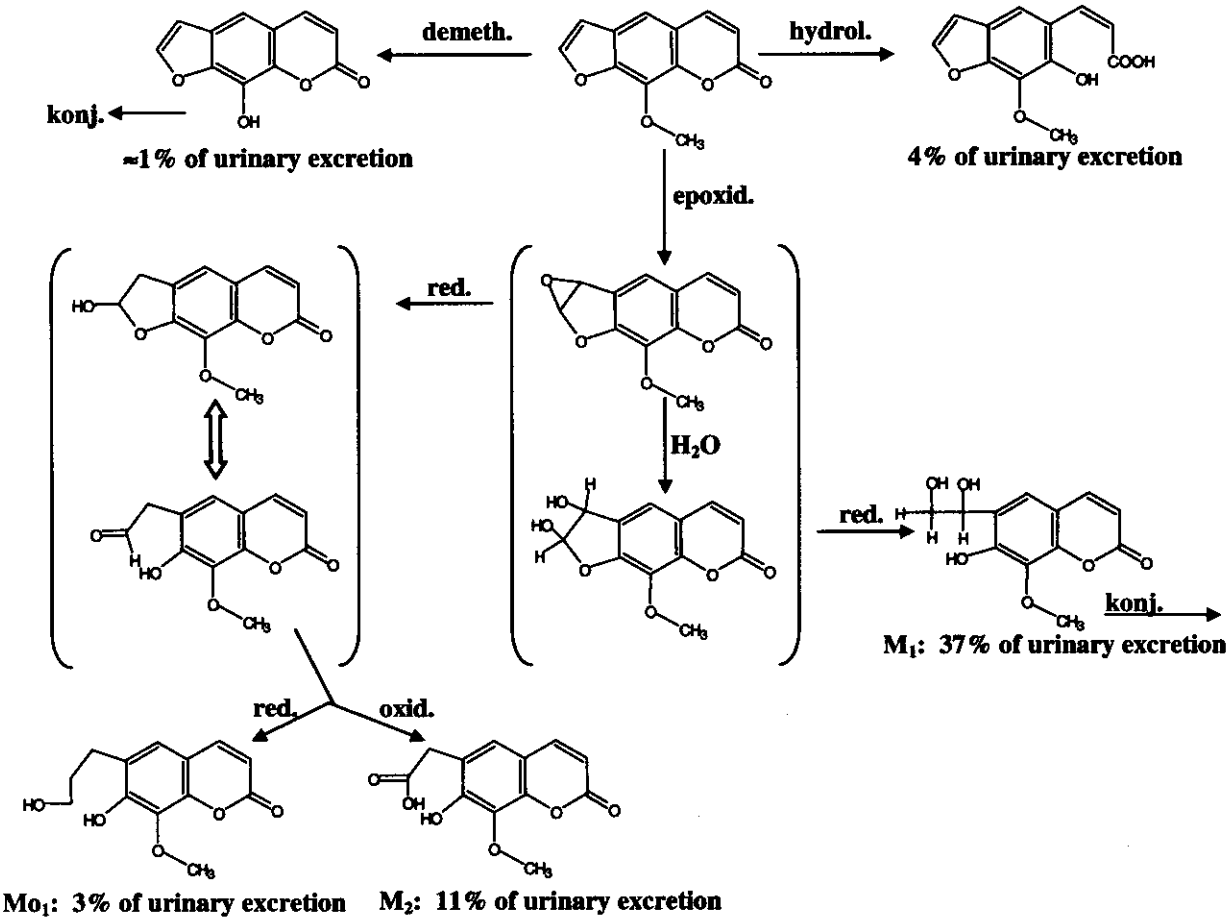

Figure 3.2: Proposed metabolic pathway of 8-MOP (Schmid *et al,* 1980).

Studies of Mays & co–workers (1987) showed that 8-MOP administered acutely is a potent inhibitor of drug metabolism *in vivo* in the rat. It inhibits the biotransformation of caffeine (CA), phenytoin (DPH) and hexobarbital (HB) but does not appear to inhibit the phase 2 metabolism of 5-(4'-hydroxyphenyl)-5-phenylhydantoin (HPPH). Upon chronic administration, 8-MOP is an inducer of the metabolism of CA but not of HB, DPH or HPPH, suggesting that the drug is a selective inducer of CYP. The pattern of induction resembles that of polycyclic aromatic hydrocarbons (PAH). Additional work has shown that 8-MOP also is a potent inhibitor of CA elimination in humans, indicating that the rat is a useful animal model for investigating interactions of 8-MOP with drug metabolism (Mays *et al,* 1987).

The intrinsic clearance and absence of measurable quantities of the unchanged drug in the urine (de Wolff & Thomas, 1986) and bile (Brickl *et al,* 1984) indicate a high metabolic clearance of 8-MOP in man. 8-MOP has a short half-life and undergoes extensive biotransformation. It has therefore been suggested to have a high degree of first-pass metabolism (de Wolff & Thomas, 1986).

3.3.3.1. Inactivation of CYP by 8-MOP

The tricyclic structure of psoralens (Figure 3.1.), allows their reversible intercalation between pyrimidine DNA bases. The furan moiety of a molecule of 8-MOP comes into contact with a pyrimidine base of one strand of DNA, the pyrone moiety of 8-MOP may come into contact with another pyrimidine base of the other strand of DNA (Fouin-Fortunet *et al,* 1986). Cross-links between opposite strands of DNA are subsequently formed which in turn may account for the known ability of psoralen to inhibit DNA synthesis and replication (Bickers *et al,* 1982).

8-MOP was initially identified as a potent, mechanism-based microsomal CYP inhibitor by Pessayre and co-workers (Kharasch *et al,* 2000). Tinel and co-workers (1987) reported that 8-MOP is activated by rat liver CYP into reactive metabolites, which covalently bind to microsomal proteins (Tinel *et al,* 1987). This covalent binding to protein requires NADPH and oxygen, suggesting that the reaction is mediated by CYP. The reaction obeyed Michaelis-Menten kinetics and was enhanced by prior treatment with β-naphthoflavone (BNF) and phenobarbital (PB) (Mays *et al,* 1989). The Michaelis-Menten mechanism for the catalysis of biological chemical reactions is one of the most important chemical reaction mechanisms in biochemistry.

The Michealis-Menten mechanism for enzyme kinetics is:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + \text{products}$$

E is the enzyme, S is the "substrate", and ES is an enzyme-substrate complex. S binds reversibly to the E in the first reaction. Enzyme velocity as a function of substrate concentration often follows the Michaelis-Menten equation.

$$V = \frac{V_{max}[S]}{[S] + K_M}$$

V is the velocity and V_{max} is the limiting velocity as substrate concentrations get very large. K_M is expressed in units of concentration, usually in Molar units. K_M is the concentration that leads to half-maximal velocity.

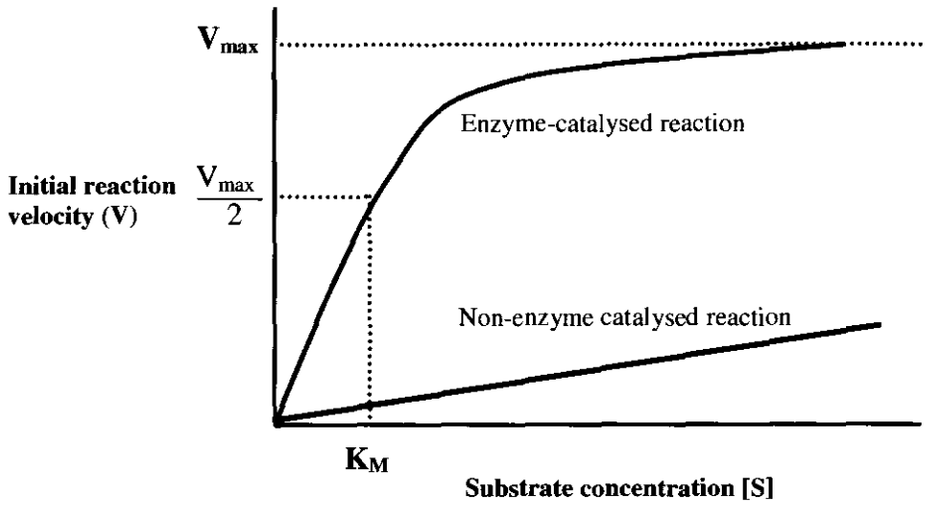

Figure 3.3: Michaelis-Menten kinetics.

As a consequence of both competitive inhibition by 8-MOP itself, and suicide inactivation by 8-MOP metabolites, Tinel & co-workers concluded that the drug is a potent inhibitor of rat liver CYP (Tinel *et al,* 1987). Inactivation of CYP by an 8-MOP metabolite may be involved also in the saturable first-pass effect of 8-MOP, whereas metabolic activation may have bearing on the mutagenicity and hepatotoxicity of 8-MOP (Fouin-Fortunet *et al,* 1986). According to preliminary data from the laboratory of Maenpaa & co-workers (1994), members of the CYP3A subfamily may be partly involved in 8-MOP metabolism in human liver microsomes.

The major site of metabolism for cyclosporin is the CYP-dependent mono-oxygenase (CYP3A4) system in the liver and the enzymes in the intestinal membranes (Fahr, 1993). CYP3A is the principle enzyme that produces the three major cyclosporin metabolites (M1, M17 and M21) in the liver, and is also found in enterocytes (Kolars *et al,* 1991).

It was subsequently shown that 8-MOP was the most potent, and a rather selective, rapid, inhibitor of human liver microsomal CYP2A6 activity (Kharasch *et al,* 2000). Because it is capable of inactivating human CYP2A6 at physiologically relevant concentrations, it carries the potential of causing a serious drug-drug interactions with any drug, compound, or toxin whose clearance is largely dependent on CYP2A6 (Koeings *et al,* 1997). The data of Maenpaa & co-workers (1994) indicates that although 8-MOP is capable of interacting with CYP2A6, it is not a substrate of CYP2A6. This is analogous to the situation with quinidine, which is a potent inhibitor of CYP2D6 but is metabolised by CYP3A isoforms (Maenpaa *et al,* 1994).

Data presented by Zhang & co-workers (2001) demonstrated that tryptamine, tranylcypromine (TCP) and 8-MOP have high specificity and relative selectivity for CYP2A6. Imitating the gene defect by chemical inhibition with *R*-(+)-TCP may be an effective way to treat nicotine dependence (Zhang *et al,* 2001).

The data of Kharasch & co-workers (2000) suggests that a single-dose 8-MOP is a partially effective but not an optimal clinical CYP2A6 inhibitor probe. Higher or multiple oral doses might achieve greater CYP2A6 inhibition (Kharasch *et al,* 2000).

3.3.3.2. Inter- and intraindividual variability

It is advisable to use doses well above the saturation process. Both inter-and intraindividual variability in plasma levels tends to be large at doses that are in the range of the saturation process. With 8-MOP the dose is limited by the onset of systemic side effects like nausea. 0.4-12 mg/kg 8-MOP are sufficient for saturation of first pass effect, only if the release rate is very quickly (Brickl *et al,* 1984).

Considerable variation of both the time (T_{max}) and the height (C_{max}) of the maximum serum concentrations of 8-MOP are observed. This variation is due to the variable absorption of 8-MOP from the different formulations and variability of the extent of first pass elimination of 8-MOP. After oral administration, 8-MOP is subjected to extensive, saturable "first pass elimination", which means that after administration of a low 8-MOP dose, none or only small amount of unchanged 8-MOP reaches the general circulation. With doses higher than the "breakthrough dose", liver enzymes are saturated and serum concentration of 8-MOP rise rapidly. Therefore no linearity exists between dose and the height of the serum concentration in the dose range around the breakthrough dose, 0.23 mg/kg (Stolk & Siddiqui, 1988).

3.3.3.3. 8-MOP and smoking cessation

The primary psychoactive substance in tobacco responsible for establishing and maintaining tobacco dependence is nicotine. It is primarily metabolised (~70%) to its inactive metabolite cotinine by the genetically variable enzyme CYP2A6. Several procarcinogens, such as tobacco-related nitrosamine 4-(methylnitros-amino)-1-(3-pyridyl)-1-butanone, aflatoxin B1, and hexamethyl-phosphoramide are also activated by CYP2A6 (Zhang *et al,* 2001). The research of Sellers & co –workers (2000) shows CYP2A6 inhibitors like 8-MOP increases nicotine

concentration in the blood. Two studies were performed on the effect of 8-MOP on nicotine metabolism and craving in smokers with normal CYP2A6 metabolism not trying to quit smoking. Those participants who received 8-MOP reported far less desire to smoke, smoked fewer cigarettes, had longer intervals between cigarettes and took fewer puffs on each cigarette (Zickler, 2000). By inhibiting the activity of CYP2A6 a potential component of a potent new treatment for nicotine dependence may be represented. This inhibition could reduce smokers' exposure to the harmful constituents of tobacco smoke while serving as part of a step-by-step program of reduction leading to cessation of smoking. Nicotine replacement therapy (NRT) is a method of smoking cessation that aims to reduce smokers' craving for nicotine. NRT is available as a gum, nasal spray, inhaler, or skin patch. Because nicotine is quickly metabolised in the liver and not enough of it gets into the bloodstream to reduce the craving, NRT does not come in a pill. If nicotine could be combined with a drug like 8-MOP, which inhibits its metabolism, oral replacements would be viable. Most patients prefer taking an oral medication, and because 8-MOP eliminates almost completely the activity of CYP2A6, which varies from person to person, its use with a nicotine pill could result in more predictable response to nicotine replacement that is possible with either patch or gum (Zickler, 2000). Zhang & co-workers (2001) has demonstrated that 30 mg of oral 8-MOP plus 4 mg of nicotine attenuated nicotine clearance, increased its bioavailability and decreased cigarette smoking by 24% (Zhang *et al,* 2001).

3.3.4. Elimination

Elimination half-life for 8-MOP is about 1 hour (Stolk *et al,* 1987). Less than 5% can be detected in the faeces and within 12 hours after oral administration more than 90% of 8-MOP can be found in the urine primarily as glucuronides or as hydroxylated moieties (Bickers *et al,* 1982). This finding might suggest that the drug belongs to the group of drugs with a liver clearance which approaches liver blood flow, in other words, 8-MOP has a high degree of first-pass metabolism (Ehrsson *et al,* 1979).

3.4. References

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4. Cyclosporine

4.1. Introduction

Cyclosporine is a neutral, lipophilic, cyclic undecapeptide compound of fungal origin (Ducharme *et al,* 1993). The drug forms the cornerstone of therapy to suppress organ rejection after transplants and reduces the manifestations of some autoimmune diseases (Kahan, 1989). In order to achieve effective immunosuppression with a minimum of nephrotoxicity, the blood concentrations of cyclosporine must be maintained within a relatively narrow range (Ducharme *et al,* 1993).

4.2. Structure and clinical formulation

Cyclosporine is extracted from *Tolypocladium inflatum Gams* with a molecular weight of 1203, (Kahan, 1989).

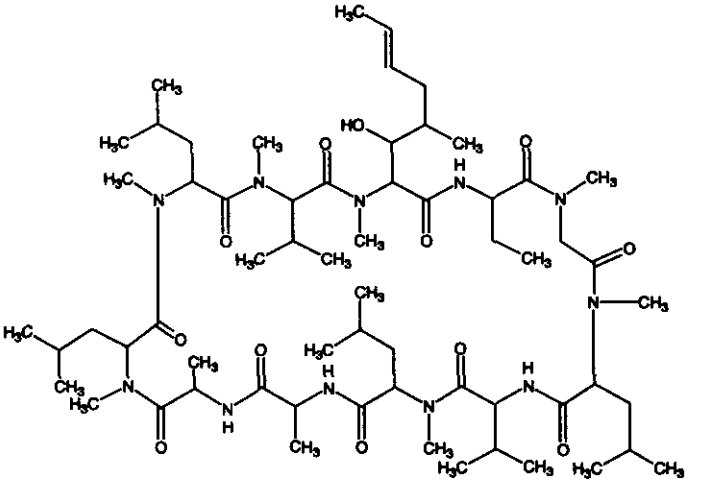


Figure 4.1: The chemical structure of cyclosporine (Kahan, 1989).

Cyclosporine is stabilised with castor oil (Cremophor) and olive oil vehicles for intravenous and oral administration, respectively. To avoid drug interactions with plasticizers in intravenous-solution bags, cyclosporine is dispensed from glass bottles (Kahan, 1989).

4.3. Pharmacokinetics

Table 4.1: Pharmacokinetic characteristics of cyclosporine. The range for peak plasma concentrations is about 150 to 1000 µg/L, and the dose 5 to 12 mg/kg/day (Scott & Higenbottam, 1988).

Parameter	Value
Absorption	≈ 8 - 60%
First-pass metabolism	≈ 10 – 27%
Enterohepatic recirculation	Nil (metabolites only)
Hepatic metabolism	≈ 99%
Vd_{ss}	1.8 – 13.8 L/kg
$t_{½\alpha}$	0.1 – 1.7 hours
$t_{½\beta}$	– 15.8 hours
Billiary excretion	> 90%
Bound to plasma lipoproteins (mainly HDL, LDL)	25 – 30%
Bound to other plasma proteins (α_1-acid glycoprotein, albumin)	5%
Found in erythrocytes	50%
Found in leucocytes	5 – 20%
Free drug	5%

Wideman (1983), analysed 245 oral pharmacokinetic profiles in 87 patients, ranging for 10- to 24-hour studies. A mean time of first serum peak was 3.46 ± 1.89 h, with the mean peak level of 1241 ± 841.2 ηg/ml (Wideman, 1983). The cyclosporine levels in serum are illustrated in figure 4.2.

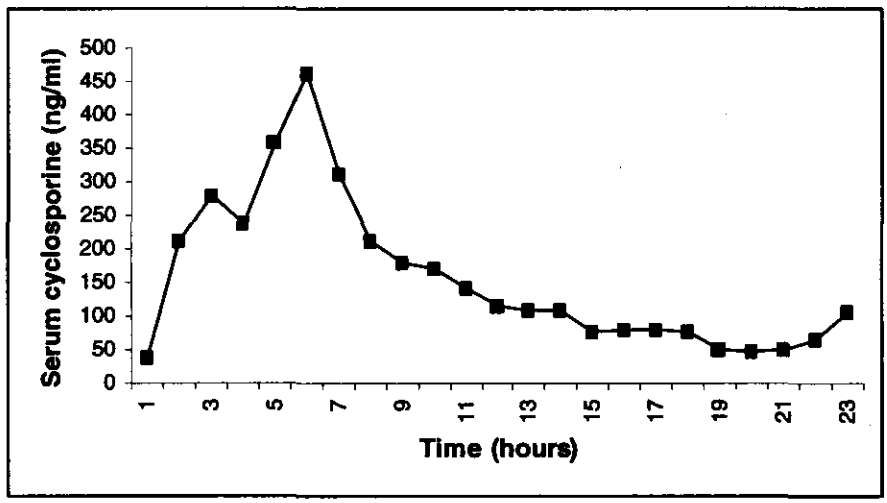


Figure 4.2: Cyclosporine levels in blood (serum) following an oral dose (Wideman, 1983).

4.3.1. Absorption

Absorption of cyclosporine occurs predominantly in the small intestine where it's also metabolised (Fahr, 1993). After oral administration the systemic absorption of cyclosporine is poor, highly variable (Jones, 1997), slowly and incompletely (Fahr, 1993). After absorption from the intestine via the lymphatics, the blood circulation carries cyclosporine to the liver, where it is extracted and excreted in the bile and eliminated through or reabsorbed from the intestine. Reabsorption is termed enterohepatic cycling or hepatic recirculation (Wideman, 1983).

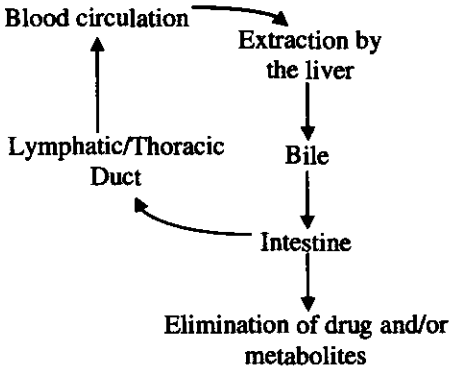


Figure 4.3: Enterohepatic cycling (Wideman, 1983).

The variability in the relative bioavailability is quite high, even in healthy volunteers (Fahr, 1993). Bioavailability is defined as the relative proportion of the administered dose that enters the systemic circulation and the rate at which the drug appears in the bloodstream (Wideman, 1983). The variable absorption of cyclosporine may account for the highly variable bioavailability and the fact that the liver mainly eliminates cyclosporine (Fahr, 1993). These variations probably reflect differences in the ability of intestinal chyme to disperse cyclosporine from its vehicle. The absorption process is adversely affected by biliary diversion, cholestyramine therapy, cholestasis, and slow gastric emptying; increased gastrointestinal motility; steatorrhea and reduced pancreatic exocrine secretion. The presence of normal serum low-density lipoprotein levels promote the absorption of the drug (Kahan, 1989). Other factors such as co-medication, type of transplant, amount and composition of bile in the gut, the lipophilicity of cyclosporine (Fahr, 1993) and the lack of dispersing the drug formulation in a drink (Grevel, 1988) may also influence the variability in absorption (Fahr, 1993).
The effects that can influence absorption are summarised in table 4.2.

Table 4.2: Factors affecting cyclosporine absorption (Lemaire *et al,* 1990).

Factor	Observation
Bile flow	Increased absorption after clamping of external bile drainage
Food	Effect on absorption controversial
Lymph	Lymphatic absorption in rat: 0,4% of dose
Duration of therapy	Increased absorption with time
Gastrointestinal status	Decreased absorption by gastrointestinal dysfunction

The extraction capability of the liver can be another source of variation. Cyclosporine appears in the blood after 0 to 0.9 hours and absorption half-life ranges from 0.5 to 2 hours (Fahr, 1993). Peak blood or plasma concentrations are normally reached within 3 to 4 hours after administration (Lemaire *et al,* 1990).

4.3.2. Distribution

Due to its high lipophilicity, cyclosporine distributes readily across most of the biologic membranes and is extensively distributed in the body (Fahr, 1993).

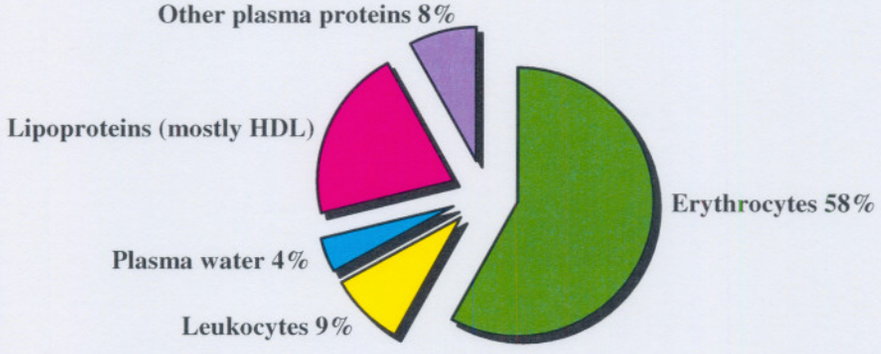


Figure 4.4: Distribution of cyclosporine in whole blood at room temperature (Fahr, 1993).

Inside the erythrocytes cyclosporine is bound to a cytosolic protein named cyclophylin. The blood distribution of cyclosporine depends on drug concentration, temperature, hematocrite and lipoprotein concentration (Lemaire *et al,* 1990). Cyclosporine is primarily bound to lipoproteins of high, low or very low density (Kahan, 1989) in the plasma and may change the lipoprotein concentrations in blood by itself (Lemaire *et al,* 1990). The toxic effects of cyclosporine on the central nervous system are enhanced if serum cholesterol levels are below 3.1 mmol/L. Conversely, hypertriglyceridemia may reduce drug side effects (Kahan, 1989). Variation in lipoprotein concentration is large within a patient population and one can expect important inter-

and intra-individual variations of the overall plasma protein binding and blood distribution of cyclosporine (Lemaire *et al,* 1990). The liver is the major depot for the drug, followed by the pancreas, fat, blood, heart, lung, kidney, and neural and muscular tissue (Figure 4.5.) (Kahan, 1989).

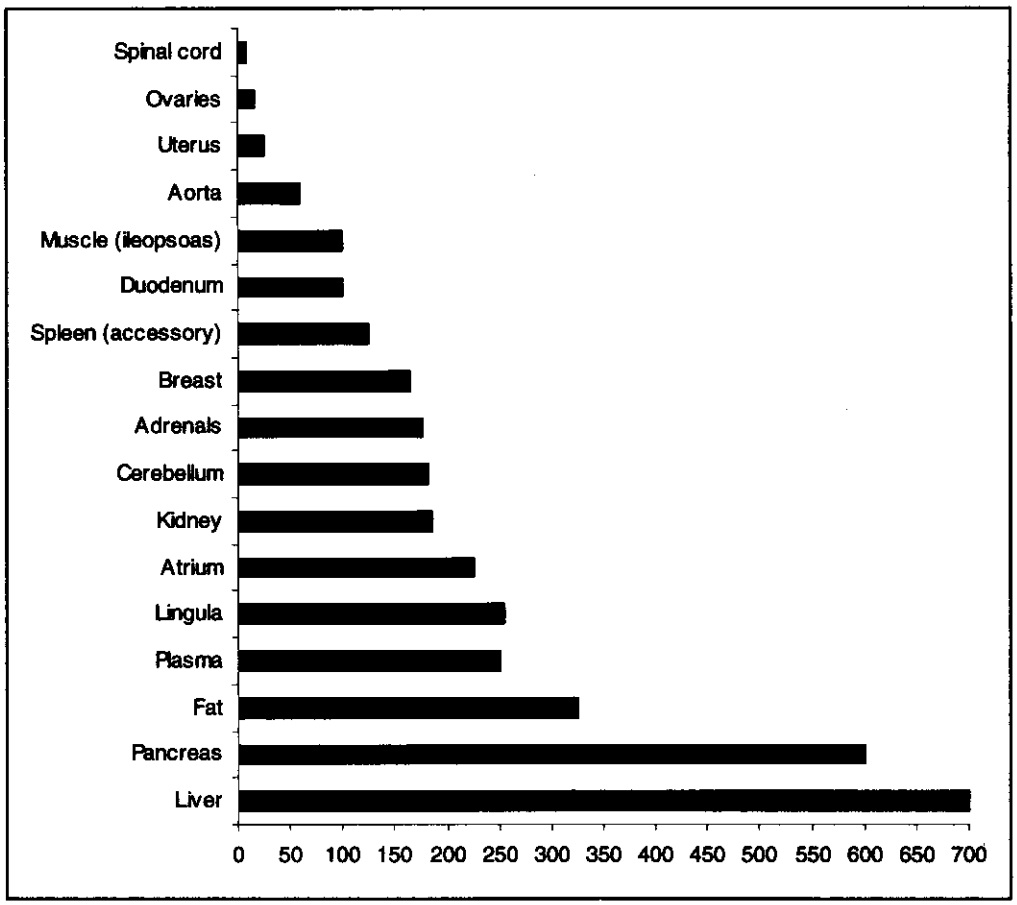


Figure 4.5: Cyclosporine tissue levels expressed as ηg/g tissue wet weight determined on samples obtained from various organs at immediate postmortem examination (Kahan *et al,* 1983).

Nonsignificant concentrations of cyclosporine were found in brain and cerebrospinal fluid and significant amounts were found in placenta en breast milk (Lemaire *et al,* 1990). The low passage of cyclosporine through the blood-brain barrier is surprising because of the lipophilicity of the drug and the long durations of treatment. Pharmacokinetic studies also showed that despite the high lipophilicity, the distribution of cyclosporine is restricted to lean body mass (Fahr, 1993).

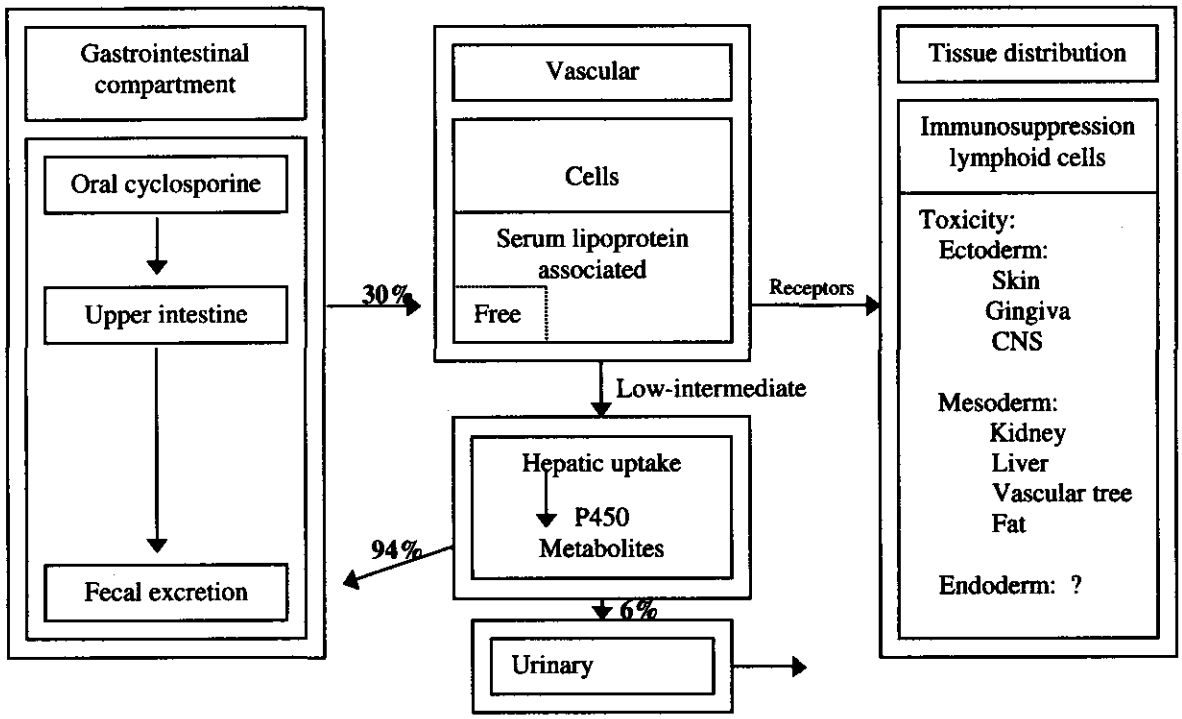


Figure 4.6: Distribution and elimination of cyclosporine. CNS denotes central nervous system (Kahan, 1989).

The drug is absorbed from the gastrointestinal tract into the vascular compartment, where the major drug fraction becomes cell-associated, and the largest serum fraction binds to lipoproteins, with only a small "free" fraction. The tissue distribution of the drug causes immunosuppression of lymphoid cells on the one hand, and toxicity to ectodermal and mesodermal structures on the other. After the liver takes up the drug, it is metabolised by the cytochrome P450 system, producing metabolites that are primarily excreted in the bile and to a small extent in the urine (Kahan, 1989).

4.3.2.1. The effect of cyclosporine on the cellular distribution and content of cyclophilin

Cyclophilin is the major intracellular binding protein for the immunosuppressive drug cyclosporine (Ryffel *et al,* 1991). Cyclophilin share amino acid sequence identity with the enzyme peptidylprolyl cis-trans isomerase and is the leading candidate for the receptor responsible for cyclosporine biological effects. Cyclosporine and the metabolites bind to cyclophilin (McDonald *et al,* 1992). Its presence in many eukaryotic cells and conservation of structure suggests that cyclophilin may play an important role in cell physiology, maybe even in the early events of T cell activation (Fahr, 1993). Although localised to the cytoplasmic compartment, cyclophilin is also associated with mitochondria, endoplasmic reticulum, Golgi

areas and the nuclear membrane, as well as cells form kidney, liver and ileum (McDonald *et al,* 1992). Recent findings by McDonald & co-workers (1992) suggest that cyclosporine increases the amount of cyclophilin in tissue (Fahr, 1993).

4.3.2.2. Difference between whole blood and serum concentrations

After oral administration of 300 mg ^{3}H-Cyclosporine in olive oil to healthy male volunteers, the plasma peak level of total radioactivity, was 585 ± 131 ηgEq/ml 3.7 hours after dosage. In blood cells, the radioactivity amounted to 1304 ± 245 ηgEq/ml. The metabolites detected in plasma and blood cells are metabolites M1, M8, M10, M17, M18, and M21. M17 is the major one and is mainly distributed in blood cells. The in vitro distribution of the major metabolites are similar to that of cyclosporine. Except M21, a minor metabolite, all the metabolites are strongly taken up by blood cells. M17 does not influence the distribution of cyclosporine in blood. All metabolites are less bound to plasma protein than cyclosporine itself and the range for the metabolites is from 24% (M8) to 87% (M21). Cyclosporine binds 93% to human plasma proteins at 37 °C (Maurer & Lemaire, 1986).

Cyclosporine is the main component of the metabolite pattern in plasma after oral administration. The 24-hour AUC of the parent drug represents 52% of the AUC of the cyclosporine and metabolites. M17 accounted for 13% of the total, the remainder ranged from 3% to 7% (Figure 4.7.). In blood cells, cyclosporine is still the major component (27.4% of the 24-hour AUC of total radioactivity), but M17 is quite as important: 24.4%. M1 accounts for 13.8%. M10 and M18 are not found in blood cells. Due to the preferential distribution of cyclosporine and its metabolites into blood cells, the levels of cyclosporine or its metabolites are higher in blood cells than in plasma (Maurer & Lemaire, 1986).

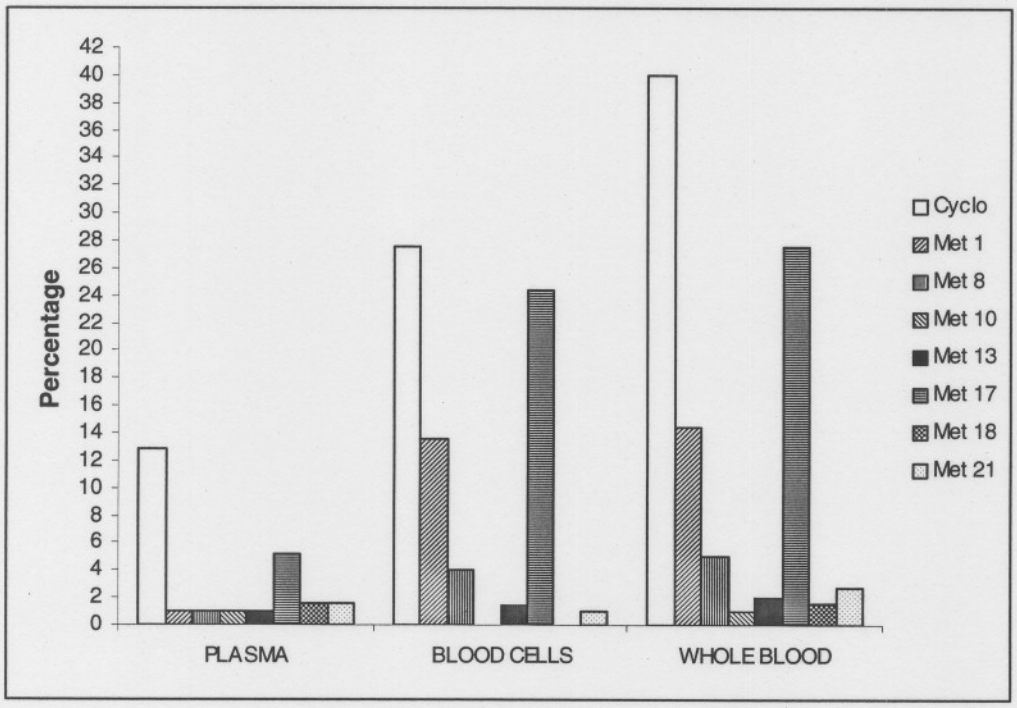


Figure 4.7: Metabolite patterns in plasma blood cells after an oral dose of 300 mg [^{3}H] cyclosporine in male healthy volunteers (Maurer & Lemaire, 1986).

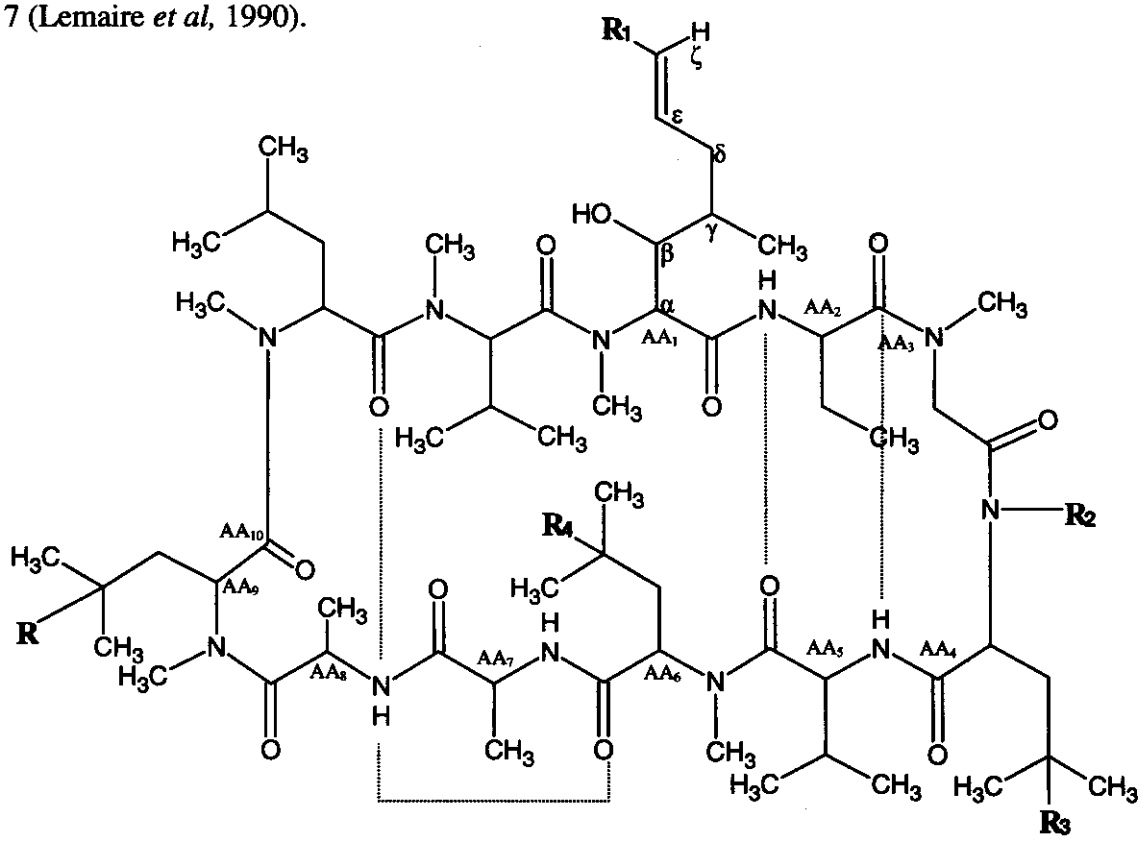
4.3.3. Metabolism

The major site of metabolism for cyclosporine is the cytochrome P450-dependent mono-oxygenase (CYP3A4) system in the liver and the enzymes in the intestinal membranes (Fahr, 1993), where the degradation of cyclosporine to its metabolites is NADPH dependent (Maurer, 1985). Interindividual variability in intestinal CYP3A4 activity has been demonstrated and might explain some of the interpatient vairability in absorption following oral cyclosporine administration (Jones, 1997). In humans, the main metabolic pathways of cyclosporine are mono- and dihydroxylation, and *N*-demethylation. All other drugs metabolised by this system may potentially interfere with cyclosporine metabolism (Fahr, 1993), see Chapter 2. The cyclic nature of the changes in hepatic mono-oxygenase activity that was found by Maurer (1985) was suggested to be caused by a metabolite of cyclosporine that acts as a "suicide substrate" and inactivates cytochrome P450 in a cyclical manner (Maurer, 1985). Being a peptide, it would be expected that cyclosporine would be extensively metabolised in the gastrointestinal tract by enzymes and intestinal flora. However, 7 of the amino acids of cyclosporine are *N*-methylated and may therefore delay but do not completely prevent degradation of the drug in the gastrointestinal tract (Fahr, 1993). While it was thought that metabolism occurred primarily in the liver, the importance of intestinal metabolism was established when metabolites of

cyclosporine were found in portal blood following instillation of cyclosporine into the intestine during the anhepatic phase of liver transplantation (Kolars *et al,* 1991). Kolars & co-workers (1991) found unequivocal and striking extrahepatic metabolism of cyclosporine, which almost certainly took place in the small intestine. They suggest that intestinal metabolism following oral administration of cyclosporine may even exceed hepatic metabolism (Jones, 1997). The principal enzyme (cytochrome P4503A) that produces the three major cyclosporine metabolites (M1, M17 and M21) in the liver is also found in enterocytes (Kolars *et al,* 1991).

4.3.3.1. Amount and number of metabolites

Claims for the number of metabolites vary (Fahr, 1993). More than 25 metabolites have been found in human blood, bile and urine; about 12 of them have been structurally identified with nuclear magnetic resonance and mass spectroscopy (Lemaire *et al,* 1990). All metabolites seem to retain their cyclic oligopeptide structure, conjugation of cyclosporine or of the formed metabolites not being important in humans (Fahr, 1993). The main component in plasma is unchanged CyA, whereas in erythrocytes, metabolites 1 and 17 are quite important; this difference in metabolite patterns may be attributed to the higher plasma protein binding of unchanged CyA. In urine, the main component is metabolite 17, whereas unchanged CyA represents only 0.1% of the administered dose. In bile, only traces of parent drug were detected; a major metabolite in human bile is the acid metabolite resulting from an oxydation of metabolite 17 (Lemaire *et al,* 1990).



Metabolite no.	R	R_1	R_2	R_3	R_4	Other modification	Molecular weight
Cyclosporine	H	CH_3	CH_3	H	H		1202.64
1	OH	CH_3	CH_3	H	H		1218.64
8	OH	CH_2OH	CH_3	H	H		1234.64
9	OH	CH_3	H	H	OH		1220.62
10	OH	CH_3	CH_3	OH	H		1234.64
13	Hydroxylated and N-demethylated derivative of cyclosporine						1204.62
16	OH	CH_3	CH_3	H	OH		1234.64
17	H	CH_2OH	CH_3	H	H		1218.64
18	H	CH_2OH	CH_3	H	H	CH-O-CH-CH_2 of AA1 β ε ζ	1218.64
21	H	CH_3	H	H	H		1188.62
25	H	CH_2OH	H	H	H		1202.64
26	OH	CH_2OH	CH_3	H	H	CH-O CH-CH_2 of AA1 B ε ζ	1204.62
203-218	H	COOH	CH_3	H	H		1234.64

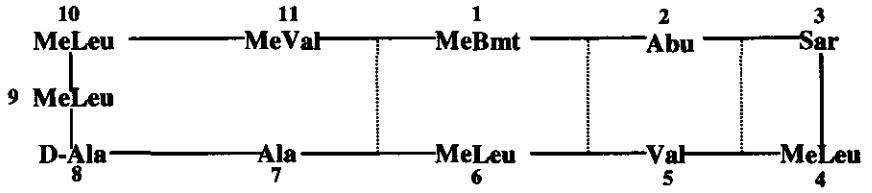


Figure 4.8: Structure of isolated metabolites of cyclosporine – top (Maurer & Lemaire, 1986). Alignment of amino acids of cyclosporine – bottom. The unique structure at position 1 is MeBmt, a novel β-hydroxy, unsaturated, 9-carbon amino acid ((4R)-4-[(E)-2-butenyl]-4, N-dimethyl-L-threonine). Abu denotes α-amino-butyric acid; Sar, sarcosine; MeLeu, N-methyl-L-leucine; Val, valine; Ala, L-alanine; D-Ala, alanine; and MeVal, N-methyl-L-valine.

All the amino acids in the structure have the "S" configuration of natural L amino acids with the exception of the D-alanine in position 8, which has the "R" configuration. The amino acids 1, 3, 4, 6, 9, 10 and 11 are *N*-methylated. Ten of the amino acids are known aliphatic amino acids, characterised upon acidic hydrolysis of cyclosporine. Amino acid 1, a β-hydroxy amino acid, also called C-9 amino acid, is unknown (Maurer, 1985).

4.3.3.2. Variability in the oral pharmacokinetics of cyclosporine

There are marked interindividual differences in the concentration and activity of hepatic CYP3A4. Thummel & co-workers (1994) showed that variation in liver content of CYP3A4 in liver transplant recipients accounted for 66% of the interpatient variation in the clearance of intravenously administered cyclosporine. In contrast, variation in liver CYP3A4 activity does not appear to account for a majority of the interpatient variability in pharmacokinetics when

cyclosporine is administered orally. Lown & co-workers (1997) showed that variation in liver CYP3A4 activity predicted only about one third of the interpatient variability in the oral clearance (CL/F) of cyclosporine in kidney transplant patients. The basis for the remaining variation in CL/F is unknown. One possible explanation may be related to the abundant expression of CYP3A4 in small bowel epithelial cells (enterocytes). Several studies have indicated that intestinal CYP3A4 is responsible for significant first-pass metabolism of orally administered cyclosporine. Lown & co-workers (1997) have found that the enterocyte content of CYP3A4 protein correlates with its catalytic activity (midazolam 1'-hydroxylation) and varies up to ten-fold among patients. Intestinal and liver CYP3A4 expression do not appear to be co-ordinately regulated. It is possible for an individual to have relatively high liver CYP3A4 activity while having relatively low intestinal CYP3A4 activity or *vica verca*. Another potential source of variation in oral cyclosporine pharmacokinetics may be related to the expression of p-glycoprotein. As with CYP3A4, there is significant interindividual variation in the intestinal expression of p-glycoprotein. Given its transport function, high intestinal levels of p-glycoprotein may interfere with drug absorption and contribute to the variation in cyclosporine oral pharmacokinetics (Lown *et al,* 1997).

Lindholm & co-workers (1988) measured total and unbound plasma concentrations of cyclosporine in seven healthy men after single oral doses (12 mg/kg) on two occasions, two weeks apart. They reported an up to two-fold intraindividual and a more than three-fold interindividual variation in plasma AUC of both total and unbound cyclosporin after oral administration (Table 4.3.).

4.3.4. Elimination

The major route of elimination of cyclosporine metabolites is biliary excretion. Most of the drug is ultimately excreted in the faeces after some enterohepatic re-circulation of cyclosporine metabolites. The clearance rate in young people is higher and they need more frequent, larger, doses. Drug clearance rates are slower in patients with decreased levels of serum low-density lipoprotein triglyceride and cholesterol. In the presence of elevated serum levels of bilirubin or alanine aminotransferase, longer dosing intervals are necessary but not with higher levels of aspartate aminotransferase, lactate dehydrogenase, or alkaline phosphatase. Renal excretion is a minor pathway of elimination for cyclosporine (Lemaire *et al,* 1990), (6% of the dose appears in the urine), therefore renal failure does not alter cyclosporine elimination (Kahan, 1989).
Factors affecting elimination can be seen in table 4.4.

Table 4.3: Pharmacokinetic data for total and unbound cyclosporine in plasma after single doses (12 mg/kg) on two occasions (Lindholm *et al,* 1988).

Study period	1 st		2 nd		
Subject	**AUC (ηg.ml^{-1}.h)**	**$t_{½}$ (h)**	**AUC (ηg.ml^{-1}.h)**	**$t_{½}$ (h)**	**AUC1/AUC2**
Total drug					
1	8040	4.8	5810	4.8	1.38
2	4120	1.9	5310	2.8	0.77
3	14320	3.3	7480	3.8	1.91
4	5880	3.8	4100	2.9	1.43
5	4110	2.3	3310	1.8	1.24
6	5200	3.0	5050	6.4	1.03
7	4340	2.1	4520	2.1	0.96
Mean	6570	3.0	5080	3.5	1.25
SD	3690	1.0	1340	1.6	0.38
Unbound drug					
1	129	8.5	83	6.2	1.55
2	70	1.9	85	3.0	0.82
3	231	3.9	136	4.7	1.70
4	101	6.9	60	3.9	1.68
5	59	2.2	64	2.0	0.92
6	82	3.5	99	4.2	0.83
7	59	2.7	73	2.8	0.81
Mean	104	4.2	86	3.8	1.19
SD	61	2.5	26	1.4	0.43

Table 4.4: Factors affecting cyclosporine elimination (Lemaire, *et al,* 1990).

Factor	Observation
Age	Lower clearance in old patients
Sex	Lower clearance in woman
Liver disease	Lower clearance in liver insufficients
Obesity	No significant differences between obese and non-obese patients when dose-normalised on ideal body weight
Circardian variations	Clearance variation between day and night

4.3.5. Drug interactions

A number of drugs can influence the metabolism of cyclosporine. As discussed in Chapter 2, the intestinal and/or hepatic CYP3A4 enzymes can be induced or inhibited which can result in the elevating or lowering of blood cyclosporine concentrations (Jones, 1997). P450 serves as a

primary component of the oxidative enzyme system, and when certain drugs interact to increase the activity of this system, it represents an increased concentration of the P450, known as enzyme induction. This leads to increased biotransformation of the cyclosporine, and therefore decreased the effect of the drug (Wideman, 1983). Steroids such as dexamethasone, rifampin, and phenobarbital (Kronbach *et al,* 1988) increase enzyme activity, subsequently increasing formation of P450. It also increase hepatic blood flow and bile flow, all of which combine to cause decreased blood levels of cyclosporine (Wideman, 1983). On the other hand, macrolide antibiotics, dihydropyridine, calcium antagonists, quinidine and benzphetamine, erythromycin, and steroids such as testosterone and estradiol or dexamethasone are inducers or substrates of cytochrome P4503A3 and have the potential of altering cyclosporine blood levels. Substrates that are metabolised with high affinity by this enzyme (e.g., erythromycin) presumably prolong the elimination of cyclosporine by competing for the same site of metabolism (Kronbach *et al,* 1988). Giving drugs such as furosemide, aminoglycosides, ketoconazole and amphotericin B with cyclosporine can increase the risk of nephrotoxicity. Interactions with digoxin have also been reported where cyclosporine appears to reduce both apparent volume of distribution of digoxin and its total plasma clearance. When the cephalosporins cefuroxime, cephradine, claforan or cetazidime are given to patients receiving cyclosporine, a slight increase in serum liver enzymes may occur, but there is no fall in renal function (Scott & Higenbottam, 1988). Table 4.5. is a summary of the most important interactions.

Cyclosporine is expensive to provide and monitor and the magnitude of these costs may preclude its use (Jones, 1997). There has been much interest in the co-administration of cyclosporine with inhibitors of CYP3A4 in order to increase concentrations in patients having difficulty attaining therapeutic levels and to decrease the cost of therapy with this expensive drug (Ducharme *et al,* 1993). Drugs which alter blood cyclosporine concentrations were initially seen as relatively contraindicated, but once the economic potential was realised, deliberate co-prescription of drugs which allow the dosage of cyclosporine to be reduced while maintaining blood cyclosporine concentrations within the therapeutic range were soon advocated (Jones, 1997).

Table 4.5: Drug interactions with cyclosporine manifesting as alterations in blood concentrations of cyclosporine (Scott & Higenbottam, 1988).

Blood cyclosporine concentration INCREASE	Blood cyclosporine concentration REDUCTION
Acyclovir	Barbiturates
Danazol	Carbamazipine
Diltiazem	Cilastatin
Doxycycline	Corticosteroids
Erythromycin	Dilantin
Ketoconazole	Phenobarbitone
Methyltestosterone	Phenytoin
Metoclopramide	Rifampicin
Nicardipine	Sandostatin
Norethisterone	Sulphadimidine / trimethoprim
Oral contraceptives	
Steroids (prednisone, prednisolone)	
Thiazide diuretics	
Verapamil	
Warfarin	

In 2001, Akhlaghi & co-workers compared cyclosporine pharmacokinetics by using the conventional formulation (Sandimmune) and switched to the microemulsion (Sandimmune Neoral) formulation, in stable heart transplant recipients receiving various cyclosporine metabolic inhibitors (Table 4.6.) (Akhlaghi *et al,* 2001).

In every group studied, administration of cyclosporine in its microemulsified form increased the rate of cyclosporine absorption. This formulation, however, did not increase cyclosporine bioavailability in patients given either ketoconazole, diltiazem or a combination of ketoconazole and diltiazem, in whom absorption already appeared to be optimised (Akhlaghi *et al,* 2001).

Table 4.6: Cyclosporine dose, area-under-the-concentration-time curve at steady state (AUC), maximum concentration (C_{max}), time to reach maximum concentration (t_{max}), and absorption half-lives ($t_{½}$) for cyclosporine administered as the conventional or microemulsified formulation measured in whole blood (Akhlaghi *et al,* 2001).

	Concomitantly administered drugs			
	Group A: cyclosporine (n=11)	**Group B: cyclosporine+Diltiazem (n=11)**	**Group C: cyclosporine+Ketoconazole (n=13)**	**Group D: cyclosporine+Diltiazem+Ketoconazole (n=12)**
Cyclosporine dose (mg/kg per day)	3.90 ± 1.03	2.98 ± 0.64	0.79 ± 0.27	0.90 ± 0.54
AUC (µg hour/liter)				
Sandimmune	3655 ± 1120	3605 ± 1.79	3601 ± 933	4070 ± 1099
Neoral	4911 ± 935	4747 ± 923	3703 ±772	4369 ± 993
C_{max} (µg/liter)				
Sandimmune	827 ± 204	816 ± 183	480 ± 164	574 ± 192
Neoral	1147 ± 307	1288 ± 415	532 ± 93	652 ± 204
ΔC_{max} (%)	+ 43%	+ 59%	+ 17%	+ 15%
T_{max} (hours)				
Sandimmune	2.27 ± 0.47	2.09 ± 0.54	2.61 ± 0.96	2.58 ± 0.90
Neoral	1.55 ± 0.52	1.45 ± 0.69	2.00 ± 0.57	2.33 ± 0.49
Δt_{max} (%)	- 32%	- 26%	- 18%	- 2%
$t_{½}$ (hours)				
Sandimmune	2.72 ± 1.45	1.44 ± 1.16	1.65 ± 1.22	1.81 ± 1.09
Neoral	0.51 ± 0.28	0.58 ± 0.62	0.67 ± 0.34	1.07 ± 0.57

Grapefruit juice was serendipitously shown to increase the bioavailability of some dihydropyridine calcium channel blockers when Bailey & co-workers (1994) used it as a diluent for alcohol in a drug interaction study. It has also been shown to interact with cyclosporine, terfenadine, midazolam and caffeine (Jones, 1997). Grapefruit juice increases the oral bioavailability of a number of drugs (Edwards *et al,* 1999). The presumed mechanism of this effect is inhibition of the extensive first-pass metabolism of these compounds by cytochrome P450 enzymes of the CYP3A subfamily (Ducharme *et al,* 1995) in the gut wall and liver (Edwards *et al,* 1999). Many lines of evidence point to the intestine, rather than the liver as the major site of this interaction *in vivo.* Firstly, the metabolism of some drugs occurs in the intestine itself, for example that of midazolam and cyclosporine. Secondly, CYP3A4 is abundantly expressed in small bowel enterocytes. Thirdly, grapefruit juice does not influence

the clearance of drugs when they are administered intravenously (Eagling *et al,* 1999). Because systemically available cyclosporine is almost entirely eliminated by hepatic metabolism and grapefruit juice had no effect on intravenous cyclosporine, the increase in plasma concentrations and bioavailability with oral dosing is the result of an effect on the gut rather than the liver (Ducharme *et al,* 1995). Reduced gut wall metabolism is consistent with the observation that enterocyte CYP3A4 protein concentration is reduced after ingestion of grapefruit juice. 6',7'-Dihydroxybergamottin, a furanocoumarin identified and isolated from grapefruit juice, is a mechanism-based or suicide inhibitor of CYP3A4. It shares this characteristic with other furanocoumarins, including bergamottin, 8-methoxypsoralen (methoxsalen) and 5-methoxypsoralen (bergapten) (Edwards *et al,* 1999).

In table 4.7. is a summary of the pharmacokinetic parameters obtained from clinical studies where an amount of drugs were taken with grapefruit juice.

Table 4.7: Pharmacokinetic parameters of some drug-grapefruit juice interactions

Drug	Pharmacokinetic parameters		
	AUC	C_{max}	t_{max}
Caffeine	inc. 28%	--	--
Cyclosporine	**inc. 60%**	**inc. 43%**	**inc. 31%**
Diltiazem	NS	NS	NS
Felodipine	inc. 151%	inc. 123%	inc. 91%
Midazolam	inc. 52%	inc. 56%	inc. 79%
Nifedipine	inc. 134%	NS	inc. 50%
Nisoldipine	inc. 76%	inc. 250%	inc. 53%
Nitrendipine	inc. 125%	inc. 106%	NS
Quinidine	NS	NS	inc. 106%
Terfenadine	--	inc. 62%	--
Triazolam	inc. 48%	inc. 30%	inc. 67%

inc. = significant increase; AUC = area under the concentration time curve; C_{max} = maximum plasma concentration; t_{max} = time to C_{max}; NS = no significant change.

Ducharme & co-workers (1993) performed a study where eleven medically stable patients (seven males, 4 females) receiving cyclosporine following kidney transplantation, were instructed to take their usual dose of cyclosporine with water for 1 week, (phase 1), with grapefruit juice (8 ounces) for 1 week (phase 2) and again with water for 1 week (phase 3) (Ducharme *et al,* 1993).

Figure 4.9. illustrates the individual trough cyclosporine concentrations in whole blood for each of the three phases of the study.

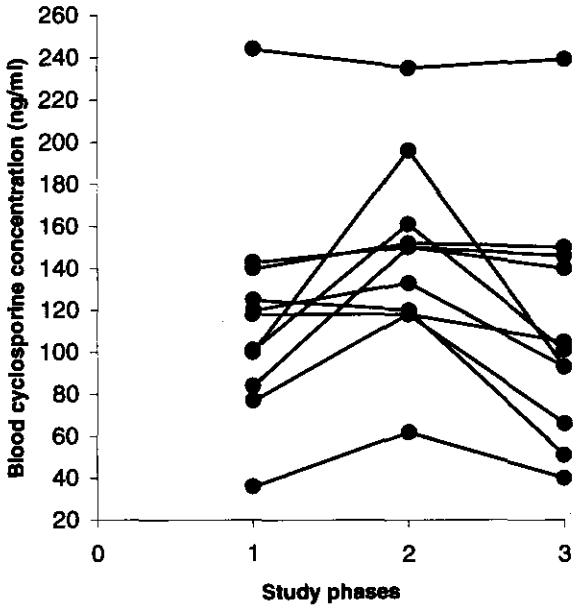


Figure 4.9: Individual trough cyclosporine concentrations in blood for each of the three phases of the study (phase 1 = water; phase 2 = grapefruit juice; phase 3 = water). Concentrations in phase 2 were significantly higher than in either phase 1 or 3 (Ducharme *et al,* 1993).

4.3.6. The role of P-glycoprotein

Cyclosporine is transported by human p-glycoprotein site (Edwards *et al,* 1999). It was clearly indicated by Schinkel & co-workers (1994) that p-glycoprotein played a significant role in the overall brain penetration and elimination of cyclosporine (Lin *et al,* 1999). The protective function of p-glycoprotein is shared with cytochrome P450 enzymes such as CYP3A4, and many compounds have been identified as substrates or inhibitors of both CYP3A4 and p-glycoprotein. Fricker & co-workers (1996) found that after administration of cyclosporine at different sites in the gastrointestinal tract, the absorption of cyclosporine was highly correlated with the quantity of p-glycoprotein messenger ribonucleic acid (mRNA) at that site (Edwards *et al,* 1999).

Cyclosporine analogues, PSC 833 and cyclosporine A were found to inhibit the drug-activated and the basal ATPase activity of p-glycoprotein with high affinity. It was suggested that both compete with the ATPase stimulators for p-glycoprotein-ATPase by interaction with the same or overlapping binding site(s). The ATPase inhibitory effects of these cyclosporine analogues were independent of their being transported by p-glycoprotein (Watanabe *et al,* 1997). The affinity for

cyclosporine increased markedly with the substitution of Glycine^{185} for Valine^{185}, suggesting that amino acid 185 was involved in the cyclosporine interactions with p-glycoprotein (Rao, 1995). It is not clear why certain compounds acted as ATPase stimulators and others as inhibitors. Cyclosporine, PSC833, verapamil, vinblastine and doxorubicin modulated ATP hydrolysis without modulating binding of ATP. One difference in the chemical structure between the cyclosporine analogues (cyclosporine A and PSC 833) and the stimulators was that the stimulators were all cations whereas the cyclosporine analogues were electronically neutral (Watanabe *et al,* 1997). In a study with a selected series of anthracycline analogues with different positive charges, it was showed that the positively charged analogues were better distinguished by p-glycoprotein that their neutral analogues. This study also showed that analogues with increasing lipophilicity, regardless of charge, were able to inhibit drug binding to p-glycoprotein with more efficiency (Lampidis *et al,* 1997). Therefore, it is possible that electronic charge and lipophilicity are important factors for determining the stimulation or inhibition by p-glycoprotein-ATPase (Watanabe *et al,* 1997).

One classification for substrates of p-glycoprotein is to divide them into compounds that interact with p-glycoprotein and activate p-glycoprotein-ATPase and compounds that interact with high affinity with p-glycoprotein, but fail to activate p-glycoprotein-ATPase (Rao & Scarborough, 1994). Some examples of classes of agents that interact with p-glycoprotein are summarised in table 4.8.

It was demonstrated that only those compounds that stimulated ATPase activity are transported by p-glycoprotein (Homolya *et al,* 1993). Cyclosporine and tacrolimus (FK506) are substrates for p-glycoprotein to transport in cultured epithelia layers (Saeki *et al,* 1993). Whereas the measure transepithelial fluxes of FK506 strongly supported this contention, the cyclosporine fluxes were far less convincing, being very near background levels. Cyclosporine did not activate p-glycoprotein-ATPase activity; therefore there was no obvious source of energy for the active transport process. Thus it seemed unlikely that cyclosporine can be actively transported by p-glycoprotein (Rao & Scarborough, 1994). However, by using Caco-2 cells, Augustijn & co-workers (1993) and Fricker & co-workers (1996) demonstrated that cyclosporine is indeed actively transported by p-glycoprotein.

Table 4.8: Agents that interact with p-glycoprotein (Seelig, 1998).

Substrates for p-glycoprotein	Agents that inhibit p-glycoprotein
Antimicrotubule drugs, e.g. colchicine, podophyllotoxin	Calcium channel blockers, e.g. verapamil, nifedipine, azidopine, dihydroperidines
Anthracyclines, e.g. doxorubicin	Anti-arrhythmics, e.g. quinidine, amiodarone
Epipodophyllotoxins, e.g. etoposide	Antihypertensives e.g. reserpine
Antibiotics, e.g. actinomycin D	Antibiotics, e.g. hydrophoid cephalosporins
Others, e.g. mitomycin C, taxol, topotecan, mithramycin	Antihistamines, e.g. terfenadine
	Detergents, e.g. Tween-80
Vinca alkaloids, e.g. vinblastine	Immunosuppressants, e.g. **cyclosporine**, FK506, rapamycin
Protein synthesis inhibitors, e.g. puromycin, emetine	Steroid hormones, e.g. progesterone
	Diterpenes, e.g. forskolin
DNA intercalators, e.g. ethidium bromide	Modified steroids, e.g. tirilaza, tamoxifen
Toxic peptides, e.g. valinomycin, gramicidin D, N-acetyl-leucyl-norleucinal (ALLN)	Lipophilic cations, e.g. tetraphenylphosphonium
	Antidepressants, e.g. tioperidone
	Antipsychotics, e.g. phenothizines

Increased levels of MDR1 and p-glycoprotein were observed in the human colon carcinoma cell line, LS 180, and its drug-resistant subline after treatment with verapamil, nifedipine, nicardipine, diltiazem and cyclosporine. Quinidine and chlorpromazine did not increase the levels of MDR1 and p-glycoprotein (Herzog *et al,* 1993). Verapamil, nifedipine, cyclosporine, quinidine and chlorpromazine are p-glycoprotein antagonists (Gottesman & Pastan, 1993).

Treatment with cyclosporine at therapeutic concentrations also increased p-glycoprotein expression and transport function in human arterial endothelial and rat proximal tubule cells. However, treatment with another immunosuppressive drug, FK506, did not change p-glycoprotein expression at therapeutic concentrations, but at higher supratherapeutic concentrations of FK506, p-glycoprotein expression was also increase in both cell lines (Hauser *et al* 1998). After rats were treated with cyclosporine, p-glycoprotein levels were increased in the kidney, intestine, liver, stomach, heart, lung, testis and spleen. However, the administration of cyclosporine did not alter the level of p-glycoprotein expression in brain tissue. The induction of p-glycoprotein overexpression was a reversible process, since after cessation of cyclosporine administration, p-glycoprotein levels steadily declined in all tissues (Jetté *et al,* 1996).

Cyclosporine and MS-209 specifically bind to p-glycoprotein, thereby reducing drug transport mediated by this compound. Inhibition by cyclosporine was assessed by Terao & co-workers

(1996) by simultaneous intravenous administration of cyclosporine while measuring intestinal absorption by the in-situ jejunal loop method. Cyclosporine is an effective inhibitor of p-glycoprotein with a relatively low inhibitory constant of about 0.08 μM. From the pharmacokinetic parameters reported and the plasma-unbound fraction of cyclosporine at steady-state in rats, the expected intestinal epithelial-free concentration of cyclosporine at steady-state is more that 0.4 μM according to the dosage schedule used in their study. Accordingly, the function of p-glycoprotein-mediated transport of the test compounds should be significantly disrupted by cyclosporine. Intestinal absorption of acebutalol and vinblastine increased 2.6 and 2.2 times, respectively, in response to administration of cyclosporine, whereas acetamide absorption was unaffected. Accordingly, it can be said that p-glycoprotein contributes significantly to the reduction of intestinal epithelial permeability (Terao *et al,* 1996).

4.4. Pharmacological actions

The pharmacological effects of cyclosporine are mostly immunosuppressive and can be summarised as follows:

4.4.1. Effects on T cells

Cyclosporine reversibly inhibits T-cell-mediated allo- and autoimmune responses. The inhibition of helper-inducer and cytotoxic T-cell activities by cyclosporine, along with its promotion of suppressor-cell function, causes immunoregulatory disequilibrium, which sets the stage for unresponsiveness rather than immunity (Kahan, 1989).

4.4.2. Effects on thymus

Cyclosporine not only decreases the total number of thymocytes but also aborts intrathymic T-cell development (Kahan, 1989).

4.4.3. Effects on β cells

The conclusion that cyclosporine spares T-cell-independent, antibody-mediated immunity is based on the failure of the drug to inhibit antilipopolysaccharide (Type I) responses. However, cyclosporine inhibits B-cell responses to some T-cell-independent (Type 2) antigens – namely,

dinitrophenyl-Ficoll and dinitrophenyl-dextran - to polyclonal anti-immunoglobulin antibodies, particularly anti-μ, and to calcium ionophores (Kahan, 1989).

4.4.4. Effects on accessory cells

Accessory cells represent a unitary target that would account for the cell selectivity of cyclosporine, since they have a mandatory role in activating helper and cytotoxic but not suppressor T lymphocytes. The only appreciable effect of cyclosporine on accessory cells is disruption of lymphokine-dependent T-lymphocyte-macrophage interactions amplifying the effector limb of the immune response (Kahan, 1989).

4.5. Uses of cyclosporine

The management of transplant patients relies heavily on the use of cyclosporine to prevent graft rejection. The drug has an effect on the *Toxoplasma gondii* organism, making it particularly suitable as an immunosuppressant in patients infected with this organism after transplantation. Remission of Hodgkin's disease has been reported with cyclosporine. Disorders with an immunological basis, such as rheumatoid arthritis, Sjogren's syndrome, Crohn's disease, systemic sclerosis, Grave's ophthalmopathy, Behcet's disease, aplastic anaemia and skin diseases such as bullous pemphigoid and psoriasis may also respond to cyclosporine administration. It has possible value in lepromatous leprosy (Scott & Higenbottam, 1988) and ill-defined antiparasitic effects in schistosomiasis and malaria. Minimal-change focal, membranous and IgA nephropathies, are also sensitive to cyclosporine treatment (Kahan, 1989).

4.6. Toxic effects

4.6.1. Renal toxicity

Cyclosporine nephrotoxicity is clinically characterised by fluid retention and dependent oedema, increasing serum creatinine and urea concentrations, reduced creatinine clearance, and sometimes a hyperchloraemic, hyperkalaemic, metabolic acidosis. Uncommonly proteinuria can occur and the incidence of azotaemia in the first 6 months of cyclosporine treatment ranges from 45 to 100%, increasing with the duration of exposure (Scott & Higenbottam, 1988). The vasoconstrictive mechanism of action of cyclosporine is controversial (Kahan, 1989). The renal dysfunction is not usually progressive. The morphological changes in the kidney are

intracellular vacuoles and eosinophil inclusions, in the epithelium of the proximal convoluted tubules. These vacuoles may be intracellular accumulations of cyclosporine or swollen endoplasmic reticulum, giant mitochondria may be seen using electron microscopy. Necrosis of the epithelium is very uncommon, but interstitial fibrosis and glomerular sclerosis may occur. Arteriopathy can also be found and this effect may be related to a reduction in renal blood flow and glomerular filtration rate. These are probably the major factors in the development of acute cyclosporine nephrotoxicity, rather than drug accumulation within the tissue. Plasma renin activity is variably affected by cyclosporine, with reports of hypoaldosteronism with reduced renin, hypoaldosteronism with increased renin, and of normal renin and aldosterone values (Scott & Higenbottam, 1988).

The main side effects of cyclosporine on renal function are summarised in table 4.9.

Table 4.9: Effects of cyclosporine on renal function (Scott & Higenbottam, 1988).

Reduced	Increased
Glomerular filtration rate	Fractional proximal absorption
Urine volume	Plasma renin activity
Sodium excretion	Serum potassium
Potassium excretion	

4.6.2. Vascular and haematologic complications

Systemic hypertension often develops within a few weeks of beginning cyclosporine treatment. This adverse effect is one of a variety of vasoconstrictive and/or vessel damaging effects the drug appears to have (Scott & Higenbottam, 1988). Cyclosporine increases ADP-induced platelet aggregation, thromboxane A_2 release, thromboplastin generation, and factor VII activity (Kahan, 1989). In addition, cyclosporine has a vasopressor effect and also may block the formation of prostacyclin in endothelial cells by inhibiting prostacyclin synthetase (Scott & Higenbottam, 1988). This may explain the arteriolopathy and platelet adhesion that result in glomerular thromboses. Atherogenic changes in serum lipids, with increased cholesterol, apolipoprotein B, and triglyceride concentrations, may exacerbate the adverse effects of the drug on endothelium.

Hypersensitivity to the olive-based and castor-oil-based drug vehicles produced a spectrum of clinically reactions, ranging from mild flushing and hypertension to hypotension, crushing chest pain, dyspnea, and respiratory distress after oral and particularly intravenous bolus administration of cyclosporine (Kahan, 1989).

4.6.3. Neurologic and dermal complications

Although cyclosporine is not believed to cross the blood-brain barrier, neurologic side effects occur in about 20 % of transplant patients.
The most common neurological side effects can be seen in table 4.10.

Table 4.10: Neurological side effects of cyclosporine (Scott & Higenbottam, 1988).

Common	Uncommon
Tremor	Encephalopathy
Burning palmar and plantar paresthesias	Cerebellar disorers
Headache	Spinal cord syndromes
Flushing	Expressive aphasia
Depression	Amnesia
Somnolence	Mania
Irritability	
Confusion	
Neuropathies	
Siezures	
Visual hallucinations	

Seizures of new onset may be triggered by hypocholesterolemia, hypertension (particularly in children), intravenous methylprednisolone therapy, hypomagnesemia, infection, haemorrhage, or cerebral infarction. Visual disorders, paresis, disorientation, and coma improve when cyclosporine treatment is discontinued, but recur when it is reinstituted (Scott & Higenbottam, 1988).

4.6.4. Hepatic toxicity

Cholestasis with hyperbilirubinemia (Kahan, 1989), raised levels of alakaline phosphatase and aminotransferases can occur within the first few weeks of treatment with cyclosporine in some patients (Scott & Higenbottam, 1988). Cyclosporine alters calcium fluxes across hepatocyte cell membranes in vitro, elevates serum bile acids, and decreases bile flow (Kahan, 1989). Morphological changes have been seen when cyclosporine and oral contraceptives were combined (Scott & Higenbottam, 1988).

4.6.5. Respiratory side effects

Acute respiratory distress syndrome is the main respiratory side effect during cyclosporine use. Several cases of pulmonary oedema, for which a capillary leak syndrome, probably related to the

cremophor EL vehicle for the intravenous form of cyclosporine, has been postulated (Scott & Higenbottam, 1988).

4.6.6. Cardiac toxicity

There are reports of 'benign' myocardial fibrosis and of pericardial effusion. Scattered interstitial infiltrates of lymphocytes are commonly seen on endomyocardial biopsies of heart transplant patients taking cyclosporine and may be unrelated to rejection. Amorphous fibrous material may be seen in the interstitium (Scott & Higenbottam, 1988).

4.6.7. Ophthalmic toxicity

Toxic retinopathy has been reported in a patient on cyclosporine with a loss of visual acuity, cotton wool spots, intraretinal and preretinal haemorrhages and macular oedema. The patient was normotensive and an embolism in a branch of the retinal artery has also been described (Scott & Higenbottam, 1988).

4.6.8. Metabolic and humoral toxicity

Metabolic changes induced by cyclosporine include hyperkalaemia, hypomagnesaemia, hyperchloraemic metabolic acidosis, hyperuricaemia, hypercholesterolaemia hypertriglyceridaemia and glucose intolerance.

Cyclosporine competes with prolactin for prolactin lymphocyte receptors and also blocks production of thyroid receptor antibodies in Grave's disease (Scott & Higenbottam, 1988). Cyclosporine occasionally produces hyperglycaemia (Kahan, 1989). In high doses cyclosporine reduces insulin production in pancreatic β cells, in standard doses cyclosporine can reduce insulin requirements in recent onset insulin-dependent diabetes mellitus (Scott & Higenbottam, 1988). Cyclosporine decreases testosterone levels, causing gynecomastia in men and impairing spermatogenesis or sperm maturation in rats, with direct damage to Leydig-cell mitochondria (Kahan, 1989).

4.6.9. Gastrointestinal complications

The frequently reported anorexia, bloating, nausea, or vomiting that follows the ingestion of the unpalatable suspension of oral cyclosporine may be ameliorated by placing the drug into soft

gelatin capsules immediately before use. Cyclosporine has no direct toxic effects on the structure of function of gastrointestinal mucosa. Acute pancreatitis is no more frequent or severe when transplant recipients area given cyclosporine than when they receive other immunosuppressive agents (Kahan, 1989).

4.6.10. Effect on bone

Elevated serum levels of the skeletal alkaline phosphatase isoenzyme may be the cause of increased bone formation. The remodelling activity is also enhanced. In rats, cyclosporine causes dose- and duration-dependent osteoporosis with high turnover. All of these effects are independent of serum calcium, magnesium, and calciotropic hormone levels (Kahan, 1989).

4.6.11. Cyclosporine-related malignancy and infections

Both malignancy, particularly of the skin and lymphoreticular system and opportunistic infections, particularly cytomegalovirus, herpes simplex (and zoster) and *Pneumocystis carinii,* can develop whilst patients are treated with cyclosporine (Scott & Higenbottam, 1988).

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5. Analytical methods

The plasma blood sample levels of both methoxsalen and cyclosporine were determined in this study. Methoxsalen was analysed at the Department of Pharmacology, Potchefstroom University, using a high-pressure liquid chromatographic method (HPLC) and cyclosporine using a fluorescence polarization immunoassay method (FPIA). The cyclosporine analysis was performed at the Department of Pharmacology, MEDUNSA. These two methods will be discussed in this chapter.

5.1. Method validation

Method validation has to do with the certainty that a process of procedure is performing consistently and is capable of delivering previously specified results accurately. Method validation ensures that results are reliable and reproducible (Shah *et al,*1992).

In the development of HPLC methods and specific with the method used in this study, the following is recorded during validation:

5.1.1. Linearity

Linearity is the capability of the analytical method to produce results that are directly proportional to the concentration of the analyte before or after mathematical transformation. The linear range is generally taken as the range over which the procedure has been demonstrated to give linear detector response and in the range of the expected levels (Shah *et al,* 1992).

5.1.2. Range

The range is the interval between the lowest and highest concentration of the analyte that has been determined with the prescribed accuracy, precision and linearity (Jordaan *et al,* 2000).

5.1.3. Accuracy

Accuracy is the extend to which the experimental results correlate with the theoretical values (Shah *et al,* 1992). A procedure is accurate if, on the average, the method provides the true answer. Accuracy can also be determined as percentage recovery. Both blank serum and blank

water samples were spiked with a known concentration of 8-Methoxypsoralen (8-MOP). The spiked samples, serum and water were extracted and analysed. The difference between the water samples (theoretical value) and the experimental values of the spiked serum samples is an indication of the extraction efficiency or recovery.

5.1.4. Precision (repeatability)

Precision means that the individual results of a specific method can be repeated after multiple sampling of the same sample and under certain presented conditions. Repeatability can also be defined as the variability within a given laboratory and can be affected by such factors as the analyst, environmental conditions or laboratory equipment (Jordaan *et al,* 2000). Repeatability can be further subdivided into within-day / intra-assay repeatability and between-day / inter-assay repeatability (Shah *et al,* 1992).

5.1.5. Specificity

Specificity can be defined as the ability of an assay to assess unequivocally the analyte of interest in the presence of compounds, which might be expected to be in the sample (Jordaan *et al,* 2000).

5.1.6. Ruggedness/robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal use (Jordaan *et al,* 2000).

5.1.7. Limit of detection

The limit of detection means the lowest amount that can be differentiated reliably from background levels, but not necessarily quantified by an analytical procedure (Shah *et al,* 1992). It can be determined as the amount of analyte that will give a peak twice the height of the baseline noise.

5.1.8. Limit of quantification

The limit of quantification can be defined as the lowest amount of analyte that can be detected and quantified with a stated level of confidence as measured by precision and accuracy (Shah *et al,* 1992).

5.2. HPLC method

Analysis of 8-MOP in pig plasma.

5.2.1. Experimental procedures

5.2.1.1. Materials

8-Methoxypsoralen and 4,5',8-trimethylpsoralen were bought from SIGMA CHEMICAL CO., P.O. Box 14508 ST. Louis, MO 63178 USA.

5.2.1.2. Instrumentation

8-MOP was analysed with a fully automated HPLC-system (G2175 AA 3D Chemstation) consisting of the following:

- Agilent 1100 Series Autosampler (200*343*435 mm).
- Agilent 1100 Series G1322A vacuum degasser (30W).
- Agilent 1100 Series G1311A Quaternary pump.
- Agilent 1100 Series G1315B Diode.
- Multiple wavelength detector (DAD), operated at 254 and 300 nm.
- Personal computer for data manipulation. Product number: G21765AA from Agilent technologies.
- Luna C18 (250×4.6 mm, 5 µm), reversed phase Phenomenex column.

5.2.1.3. Glassware

The glassware were washed with 7XPF cleaning solution from ICN biochemical technologies inc., rinsed with de-ionised, distilled, filtered water to remove all impurities as well as acetone-water before drying at 100 °C for 15 minutes.

5.2.2. Standard solutions of 8-MOP

A mother and daughter solution were prepared. The mother solution were freshly prepared each week and stored at 0 °C. The daughter solutions were freshly prepared from the mother solution each day. Each standard mother solution was prepared by weighing 1 mg of 8-MOP and diluting it to 10 ml with 2 ml HPLC-grade acetonitrile and 8 ml distilled water in a 10 ml volumetric flask giving a concentration of 100 µg/ml. The daughter solution was prepared by diluting 100 µl of the mother solution to 10 ml with distilled water giving a concentration of 1000 ηg/ml.

5.2.3. Standard solutions of TMP

A standard solution was prepared by weighing 1 mg of TMP and diluting it to 10 ml with HPLC-grade acetonitrile in a 10 ml volumetric flask giving a concentration of 100 µg/ml. Of this solution, 100 µl were diluted to 10 ml with HPLC-grade acetonitrile in a 10 ml volumetric flask giving a concentration of 1000 ηg/ml. 300 µl of this solution were added to each 500 µl plasma sample as internal standard to acquire a concentration of 1000 ηg TMP/ml plasma.

5.2.4. Liquid extraction

300 µl of the internal standard (1000 ηg TMP/ml plasma) and 2 ml of HPLC-grade acetonitrile were added to the 500 µl plasma sample in a centrifuge tube and shaken for 2 minutes at room temperature. The sample was centrifuged for 10 minutes at 1000 *g*. The clear supernatant was transferred into another tube were 3 ml of a 60:40 *n*-Hexane:Chloroform were added. The samples were shaken for 2 minutes, centrifuged at 1000 *g* for 10 minutes and then the clear organic phase were transferred to another tube and evaporated at 40 °C in a water bath under dry nitrogen. Prior to HPLC the residue was redissolved in 125 µl of the mobile phase (45:55 ammonium acetate buffer pH 5:acetonitrile HPLC-grade) and 100 µl were injected.

5.2.5. Standard curves

The ratio ($AUC_{8\text{-}MOP}/AUC_{TMP}$) was measured in this study, where 8-MOP ($AUC_{8\text{-}MOP}$) is the assayed compound and TMP (AUC_{TMP}), the internal standard. The standard curves were constructed by plotting the ratios against the 8-MOP concentrations. To construct a standard curve, *nine* pig plasma samples were required. *One* of these plasma samples served as a blank (Blank 1) and was prepared according to the method described in section 5.2.4.

The tables in Appendix 1 of linearity demonstrate the origin of the values for the x- and y-axes of the standard curve and figure 5.1. is an example of typical standard curves utilised in the study. The curves is defined by the following equation:

$$y = mx + c$$

Where

y = $AUC_{8\text{-}MOP}/AUC_{TMP}$

x = concentration of 8-MOP in ηg/ml

m = slope of linear curve

c = y-intercept of linear curve

5.2.6. Validation results

The raw data as compiled for validation can be seen in Appendix 1.

5.2.6.1. Linearity and range

The concentration range of the linear curves was chosen the same as that for the standard curves namely 5.0 to 1000 ηg/ml.

Acceptance criteria for linearity differ from researcher to researcher. The correlation coefficient of the best linear least squares regression model should usually be between 0.98 and 1.00. Since we analysed biological materials, we set our acceptance level at 0.98.

The procedure and calculations for determining linearity was exactly the same as for the standard curves, assessing nine pig plasma samples: one blank and eight standards with different concentrations (5 ηg/ml, 10 ηg/ml, 25 ηg/ml, 50 ηg/ml, 100 ηg/ml, 250 ηg/ml, 500 ηg/ml and

1000 ηg/ml plasma respectively). Table 5.2.6.1. contains the results for three linear curves. The agreement between the curves was determined by calculating a mean, SD and %CV for the regression coefficients, slopes and y-intercepts of the three curves (Figure 5.1). Since the regression coefficient of each linear curve was greater than 0.98 and the agreement between the linear curves were good (%CV = 0.0644% for the regression coefficients and 0% for the slopes), the linearity of the method was considered as acceptable for this study. The relative large CV (9.6656 %) of the y-intercept values is not significant due to the very small order of magnitude of these values.

Table 5.2.6.1: Determination of linearity within the chosen range

	Concentration range (ηg/ml)	**Regression coefficient**	**Slope**	**Y-intercept**
Linear curve 1	5-1000	0.9996	0.0018	0.0096
Linear curve 2	5-1000	0.9994	0.0018	0.0103
Linear curve 3	5-1000	0.9984	0.0018	0.0116
	Mean (n=3)	0.9991	0.0018	0.0105
	SD	0.0006	0	0.0011
	%CV	0.0644	0	9.6656

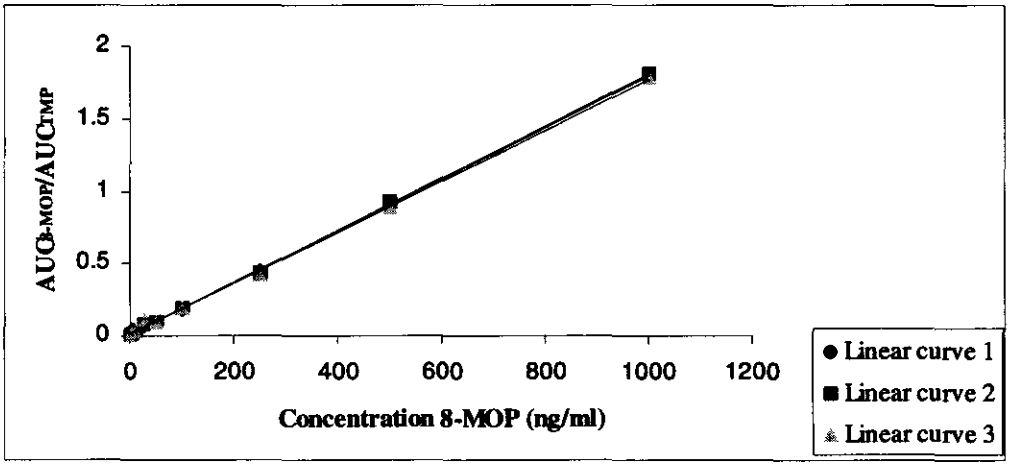

Figure 5.1: Graphic comparison of linear curve 1,2 and 3.

5.2.6.2. Accuracy

Accuracy was determined by preparing 3 replicates of four different concentrations respectively – 10 ηg/ml, 50 ηg/ml, 500 ηg/ml and 1000 ηg/ml. The true concentrations were calculated theoretically from the standard 8-MOP solutions.

The experimental concentrations were calculated as follows: The average AUC ratio of each replicate of the standard samples was substituted into the standard curve equation to calculate the corresponding concentration for each replicate. The mean, standard deviation (SD) and coefficient of variation (%CV) were determined for the calculated concentrations of the three replicates of 10 ηg/ml, 50 ηg/ml, 500 ηg/ml and 1000 ηg/ml respectively and are reported in Table 5.2.6.2. The coefficient of variation ranged between 1.11% and 37.99% and was considered as acceptable for this study.

Table 5.2.6.2. Accuracy.

Reference concentration (ng/ml)	Concentration measured ng/ml (n=3)	SD	%CV	% Recovery
10	7.9945	3.0372	37.9911	79.9452
50	45.8546	1.4871	3.2431	91.7092
500	498.8892	10.2865	2.0619	99.7779
1000	990.7305	10.9832	1.1086	99.0731

5.2.6.3. Precision

Precision (repeatability) was determined by analysing three replicates of four different concentrations respectively – 10 ηg/ml, 50 ηg/ml, 500 ηg/ml and 1000 ηg/ml. These determinations were similar to those reported for accuracy (Section 5.2.6.2.). The mean, standard deviation and coefficient of variation were determined for the AUC ratios for the three replicates of 10 ηg/ml, 50 ηg/ml, 500 ηg/ml and 1000 ηg/ml respectively and are reported in Table 5.2.6.3. The coefficient of variation ranged between 1.10% and 21.96% and was considered as acceptable for this study.

Table 5.2.6.3. Determination of precision

Reference concentration (ng/ml)	Mean ratio (n=3)	SD	%CV
10	0.0249	0.0055	21.9644
50	0.0930	0.0027	2.8771
500	0.9085	0.0185	2.0381
1000	1.7938	0.0200	1.1021

5.2.6.4. Specificity

Baseline separation can be seen as a criterium for specificity. A series of chromatograms were prepared to visually compare them for interfering peaks near the peaks of interest – 8-MOP (retention time ≅ 6.2 min) and TMP (retention time ≅ 14.0)(Figure 5.2 to 5.7).

The mobile phase (45% ammonium acetate buffer, pH 5:55% acetonitrile).

An aliquot of 100 µl mobile phase was injected into the HPLC. The resulting chromatogram (Figure 5.2.) registered a single peak at ≅1.938 min. The peak did not interfere with the peaks of 8-MOP and TMP.

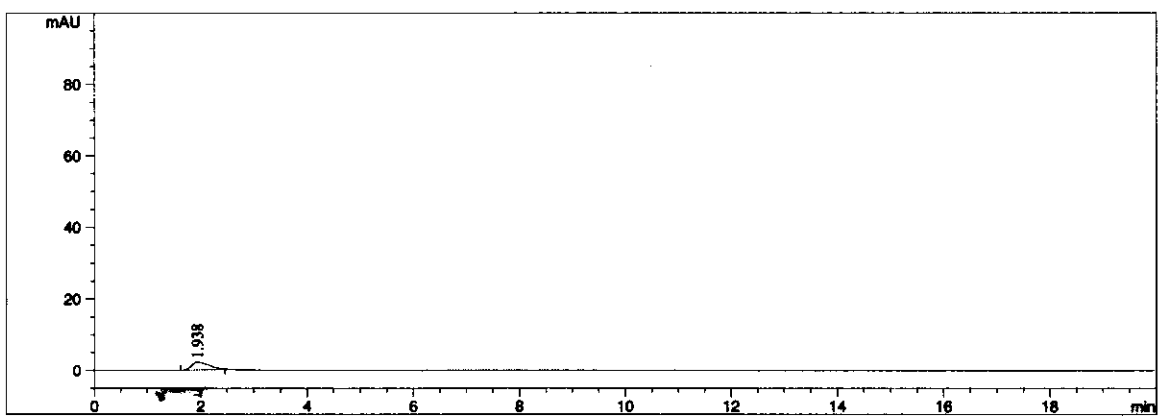

Figure 5.2: HPLC chromatogram of the mobile phase (45% ammonium acetate buffer, pH 5:55% acetonitrile).

The solvents (pure, distilled water and 100% HPLC-grade acetonitrile)

An aliquot of 100 µl pure, distilled water and 100 µl 100% HPLC-grade acetonitrile were injected into the HPLC. The latest eluting peak on the resulting chromatogram (Figure 5.3. and 5.4.) was at ≅ 2min. These peaks did not interfere with the peaks of 8-MOP and TMP.

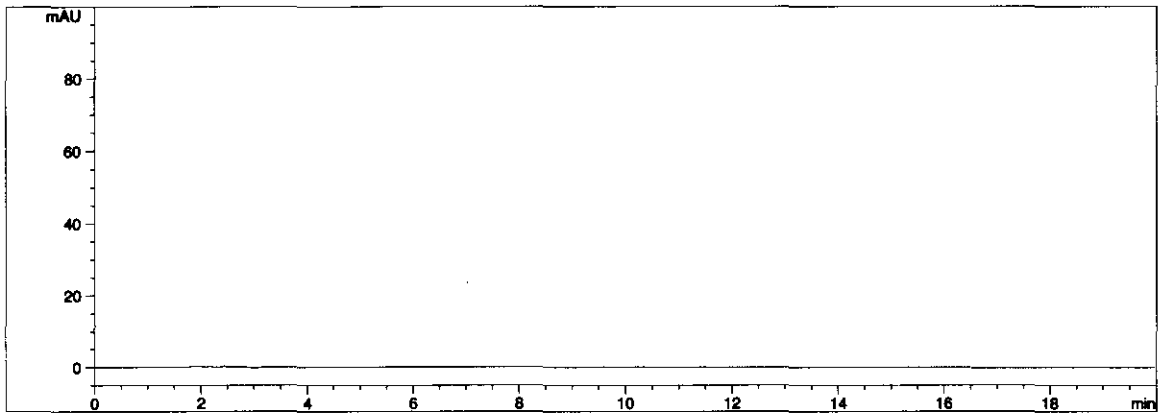

Figure 5.3: HPLC chromatogram of pure, distilled water

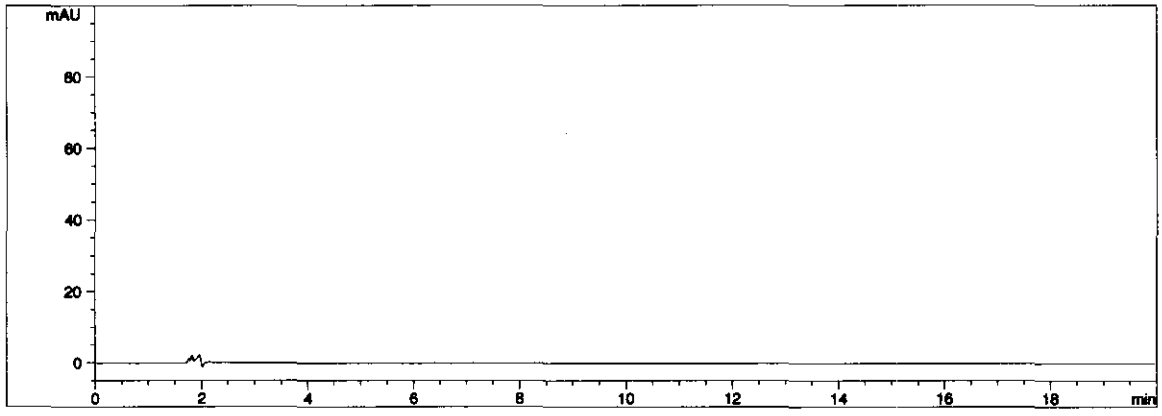

Figure 5.4: HPLC chromatograms of 100% HPLC-grade acetonitrile

The internal standard

The standard solution of TMP was prepared according to the method (Section 5.2.3.) and extracted out of plasma but with the omission of 8-MOP. A peak at ≅14.2 min was registered for TMP (Figure 5.5.). The only other peaks present were those representative of the mobile phase, solvents and plasma with the latest eluting peak before TMP at ≅3.8 min. In addition, however, small peaks were visible that were thought to be plasma-derived compounds which co-extracted with the psoralens in the *n*-hexane:chloroform phase, since they were absent in the previous chromatograms.

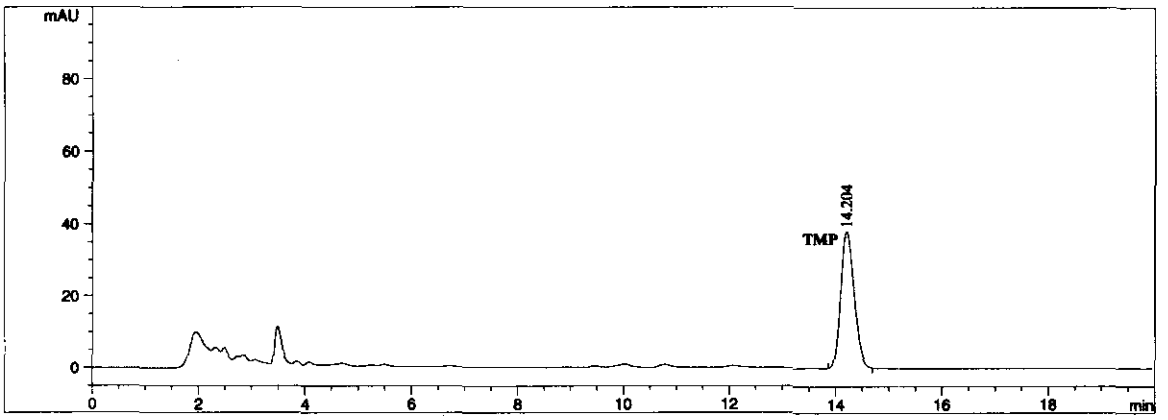

Figure 5.5: HPLC chromatogram of the internal standard (TMP) that was prepared according to the method described in Section 5.2.3. but with the omission of 8-MOP.

The assayed compound (8-MOP)

Standard daughter solutions were prepared according to the method (Section 5.2.2.). 100 µl was injected into the HPLC. A peak at ≅6.1 min was registered for 8-MOP (Figure 6). The only other peaks present were those representative of the mobile phase, and solvents with the latest eluting peak before 8-MOP at ≅1.8 min.

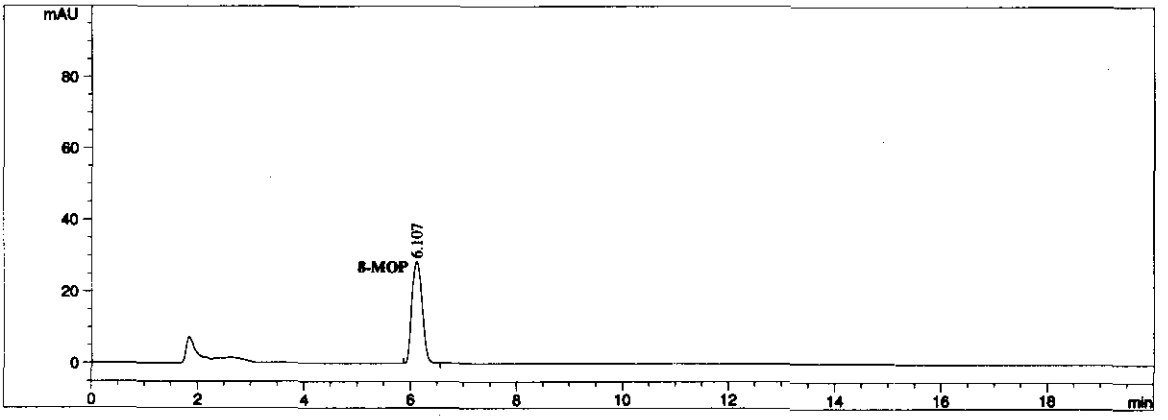

Figure 5.6: HPLC chromatogram of the assayed compound (8-MOP) that was prepared according to the method described in Section 5.2.2.

The assayed compound (8-MOP) and the internal standard (TMP) in a plasma sample

A pig plasma sample was prepared and extracted according to the method (Section 5.2.2. and Section 5.2.4.), the daughter solution was prepared by diluting 100 µl of the mother solution to 10 ml with fresh pig plasma giving a concentration of 1000 ηg/ml plasma. The internal standard (TMP) was also prepared according to the method in Section 5.2.3. The resulting chromatogram

(Figure 5.7.) exhibited all the peaks present in the previous chromatograms (Figure 2 to 6). 8-MOP eluted at ≅ 6.4 min and TMP at ≅ 14.6 min. The other peaks present were those representative of the mobile phase and solvents with the latest eluting peak before 8-MOP at ≅ 4.1 min. In addition, however, small peaks were visible that were thought to be plasma-derived compounds which co-extracted with the psoralens in the *n*-hexane:chloroform phase, since they were absent in all other chromatograms except for Figure 5.5. which was also a plasma sample.

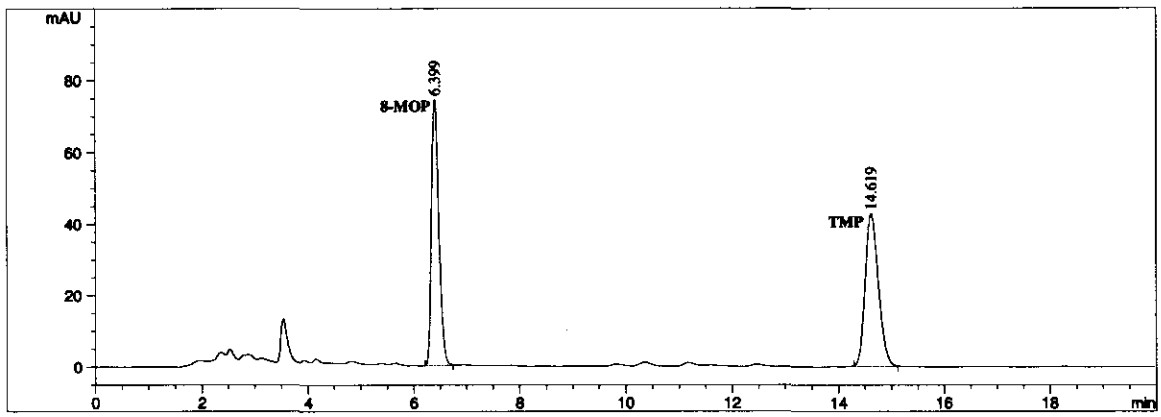

Figure 5.7: HPLC chromatogram of a pig plasma sample that was prepared according to the method described in Section 5.2.2 for 8-MOP, 5.2.3 for TMP and 5.2.4 for the extraction.

5.2.6.5. Ruggedness/robustness

Standards of 500 ηg/ml were prepared and extracted as before and the mobile phase composition (70, 55, 50 and 30% acetonitrile) and flow rate (0.5, 0.8, 1.0 and 1.3 ml/min) were varied to investigate the robustness.

Variation in mobile phase composition

The acetonitrile content of the mobile phase was increased from 30 % to 70 % (Figure 5.8.). An acetonitrile content of 30 % resulted in longer retention times and the peaks resolved poorly. An acetonitrile content of 70 % resulted in shorter retention times and improved resolution, but the peaks of interest eluted too close to the peaks of the mobile phase and solvents.

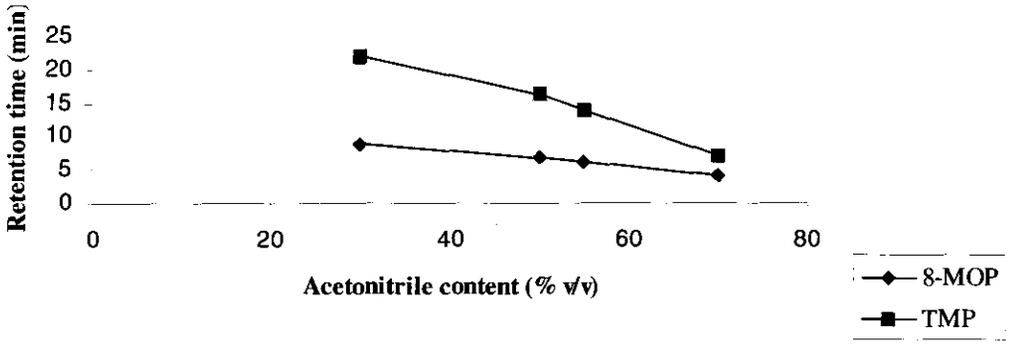

Figure 5.8: The effect of variation in mobile phase composition on the retention times of 8-MOP and TMP.

Variation in flow rate

The flow rate was varied from 0.8 ml/min to 1.3 ml/min (Figure 5.9.). An increase of the flow rate to 1.3 ml/min resulted in shorter retention times and sharper peaks, but increased column pressure. A decrease in flow rate to 0.5 ml/min resulted in longer retention times and peak broadening occurred.

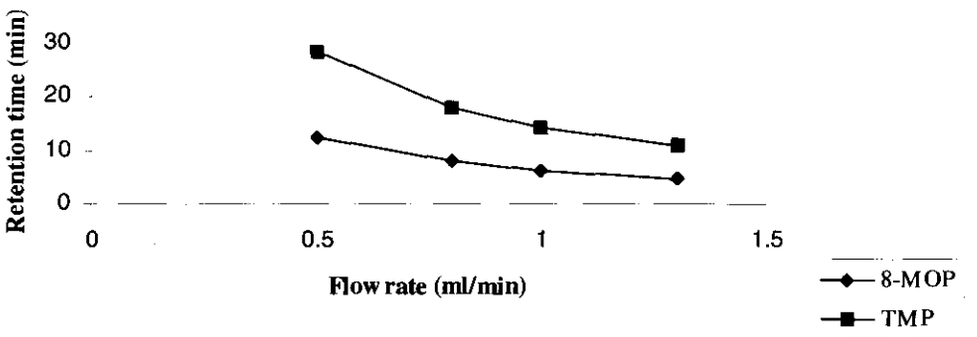

Figure 5.9: The effect of variation in flow rate on the retention times of 8-MOP and TMP.

5.2.6.6.1. Limit of detection

The lowest amount that could be detected was 5 ηg/ml for 8-MOP. The peakheight was 2-3 times that of the baseline noise.

5.2.6.6.2. Limit of quantification

The limit of detection for the method validated was 10 ηg/ml for 8-MOP.

5.3. HPLC method procedures

1. Set the flow rate at 1 ml/min.
2. Freshly prepare the mobile phase. Mobile phase A is a 0.01 M ammonium acetate buffer (pH 5), in ultra pure water, while mobile phase B consists of 100% acetonitrile. Filter through a 0.22 µm filter under vacuum and degas prior to use.
3. Add 300 µl of the internal standard (1000 ηg TMP/ml plasma) and 2 ml of HPLC-grade acetonitrile to the 500 µl plasma samples in a centrifuge tube.
4. After shaken for 2 minutes at room temperature, the sample is centrifuged for 10 minutes at 1000 *g*.
5. Transfer the clear supernatant to another centrifuge tube and add 3ml of a 60:40 *n*-Hexane:Chloroform mixture.
6. Shake the samples for 2 minutes at room temperature.
7. Centrifuge at 1000 *g* for 10 minutes.
8. Transfer the clear organic phase to another tube and evaporate at 40 °C in a waterbath under dry nitrogen.
9. Redissolve the residue in 125 µl of the mobile phase (45:55 ammonium acetate buffer, pH 5:acetonitrile, HPLC grade).
10. Inject 100 µl.

8-MOP can be detected at 254 and 300 nm but because we analyse from plasma, the peaks at 300 nm are the purest with no interfering peaks.

5.4. FPIA method

The TDx/TDxFLx system by Abbott was used to determine the Cyclosporine blood samples. Cyclosporine Monoclonal Whole Blood assay is an *in vitro* reagent system for the quantitative measurement of cyclosporine (Sandimmune®, Cyclosporine A) in human whole blood. The assay can also be used to measure cyclosporine samples in serum (Abbott, 2003).

5.4.1. Background of the method

The Cyclosporine Monoclonal Whole Blood assay utilizes Fluorescence Polarization Immunoassay (FPIA) technology. The process includes the following:

The sample, pre-treatment solution, drug (cyclosporine) antibody, and buffer are delivered to the predilution well of the FPIA sample cartridge. The pre-treatment solution displaces cyclosporine from protein binding sites. Variable unbound cyclosporine from the sample equilibrates with the fixed amount of antibody. If the concentration of the cyclosporine in the sample is high, less antibody sites remain open. An aliquot of the predilution mixture is equilibrated with cyclosporine Fluorescein Tracer in the FPIA cuvette. Cyclosporine tracer binds to open cyclosporine sites on the cyclosporine antibody. When excited by polarized vertical light, the small unbound cyclosporine tracer molecule rotates rapidly, emitting light in many different planes. The result is a decrease in the intensity of vertical polarized light. In contrast to unbound cyclosporine tracer, antibody complexes of sample cyclosporine and cyclosporine tracer are large molecules. Large molecules rotate slower and emit polarized light in the same vertical plane. The result is an increase in the intensity of the vertical polarized light. FPIA optics detects and measures the intensity of the polarized, vertical light. The change in polarized light intensity is proportional to the concentration of the cyclosporine in the specimen (Abbott, 2003).

5.4.1.1. Reagents

The reagents consist of the following:

S =Cyclosporine Monoclonal Whole Blood Antibody

T = Cyclosporine Monoclonal Whole Blood Fluorescein Tracer Solution.

P = Pretreatment Solution.

- Zinc sulfate solution.
- Surfactants in water (5 ml).

5.4.1.2. Cyclosporine Monoclonal Whole Blood Calibrators:

6 Vials (A = 10 ml; B-F = 4 ml) at the following concentrations: 0 ηg/ml, 100 ηg/ml, 250 ηg/ml, 500 ηg/ml, 1000 ηg/ml and 15000 ηg/ml.

5.4.1.3. Cyclosporine Monoclonal Whole Blood Controls:

3 Vials within the following ranges:

Vial	Cyclosporine (ηg/ml)
CONTROL L	120.00-180.00
CONTROL M	340.00-460.00
CONTROL H	680.00-920.00

5.4.2. Sample collection and preparations for testing analysis

A pre-treatment step must be performed on each cyclosporine sample (calibrators, controls, and patient samples) before testing. The pre-treatment step minimises the interference from endogenous protein-bound fluorescent compounds. The pre-treatment consists of the addition of solubilization reagent and whole blood precipitation reagent/probe wash to the sample in order to solubilise the cells and to precipitate protein respectively, followed by centrifugation to obtain a clear supernatant. The cyclosporine assay is then performed on the sample supernatant.

5.4.3. FPIA method procedures

1. Pipet 150 µl of the sample into centrifuge tube.
2. Dispense 50 µl of solubilization reagent into centrifuge tube.
3. Dispense 300 µl of whole blood precipitation reagent/probe wash into each centrifuge tube.
4. Mix on a vortex mixer for 10 seconds.
5. Centrifuge the specimens for five (5) minutes at 9.500 x g. A minimum of 150 µl of supernatant is required to perform the cyclosporine assay.
6. After centrifugation, decant the supernatant into the sample well.

5.4.4. Results

Control and cyclosporine concentrations are automatically recorded on the printout in ηg/ml.

5.4.5. Trademarks

Sandimmune® is a registered trademark of Sandoz, Inc.
Sandimmune – Kit is a product of Sandoz Ltd., Basel, Switzerland
CYCLO- Trac SP 125/RIA Kit is a product of INCSTAR Corporation, Stillwater, MN.
(Abbott Laboratories, 2001).

5.5. References

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6. Methods: Clinical study and pharmacokinetic analysis

In this chapter the clinical study and pharmacokinetic analysis of the interaction between cyclosporine and methoxsalen will be discussed.

6.1. Clinical study

The study was approved by the Ethics committee with the following number: 03M07.

6.1.1. Selection of subjects

Male volunteers, 20 to 25 years old, within 15 % of their ideal body weight (BMI = mass/length ^{2}; ideal 20-25) were randomly selected and only included in the study on dr. Dijkstra's recommendation. All the subjects were in good physical condition and healthy as judged by medical history, physical examination, and laboratory testing (biochemical en haematologic profiles). All subjects showed a normal electrocardiogram (ECG) and normal blood and urine chemistries, as determined prior to commencement of the study. The following blood and urine analysis were done:

HAEMATOLOGY (Full blood count): Haemoglobin (Hb), Red blood cell count (RBC), Haematocrit (HCT), Mean corpuscular volume (MCV), Mean corpuscular haemoglobin (MCH), Platelets (PLT), White blood cell count (WBC) (Before and after dosing with cyclosporine), White blood cell differential (WBC DIFFERENTIAL), neutrophiles, lymphocytes, monocytes, eosinophiles, basophiles.

BIOCHEMISTRY: Bilirubin total, Alkaline phosphatases, Alanine aminotransferase (SGPT) (ALT), Aspartate aminotrasferase (SGOT) (AST), Creatinine, Total protein, Albumin, Blood glucose, Total cholesterol, Potassium, Sodium, C-reactive protein (CRP).

VIROLOGY: Hepatitis B, Hepatitis C.

MICROBIOLOGY: Urine dipstick and microscopy.

The subjects were tested on three days, each separated by one week. Before participation, all subjects received written and oral information about the nature of the project and their written consent were obtained.

6.1.2. Inclusion and exclusion criteria

Exclusion criteria included history of illicit drug use or alcohol abuse, any major systemic disorder, severe allergy to a drug or food, and long-term use of any medication. Volunteers were advised to use extra sun protection and avoid contact with UVA/B rays on the day Oxsoralen® was taken. Volunteers with kidney and liver abnormalities and porphyria were excluded from this study.

6.1.3. Subject responsibilities

Limited amounts of paracetamol were allowed during the study period. The subjects had to refrain from all citrus, figs, celery, parsley or parsnip products for 7 days prior to the first experimental day, and throughout the entire study period.

Use of alcohol was prohibited for the full duration of the study period, or for at least 48 hours prior to and during experimental days. No beverages or food containing caffeine (including coffee, tea, Coca-Cola and chocolate) were allowed for 36 hours prior to, and for the complete duration of the trial. Volunteers were asked to refrain from smoking 24 hours prior to, and during the experimental days.

Subjects had to fast for 10 hours before the experiment and were only allowed intake of water four hours after methoxsalen and/or cyclosporine administration.

6.2. Study design

6.2.1. Number of subjects

Twelve healthy volunteers were divided into 3 experimental groups consisting of four subjects each for the three experimental days.

6.2.2. Study design

The study was a randomised crossover design consisting of three treatments separated by a 1-week wash-out period. The 12 volunteers were divided into 3 groups, A, B, C. Treatment one (1) single dose of 200 mg cyclosporine (Sandimmun Neoral ®), the second treatment (2) 40 mg of methoxsalen (Oxsoralen®) orally, and the third treatment (3) Sandimmun Neoral® and Oxsoralen®.

The study design can be seen in table 6.1.

Table 6.1. Illustration of the crossover study

	Group A	Group B	Group C
Day 1	1	2	3
Day 2	2	3	1
Day 3	3	1	2

The treatments were received at approximately 8 AM after an 10 hour fast. Subjects received a standardised meal 2, 5 and 8 hours after administration of the treatments.

6.2.3. Dosing

Subjects received treatments 1, 2 and 3 with 100 ml tap water. For the next 4 hours after drug intake, the subjects had to remain in the semi-recumbent position thereafter they were allowed to lie on their backs.

6.2.4. Fasting and meals

Subjects had to fast for 10 hours before the experiment (Grevel *et al,* 1988). They had to refrain from alcohol for 48 hours before drug intake. Standardised meals, without any traces of furocoumarins, were served at 4 and 10 hours after tablet intake. Water as much as desired were allowed from 4 hours after drug intake (Ehrsson *et al,* 1979). Neither beverage containing caffeine nor smoking were allowed (Bailey *et al,* 1995).

6.3. Materials

6.3.1. Methoxsalen and cyclosporine used

Sandimmun Neoral® (Novartis South Africa (Pty) Ltd, 72 Steel Road, Spartan, Kempton Park) and Oxsoralen® (Pharmaco Distribution (Pty) Ltd, Fedsure Close 2, Norwich Close, Sandton).

6.4. Sampling schedule

6.4.1. Samples for analysis of serum methoxsalen and cyclosporine

10 ml blood samples were collected by venipuncture at 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 12 and 24 hours after the dose. Blood were drawn and collected from a forearm vein in heparinised tubes (methoxsalen) and EDTA tubes (cyclosporine). The samples were centrifuged and the serum collected. Serum samples were frozen at -20°C until assay determination of methoxsalen and cyclosporine from the human plasma was done by HPLC.
Plasma samples were personally transported to MEDUNSA, for the determination of cyclosporine.

6.5. Data analysis

6.5.1. Pharmacokinetic calculations

Non-compartemental analyses were performed. Plasma C_{max}, the maximum concentration of cyclosporine and the time to reach C_{max} (t_{max}) were obtained directly from the highest observed value in the individual plasma concentration–time profiles. The pharmacokinetic parameters for cyclosporine and methoxsalen were calculated as follow (Schuirmann *et al,* 1987).

i) AUC_{0-t}: the area under the plasma concentration versus time curve, from 0 to the last measurable concentration, was calculated by linear trapezoidal method.

$$AUC_{0-t} = \sum_{i=0}^{n-1} \frac{t(i+1)-t(i)}{2}(C_i + C_{i+1}) \qquad (1)$$

N = number of data points, C = plasma concentration

ii) $AUC_{0\text{-}inf}$: the area under the plasma concentration versus time curve from 0 to infinity. $AUC_{0\text{-}inf}$ was calculated as the sum of the plasma concentration after 12 hours plus the ratio of the $AUC_{0\text{-}24}$ to the elimination rate constant:

$$AUC_{0\text{-}inf} = AUC_{0\text{-}t} + Cn / K_e \quad (2)$$

Cn = concentration at time 24 hour, K_e = elimination rate constant

iii) C_{max}: maximum measured plasma concentration. The value was obtained from the concentration-time profiles.

iv) T_{max}: time of the maximum measured plasma concentration. This was observed directly form concentrate time profiles.

v) K_e: Apparent first-order elimination or terminal rate constant calculated by means of log-linear regression using the least 3 to 5 points.

$$\text{Slope } (K_e) = \text{Ln } C½ \div t \quad (5)$$

vi) $t_{½}$: the elimination half-life or terminal half-life was calculated as follows:

$$t_{½} = 0.693 / K_e \quad (6)$$

vii) Apparent volume of distribution

$$Vd = \text{Dose} / K_e\ (AUC_{0\text{-}inf}) \quad (7)$$

viii) Clearance:

$$Cl = Vd / K_e \quad (8)$$

6.5.2. Statistical analysis

6.5.2.1. Analysis of variance (ANOVA) statistics

Log transformed values of AUC_{last}, AUC_{inf} and C_{max} were analysed by a crossover analysis (ANOVA) model. The model includes sequence, subject (sequence), product and period effects.

A 90% confidence interval of the difference was calculated. The above analysis was performed using the SAS® program by the Biostatic department of PU for CHE.
The data were not totally randomised and the 90% confidence interval was calculated with the Mann-Whitney formula.

6.5.2.2. Paired t-test

The geometric means were used to calculate clinical significance. A paired t-test was also performed. The Statistica 6 program from Stasoft® was used to perform the paired t-test.

6.5.2.3. Clinical significance

The following formula was used to calculate clinical significance (η^2);

$$\eta^2 = \frac{\frac{n-k-2}{n-k} F-1}{\frac{n-k+1}{k-1} + \frac{n-k-2}{n-k} F} \quad (9)$$

Were: n = number of volunteers used

k = 2

F = F value from ANOVA results on untransformed data

When: $\eta^2 \approx 0.01$ small clinical significance

$\eta^2 \approx 0.06$ medium clinical significance

$\eta^2 \approx 0.14$ large clinical significance

(Steyn & Viljoen, 2002).

6.6. References

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7. Pharmacokinetic results, discussion and conclusion

In this chapter the following will be discussed:

i) The pharmacokinetics of cyclosporine and the influence of methoxsalen on the pharmacokinetics of cyclosporine.

ii) The pharmacokinetics of methoxsalen and the influence of cyclosporine on the pharmacokinetics of methoxsalen.

This will be discussed as follows:

7.1. Demographic data of the volunteers.

7.2. Pharmacokinetic data of cyclosporine and methoxsalen.

7.2.1. Dosages

7.2.2. Individual plasma level profile of cyclosporine

7.2.3. Individual plasma data profile of methoxsalen

7.3. Pharmacokinetic parameters.

7.3.1. Individual pharmacokinetic parameter of cyclosporine

7.3.2. Individual pharmacokinetic parameter of methoxsalen

7.3.3. Geometric statistical parameters of cyclosporine

7.3.4. Geometric statistical parameters of methoxsalen

7.4. Discussion

7.5. Conclusion

7.6. References

7.1. Demographic data of the volunteers

The demographic data of the12 male volunteers can be seen in table 7.1.

Table 7.1: Demographic data of the12 male volunteers.

Volunteer	Weight (kg)	Length (m)	Age (years)
1	88	1.91	21
2	88	1.78	22
3	84	1.79	23
4	101	1.84	25
5	83	1.83	20
6	74	1.71	20
7	65	1.75	21
8	72	1.81	23
9	91	1.84	20
10	78	1.77	23
11	75	1.79	22
12	60	1.87	23
Average	79.9	1.8	21.9
SD	11.55	0.05	1.56
Range	60-101	1.71-1.91	20-25

Twelfth healthy male volunteers, 20 to 25 years old, within 15 % of their ideal body weight (BMI = mass/length^2; ideal 20 – 25) participated in this study after signing a consent form. The length of the individuals ranged from 1.71 to 1.91 metres with an average of 1.8 meter. The average weight was 79.9 kg and range from 60 to 101 kg. Subjects were healthy as judged by medical history, physical examination, and laboratory testing (biochemical and haematologic profiles).

14 Male volunteers were initially tested and examined but two subjects were excluded because of haematuria and the chronic use of medication, respectively.

Subjects were refrained from alcohol and medications, including over-the-counter products, throughout the study. All subjects had a normal electrocardiogram (ECG) and normal blood and urine chemistries, determined prior to commencement of the study.

7.2. Pharmacokinetic data of cyclosporine and methoxsalen

7.2.1. Dosages

The study was a randomised crossover design consisting of three treatments separated by a 1-week wash out period. The 12 volunteers were divided into 3 groups. The treatments were received at approximately 8 AM after fasting overnight (see Table 7.2 for dosages). The subjects received a standardised meal 4 and 10 hours after administration of the treatments. The crossover study design is illustrated in table 7.2.

Table 7.2: Study design.

	Week 1	Week 2	Week 3
Volunteer 1	*200 mg Cyclosporine*	**200 mg Cyclosporine + 40 mg Methoxsalen**	40 mg Methoxsalen
Volunteer 2	*200 mg Cyclosporine*	**200 mg Cyclosporine + 40 mg Methoxsalen**	40 mg Methoxsalen
Volunteer 3	*200 mg Cyclosporine*	**200 mg Cyclosporine + 40 mg Methoxsalen**	40 mg Methoxsalen
Volunteer 4	*200 mg Cyclosporine*	**200 mg Cyclosporine + 40 mg Methoxsalen**	40 mg Methoxsalen
Volunteer 5	40 mg Methoxsalen	*200 mg Cyclosporine*	**200 mg Cyclosporine + 40 mg Methoxsalen**
Volunteer 6	40 mg Methoxsalen	*200 mg Cyclosporine*	**200 mg Cyclosporine + 40 mg Methoxsalen**
Volunteer 7	40 mg Methoxsalen	*200 mg Cyclosporine*	**200 mg Cyclosporine + 40 mg Methoxsalen**
Volunteer 8	40 mg Methoxsalen	*200 mg Cyclosporine*	**200 mg Cyclosporine + 40 mg Methoxsalen**
Volunteer 9	**200 mg Cyclosporine + 40 mg Methoxsalen**	40 mg Methoxsalen	*200 mg Cyclosporine*
Volunteer 10	**200 mg Cyclosporine + 40 mg Methoxsalen**	40 mg Methoxsalen	*200 mg Cyclosporine*
Volunteer 11	**200 mg Cyclosporine + 40 mg Methoxsalen**	40 mg Methoxsalen	*200 mg Cyclosporine*
Volunteer 12	**200 mg Cyclosporine + 40 mg Methoxsalen**	40 mg Methoxsalen	*200 mg Cyclosporine*

7.2.2. Individual plasma level profile of cyclosporine

The graphs of the individual plasma levels of cyclosporine in the volunteers can be seen in figure 7.1. and the combination of cyclosporine and methoxsalen in figure 7.2. The data from which the graphs were constructed are represented in tables 7.3. and 7.4. respectively.

Table 7.3: Individual plasma levels of cyclosporine in ηg/ml.

CYCLOSPORINE

TIME	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	MEAN	STD DEV	MIN	MAX
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3.14	1.98	0.00	0.00	0.00	0.43	1.03	0.00	3.14
0.5	0.42	0.00	22.53	24.74	0.00	28.22	2.21	15.31	23.23	10.13	59.19	16.06	16.84	16.94	0.00	59.19
1	0.00	39.50	212.55	254.90	24.67	346.57	24.13	190.80	71.77	87.58	286.15	329.73	155.70	128.61	0.00	346.57
1.5	111.29	98.43	216.46	283.67	98.18	442.40	46.84	484.70	178.73	447.10	310.10	475.89	266.15	164.13	46.84	484.70
2	181.81	120.29	147.20	187.45	152.14	288.99	99.57	482.00	285.21	295.76	275.50	472.92	249.07	126.91	99.57	482.00
2.5	129.94	99.45	98.43	148.48	131.37	166.84	152.51	442.89	257.89	291.01	137.75	422.08	206.55	120.36	98.43	442.89
3	71.87	48.83	50.51	99.53	95.35	103.55	144.89	330.61	182.84	199.63	97.56	336.26	146.79	98.83	48.83	336.26
4	43.94	18.48	15.31	49.97	50.82	69.25	141.90	132.92	76.85	75.67	43.97	153.16	72.69	46.52	15.31	153.16
5	14.99	13.46	13.40	42.32	27.41	27.09	70.91	82.62	56.65	62.58	39.86	122.06	47.78	32.94	13.40	122.06
6	14.51	4.40	6.73	21.57	20.51	17.28	40.81	64.61	45.53	56.52	41.79	89.03	35.27	25.78	4.40	89.03
8	9.03	3.54	5.80	15.18	7.86	14.40	39.93	23.93	24.85	36.31	22.11	52.28	21.27	15.15	3.54	52.28
12	6.56	0.03	1.08	6.77	0.00	7.31	11.86	16.88	18.29	18.40	11.02	25.11	10.28	8.13	0.00	25.11
24	4.52	0.00	0.00	0.00	0.00	1.24	11.13	4.09	4.39	3.84	4.10	4.35	3.14	3.22	0.00	11.13

Table 7.4: Individual plasma levels of cyclosporine in the presence of methoxsalen in ηg/ml.

CYCLOSPORINE WITH METHOXSALEN

TIME	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	MEAN	STD DEV	MIN	MAX
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.5	31.59	24.32	25.47	0.00	0.00	25.74	0.00	1.73	27.55	0.00	81.95	20.37	19.89	23.44	0.00	81.95
1	78.39	83.41	195.65	33.75	40.52	367.91	21.26	180.37	78.40	101.54	329.70	357.84	155.73	129.58	21.26	367.91
1.5	110.99	133.44	217.94	275.08	125.92	478.94	44.45	453.46	180.81	460.51	339.77	540.89	280.18	169.72	44.45	540.89
2	142.73	151.19	156.04	309.99	185.15	328.67	94.39	514.29	296.60	345.84	208.23	539.91	272.75	144.31	94.39	539.91
2.5	185.05	116.58	105.02	208.24	158.68	182.58	132.98	456.35	261.63	318.48	156.39	451.85	227.82	121.49	105.02	456.35
3	160.06	76.97	98.74	159.97	119.10	117.23	130.76	302.73	198.72	232.74	109.34	383.14	174.13	91.52	76.97	383.14
4	62.47	41.91	58.03	55.52	46.58	57.37	136.69	236.34	91.13	91.78	44.62	172.12	91.21	60.77	41.91	236.34
5	40.31	38.05	22.41	40.57	37.28	37.98	59.17	116.75	58.76	79.14	37.73	127.14	57.94	33.31	22.41	127.14
6	31.92	28.05	14.31	30.29	24.85	28.48	36.32	81.17	52.54	64.16	33.21	98.80	43.68	25.43	14.31	98.80
8	26.29	21.88	7.98	10.61	17.68	16.12	33.05	21.76	28.01	38.01	20.16	55.93	24.79	13.05	7.98	55.93
12	14.47	11.20	5.03	9.53	5.05	5.51	4.85	26.64	19.38	13.02	10.09	26.85	12.64	7.93	4.85	26.85
24	9.63	9.78	0.00	0.00	0.00	0.19	0.00	2.98	6.25	0.19	3.76	4.89	3.14	3.77	0.00	9.78

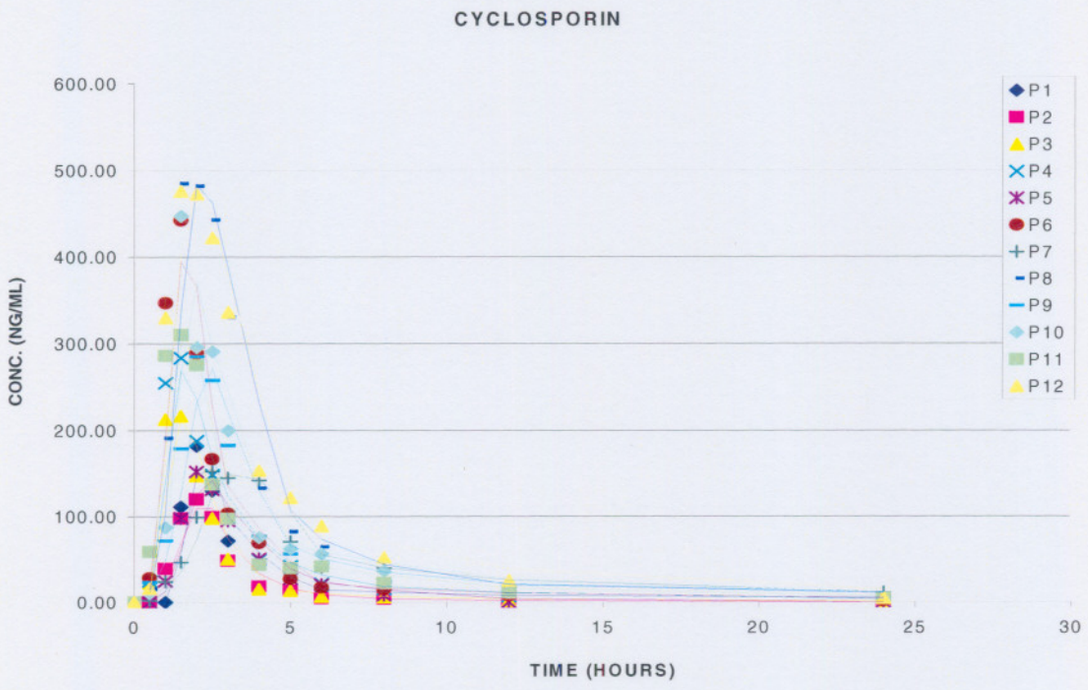

Figure 7.1: Graph representing the individual plasma levels of cyclosporine. The peak levels range from a minimum of 120.29 to a maximum of 484.7 ηg/ml. The average peak level was 296 ηg/ml.

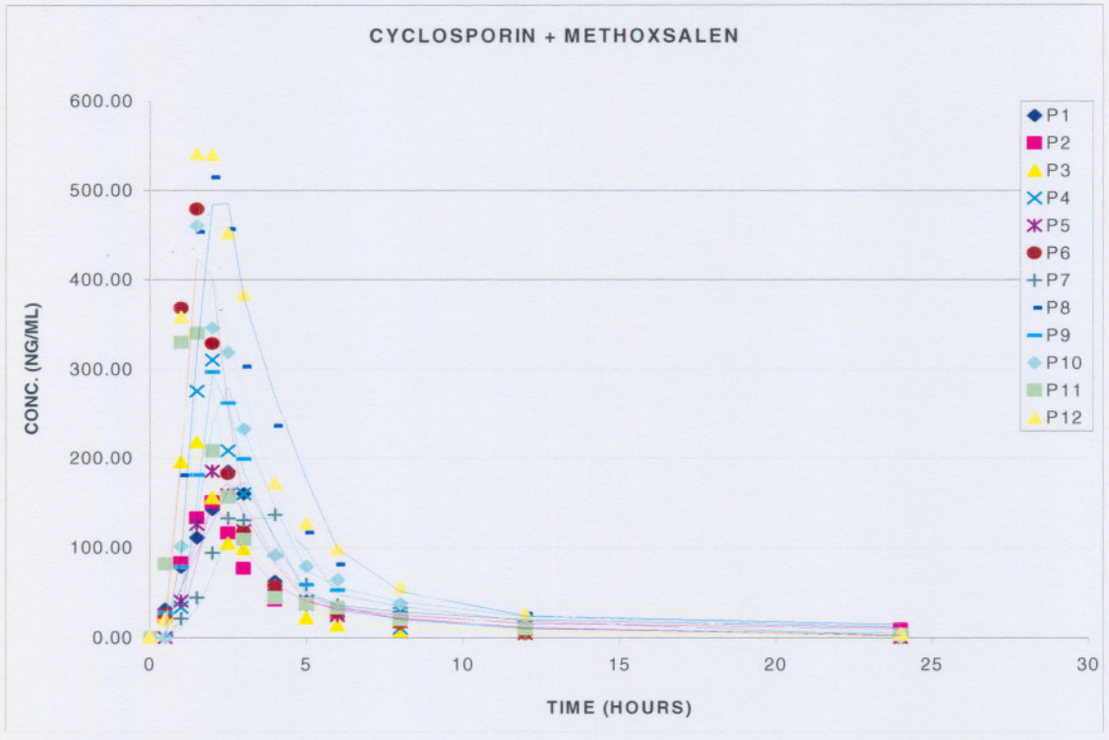

Figure 7.2: Graph representing individual plasma levels of cyclosporine in the presence of methoxsalen. The peak levels range from a minimum of 136.69 to a maximum of 540.89 ηg/ml . The average peak level was 318.08 ηg/ml.

7.2.3. Individual plasma level profile of methoxsalen

The graphs of the individual plasma levels of methoxsalen in the volunteers can be seen in figure 7.3. and the combination of methoxsalen and cyclosporine in figure 7.4. The data from which the graphs were constructed are represented in tables 7.5. and 7.6. respectively.

Table 7.5: Individual plasma levels of methoxsalen in ηg/ml.

METHOXSALEN

TIME	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	MEAN	STD. DEV	MIN	MAX
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.5	172.08	0.00	0.00	61.36	0.00	0.00	0.00	0.00	0.00	0.40	0.00	0.00	19.49	47.36	0.00	172.08
1	300.09	29.36	15.13	149.85	20.30	0.00	47.28	19.12	75.87	269.51	171.97	9.54	92.33	100.04	0.00	300.09
1.5	387.28	39.22	223.60	138.23	54.10	72.12	45.77	95.66	84.99	338.67	179.80	36.00	141.29	115.74	36.00	387.28
2	180.02	37.61	185.23	90.95	49.53	104.42	38.53	149.47	72.44	264.06	190.43	45.15	117.32	75.47	37.61	264.06
2.5	126.86	67.86	132.84	66.48	41.73	147.08	86.28	171.16	65.64	220.18	19.05	165.02	109.18	62.50	19.05	220.18
3	82.04	54.97	88.55	36.44	42.52	133.28	61.26	138.63	46.38	171.06	186.66	230.98	106.07	65.60	36.44	230.98
4	57.50	16.42	36.07	13.91	19.58	72.05	32.52	84.64	20.32	109.62	134.89	146.46	62.00	46.36	13.91	146.46
5	34.20	7.16	12.68	8.20	0.00	23.03	11.47	33.58	7.09	11.01	77.81	60.68	23.91	22.57	0.00	77.81
6	25.93	4.96	3.51	1.98	0.00	2.37	0.00	8.32	0.00	11.43	0.00	20.12	6.55	7.89	0.00	25.93
8	19.60	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	9.36	39.08	2.78	5.90	11.07	0.00	39.08
12	8.82	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.73	3.81	0.00	8.82
24	2.91	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.24	6.40	0.00	2.91

Table 7.6: Individual plasma levels of methoxsalen in the presence of cyclosporine in ηg/ml.

METHOXSALEN

TIME	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	MEAN	STD DEV	MIN	MAX
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.5	0.00	0.00	0.00	0.00	0.00	104.52	0.00	0.00	0.00	0.00	0.00	0.00	8.71	30.17	0.00	104.52
1	4.59	92.52	93.99	39.43	0.00	125.06	20.58	39.82	0.00	102.76	29.81	49.57	49.84	43.39	0.00	125.06
1.5	67.88	139.62	91.31	44.89	0.00	116.13	74.59	99.57	14.40	171.71	49.16	76.85	78.84	49.40	0.00	171.71
2	67.23	104.35	68.93	50.48	3.26	102.77	57.45	103.74	84.61	211.09	65.43	87.82	83.93	49.10	3.26	211.09
2.5	47.56	62.88	51.33	53.73	7.60	80.00	69.60	76.41	49.59	191.32	66.87	87.05	70.33	43.31	7.60	191.32
3	26.80	46.29	29.00	53.73	16.44	69.30	67.32	58.48	36.68	154.95	153.37	94.13	67.21	45.88	16.44	154.95
4	6.81	23.29	36.39	24.75	15.76	29.71	84.12	36.18	11.13	96.56	242.40	174.89	65.17	73.78	6.81	242.40
5	1.43	6.47	21.20	4.44	9.40	3.93	56.69	58.84	0.00	56.65	151.00	116.63	40.56	49.70	0.00	151.00
6	0.00	2.60	7.61	0.00	0.00	0.00	18.86	36.03	0.00	16.53	84.40	59.62	18.80	27.68	0.00	84.40
8	0.00	0.00	0.00	0.00	0.00	0.00	0.00	5.06	0.00	0.00	27.52	8.58	3.43	8.07	0.00	27.52
12	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
24	0.00	0.00	0.00	0.00	0.00	4.81	0.00	0.00	0.00	0.00	0.00	0.00	0.40	1.39	0.00	4.81

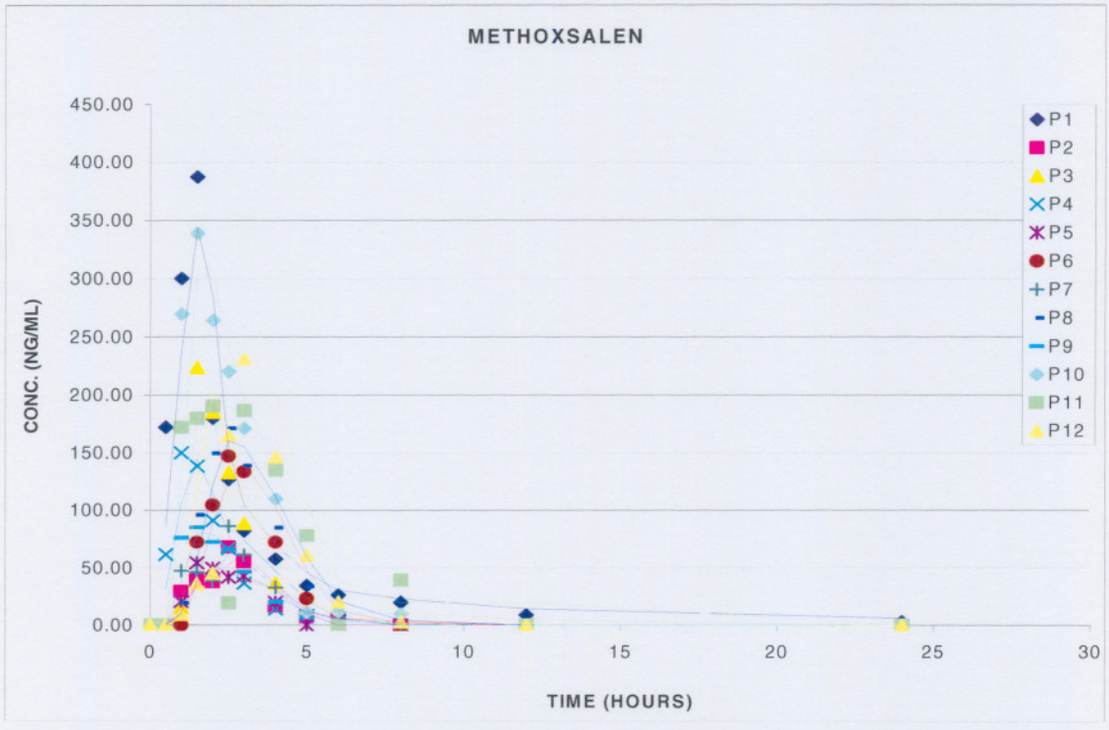

Figure 7.3: Graph representing individual plasma levels of methoxsalen. The peak levels range from a minimum of 54.1 to a maximum of 387.28 ηg/ml. The average peak level was 177.69 ηg/ml.

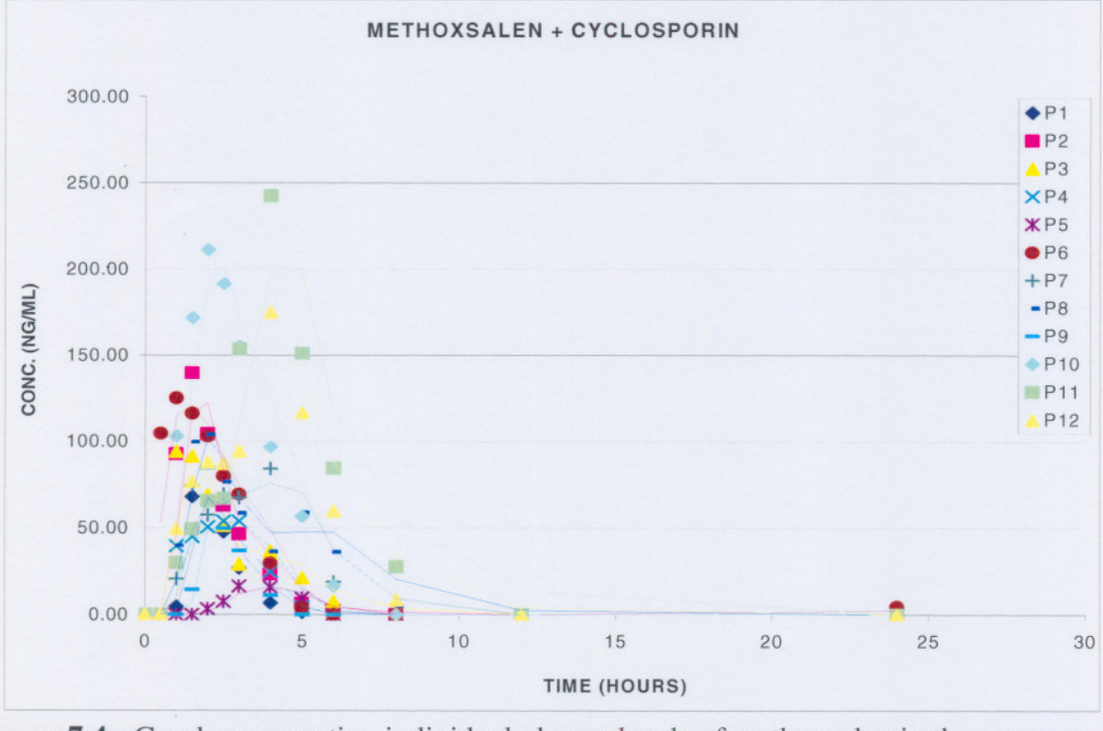

Figure 7.4: Graph representing individual plasma levels of methoxsalen in the presence of cyclosporine. The peak levels range from a minimum of 16.46 to a maximum of 242.40 ηg/ml. The average peak level was 116.46 ηg/ml.

7.3. Pharmacokinetic parameters

7.3.1. Individual pharmacokinetic parameter of cyclosporine

Table 7.7. represents the individual pharmacokinetic parameters for cyclosporine alone (reference) and the cyclosporine and methoxsalen combination (test). The table consists of the means of each paramater, standard deviation (SD), minimum value (Min) and maximum value (Max). The mean values of each parameter were compared with a paired t- test and the p-values are reported in the table.

Table 7.7: Individual pharmacokinetic parameters for cyclosporine and the combination of cyclosporine and methoxsalen.

(T) = Cyclosporine in the presence of methoxsalen

(R) = Cyclosporine

Volunteer	AUC_{0-t}(R) (ηg.h/ml)	(T) (ηg.h/ml)	$AUC_{0-\infty}$(R) (ηg.h/ml)	(T) (ηg.h/ml)	C_{max}(R) (ηg/ml)	(T) (ηg/ml)	T_{max}(R) (h)	(T) (h)	K_e(R) (h)	(T) (h)	$t_{½}$(R) (h)	(T) (h)
1	452.00	793.19	470.87	856.33	181.81	185.05	2	2.5	0.131	0.106	5.3	6.53
2	260.66	645.59	260.66	707.83	120.29	151.19	2	2	0.787	0.101	0.88	6.86
3	448.78	589.75	448.79	589.85	216.46	217.94	1.5	1.5	0.463	0.364	1.5	1.91
4	746.37	783.06	746.63	783.37	283.67	309.99	1.5	2	0.333	0.331	2.08	2.10
5	407.27	553.89	407.27	554.10	152.14	185.15	2	2	0.507	0.342	1.37	2.03
6	933.72	992.53	936.89	992.98	442.40	478.94	1.5	1.5	0.226	0.315	3.07	2.20
7	815.64	615.88	872.34	616.03	152.51	136.69	2.5	4	0.122	0.378	5.68	1.83
8	1582.05	1752.81	1594.02	1762.12	484.70	514.29	1.5	2	0.202	0.221	3.44	3.14
9	975.18	1061.20	993.31	1087.84	285.21	296.60	2	2	0.169	0.157	4.09	4.40
10	1191.75	1222.63	1211.02	1223.24	447.10	460.51	1.5	1.5	0.165	0.327	4.19	2.12
11	923.83	927.50	938.90	940.74	310.10	339.77	1.5	1.5	0.167	0.170	4.15	4.08
12	1861.88	2059.42	1877.60	2076.87	475.89	540.89	1.5	1.5	0.201	0.2	3.45	3.46
M	883.34	1000.62	896.52	1015.94	296.02	318.08	1.75	2.00	0.29	0.25	3.27	3.39
SD	589.06	475.07	487.02	473.47	136.26	148.16	0.34	0.71	0.20	0.10	1.54	1.77
Min	260.66	553.89	260.66	554.10	120.29	136.69	1.50	1.50	0.12	0.10	0.88	1.83
Max	1975.18	2059.42	1877.60	2076.87	484.70	540.89	2.50	4.00	0.79	0.38	5.68	6.86
p-value*	0.68		**0.04**		**0.004**		0.08		0.57		0.86	

p-value* as calculated from arithmetic means by paired t-test. Statistical significance; $p<0.05$.

The average mean AUC_{0-t} of cyclosporine was 958.26 ηg.h/ml with a SD of 589.06. The range was from 160.66 to 1975.18 ηg.h/ml. In the presence of the methoxsalen the average AUC_{0-t} increased to 1000.62 ηg.h/ml while the SD declined to 475.07. The range was from 553.89 to 2059.42 ηg.h/ml. The average mean $AUC_{0-\infty}$ of cyclosporine was 896.52 ηg.h/ml with a SD of 487.02. The range was from 260.66 to 1877.60 ηg/h/ml. In the presence of methoxsalen the average $AUC_{0-\infty}$ increased to 1015.94 ηg.h/ml while the SD declined to 473.47. The range was from 554.10 to 2076.87 ηg.h/ml. The average C_{max} of cyclosporine was 296.02 ηg/ml with a SD of 136.26. The range was from 120.29 to 484.70 ηg/ml. In the presence of methoxsalen the average C_{max} value increased to 318.08 ηg/ml and the SD was 148.16.

The p-values from table 7.7. indicated that the difference between the two treatments were statistical significant for $AUC_{0-\infty}$ ($p= 0.04$) and C_{max} ($p=0.004$). The median of $T_{max}(R)$ was 1.5 hours and the range was between 2 and 2.5 hours. The median of $T_{max}(T)$ was 1.5 hours and ranges from 1.5 to 4 hours.

7.3.2. Individual pharmacokinetic parameter of methoxsalen

Table 7.8. represents the individual pharmacokinetic parameters for methoxsalen alone (reference) and methoxsalen and cyclosporine combination (test). The table consists of the means of each paramater, standard deviation (SD), minimum value (Min) and maximum value (Max). The mean values of each parameter were compared with a paired t- test and the p-values are reported in the table.

Table 7.8: Individual pharmacokinetic parameters of methoxsalen and the combination of methoxsalen and cyclosporine.

(T) = Methoxsalen in the presence of cyclosporine

(R) = Methoxsalen

Volunteer	AUC_{0-t}(R) (ng.h/ml)	(T) (ng.h/ml)	$AUC_{0-\infty}$(R) (ng.h/ml)	(T) (ng.h/ml)	C_{max}(R) (ng/ml)	(T) (ng/ml)	T_{max}(R) (h)	(T) (h)	K_e(R) (h)	(T) (h)	$t_{½}$(R) (h)	(T) (h)
1	910.63	89.08	918.55	89.08	387.28	67.88	1.5	1.5	0.193	0.810	3.59	0.86
2	154.78	264.72	154.78	264.72	67.86	139.62	2.5	1.5	0.807	0.919	0.86	0.75
3	391.99	241.57	391.99	241.58	223.60	93.99	1.5	1	0.987	0.480	0.7	1.44
4	303.10	159.12	303.10	159.12	149.85	53.73	1	2	0.914	1.247	0.76	0.56
5	132.83	42.64	132.86	42.82	54.10	16.44	1.5	3	0.389	0.280	1.78	2.48
6	349.11	343.02	349.11	343.03	147.08	125.06	2.5	1	1.323	0.921	0.52	0.75
7	195.56	326.09	195.56	326.09	86.28	84.12	2.5	4	0.794	0.748	0.87	0.93
8	443.44	355.61	443.44	355.63	171.16	103.74	2.5	2	0.843	0.422	0.82	1.64
9	208.95	110.46	208.75	110.46	84.99	84.61	1.5	2	0.798	0.999	0.87	0.69
10	820.81	624.58	820.81	624.58	338.67	211.09	1.5	2	0.663	0.664	1.05	1.04
11	837.29	802.63	838.09	802.64	190.43	242.40	2	4	0.312	0.549	2.22	1.26
12	528.30	603.12	528.30	603.12	230.98	174.89	3	4	0.913	0.771	0.76	0.90
M	439.72	330.22	440.45	330.24	177.69	116.46	1.96	2.33	0.75	0.73	1.23	1.11
SD	278.04	236.58	279.37	236.57	104.88	65.97	0.62	1.13	0.32	0.27	0.89	0.54
Min	132.83	42.64	132.86	42.82	54.10	16.44	1.00	1.00	0.19	0.28	0.52	0.56
Max	910.63	802.63	918.55	802.64	387.28	242.40	3.00	4.00	1.32	1.25	3.59	2.48
p-value*	0.15		0.15		0.06		0.26		0.90		0.66	

p-value* as calculated from arithmetic means by paired t-test. Statistical significance; $p<0.05$.

The average mean AUC_{0-t} of mehoxsalen was 439.72 ηg.h/ml with a SD of 278.04. The range was from 132.83 to910.63 ηg.h/ml. In the presence of the cyclosporine the AUC_{0-t} decreased to 330.22 ηg.h/ml while the SD declined to 236.58. The range was from 42.64 to 802.63 ηg.h/ml. The average mean $AUC_{0-\infty}$ of methoxsalen was 440.45 ηg.h/ml with a SD of 279.37. The range was from 132.86 to 918.55 ηg.h/ml. In the presence of cyclosporine the average $AUC_{0-\infty}$ decreased to 330.24 ηg.h/ml while the SD also declined to 236.57. The range was from 42.82 to 802.64 ηg.h/ml. The average C_{max} of methoxsalen was 177.69 ηg/ml with a SD of 104.88. The range was from 54.10 to 387.28 ηg/ml. In the presence of cyclosporine the average C_{max} value decreased to 116.46 ηg/ml and the SD was 65.97.

The p-values from table 7.7. indicated that the difference between the two treatments were not statistical significant. The median of T_{max}(R) was 1.5 hours and the range was between 1 and 3 hours. The median of T_{max}(T) was 2 hours and ranged from 1 to 4 hour

7.3.3. Geometric statistical parameters of cyclosporine

The geometric statistical parameters of cyclosporine can be seen in table 7.9.

Test = Cyclosporine in the presence of methoxsalen

Reference = Cyclosporine

These results were calculated from the ANOVA analysis.

Table 7.9: Geometric statistical parameters of cyclosporine.

	CYCLOSPORINE		RATIO	90% CONFIDENCE INTERVAL (MANN-WHITNEY)	F	η^2
	TEST	REFERENCE				
AUC_{0-t} (ng.h/ml)	915.75 ± 474.63	762.64 ± 482.99	120.50	103 – 138	3.550	0.133
$AUC_{0-\infty}$ (ng.h/ml)	932.97 ± 473.48	774.06 ± 487.02	122.00	103 – 141	4.794	**0.191**
C_{max} (ng/ml)	285.70 ± 148.16	266.04 ± 136.26	108.00	102 - 114	13.915	**0.458**
$t_{½}$ (h)	3.03 ± 1.78	2.85 ± 1.54	95.00	68 – 122	0.001	-0.091
t_{max} (h)	1.91 ± 0.71	1.72 ± 0.34	107.50	100 - 115	2.000	0.048

The differences in $AUC_{0-\infty}$ ($\eta^2 = 0.191$) and C_{max} $\eta^2 = 0.458$) were clinical significant. The difference in AUC_{0-t} between the two treatments was in the medium to large clinical significance range. The 90% confidence interval for both AUC_{0-t} and $AUC_{0-\infty}$ fell outside the conventional 80- 125% ranges. These results confirmed the results obtained with the paired t-test (Table 7.7.). The 90% confidence interval for the C_{max} value was in the conventional range although the clinical difference was significant.

7.3.4. Geometric statistical parameters of methoxsalen

The geometric statistical parameters of methoxsalen can be seen in Table 7.10.

Test = Methoxsalen in the presence of cyclosporine

Reference = Methoxsalen

These results were obtained from the ANOVA analysis.

Table 7.10: Geometric statistical parameters of methoxsalen.

	METHOXSALEN		RATIO	90% CONFIDENCE INTERVAL (MANN-WHITNEY)	F	η^2
	TEST	REFERENCE				
AUC_{0-t} (ng.h/ml)	246.91 ± 236.58	362.24 ± 278.04	77	41 – 113	1.259	0.001
$AUC_{0-\infty}$ (ng.h/ml)	247.00 ± 236.56	362.54 ± 279.37	77	41 – 113	1.253	0.000
C_{max} (ng/ml)	96.48 ±65.97	150.12 ± 104.88	70	41 – 99	2.782	0.093
$t_{½}$ (h)	1.02 ± 0.54	1.04 ± 0.89	114.5	88 – 141	0.005	-0.091
t_{max} (h)	2.13 ± 1.13	1.87 ± 0.62	118	73 - 163	1.091	-0.011

The differences between the two treatments were not clinical significant for any of the parameters. The 90 % confidence interval for all the parameters was however lower than the conventional range of 80 to 125%.

7.4. Discussion

By serendipity it was found that grapefruit juice could increase the oral bioavailability of a number of drugs metabolised by the CYP3A4 system (Bailey *et al,* 1998). A few of these drugs reported in the literature are cyclosporine, terfenadine, midazolam and lovastatin (Fukuda *et al,* 2000).

The particular components in grapefruit juice responsible for the interaction and the exact mechanism of the interaction have not been fully elucidated (Ho *et al,* 1998). However, the selective down regulation of CYP3A4 content in the small intestine as proved by a decrease in immunoreactive CYP3A4 content in enterocytes, is one of the suggested mechanism (Ohnishi *et al,* 2000). From the recent findings on the inhibitory activity of nonflavonoid compounds, it is probably that psoralens, mainly 6'7'-dihydroxybergamottin, in grapefruit juice are the components responsible for the inhibition of the enzymes (Fuhr, 1998). Dihydroxybergamottin, is a furancoumarin and mechanism-based or suicide inhibitor of CYP3A4 in the liver and enterocytes. It shares this characteristic with other furancoumarins, including bergamottin, 8-MOP and 5-MOP (Edwards *et. al,* 1999).

It was subsequently shown that 8-MOP was a potent, and rather selective, rapid inhibitor of human liver microsomal cytochrome activity and is also capable of inhibiting most of the CYP isoforms (Kaharasch *et al,* 2000). 8-MOP is therefore a potent suicide inhibitor of the enzyme system (Labbe *et al,* 1989). Studies of Mays & co-workers (1987) showed that 8-MOP inhibits the biotransformation of caffeine, hexobarbital and 5,5-diphenylhydantoin.

In many cases, where CYP is inhibited, p-glycoprotein inhibition may also occur (Kim *et al,* 1999). We speculate that 8-MOP may then also act as an inhibitor of p-glycoprotein, therefore higher concentrations of cyclosporine stays in the body.

The present study was conducted to investigate the possibility that 8-MOP could also inhibit the metabolism of cyclosporine and increased the bioavailability of the drug. The average AUC $_{0-\infty}$ of cyclosporine increased from 896.52 ηg.h/ml to 1015.94 ηg.h/ml (11%), AUC $_{0-t}$ from 958.26 ηg.h/ml to 1001.62 ηg.h/ml (4%) and C_{max} from 486.70 to 540.89 ηg/ml (10%) in the present study. The t_{max} values increased from 1.75 hours to 2.0 hours and the half-life from 3.27 to 3.39 (3%). The increase in $AUC_{0-\infty}$ and C_{max} was the only statistical significant results (p = 0.04 and 0.004 respectively) in table 7.6. These results were confirmed with another statistical test were

both the parameters shown clinical significance (0.191 and 0.458 respectively for $AUC_{0-\infty}$ and C_{max}) in table 7.9. Although an increase was observed it was lower than increases reported in the literature when cyclosporine was combined with grapefruit juice. Ku & co-workers 1998 and Yee *et al*, 1995 documented an increase of 45% and 30% respectively in AUC when grapefruit juice was added to cyclosporine. The reasons for the discrepancy between the results in this study and the literature can be one of the following:

Grapefruit juice versus 8-MOP: It is speculated in the literature that a number of compounds in grapefruit juice could be responsible for the effect and not the furancoumarin alone.

The dose of 8-MOP was below the saturation dose: It is advisable to use 8-MOP doses well above the saturation process. Both inter- and intraindividual variability in plasma levels of 8-MOP tends to be high at doses in the range of the saturation process. With 8-MOP the dose is limited by the onset of systemic side effects like nausea. 0.4 – 12 mg/kg 8-MOP are sufficient for saturation of first-pass effect, only if the release rate is very quickly (Brickl *et al*, 1984). In this study the dose was 2.5 mg/kg, which is relatively low. Considerable variation of both the time (t_{max}) and the height (C_{max}) of the maximum serum concentrations of 8-MOP were observed in the literature and this study. After oral administration, 8-MOP is subjected to extensive, saturable 'first-pass elimination", which means that after administration of a low 8-MOP dose, none or only small amounts of unchanged 8-MOP reaches the general circulation. With doses higher than the "breakthrough dose", liver enzymes are saturated and serum concentrations of 8-MOP rise rapidly (Stolk & Siddiqui, 1988).

Large interindividual variation in enzyme activity between individuals: Thummel & co–workers (1994) showed that variation in liver content of CYP3A4 in liver transplant recipients accounted for 66% of the interpatient variation in the clearance of intravenously administered cyclosporine. In contrast, variation in liver CYP3A4 activity does not appear to account for a majority of the interpatient variability in pharmacokinetics when cyclosporine is administered orally. Lown & co-workers (1997) demonstrated that variation in liver CYP3A4 activity predicted only about one third of the interpatient variability in the oral clearance (CL/F) of cyclosporine in kidney transplant patients. The basis for the remaining variation in CL/F is unknown. One possible explanation may be related to the abundant expression of CYP3A4 in small bowel epithelial cells (enterocytes). Several studies have indicated that intestinal CYP3A4 is responsible for significant first-pass metabolism of orally administered cyclosporine. Lown & co-workers (1997) have found that the enterocyte content of CYP3A4 protein correlates with its catalytic

activity (midazolam 1'-hydroxylation) and varies up to ten-fold among patients. Intestinal and liver CYP3A4 expression does not appear to be co-ordinately regulated. It is possible for an individual to have relatively high liver CYP3A4 activity while having relatively low intestinal CYP3A4 activity or *vica verca.*

P-glycoprotein: As with CYP3A4, there is significant interindividual variation in the intestinal expression of p-glycoprotein. Given its transport function, high intestinal levels of p-glycoprotein may interfere with drug absorption and contribute to the variation in cyclosporine oral pharmacokinetics (Lown *et al,* 1997).

The standard deviation on the pharmacokinetic parameters in this study declined when 8-MOP was added. The SD on AUC_{0-t} declined by 19% and on $AUC_{0-\infty}$ and C_{max} by 3 and 5 % respectively. Although the decrease was small it can be postulated that the interindividual variation declined due to the inhibition of the enzymes both in the liver and the intestine. It was postulated in the literature that the increase in bioavailability and decrease in variability is mainly due to the reduction in gut wall metabolism (Ducharme *et al,*1995).

An interesting observation of the study was the fact the 8-MOP levels decrease when the drug was combined with cyclosporine. The AUC_{0-t} values decreased from 439.72 to 330.22 (24%), $AUC_{0-\infty}$ values from 440.45 to 330.24 (24%) and C_{max} values from 177.69 to 116.46 (34%) (Table 7.8.). The 90% confidence intervals as calculated by Mann-Whitney were below the conventional ranges. The difference was however not statistical significant for any of the parameters. These results confirm the observation from the literature and this study that the absorption of 8-MOP is highly variable.

The interaction between cyclosporine, 8-MOP and the p-glycoprotein played also an important role. Cyclosporine is a p-glycoprotein inhibitor (Gottesmann & Pastan, 1993). If 8-MOP is a substrate for p-glycoprotein, this would mean that higher concentrations of 8-MOP would remain in the body when given together with cyclosporine resulting in higher plasma levels. The results shown differently. The lower 8-MOP levels is either due to inconsistent absorption or that the drug is eliminated by another transport system. We suggest that the formulation of cyclosporine may decrease the absorption of 8-MOP, therefore, lower concentrations. We can see (Table 7.10.) that the 90% confidence interval changed, which indicated lower absorption of 8-MOP.

In the newer literature authors suggested more transport systems than p-glycoprotein. The multidrug resistance-associated protein (MRP) is a membrane bound glycoprotein. Like p-glycoprotein, MRP can cause resistance to a range of hydrophobic drugs (Zaman *et al,* 1994). Organic anion transporting polypeptides OAT1, OAT2 and OAT3 are a group of members of the OAT-family and mediate the uptake of a variety of anionic compounds (Hosoyamada *et al,* 1999, Race *et al,* 1999, Sekine *et al,* 1998). A third transport system is the organic cation transporter family (Holm, 2003).

It can also be speculated that another transport system takes over the elimination of 8-MOP when cyclosporine inhibits p-glycoprotein.

7.5. Conclusion

Twelfth healthy male volunteers participated in this study after signing a consent form. They received 200 mg cyclosporine (Sandimmune®), 40 mg methoxsalen (Oxsoralen®) or both at 3 different occasions, with a 1week washout period between every treatment. Blood were drawn at 13 time intervals and stored at –20°C until analysed. The methoxsalen plasma levels were determined with a validated HPLC method and the cyclosporin levels with a FPIA method.

The main findings and trends observed in the study were as follows:

The $AUC_{0-\infty}$ and C_{max} values increased clinical significantly when 8-MOP was added to the cyclosporine (p = 0.04 and 0.004 respectively). 8-MOP is a psoralen and share the same characteristics as the furancoumarin, bergamottin (Edwards *et al,* 1999), which in turn is partly responsible for the grapefruit juice-drug interactions (Fuhr, 1998). We concluded that the same inhibitory effect on the CYP enzyme system may took place with methoxsalen. Cillié (2003) also insinuated that bergamottin possessed *in vitro* activity as a CYP3A4 inhibitor, and could attribute to the grapefruit juice interaction (Cillié, 2003).

Cyclosporine decreased the plasma levels of 8-MOP, but these results were neither statistical, nor clinical significant. The reduction in levels could be due to:

* The combination of cyclosporine and 8-MOP, with their different, soft gel and hard capsule formulations.
* Poor absorption of 8-MOP, and thus poor bioavailability (Criss *et al,* 2002).
* Cyclosporine is a p-glycoprotein inhibitor (Gottesmann & Pastan, 1993). If 8-MOP is a substrate for p-glycoprotein the levels had to be higher. The fact that the levels decreased could mean that methoxsalen used another transport system. The individual pharmacokinetic results obtained were highly variable among individuals for 8-MOP.

Unresolved issues and future work:

- From the data and findings we suggest that a combination formulation of cyclosporine and methoxsalen can be beneficial. The variability in methoxsalen absorption is however a problem and must be addressed before such a dosage form can be viable.

- Investigations of the possibility of genetic polymorphism for the metabolism of cyclosporine and methoxsalen.

7.6. References

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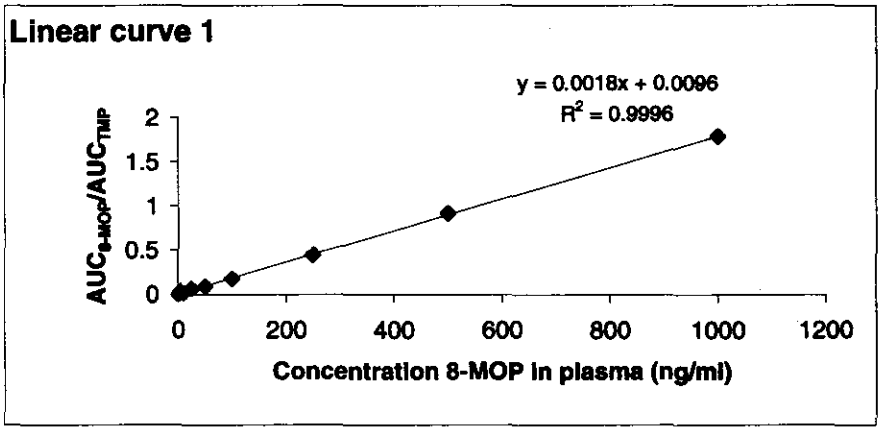
APPENDIX 1

1. LINEARITY

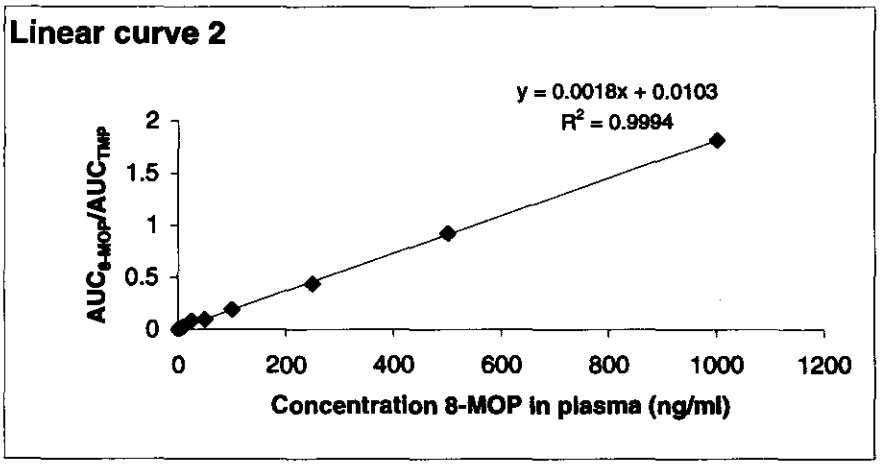
Linear curve 1

Sample	HPLC parameter			AUC ratio (y-axis)	Concentration (ng/ml) (x-axis)
	8-MOP / TMP	AUC	R_T (min)		
Blank	8-MOP	0	-	0	0
	TMP	690.99823	14.219		
1	8-MOP	26.56106	6.073	0.038460789	5
	TMP	690.60101	14.177		
2	8-MOP	14.56165	6.240	0.020686084	10
	TMP	703.93457	14.170		
3	8-MOP	46.67262	6.242	0.066946024	25
	TMP	697.16791	14.130		
4	8-MOP	61.32235	6.249	0.091736481	50
	TMP	668.46198	14.143		
5	8-MOP	116.27612	6.221	0.179056709	100
	TMP	649.38153	14.059		
6	8-MOP	297.04657	6.242	0.443984243	250
	TMP	669.04755	14.160		
7	8-MOP	615.62323	6.201	0.915016786	500
	TMP	672.79993	14.077		
8	8-MOP	1236.68958	6.214	1.7893714	1000
	TMP	691.13074	14.048		



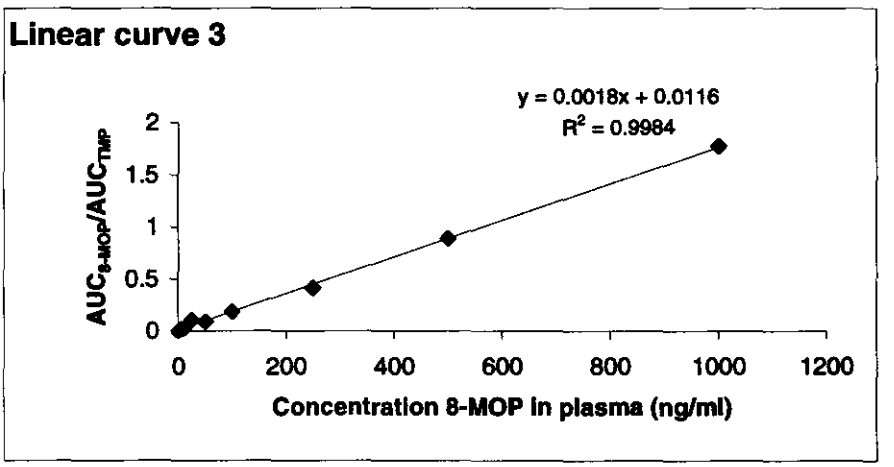
Linear curve 2

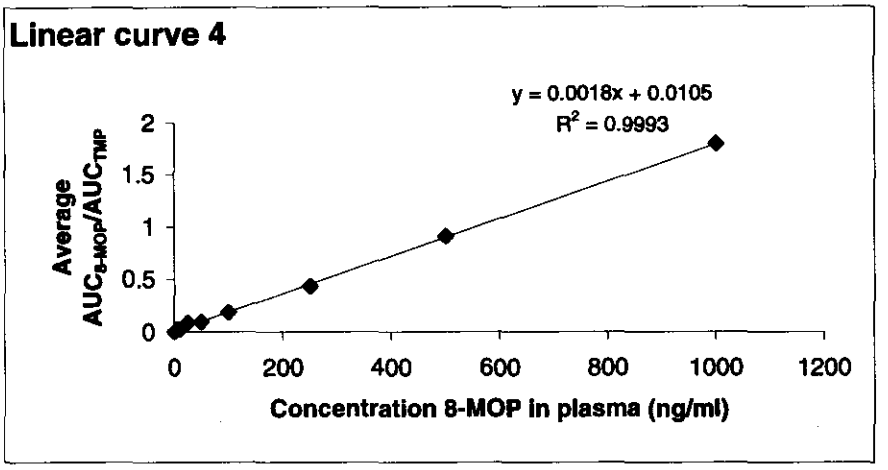
Sample	HPLC parameter			AUC ratio (y-axis)	Concentration (ng/ml) (x-axis)
	8-MOP / TMP	AUC	R_T (min)		
Blank	8-MOP	0	-	0	0
	TMP	710.05890	13.918		
1	8-MOP	11.45381	6.172	0.015892518	5
	TMP	720.70453	13.961		
2	8-MOP	22.68825	6.164	0.031070421	10
	TMP	730.22021	13.901		
3	8-MOP	61.34933	6.138	0.086037364	25
	TMP	713.05450	13.834		
4	8-MOP	59.40360	6.169	0.096116976	50
	TMP	618.03442	13.884		
5	8-MOP	119.78677	6.117	0.190462508	100
	TMP	628.92572	13.753		
6	8-MOP	271.76837	6.095	0.435282015	250
	TMP	624.35010	13.671		
7	8-MOP	570.29687	6.074	0.92287737	500
	TMP	617.95520	13.732		
8	8-MOP	1170.25806	6.137	1.815428025	1000
	TMP	644.61823	13.791		



Linear curve 3

Sample	HPLC parameter			AUC ratio (y-axis)	Concentration (ng/ml) (x-axis)
	8-MOP / TMP	AUC	R_T (min)		
Blank	8-MOP	0	-	0	0
	TMP	693.01563	13.357		
1	8-MOP	15.93847	5.968	0.022074318	5
	TMP	722.03680	13.448		
2	8-MOP	15.81149	6.004	0.022913873	10
	TMP	690.04004	13.398		
3	8-MOP	79.14839	5.948	0.109767825	25
	TMP	721.05273	13.288		
4	8-MOP	60.62897	5.972	0.091261427	50
	TMP	664.34387	13.354		
5	8-MOP	127.38921	5.933	0.184921713	100
	TMP	688.88184	13.234		
6	8-MOP	282.17838	5.955	0.416290485	250
	TMP	677.84009	13.284		
7	8-MOP	626.56360	5.990	0.887607702	500
	TMP	705.90149	13.349		
8	8-MOP	1139.21204	5.946	1.776645047	1000
	TMP	641.21533	13.272		





Determination of linearity within chosen range

	Concentration range (ng/ml)	Regression coefficient	Slope	Y-intercept
Linear curve 1	5-1000	0.9996	0.0018	0.0096
Linear curve 2	5-1000	0.9994	0.0018	0.0103
Linear curve 3	5-1000	0.9984	0.0018	0.0116
	Mean (n=3)	0.999133	0.0018	0.0105
	SD	0.00064291	0	0.001014889
	%CV	0.064346788	0	9.665609524

2. ACCURACY

Accuracy according to linear curve 4:

Slope (m) of linear curve 4: 0.0018

Y-intercept of linear curve 4: 0.0105

2 = 10 ng/ml

Sample	HPLC parameter			AUC ratio	Concentration according to linear curve equitation (ng/ml)
	8-MOP/TMP	AUC	R_T (min)		
1	8-MOP	14.56165	6.240	0.020686084	5.658935556
	TMP	703.93457	14.170		
2	8-MOP	22.68825	6.164	0.031070421	11.42801167
	TMP	730.22021	13.901		
3	8-MOP	15.81149	6.004	0.022913873	6.896596111
	TMP	690.04004	13.398		

Average of concentration (ng/ml): 7.994514446

SD of concentration (ng/ml): 3.03720732

%CV of concentration (ng/ml): 37.99114181

4 = 50 ng/ml

Sample	HPLC parameter			AUC ratio	Concentration according to linear curve equatation (ng/ml)
	8-MOP/TMP	AUC	R_T (min)		
1	8-MOP	61.32235	6.249	0.091736481	45.13137833
	TMP	668.46198	14.143		
2	8-MOP	59.40360	6.169	0.096116976	47.56498667
	TMP	618.03442	13.884		
3	8-MOP	60.62897	5.972	0.091261427	44.86745944
	TMP	664.34387	13.354		

Average of concentration (ng/ml): 45.85460815

SD of concentration (ng/ml): 1.487097621

%CV of concentration (ng/ml): 3.243071266

7 = 500 ng/ml

Sample	HPLC parameter			AUC ratio	Concentration according to linear curve equatation (ng/ml)
	8-MOP/TMP	AUC	R_T (min)		
1	8-MOP	615.62323	6.201	0.915016786	502.5093256
	TMP	672.79993	14.077		
2	8-MOP	570.29687	6.074	0.92287737	506.8763167
	TMP	617.95520	13.732		
3	8-MOP	626.56360	5.990	0.887607702	487.2820567
	TMP	705.90149	13.349		

Average of concentration (ng): 498.889233

SD of concentration (ng/ml): 10.28652315

%CV of concentration (ng/ml)::2.061885178

8 = 1000 ng/ml

Sample	HPLC parameter			AUC ratio	Concentration according to linear curve equatation (ng/ml)
	8-MOP/TMP	AUC	R_T (min)		
1	8-MOP	1236.68958	6.214	1.7893714	988.2618889
	TMP	691.13074	14.048		
2	8-MOP	1170.25806	6.137	1.815428118	1002.737843
	TMP	644.61823	13.791		
3	8-MOP	1139.21204	5.946	1.776645047	981.1916928
	TMP	641.21533	13.272		

Average of concentration (ng/ml): 990.7304749

SD of concentration (ng/ml): 10.98314982

%CV of concentration (ng/ml): 1.108591095

Determination of accuracy

Reference concentration (ng/ml)	Concentration measured ng/ml (n=3)	SD	%CV	% Recovery
10	7.994514446	3.037207	37.9911378	79.94515
50	45.85460815	1.487098	3.243071266	91.70922
500	498.889233	10.28652	2.061885178	99.77785
1000	990.7304749	10.98315	1.108591095	99.07305

3. PRECISION

2 = 10 ng/ml

Sample	HPLC parameter			AUC ratio
	8-MOP/TMP	AUC	R_T (min)	
1	8-MOP	14.56165	6.240	0.020686084
	TMP	703.93457	14.170	
2	8-MOP	22.68825	6.164	0.031070421
	TMP	730.22021	13.901	
3	8-MOP	15.81149	6.004	0.022913873
	TMP	690.04004	13.398	

Average of AUC ratio: 0.024890126
SD of AUC ratio: 0.005466973
%CV of AUC ratio: 21.96442477

4 = 50 ng/ml

Sample	HPLC parameter			AUC ratio
	8-MOP/TMP	AUC	R_T (min)	
1	8-MOP	61.32235	6.249	0.091736481
	TMP	668.46198	14.143	
2	8-MOP	59.40360	6.169	0.096116976
	TMP	618.03442	13.884	
3	8-MOP	60.62897	5.972	0.091261427
	TMP	664.34387	13.354	

Average of AUC ratio: 0.093038295
SD of AUC ratio: 0.002676776
%CV of AUC ratio: 2.877069061

7 = 500 ng/ml

Sample	HPLC parameter			AUC ratio
	8-MOP/TMP	AUC	R_T (min)	
1	8-MOP	615.62323	6.201	0.915016786
	TMP	672.79993	14.077	
2	8-MOP	570.29687	6.074	0.92287737
	TMP	617.95520	13.732	
3	8-MOP	626.56360	5.990	0.887607702
	TMP	705.90149	13.349	

Average of AUC ratio: 0.908500619
SD of AUC ratio: 0.018515742
%CV of AUC ratio: 2.038054968

8 = 1000 ng/ml

Sample	HPLC parameter			AUC ratio
	8-MOP/TMP	AUC	R_T (min)	
1	8-MOP	1236.68958	6.214	1.7893714
	TMP	691.13074	14.048	
2	8-MOP	1170.25806	6.137	1.815428118
	TMP	644.61823	13.791	
3	8-MOP	1139.21204	116.03389	1.776645047
	TMP	641.21533	13.272	

Average of AUC ratio: 1.793814855
SD of AUC ratio: 0.01976967
%CV of AUC ratio: 1.102102034

Determination of precision

Reference concentration (ng/ml)	Mean ratio (n=3)	SD	%CV
10	0.02489	0.005466973	21.96442477
50	0.093038295	0.002676776	2.877069061
500	0.908500619	0.018515742	2.038054968
1000	1.793814855	0.01976967	1.102102034